Chapter 8: DNA Sequencing: Identification of Novel Viral Pathogens

Chapter Overview

In addition to the value of DNA sequencing for identifying genes and examining whole genomes, new technologies now permit "deep sequencing" of transcriptomes, metagenomes, and environmental samples. Bioinformatics is essential for assembly of short sequences into complete gene or genome sequences and for applications that use the short sequences themselves. By completing the projects in this chapter, students will understand how sequence data are read, some uses of sequences produced by high-throughput next-generation sequencing methods, the problem of sequence assembly, algorithmic approaches to constructing a full-length sequence from an array of short sequences, and the use of coverage as a measure of assembly quality. Additionally, students in programming courses will write programs to create test sequence data with a desired level of coverage and write a miniassembler program.

- **Biological problem**: Identification of unknown causes of viral disease
- **Bioinformatics skills**: Manipulating and mapping short sequence reads, assembling sequences into contigs, measures of quality
- **Bioinformatics software**: Galaxy, Megablast, SRA and Trace databases, CAP assembler
- **Programming skills**: Generating random string fragments, Overlapping strings, Traveling Salesperson Problem

Understanding the Problem: Deep Sequencing of Clinical Samples

*It might surprise you to know that diarrhea is the second most common cause of death in children under age 5, killing an estimated 2 million children worldwide each year. Although many people in countries with access to clean drinking water and reliable sanitation may consider this disease a mere annoyance, globally, billions of people lack these basic services. Indeed, diarrhea is third among causes of death for both children and adults in low-income countries, accounting for nearly 7% of fatalities. Most deaths from diarrheal disease result from dehydration, and the chronic or recurrent diarrhea common in many parts of the world is also an important cause of malnutrition. In recent years, several new causes of diarrhea havebeen identified, including cosavirus, klassevirus, and an entirely new genus of parvoviruses. Importantly, these new viruses have been identified not by traditional culture methods but by metagenomics (see References and Supplemental Reading). New "deep sequencing" methods (***Figure 8.1***) applied to any and all DNA found in a human clinical sample not only tell us about what bacteria and viruses are present but have led to the identification of previously uncharacterized species, including novel pathogens.*

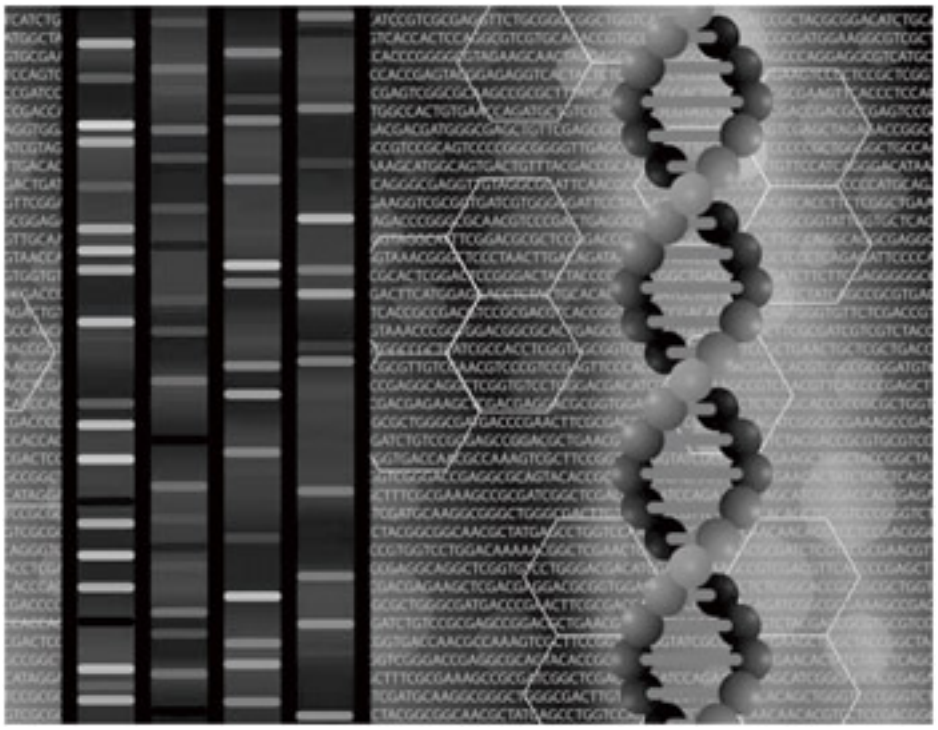
Figure 8.1: Automated sequencing of shotgun sequences and high-throughput next-generation techniques have enabled advances in genome and metagenome sequencing. A computer-generated image of automated sequencing output is shown here. © The Biochemist Artist/ShutterStock, Inc.

Identification of the specific microbe responsible for a given disease has been a difficult problem ever since Robert Koch and Louis Pasteur pioneered the germ theory of disease in the late 1800s. Indeed, given an uncomplicated case of diarrheal disease, it is more efficient for a physician to simply treat dehydration and determine whether antibiotic intervention is warranted than to pursue time-consuming and expensive procedures to identify a specific causative organism. The same is true for many other common diseases—upper respiratory syndromes, fevers, skin problems, and so on. Thus, the full spectrum of pathogens that can cause these diseases remains undetermined, and this is particularly true for viral pathogens because of the difficulty of isolating and culturing unknown viruses. Unexpectedly, DNA sequencing has become an unexpected resource for solving problems of this kind and for examining genomes, measuring gene expression, characterizing ecosystems, and more.

Initially, sequencing was limited by technology and cost to individual genes of interest cloned into plasmid vectors but quickly progressed to sequencing of entire genomes, potentially allowing researchers to define all the functions of a cell and even an entire organism in terms of its genes and their interactions with the environment. The publicly funded International Human Genome Project (IHGP) began in 1990 with a plan to obtain the complete sequence of the human genome—3,000,000,000 nucleotides of information—by mapping and sequencing an ordered set of genome segments. Eight years later, a competitor, Celera Genomics, a private company headed by Dr. Craig Venter, entered what became an acrimonious race. Despite the IHGP's sizeable head start, both groups announced draft genome sequences in 2000. The key to Celera's success was to eliminate the time required to develop orderly arrays and simply sequence random genome fragments, relying on bioinformatic techniques and computational power to assemble these short "shotgun" sequences into complete chromosome sequences (see References and Supplemental Reading). Further advances in sequencing technology have taken this approach to the extreme: so-called next-generation sequencing techniques generate huge numbers of sequences in parallel, but they are as short as tens of bases each. Sophisticated assembly software can join these bits of sequence into full-length DNA sequences with a high degree of accuracy. With these technologies constantly pushing the boundaries of faster, cheaper sequencing, in what new ways might we use DNA sequencing?

Bioinformatics Solutions: Assembly and Mapping of Short Sequence Reads

DNA sequencing is the process of determining the order of the nucleotides that make up a piece of DNA. This is the laboratory technique that generates not only all the DNA sequences you've been working with throughout this text (for more detail on sequencing techniques, see the BioBackground section at the end of the chapter) but most of the amino-acid sequences as well, because computational "translation" of a nucleotide sequence is much faster and cheaper than directly sequencing a protein. Although the human genome was not the first to be sequenced (among cellular organisms, that honor belongs to the yeast *Saccharomyces cerevisiae*), it has generated the most interest: Its far-reaching potential has been compared with the invention of the printing press. We remain a long way from knowing the function of every gene in the human genome, but we have all the raw data: the nucleotide sequences of all 23 distinct human chromosomes and all the 20,000+ genes they carry.

Although dideoxy sequencing was used in both cases, Celera genomics was able to complete the sequencing of the human genome in a fraction of the time required by the IHGP by pioneering a faster **shotgun sequencing** technique (**Figure 8.2**). The Celera approach was fast because many DNA fragments could be sequenced at once, but it created a major computational problem because it produced many short DNA sequences whose relationship to each other was unknown. With algorithms capable of accurately assembling these sequences into the sequences of complete chromosomes, Celera opened the door to rapid genome sequencing. As this technique gained momentum, dozens of other genomes were completed, including bacteria, vertebrate and invertebrate animals, plants, fungi, and viruses.Huge benefits have already been reaped from genome sequencing, including better understanding of biological processes, identification of genes responsible for disease, development of improved therapies, and industrial and agricultural applications.

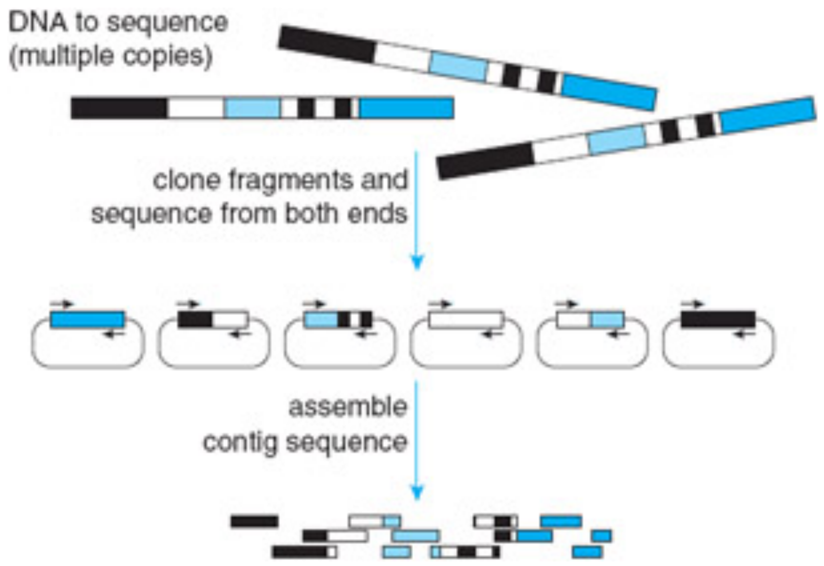

Figure 8.2: Schematic representation of shotgun sequencing. The DNA to be sequenced is fragmented, random fragments are cloned into plasmids, and the fragments are then sequenced from both ends. Computational assembly of many fragments allows the complete sequence of the original DNA to be reconstructed.

However, translating shotgun sequence data into quality genome sequence requires high**coverage**: Each segment of the genome must be sequenced many times over to generate enough overlapping fragments to assemble the complete genome. The advent of next-generation sequencing techniques (see BioBackground) drastically increased the rate at which sequence could be obtained. In 454 sequencing, for example, a million individual sequence reads can be done in a single run, and Illumina and SOLiD technology can multiply that by 1,000 times. However, in maximizing data throughput, these techniques sacrifice **read length**, or the lengths of the DNA sequences they identify. Read lengths in dideoxy sequencing can be 800 nucleotides long or longer, but that number drops to 500 nucleotides for 454 sequencing and less than 100 nucleotides for Illumina and SOLiD. These sequencing techniques are therefore only as good as the bioinformatics software that allows us to analyze and interpret them.

The short sequences generated by next-generation sequencing are used in two general ways: assembled into genomes or used directly to identify RNAs, organisms, or functional segments. Genome assembly has progressed to the point that we can begin to contemplate applications such as the rapid and inexpensive determination of each *individual* human's complete genome sequence. Meanwhile, short sequence reads from cellular, environmental, or clinical samples are used to determine the complete set of mRNAs produced in a given tissue or under a given condition (**RNA-seq**) or to identify all the organisms present in a particular environment without the need to isolate or culture them (**metagenomics**). These latter applications are often referred to as **deep sequencing** techniques. Deep sequencing of DNA present in a stool sample, throat swab, or skin wash can be used to identify the microbes normally present in the human body (the **microbiome**) as well as any pathogenic organisms that may be present. In the future, doctors may be able to use deep sequencing to take a microbial "census" of patient tissues. Applications like these depend on bioinformatics to provide algorithms for reliable assembly of massive amounts of fragmentary data into meaningful sequences and for mapping sequence reads relative to genomes.

In this chapter's projects, you will examine and use some of these bioinformatics applications to work with sequencing data. You will see how DNA sequencing data are presented, identify viruses from short sequence reads, experiment with assembly programs, and (if your course includes programming) write your own miniassembly program.

BioConcept Questions

1. In Sanger sequencing, why does a newly synthesized strand of DNA terminate when DNA polymerase inserts a dideoxy nucleotide? How are these terminated DNA strands used to "read" the nucleotide sequence of the original DNA molecule?
2. Why is shotgun sequencing so much faster than the directed approach originally taken by the IHGP? Why is it more dependent on computer power and bioinformatics?
3. If the entire human genome were cleaved into a single set of small, non-overlapping fragments, we could not determine the genome sequence by sequencing the fragments. Explain why this is the case.
4. How do next-generation sequencing techniques extend and improve on the shotgun sequencing technique? What are their disadvantages?
5. Complex genomes often contain many repeated sequences. For example, there are many STR (short tandem repeat) sites in the human genome, where a short sequence such as GATA might be repeated anywhere from a few to dozens of times. Why would an STR region potentially pose a problem for sequencing? Are next-generation techniques more or less susceptible to errors resulting from repeated sequences than older technologies?

Understanding the Algorithm: Determining Overlap in Sequence Assembly

Learning Tools

Download To better understand the problem of sequence assembly and the importance of the depth of coverage, you can download **Assembly exercise.pdf** from the *Exploring Bioinformatics* website. This file contains three copies of a short sequence representing threefold sequencing coverage that can be printed, cut into pieces, and reassembled.

In this chapter's Web Exploration, you will gain experience using both sequence assembly tools and alignment-based tools for metagenomic analysis of short sequence reads from a clinical sample. In the Guided Programming Project and On Your Own Project, we focus on programs for assembling sequences, so this section explores assembly algorithms.

The problem of **sequence assembly** is similar to the problem you would encounter if you ran this page through a paper shredder. Each fragment of the page might contain just a few words or perhaps just a handful of letters, and reassembling the complete text would be a daunting task. In shotgun sequencing, however, each part of the genome is represented more than once, as if you made several copies of this page and then shredded them together. Therefore, you might find pieces with `ach fr` and `ent o` but then you might discover a piece with `Each fragm` and another with `nt of th` that came from different copies of the page. Even if you never found the missing piece with agm that fits between the first two, you could conclude that a segment of the original text read `Each fragment of th`. In this way, overlapping short DNA sequences can allow us to build up the original sequence of a long piece of DNA (**Figure 8.3**).

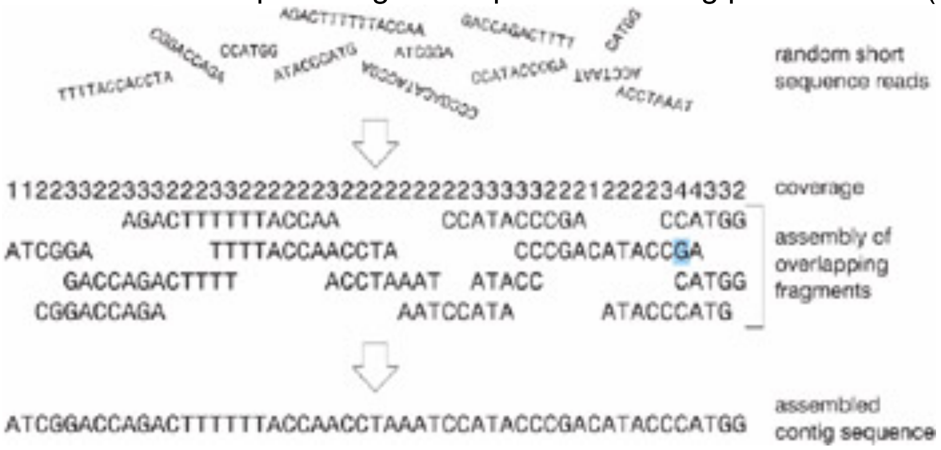

Figure 8.3: Assembly of short sequence reads into a longer contiguous sequence (contig). Overlaps are used to order the fragments, and coverage shows how often each nucleotide in the contig has been sequenced, a measure of the quality of the assembly. The highlighted G nucleotide appears to be a sequencing error.

A **sequence read** is a single piece of data from a DNA sequencing reaction; whether it is an 800-nt fragment from Sanger sequencing or a 40-nt fragment from a next-generation platform, it can be represented as a string of nucleotides. Thus, given a large number of strings representing nucleotide sequences, a sequence assembly program looks for overlaps to decide which strings should be joined together. Joining sequence fragments builds longer sequences called **contigs**, and when enough overlaps have been found, the contig represents an entire mRNA, plasmid, or chromosome. This process is far from trivial, however: Overlaps may be small, and the repeated sequences common to complex genomes (more than 50% of the human genome consists of repeated sequences of various kinds) introduce the possibility of misassembly. Furthermore, a fragment could come from either strand of the DNA molecule, so the assembler has to try assembling a given fragment and also its reverse complement. Furthermore, inaccuracy in the sequencing reactions themselves or in the base-calling software (see BioBackground) means that it may be unclear whether an overlap is genuine.

Sequence assembly is therefore one of the most complex bioinformatics problems. In fact, it is still considered an open problem—one not completely solved—because no existing algorithm can reassemble fragments with complete accuracy in all situations.

We are thus forced to rely on technical solutions to increase accuracy, such as continuing to sequence random fragments until every section of the target DNA has been sequenced multiple times.Then, errors become noticeable as bases fail to align properly (Figure 8.3). The assembly program keeps track of **coverage**—the number of times the nucleotide at each position hasbeen sequenced—as a measure of the reliability of the assembly at each point.

To see how an assembly algorithm would work, let's consider the problem of assembling just two fragments. If two fragments overlap, the "suffix" (right or 3′ end) of one fragment must overlap the "prefix" (left or 5′ end) of the other fragment so that the base positions in the overlapping region match (**Figure 8.4A**). Then, the two fragments can be merged. However, what if there is more than one way they could overlap? Consider the sequences `AATGCCTGA`and `TGACGAGTTAATGC`: These could overlap in two different ways, as shown in **Figure 8.4B**. Which is the correct one? A common initial criterion for an assembly program is simply to choose the largest overlap as the one that most likely represents a correct assembly. Assuming the sequences are not identical and neither is a substring of the other, the longest possible overlap is one less than the length of the shorter sequence. We can therefore start with this maximum length and see if we can find an overlap this long. If not, we can look for an overlap one base shorter and so on, stopping the search as soon as a matching overlap is found. Then, we know we have identified the longest possible overlap and can merge the sequences (Figure 8.4B). Algorithmic steps to accomplish this are as follows.

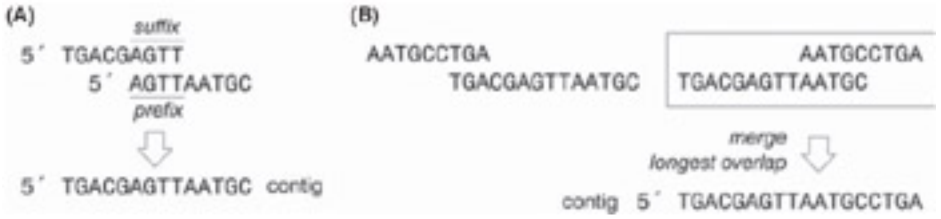

Figure 8.4: Assembly of two fragments with (A) an unambiguous overlap allowing the two to be merged into a contig, or (B) an ambiguous overlap requiring the assembly program to make a decision; here, the longest overlap is chosen as the most likely correct assembly.

Algorithm

Determining Largest Overlap Algorithm

1. Start with two sequences: s1 and s2.
2. Set *n* = size of the smallest sequence - 1 (*n* will represent the largest overlap).
3. Compare n suffix characters from s1 with n prefix characters from s2. Also compare n suffix characters from s2 with n prefix characters from the s1.
4. Count matching bases in the prospective overlap region. If the number of matches in either set equals n, the largest overlap has been found: merge sequences to yield the contig sequence.
5. If the number of matches is less than n, subtract 1 from n. If n is 0, there is no overlap; otherwise, go to step 3.

Given the sample sequences provided, this algorithm would first look at eight-base overlaps (the short sequence is nine nucleotides) and then seven and six. At *n* = 5, a match would be found with `AATGC` in the prefix of the short sequence matching `AATGC` in the suffix of the long sequence (Figure 8.4B), and the two would merge to form the contig`TGACGAGTTAATGCCTGA`.

Of course, real sequence assembly is much more complicated: We have not considered the opposite strand or allowed for possible imperfect matches due to sequencing errors, and we have considered only two fragments, instead of the millions or even billions that can result from next-generation sequencing. The exhaustive matching of pairs of fragments will quickly become so computationally intensive as to be impractical, so heuristics must be used. One heuristic solution is a "greedy" algorithm: Given the choice of overlapping fragment A with fragment B, B with C, or C with A, the program makes the "educated guess" that the largest overlap is the best and proceeds without trying every possibility. The On Your Own Project provides a more detailed explanation of using this heuristic for sequence assembly.

Test Your Understanding

1. Suppose two sequence reads give `GGGGCAGGCC` and `GCCCCGG`. What would be the sequence of the contig produced using the algorithm just given?
2. Now suppose you would like your algorithm to account for the possibility that the sequences could come from either strand of the DNA. How would you modify the algorithm to accomplish this? Would the contig resulting from the two sequences in question 1 change as a result?
3. The algorithm presented assumes that the strings cannot be identical and that one cannot be contained completely within the other (one cannot be a substring of the other). But this is a somewhat arbitrary constraint, particularly when comparing a short sequence with a longer contig that has been built. How would you change the algorithm to allow for substrings and identical sequences?
4. Real sequencing data are "noisy:" They can contain incorrect characters due to sequencing errors (for example, the accuracy of most next-generation methods decreases as the fragment length increases) or to ambiguities leading to incorrect base-calling. How would you modify the algorithm so that a perfectly matching overlap is not required but merely one that exceeds some threshold value? How would incorporating this change affect the number of comparisons that must be made between two sequences?

Chapter project: Identifying Viruses Through Metagenomic Analysis of Clinical Samples

To sequence, for example, the human genome, one might imagine extracting DNA from a sample of human cells free from contamination by bacteria or other sources of nonhuman DNA. However, what if we were to extract and sequence DNA of *any* kind that might happen to be in a soil sample, water sample, or stool sample? The resulting sequence would give us information about the genomes of all the different organisms present in that environment: We call this mixture a **metagenome**. This information could be used in a number of ways: We might use specific primers to sequence only diagnostic DNA segments, such as the genes for ribosomal RNA that are present in every organism and commonly used in phylogenetic analysis (seeChapters 6 and 7). Or, a biotech company might try to get a broad sample of protein coding genes and look for novel enzymes that might have practical applications. Or, we might use the metagenome to find evidence of microbes that live in association with humans, potentially proceeding from there to build a complete genome of a previously unknown organism. This is how several new viruses that cause diarrheal disease were actually identified, and we use some of these same techniques in this chapter's projects.

Learning Objectives

- Understand how short, random DNA sequences can be assembled to generate sequences of genes and genomes
- Appreciate the difficulty of accurate assembly and the dependence of sequencing on strong, efficient bioinformatics algorithms
- Gain experience with metagenomic uses of next-generation sequencing
- Know the various sources of inaccuracy, biological and computational, in sequence assembly and how quality data and coverage can increase accuracy
- Understand how to produce test data that simulate sequence reads and the value of these simulated data

Suggestions for Using the Project

The Web Exploration Project for this chapter allows students to deal with DNA sequence data in three distinct ways; the three parts of this project can be used independently depending on the focus desired by the instructor. The Guided Programming Project leads students to write code to generate simulated sequence data that are then used with the miniassembler in the On Your Own Project; instructors can provide either or both of these solutions in finished form for use in nonprogramming courses.

Programming courses:

- Web Exploration: See the output of Sanger sequencing data and understand base-calling, assemble a small sequence read dataset, and map metagenomic sequence data to known organisms. Parts I, II, and III can be used independently.
- Guided Programming Project: Develop a simulator to produce test data resembling the output of various sequencing platforms.
- On Your Own Project: Understand greedy algorithms for heuristic assembly of sequence data; develop a miniassembler to assemble sequencing data.

Nonprogramming courses:

- Web Exploration: See the output of Sanger sequencing data and understand base-calling, assemble a small sequence read dataset, and map metagenomic sequence data to known organisms. Parts I, II, and III can be used independently.

- Guided Programming Project: Download executable code for a sequence data simulator and use it to further experiment with the Web-based assembler.
- On Your Own Project: Understand greedy algorithms for heuristic assembly of sequence data; download executable code for a miniassembler and test with data from the Guided Programming Project and/or Web Exploration.

Web Exploration— Analysis of Virus Sequences in the Human Metagenome

The Web Exploration for this chapter is divided into three independent parts. In the first section, we look at sequence traces for dideoxy sequencing of a virus genome to better understand the nature of automated DNA sequence, how base-calling works, and some potential sources of error in sequence data. We then use a small sample of actual next-generation sequencing data taken from a metagenomic experiment to identify the organisms present in a stool sample based on short, random DNA sequence reads. Finally, we use an assembly program to see how sequence reads can be built into a contiguous virus genome sequence.

Part 1: DNA Sequence Traces and Base-Calling

Link Automated dideoxy sequencing (see BioBackground) was one of the major innovations that made genome sequencing possible. However, it changed the nature of raw sequence data from bands on a gel to a computerized record of light wavelengths and intensities. These data can be output as an **electropherogram**, more commonly called a **DNA trace** (**Figure 8.5**), in which the fluorescence emitted by each dideoxynucleotide is represented by a color and the intensity represented by peak height. A researcher can examine the trace by hand to determine the sequence (e.g., a T for each red peak). However, this is extremely tedious even for a short sequence and certainly impossible for an entire genome. Thus, sequencing software also includes a **base-calling** program (**Phred** is a popular example) that interprets the color and intensity data and outputs an actual sequence of nucleotides.

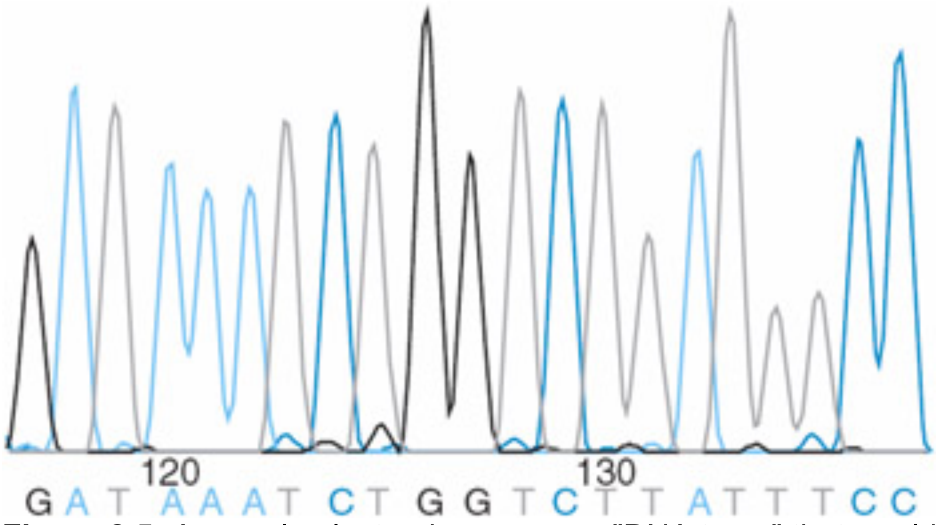

Figure 8.5: A sample electropherogram or "DNA trace" that would be generated by automated Sanger sequencing. The different color shades represent the four distinct

fluorescent nucleotides detected, while the peak heights represent the intensity of detection of that particular fluorescence. At the bottom of the figure is the DNA sequence as determined by an automated base-calling program.

Link Today, dideoxy sequencing is done inexpensively by many companies and universities. A researcher submitting DNA to be sequenced usually receives not only FASTA-formatted sequence files but also the sequence trace itself. Although the base-calling programs have good accuracy, there are always ambiguities: Is a broad peak one base or two? Is a weak peak an actual base or an artifact? In a small sequencing project, the reliability of the sequence can be improved by checking the accuracy of the base-calling using a trace viewer. **Chromas** is a commonly used desktop trace viewer that comes in a free "light" version. For our purposes, however, we can look at some sequence traces stored in the **NCBI Trace Archive**, a database of dideoxy sequencing projects.

Navigate to the Trace Archive database. A difficulty in using this database is that it does not use the standard NCBI Entrez search interface. To locate some sequences to examine, click the tab labeled `Obtaining Data` and then the option `Registered Species` to see a list of species for which there is sequence in the database. You should find an entry for `Human Gut Metagenome`; clicking this entry creates a query in the search field above; click `Submit`to see the results. These sequences come from a metagenomic project in which DNA taken from the human gut (via a fecal sample) was sequenced to identify the microbial species present. You can see that the data consist of a great number of comparatively long reads. Although they have already been edited to remove the least reliable data from each sequence, you may be able to see some spots where bases could not be accurately determined, indicated by N.

Change the display to show the sequencing traces rather than the FASTA file. Click and drag the trace itself or click in the bar just above it to move through the sequence. It should be clear how the quality of the sequence changes along the read, from tall but indistinct initial peaks to a region where the sequence is very easy to read, to much lower peaks farther on. Notice that the base-calling software can determine bases far past where we can distinguish peaks (though a more sophisticated trace reader allows changing the scale to increase the viewable size of the peaks). Examine any Ns that occur in the sequence; can you manually call the base that the software could not call? Look for some runs of bases, such as three or more Gs or As in a row; can you see why these can be hard to call? Do you agree with the base-calling program? What other areas of the sequence appear to be difficult to determine precisely? You can also change the display to show quality, an estimate of reliability for each nucleotide, or to show information about the sequence run.

Web Exploration Questions

1. Looking at the DNA sequence traces, what conditions appear to cause the base-calling program to output N rather than designating a specific base?
2. How many nucleotides of sequence was the base-calling program able to read for the traces you examined?

3. Why does the lowest quality sequence occur at the beginning and the end of the sequence run?
4. Although each dideoxy sequencing run produces a sequence trace, in a large metagenomic or genome sequencing project, it would not be practical to examine each trace and manually assign difficult bases. How can the sequences returned by an automated base-caller be used reliably in such a project?

Part II: Metagenomic Analysis of the Human Virome by Next-Generation Sequencing

Link To sequence a genome, many-fold coverage of every nucleotide is necessary for high accuracy. However, there are many uses of sequencing in which *individual* reads provide valuable information. Notably, for a metagenomic project intended to sample all the organisms in an environment, individual reads can be compared with sequence databases to identify known organisms or distinguish novel ones. Data from many such projects can be found in public databases such as NCBI's **Sequence Read Archive** (SRA) database. One example is a project led by Gary D. Wu at the University of Pennsylvania in which DNA from fecal samples was sequenced with the intent of examining the microbial population (**microbiome**) of the human gut under various dietary conditions. Although the original intent of the study was to relate the microbiome to Crohn's disease, these same data were also mined to examine the **virome**, or viral population, of the gut. Here, we examine a small sample of data from this research project to see how metagenomic data can be analyzed.

Link Although there are many freely available programs, most software for analysis of next-generation sequencing data must be downloaded and run on a desktop computer, because of the complexity of working with millions or billions of short sequences (see More to Explore, for some programs you might be interested in using). Indeed, our reason for examining only a portion of the available metagenomic data is to keep processing time reason- able for a course project. One notable Web-based tool that can be used for metagenomic analysis (as well as many other kinds of sequence data analysis) is **Galaxy** (see References and Supplemental Reading), a flexible interface that can be used to run many different kinds of bioinformatics programs.

On the Galaxy main page (try `Galaxy sequence analysis` if you are using a search engine to find it), you will see three panes: a tool list on the left, a pane with parameters for the current tool in the center (initially, some available tutorials are displayed here), and a history pane on the right showing pending and completed analyses. Let's start by downloading data from the virome study. In Galaxy, this can be done by accessing EBI's interface to the SRA database: In the tool pane, choose `Get Data` and then `EBI SRA` to open a search interface in the second pane. The accession number for the data from the virome project is `SRS072363`; enter this in the search box and submit the search. You should see the SRA database entry for a sample from one specific subject in the study; at the bottom of the page, you should see listings for two specific files of Illumina sequencing data in FASTQ format. The far-right column in the file listing table is headed `Galaxy`; click on file 1 in this column to import these data into Galaxy. You will see a message in the center pane and then a task added to the history pane; the task will turn yellow when the server starts on it and green when it is complete. Once complete, you can click the task to see a "preview" of the data it contains within the history pane or the eye icon to see the data file itself in the center pane. Notice in the description of this file the large number of sequences it contains.

Next-generation sequencing techniques automate sequencing and base-calling even more fully than in automated dideoxy sequencing. Although raw sequence data can be viewed (454 sequencing, for example, generates a flowgram similar in principle to an electropherogram), the enormous number of reads and the automation drastically decrease the value of any manual examination of the data. Instead, it is common to summarize both the called bases and data on the quality of the read within a single file in FASTQ format (**Figure 8.6**). Like a FASTA file, a FASTQ sequence file starts with a comment line, in this case beginning with @, to identify the sequence. The next line of the file is the sequence itself. There is then a line starting with + where an additional comment may optionally be added. The last line gives a quality score for each nucleotide, encoded as an ASCII character. The quality score range depends on the sequencing software; older Illumina software used a quality score from -5 to 62, whereas Sanger format uses a quality score based on the Phred algorithm, from 0 to 93.

```
@EAS100R : 3: 90 : 836 : 2213#0 TCGATGATTTGGGGTTCAATCCATTTGTTCAA
%%%%)!''*((((***+))%)**55CCF>>>>>
```

Figure 8.6: Example of next-generation sequencing data in FASTQ format. The first line is a comment marked by @ and identifies the sequence (including the instrument, run, specific cell, etc.). The second line is the sequence itself. The third line is an additional comment line marked by +, and the fourth line is the quality score for each base encoded by calculating a Phred quality score, adding 33 and using the ASCII character corresponding to that number (so, % = ASCII 37 = Phred 4;C = ASCII 67 = Phred 34).

Converting the imported data to Sanger FASTQ format is needed for many of the Galaxy tools; to accomplish this, find `FASTQ Groomer` under `NGS` (next-generation sequencing) `QC and manipulation` in the tools pane. Notice that this tool will work on an item from your history, in this case the imported sequence data. Be sure the input data type is `Sanger` (your data are from Illumina 1.8, which uses the same FASTQ quality score system as FASTQ files for Sanger sequencing) and execute the task. You can expect this task to take a fairly long time to process (maybe hours if the load on the server is high). However, you can put additional tasks into the queue while you are waiting and they will be completed in order once this step is done.

If you look at the actual sequences in the imported or groomed data file, you will notice that many are runs that consist only of Ns, indicating that no useful sequence data were obtained. Others may be very short, and others may have very low-quality scores. Let's limit our analysis for this project to runs that yielded a reasonable amount of good-quality sequence. To do so, look in the same category of tools for `Filter FASTQ reads by quality score and length`. Use the FASTQ Groomer output and set minimum length to 50 nucleotides and the quality cut-off value to 20, which represents a 99% probability that the base has been called correctly. Run the analysis and note how many sequences were discarded.

Now that we have used the quality data to develop a subset of sequences we want to pursue further, we can convert the FASTQ data to a simple FASTA file of sequences with an identifying line. The complex identifiers in the FASTQ file are not really needed; let's give each of our sequences a simple identifier like GutVirome-1, GutVirome-2, and so on. Galaxy has tools for manipulating complex genome files that perform these actions easily. First, convert the FASTQ data to a table, using the `FASTQ to Tabular` converter tool. The output is in columns: sequence identifier, the sequence itself, and the quality data. Now add a column to the table, using the `Add column` tool found under `Text manipulation`. In the`Add this value` field, type `GutVirome` and then change `Iterate` to `Yes`; this adds a column of data containing sequentially numbered labels as suggested earlier. Finally, generate the FASTA file using `Tabular-to-FASTA` (under `FASTA manipulation`), with the new fourth column (c4) as the title column and the second column (c2) as the sequence column. The resulting FASTA data should look very familiar to you.

Using the FASTA file, we can now do the actual metagenomic analysis. We want to compare each remaining sequence read with the entire database of known sequences and identify the source of the sequence: human DNA, known or novel bacterial species, known or novel virus, and so on. Galaxy includes MegaBLAST as a tool that can perform this search; essentially, it will carry out a BLAST search for every sequence in your FASTA file, using parameters optimized to allow for small differences due to sequencing errors. Choose `Megablast` under`NGS: Mapping`, set the FASTA data as input, `nt` as the target database, a word size of 16, and a minimum percent identity threshold of 80%. Note at the bottom of this pane how the MegaBLAST output will look and then execute the database search. This process may also take some time; when it is complete, you should see that the number of lines has grown drastically, because any of the sequence reads can match multiple database sequences.

How can one deal with such a large set of results—to say nothing of the enormous amount of data we would have obtained had we started with all the sequence data from all the study subjects? One way to summarize the results is by retrieving from the database the taxonomic information (species, genus, family, order, etc.) for each matched sequence. Results can then be grouped on this basis to reveal whether the sequenced DNAs belong to viruses, bacteria, human cells, or other organisms—even those that do not match a known species can be classified into larger groups. Under `Metagenomic analyses`, choose `Fetch taxonomic representation`. Set the name column to c1 (the identifier you gave the sequences) and the GIs column (GenBank gene identifier) to c2; these accession numbers will be used to retrieve the taxonomic information. Run the analysis.

There are now a number of possible ways to examine the data further. To look at all the virus sequences in the dataset, for example, filter the data to show only the lines in which the Superkingdom column contains viruses. Similarly, well-chosen filters can allow you to look at bacteria or fungi or other organisms (you may need to look at some sample data to decide on filter terms). Another way to look at the data is to generate a phylogenetic tree of the organisms identified by the alignments: first run `Find lowest diagnostic rank` on the taxonomic data and then `Draw phylogeny` to get a PDF file showing the tree.

Web Exploration Questions

5. How does the number of viral sequences found in the sequence runs you analyzed compare with the number of bacterial sequences? Are there fungal sequences? Protists? Do these relative numbers make sense in terms of the human gut environment and the roles of these organisms?
6. Some of the species represented among the gut sequences might seem surprising. What seemingly unlikely species were identified, and what are some possible reasons for these results?
7. What are the most commonly found viral sequences? Why is this the case?
8. How could viruses that are normal residents of the gut community be distinguished from those that might be pathogens?
9. How could novel viruses be distinguished from related viruses that have already been characterized?

Part III: Assembling the Sequence of a Novel Virus

Whereas metagenomic analysis can be conducted using individual short sequence reads resulting from next-generation sequencing of clinical samples, determining the genome sequence of any organism requires assembly of sequence reads into contigs with sufficient depth of coverage to detect and correct errors. The depth of coverage required is lower for sequencing methods producing long reads and much higher for techniques producing very short reads. Once a genome of interest has been identified from a metagenomic sample, it may be possible to identify enough reads from that genome to begin assembling its sequence. With the identification of portions of the genome, specific primers can be designed based on the now known sequence and used on the same metagenomic DNA samples to fill in the gaps in the genome. This process has been used to identify a number of novel pathogenic human viruses in recent years. For example, klassevirus, a new human virus in the picornavirus family, was identified in this manner from stool samples taken from children with diarrhea who tested negative for known diarrheal viruses (see References and Supplemental Reading). Viruses such as these may turn out to be important causes of human disease that have escaped detection until now.

Link As with metagenomic analysis, most assembly programs that can handle genomic sequence data, especially next-generation sequencing data, are intended to run on powerful desktop machines (see More to Explore for some desktop programs you could use for assembly). For this project, we use **EGassembler**, a Web implementation of the CAP3 (*c*ontig *a*ssembly *p*rogram) assembler (see References and Supplemental Reading). From the *Understanding Bioinformatics* website, you can download **reads.txt**, a file that contains 2,500 simulated 454 sequencing reads in FASTA format, representing the genome of an unknown virus identified in metagenomic samples. These sequences range in length from 100 to 500 bases and contain between 1 and 10 random substitutions or single-nucleotide deletions each, representing the errors inherent in sequencing data.

Download Navigate to the EGassembler page and either upload the sequence file or copy and paste the sequences into the input field. Notice that in addition to the CAP3 assembler itself, EGassembler includes software to scan for low-quality sequence (e.g., sequences containing many Ns) and remove sequences matching databases of organelle and cloning vector DNA as well as highly repetitive sequences. For our purposes, turn off the options other than sequence cleaning and the assembly step itself and then run the program. You should immediately see the results of sequence cleaning; you can view a.cln file to identify reads that were discarded and then examine these reads in the original sequence file.

In a few minutes, the link to the results should become functional. From the results page, you can view (1) the contig or contigs that resulted from the assembly of your sequence reads; (2) any "singletons," which are reads that could not be assembled into the contigs or that were not used in creating the contig; and (3) an alignment of the individual sequence reads showing how they led to the generation of the consensus contig sequence.

Web Exploration Questions

10. How many sequence reads were rejected in the sequence cleaning process? Can you determine why they were rejected?
11. Use BLAST to compare your contig sequence with known sequences in GenBank. The assembled sequence should match one known sequence with a high degree of similarity. What have we sequenced? How long is its genome?
12. Because next-generation sequencing produces random short reads, there is no guarantee that even 2,500 reads would be sufficient to completely sequence a particular genome. Did the sequence reads you assembled cover the entire genome or do gaps remain? To fill any gaps, would it make sense to simply run more sequencing reactions, or are there other approaches that should be considered?
13. Looking at the contig alignment file in the EGassembler results, you should be able to see hundreds if not thousands of small sequencing errors among the sequence reads. Was the assembler able to generate a correct contig sequence (as compared with the known sequence in the data-base) despite these errors? Explain how the sequence errors were accurately corrected. Were all errors caught, or did some remain in the final contig sequence?
14. You used the default parameters for the CAP3 assembler in your EGassembler run. In a real sequencing project, however, you might want to change variables such as the overlap percent identity cut-off (the minimum percentage of nucleotides that must be identical in the overlapping region of two fragments). By default, CAP3 is quite tolerant of sequencing errors (and in fact automatically compensates for some of the common problems of high-throughput sequencing, such as low-quality sequence at the beginning and end of fragments). To see

how these parameters affect the assembly, try setting the overlap percent identity cut-off to 100%. What happens to your contig? Does the quality of your alignment change? (You can choose Step-by-Step Assembly at the top of the page to access more parameters.)

More to Explore: Sequencing Tools

DNA sequencing has become such an important part of molecular biology and bioinformatics that a large number of software tools for analyzing sequencing information are available, both proprietary and otherwise. As mentioned previously, the sizes of data files containing millions or hundreds of millions of sequencing reads and the processing power required to analyze them reduce the desirability of Web-based interfaces, so many of the freely available programs must be down-loaded and installed on one's own computer. **Table 8.1** lists a number of sequence analysis programs that you might be interested in working with in the future.

Table 8.1: Some sequence assembly and analysis software.

Open table as spreadsheet

Program	Description
Sequence assembly	
Velvet	Assembler optimized for very short sequence reads
Oases	Extension of Velvet for transcriptome assembly
IDBA-UD	Assembler optimized for uneven coverage
SSAKE	Short-read assembler based on a greedy algorithm
CABOG	Celera software for small and large genome assembly
SOAPdenovo	Assembler capable of human genomesize assembly
Mapping of sequence reads to reference genomes (metagenomics)	
Bowtie	Fast alignment of sequence reads to human genome
BWA	Aligns sequence data with a reference sequence
MAQ	Maps sequence reads and identifies variants
SOAPaligner	Maps short oligonucleotides onto reference sequences

Guided Programming Project: Sequencing and Assembly

The goal of this guided project is to better understand sequencing data and how they are handled computationally in two ways: by developing a program to generate fragments of a known sequence that effectively simulate actual sequencing data and by using a simple assembly algorithm to assemble pairs of error-free sequence reads. In the On Your Own Project, you will carry this further, developing a miniassembly program capable of a more complex assembly.

Simulating Sequencing Data

The accuracy of any sequence assembly or metagenomic read-mapping program must be tested, and it is often convenient to have a set of test data that closely matches real sequencing data but has a known solution. Instead of using a contig assembled from actual sequencing data (which could be subject to assembly errors), a sequence simulation algorithm is commonly used to generate test fragments of a known DNA sequence that are designed to mimic the results of a particular sequencing platform (see References and Supplemental Reading). These simulated sequence reads should be random segments of the known sequence (representing random "shotgun" sequence data) whose size is appropriate for the sequencing technology being simulated (**Table 8.2**); we can ask the user to supply a desired minimum and maximum fragment length. Simulated sequencing errors and variable sequence quality can be introduced to increase the realism of the simulation.

Table 8.2: Read lengths for major sequencing technologies.
Open table as spreadsheet

Sequencing Platform	Typical Read Length	No. of Reads per Run
Sanger	500–900 bp	1–96
454	200–300 bp	400,000
Solexa	36 bp	3.4 million
Illumina	100 bp	3 billion
SOLiD	35 bp	1.7 billion

In a real sequencing project, fragments of the DNA to be sequenced are produced by random processes. Thus, our program should randomly choose a substring of the input DNA string that falls within the specified size range. However, we need to make sure we generate enough overlapping fragments to cover the whole genome. The original shotgun sequencing genome projects tried to achieve about eightfold coverage of the entire original sequence: that is, each base position in the original sequence should appear in at least eight fragments. Next-generation sequencing methods, with their shorter reads, typically work with 30-fold coverage, while an application such as identifying rare mutations with a high degree of confidence may require 1,000-fold coverage. We should allow the user to input a desired coverage value, simulating the ability of a user to "tune" the coverage in a sequencing project. The pseudocode that follows describes an algorithm to simulate sequence reads by generating fragments of an input sequence to achieve a desired coverage level.

Algorithm

Sequence Read Simulator: Generating Fragments for Sequence Assembly

Goal: To generate random fragments from an input sequence.

Input: A single nucleotide sequence, user-defined minimum fragment size, maximum fragment size and coverage fold

Output: A set of fragments

Note: substring is assumed exclusive, thus substring(1,4) includes positions 1, 2, 3 only

```
// Initialization
Input the sequence: s1
Input the minimum and maximum fragment size: fMin, fMax
Input the coverage fold expected: fold
for each i from 0 to length of s1 - 1
      coverage[i] = 0 // holds coverage count of nucleotides

// STEP 1: Generate a set of fragments for the input sequence
numFrags = 0
do
   randLength = random number between fMin and fMax, inclusive
   randStart = random number between 0 and (length of s1-
   randLength)
   frags[numFrags] = s1.substring(randStart,
   randStart+randLength)
   numFrags++
   // update coverage
   for each i from randStart to (randStart+randLength-1)
       coverage[i]++
while (!coverageMet(coverage, fold))
output frags
// function to determine if coverage met
function coverageMet(coverage, fold)
   i = 0
   met = true // assume coverage met
   while (i < coverage length and met==true)
   if coverage[i] < fold
      met = false
   i++
   return met
```

Assembling Paris of Sequence Reads

The goal of an assembly program is to produce one contig from a set of sequence fragments; here, we implement one small but important step in the assembly process: assembling each*pair* of fragments in a set of sequence reads into a contig using the overlap algorithm (see Understanding the Algorithm). The program will need to use a nested loop to iterate through the set of sequence reads, attempting to overlap each fragment with every other fragment and looking for the largest overlap. The output for each pair of fragments should include the original fragments, the resulting contig, and the number of characters in the overlapping region. The following pseudocode shows a solution for finding the largest overlap.

Algorithm

Fragment Overlap Generator: Finding Overlaps Between Pairs of Fragments

Goal: To determine the largest overlap between pairs of fragments **Input:** Set of fragments

Output: The fragments, the resulting contig, and length of the overlapping region for each pair of fragments in the input file

---

```
// Initialization
Input the fragments and store in an array: frags
numFrags = number of fragments read
// STEP 1: Determine overlap for each pair of fragments

for each i from 0 to numFrags-1
  for each j from i+1 to numFrags
    f1Len = length of frags[i]
    f2Len = length of frags[j]
    minLen = minimum of f1Len and f2Len
    overlap = 0
    frag1 = frags[i]
    frag2 = frags[j]
    k = minLen - 1
    while k >= 1 and overlap == 0
      // compare suffix of frag1 to prefix of frag2
      if frag1.substring(f1Len-k, f1Len) == frag2.substring
        (0, k)
        // create contig
        contig = frag1.substring(0, f1Len-k) + frag2
        overlap = k
        output frag1, frag2, contig, overlap
      else if frag2.substring(f2Len-k, f2Len)
        == frag1.substring(0, k)
        contig = frag2.substring(0, f2Len-k) + frag1
        overlap = k
        output frag1, frag2, contig, overlap
      k--
```

Putting Your Skills Into Practice

1. Implement the Sequence Read Simulator and Fragment Overlap Generator programs described in the pseudocode, using whatever language is used in your course. Test your programs with a short sequence to validate them, but note that it will be difficult to obtain adequate coverage if your sequence is too short.

2. **Download** Experiment with different short sequence lengths, coverage values, and minimum/maximum fragment sizes. How many "sequence reads" did it take to get the level of coverage you specified? How does that change if you change the fragment size? Was your overlapping assembly program able to match the correct fragments to generate a set of contigs found in the original sequence? Then, try running your program on a larger sequence, such as the klassevirus genome sequence you can download from the *Exploring Bioinformatics*website.
3. Modify your sequence read simulator to output the coverage values for your sequence. Where in the sequence do the highest coverage values occur? Can you explain this pattern? Does the pattern change if you change the fragment length or coverage parameters or the size of the input sequence? Does this pattern accurately simulate what would happen in a real sequencing experiment, or is it merely a computational artifact? If you wanted more even coverage, how could you modify your program?
4. You may have noticed that it is possible for your sequence read simulator to generate a fragment that is a substring of (entirely contained within) another fragment. Fragments that are substrings of other fragments are considered "singletons" and are often eliminated from the assembly process, because they do not add any additional information and can even decrease the efficiency of the assembly process. Modify either program to remove all fragments that are entirely substrings of other fragments so they are not used when finding overlaps.
5. The real sequencing process is prone to misreads; these occur with high frequency at the beginning and end of a sequence read, where sequencing is difficult for technical reasons, but can be found randomly throughout the sequences when the data sent to the base-calling software is ambiguous. Sometimes the sequencing reactions fail and a particular fragment is unreadable (usually represented by all Ns). Make your sequence read simulator more realistic by modifying its code to introduce random changes (inserted, deleted, or changed base) or Ns at a low rate. (For a more challenging exercise, make the likelihood of such changes higher at the ends of the sequence.) Then, modify your overlap generator so it looks for matches that exceed some configurable threshold but does not require an exact match (for example, if the matching threshold is 75%, then at least 75% of the characters in the overlapping region must match). Can you still get accurate assembly?
6. Our simulator program only considered a single input string, representing one DNA strand. Real sequencing data could come from either strand, and in fact pairs of sequences from opposite ends of a DNA fragment, one from each strand, are often generated. The assembler cannot know in advance which strand a fragment came from, so it would have to try each fragment *and* its inverse complement to determine which assembled best. Modify your simulator so it chooses whether to output a selected fragment or its reverse complement (use your code from Chapter 2) and your fragment generator so it will try both strands.
7. Repeated sequences pose a major problem for sequence assembly programs (indeed, some repeat-intensive regions of the human genome, such as the areas

around centromeres, have yet to be sequenced). Test your overlap generator program with the following sequences (assume their positions relative to the original sequence are as shown). Considering the results, discuss the difficulty the repeat problem presents in determining a best overlap. Keep in mind that the length of a repeated sequence can often be much longer than the possible size of a fragment read.

```
Original sequence:      GGATAGATATATATATATATCGACTTC

Test fragment set 1:    GGATAGATATAT
                                        ATATGCACTTC
Test fragment set 2:    GGATAGATATATATAT
                            GGATAGATATATATAT
```

On Your Own Project: A Mini-Assembly Program

The Guided Programming Project introduced you to the problem of finding overlaps between pairs of fragments, and the Putting Your Skills Into Practice exercises should have helped you recognize the additional complexity introduced by sequencing errors and repeated sequences. In this project, we develop a miniassembly program capable of assembling multiple overlapping fragments into a contig using a "greedy" algorithm based on the traveling salesperson problem. We also look at the role of coverage in correcting errors and in determining which overlap is best when multiple options exist. Instructors of nonprogramming courses can download a **completed miniassembly program** from the *Exploring Bioinformatics* website that students can use in completing the exercises under Programming the Solution later in the chapter.

Understanding the Problem

Assembling a contig requires identifying overlaps among sequence reads and then determining how best to piece together the overlapping fragments. However, a single fragment may overlap with many other fragments, making it difficult to choose which pair to merge. **Table 8.3** shows a simple example: For each fragment in a simple hypothetical sequencing project, the fragments that can overlap its suffix are shown along with (in parentheses) the length of each overlap. This output could be produced by a simple modification of the program you wrote for the Guided Programming Project. The suffix of fragment 1, for example, overlaps the prefixes of four other fragments: fragment 2 by three characters (TTG) and fragments 3, 4, and 7 by one character each (G in each case).

Table 8.3: Overlaps for a hypothetical set of sequence reads.
Open table as spreadsheet

Fragments	Overlaps (Length)
1. TACCTTG	2 (3), 3 (1), 4 (1), 7 (1)
2. TTGAT	1 (1), 3 (3)

Table 8.3: Overlaps for a hypothetical set of sequence reads.
Open table as spreadsheet

Fragments	Overlaps (Length)
3. GATATGG	4 (2), 7 (1)
4. GGAG	3 (1), 7 (1)
5. CTCTA	1 (2), 6 (3)
6. CTAGT	1 (1), 2 (1)
7. GCTCT	1 (1), 2 (1), 5 (4), 6 (2)

Once the overlaps are identified, how do we merge the fragments? One approach is to simply start with the first fragment, merge with a matching one (fragment 2 in Table 8.3 would work), choose another fragment that matches the growing contig (fragment 3 in this case), and so on until all fragments are chosen. In the example, fragments 7, 5, 6, 1, 2, 3, and 4, merged in that order, would form a contig. But how did we know where to start? Would other choices have given a different path or led us to a dead end?

To develop an algorithmic solution to this problem, let's look at the data in the form of a**graph**, which in computer science is a data structure showing relationships among elements:**Figure 8.7** shows a graph of the data in Table 8.3. Each fragment is represented by a numbered node, with directional arrows representing overlaps between fragments. The suffix of the fragment at the tail of the arrow overlaps the prefix of the fragment at its head, and the arrow is labeled with the length of the overlap. The contig is then generated by finding a path in the graph that passes through each node once.

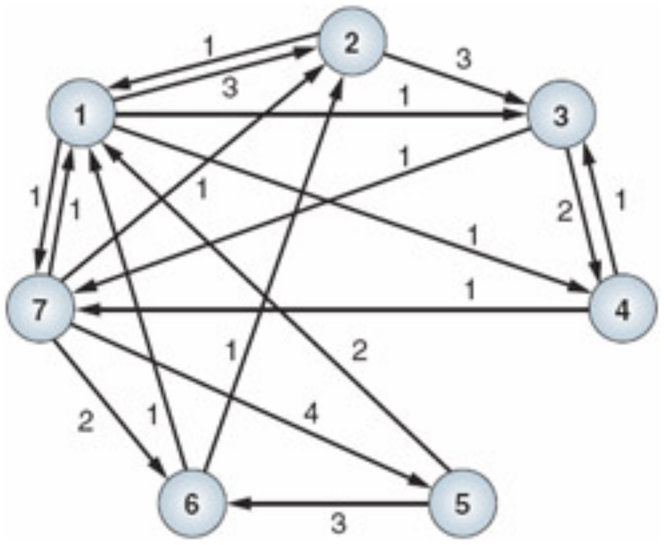

Figure 8.7: Graph representing overlaps between fragments as paths between nodes.

The assembly problem is closely related to a very famous problem called the **Traveling Salesperson Problem** (TSP), usually described as finding the shortest flight path between a set of cities so that each city is visited only once and the path begins and ends in the same city. The possible flight paths and distances are fixed, so the problem can be represented as a graph where cities are nodes and arrows are flight paths, much like Figure 8.7. The good news is that there is a solution to the TSP, but the bad news is that it can take an enormous amount of computational time to find it: if *n* is the number of cities (or sequences), the number of possible paths is *n*!. This is a truly huge number if we consider the 3 billion–base pair human genome covered 30-fold by 100-base pair sequencing reads! Worse, we do not know which fragment comes first and (if it is a new sequence we are assembling) we have no way to verify the correctness of the solution, unlike the traveling salesperson, who at least knows the starting and ending city and that the goal is the shortest path. Fortunately, as you saw previously in Understanding the Algorithm, using heuristics will help.

A **greedy** algorithm is one way to solve the TSP in a reasonable time. This is a heuristic that when faced with a decision "greedily" chooses the option that appears to best serve its goals. Because the goal of the TSP is the shortest path, a greedy algorithm would always choose the arrow with the shortest distance at any decision point. Unfortunately, this approach does not guarantee a solution: It is possible to arrive at a node with no arrows leading away from it.

For the assembly problem, because we are unable to determine in advance which overlap is the correct one (i.e., the one that leads to assembling the original sequence) at any node, we could greedily choose the arrow representing the longest overlap. In this problem, we do not have a predetermined starting node, but we can be greedy here as well and start with the largest overlap among all the pairs of fragments. But does this make sense biologically? As the length of the overlap increases, the probability that it is genuine and not a chance match increases: A fragment that ends in A will overlap *any* fragment that starts with A (one of four just based on chance), whereas a fragment ending in ACTG will find a chance match just one time in 256, and the probability of a chance eight-base overlap is only one in 65,536. Therefore, by always greedily choosing the largest overlap, we can reasonably expect to end up with the shortest common **superstring**—a string that includes all the fragments in the smallest total number of characters, which would be our contig.

Remember, however, that the greedy algorithm does not guarantee finding the original sequence; sequencing errors and repeated sequences are problematic because they make it more difficult to correctly determine the overlaps in the first place. Because we do not know the original sequence, we cannot be sure our program has found the correct solution (though successful testing with good simulated data will increase our confidence), and error correction becomes very important. There are three general ways to correct errors (**Figure 8.8**).**Preprocessing** error correction means fixing problems in the data *before* processing that data; this might be done by improving sequencing techniques, increasing read lengths to reduce the impact of repeats, hand-calling bases in questionable areas (in a small enough project), or analyzing the output data and eliminating reads or regions with poor quality scores. **Inprocessing** modifies an algorithm to better handle errors in the data, such as setting a threshold match value in the assembly algorithm to deal with misreads, which we can easily implement (see Putting Your Skills Into Practice, exercise 5). An inprocessing solution for repeated regions is to use **matepair reads**, pairs of sequence reads from the two ends of a DNA fragment. If these reads aren't found on opposite strands within a short distance of each other in the final assembly, the assembly is incorrect. **Postprocessing**validates the output *after* the algorithm has run; taking this approach, we will use coverage statistics to identify possible areas of misassembly resulting from repeated regions.

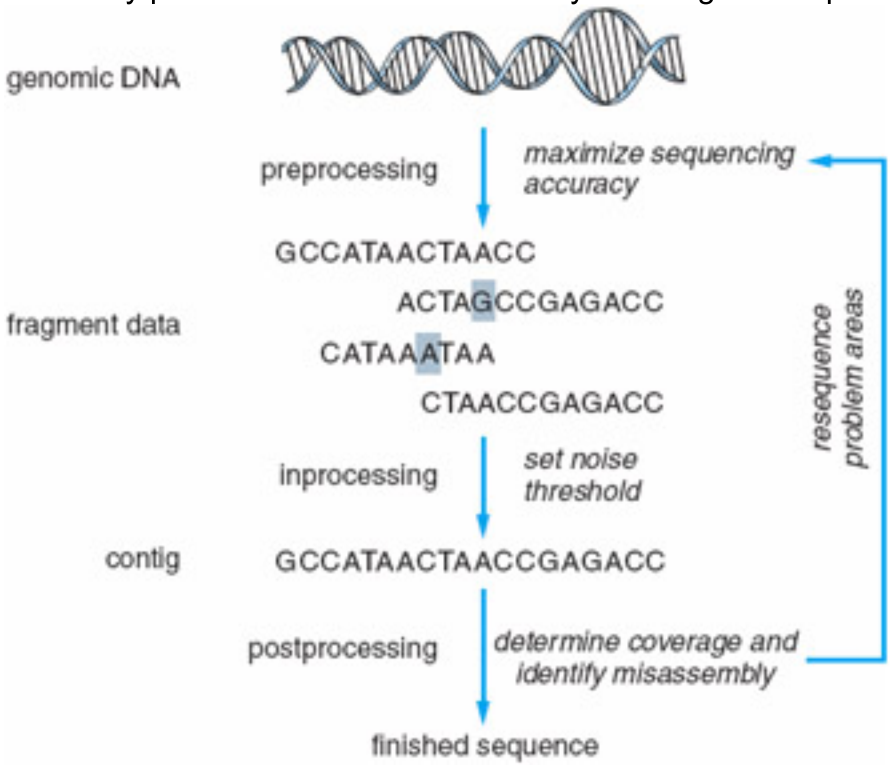

Figure 8.8: Schematic illustration of the sequence assembly and validation process.

Given the sequence fragments and resulting contig sequence in **Figure 8.9A**, even with a misread (GTCTA, rather than GTCTC), a consensus sequence can be successfully built. However, even though all the fragments overlapped, the contig does not match the original sequence: fragment 3 was misassembled due to the repeated sequence TCGTAG. How could we recognize this without knowing the original sequence? Examining coverage is one method, because coverage should be relatively constant across the sequence. Repeated sequences can match more fragments than they should, producing a high-coverage peak (**Figure 8.9B**) that could be flagged as a possible location of misassembly. Determining coverage for each base position is simply a matter of counting the number of fragments that overlap that position; in Figure 8.9A, coverage values would be (1,1,2,3,3,2,3,2,2,1,2,1,1,2, 2,1,1) for an average of 1.8. The repeat region shows noticeably higher coverage even in this small sample. Regions with low coverage values, on the other hand, should be considered unreliable simply because they may not have been sequenced enough to correct misreads orother problems. Statistical calculations can be done to establish minimum and maximum coverage values for high reliability.

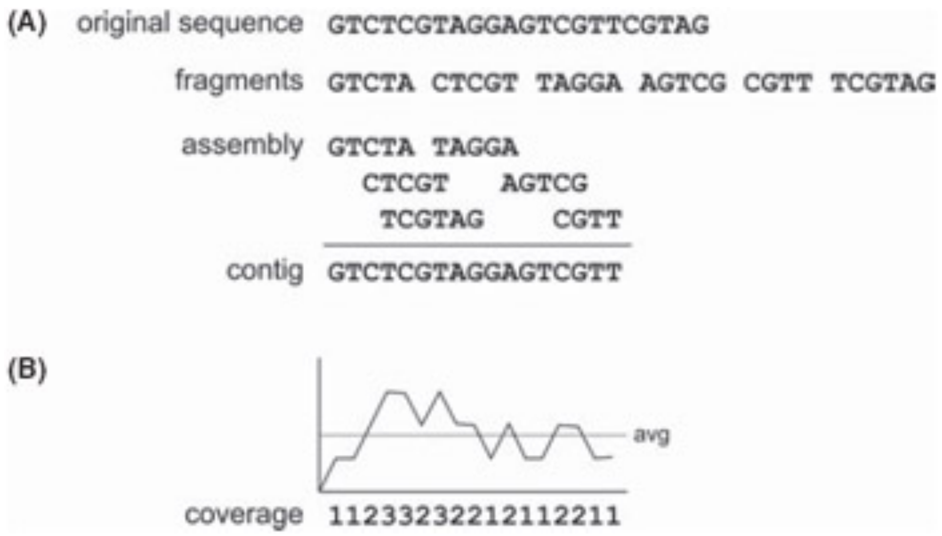
(A)
original sequence GTCTCGTAGGAGTCGTTCGTAG
fragments GTCTA CTCGT TAGGA AGTCG CGTT TCGTAG
assembly GTCTA TAGGA
CTCGT AGTCG
TCGTAG CGTT
contig GTCTCGTAGGAGTCGTT
(B)
avg
coverage 11233232212112211

Figure 8.9: Sample sequencing project: (A) Fragments are generated, sequenced, and assembled, but a repeated sequence results in misassembly; (B) Peak of coverage shows possible location of misassembly.

Solving the Problem

At this point, you should be able to see how an algorithm would be built to tackle the difficult problem of sequence assembly using our TSP-based "greedy" approach. First, use what you learned from the Guided Programming Project to determine the overlaps, implementing a threshold percentage for matching the overlaps (see Putting Your Skills Into Practice, exercise 4) and think carefully about how to organize and store the overlap information so that it is easy to retrieve as you begin merging sequences.

Figure 8.10 steps through the process of merging the test sequences in Table 8.3 using their overlap data and a greedy algorithm. The arrow linking nodes 7 and 5 has the largest overlap (**Figure 8.10A**, blue arrow), so using the greedy algorithm, our first merge is the suffix of 7 with the prefix of 5. Remember that our final path must visit each node only once; node 7 now leads to node 5 and therefore cannot lead to any other node, so we can eliminate any other arrows leading away from node 7 (Figure 8.10A, dashed arrows). In sequence terms, we have overlapped `GCTCT` with `CTCTA` to give the contig `GCTCTA`, so we cannot overlap the 3′end of GCTCT with any other fragment. Similarly, node 5 has now been visited, so we can eliminate any other arrows leading into node 5 (there are none in this case).

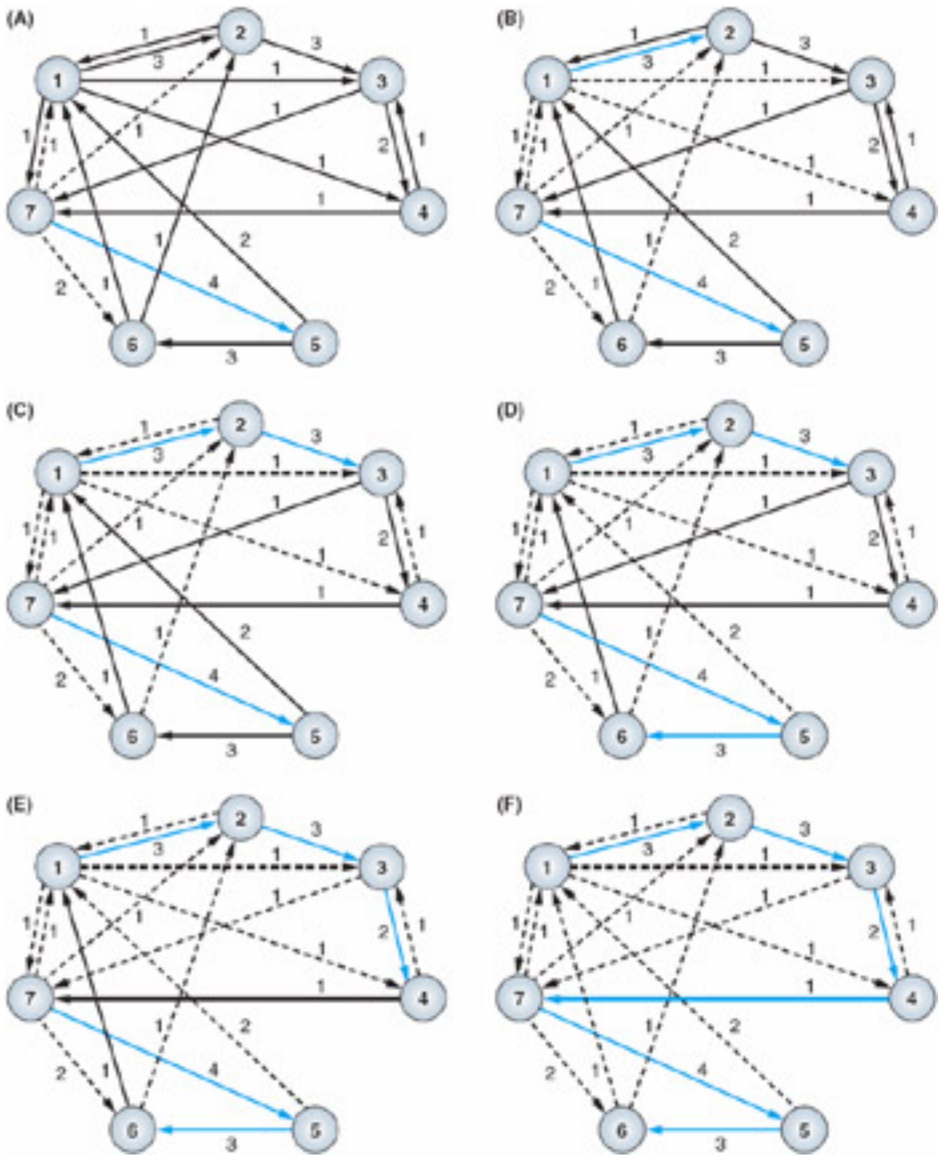
Figure 8.10: Steps in finding a path to a sequence alignment. From left to right and top to bottom, each graph shows a link (heavy arrow) between two sequence fragments that would be chosen using a "greedy" algorithm. Dashed arrows show paths that can be discarded once a choice is made.

Now, we choose the next-longest available overlap. The overlap is 3 nt for 1→2, 2→3, and 5→6, so we could choose any of these; by simply taking the first one, we would choose 1→2 (**Figure 8.10B**, blue arrow). Again, other paths leading away from 1 or into 2 are eliminated (dashed arrows). Proceeding in this fashion, we would choose the paths from 2→3 (**Figure 8.10C**) and then 5→6 (**Figure 8.10D**), eliminating potential choices as we proceed. Now the longest overlap remaining is between 3 and 4 (**Figure 8.10E**); once this is chosen, the only remaining paths are 4→7 and 6→1. Again, arbitrarily choosing the first one gives the result in**Figure 8.10F**: a complete path through all seven nodes in the order 1, 2, 3, 4, 7, 5, 6. Once you have the final path, you can easily obtain the final contig by overlapping the fragments in order of the path. Therefore, in our example, you would overlap fragment 1 with fragment 2. The resulting contig would then overlap with fragment 3. That contig would overlap with fragment 4 and so on. This corresponds to the assembly of all the fragments into the contig`TACCTTGATATGGAGCTCTAGT`. Note, however, that the algorithm gives the path but does not specify *how* the developing contig overlaps with the next fragment, which will not necessarily be by the same number of nucleotides as the original fragments.

Programming the Solution

Now you should have enough information to extend your overlap-finding program to become a full-fledged mini-assembler, using the "greedy" algorithm as described here. As each fragment is chosen, keep track of where it fits in the growing contig so you can calculate coverage; use this information to flag any unreliable sequences or likely repeats once you have built your contig. Check manually to see if your algorithm can correctly assemble a set of fragments with good overlaps based on a short test sequence before you start implementing your solution in your language of choice.

Download Test your program on the short sequences in Table 8.3; do you get the contig described earlier? Then use your **sequencing simulator program** (instructors can download this from the *Exploring Bioinformatics* website for nonprogramming courses) to generate fragments for some longer sequences (try 200 nucleotides or so at a time from the**klassevirus sequence**, for example) with more coverage. Does your program correctly assemble the fragments? How much coverage is necessary for it to do so reliably? How does the average fragment length affect its accuracy? Does the program ever fail to find a solution (and did you think to have it let the user know of this failure)?

To see how your program handles repeats, introduce some into your test sequences—for example, put 10 consecutive repeats of GCATC in the middle of a 100- or 200-nucleotide sequence, generate fragments that are 10 or 20 nucleotides long, and then see how your program handles the assembly and whether your coverage values correctly identify problem areas. Then try a more realistic sequence, such as the complete klassevirus genome. Does your program work equally well here, or does it encounter problems? What do your coverage values tell you about the reliability of various regions of your contig sequence?

Connections— The Future of Genome Sequencing

The rate at which genes, genomes, and metagenomes can be sequenced continues to expand rapidly, whereas the cost continues to decline. As a result, sequencing is being used in ways we never previously imagined. Not only will the sequencing of individual human genomes soon become practical (the so-called $1,000 genome is nearly within the reach of several companies as of this writing), but we are sequencing the genomes of the entire human microbiome and applying sequencing technology to the identification of targets for transcription factors, mutations resulting in complex genetic disorders, and genetic diversity of endangered animals. As sequencing moves from the research lab to the hospital lab, we will see it used for genetic screening, cancer diagnosis, and preimplantation diagnosis of embryos. Individualized medicine will likely become a reality, with drugs tailored to the individual genetic makeup of a particular patient. Ecologists, evolutionary biologists, pathologists, forensic scientists, and many others will also benefit.

Of course, just obtaining the sequence is not the end of the story. To make the sequence useful, improved bio-informatics techniques to identify genes (Chapters 9 and 10) are needed, especially as small RNAs and other unexpected findings challenge our definition of genes. Sequence alignment will also continue to be a major player as genomics moves increasingly into the interpretation phase. And along with the rapid pace of scientific change will come a need to consider the wise and ethical use of these vast volumes of data: Should we diagnose genetic diseases we cannot yet treat? Should insurance companies have access to risk data based on sequence analysis? What would constitute fair and equitable access to new medical technologies that may be highly effective but at least initially extremely expensive? Continued advances in sequencing technology will no doubt provide both new answers and new questions in the near future.

BioBackground: Sequencing DNA

It is helpful for both developers and users of sequence analysis software to understand how DNA sequencing is done. The strengths and limitations of a particular sequencing technology affect the nature and quality of the DNA sequences obtained, which in turn impact how those sequences should be treated by assembly or mapping software. This section does not attempt to be a complete manual on DNA sequencing, but we discuss three commonly used sequencing platforms to aid in understanding how the fragments analyzed by sequencing software are obtained. Good sources of further information are listed in References and Supplemental Reading.

Automated Sanger Sequencing

The sequencing method developed by Fred Sanger in 1975 was not the first, but it was far better suited to the rapid sequencing of long DNAs than the laborious chemical cleavage methods that preceded it. The technique became widely used and by the time the human genome project began had been improved by the use of fluorescent nucleotides and automated.

Sanger sequencing (or **dideoxy sequencing**) harnesses DNA polymerase, the enzyme that normally replicates DNA in the cell. The DNA molecule to be sequenced serves as the template for DNA polymerase, and a short single-stranded **primer** binds to the template and serves as the starting point. DNA polymerase can then synthesize multiple copies of a single strand of DNA complementary to the template (**Figure 8.11**). However, there is a twist: In addition to providing ordinary nucleotides (**dNTPs**) to be joined into the new DNA strand, fluorescent **dideoxy nucleotides** are added. Dideoxy nucleotides lack the 3′ –OH group to which the next nucleotide in the chain would be joined; when a dideoxy nucleotide is added to a growing DNA strand, synthesis stops. Thus, if low concentrations of dideoxy A, C, G, and T nucleotides (**ddNTPs** for short), each fluorescing a different color, are added to a reaction containing polymerase, primer, template, and dNTPs, a set of DNA fragments will be generated, each of which ends in a dideoxy nucleotide that can be identified by its fluorescence.

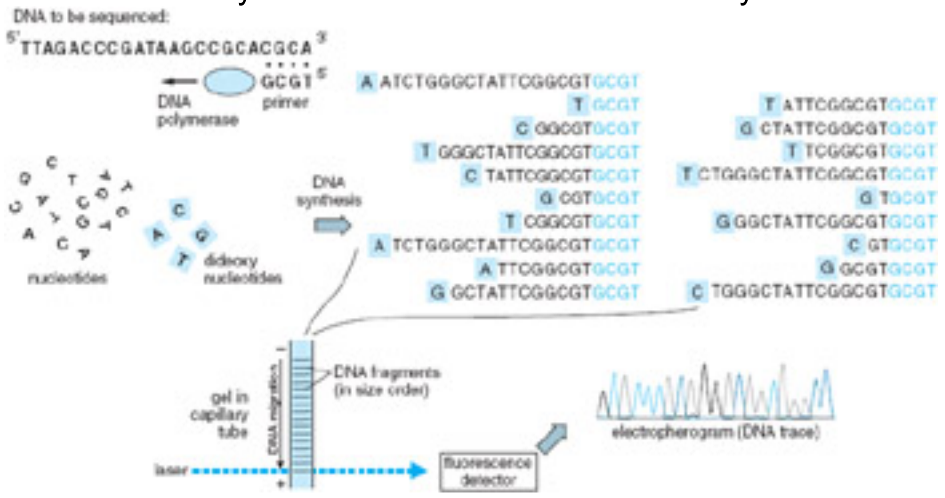

Figure 8.11: Sequencing DNA by the Sanger (dideoxy) method. Dideoxy nucleotides that terminate fragments are shown in boxes.

In automated Sanger sequencing, the fragments are placed on a gel-like matrix in a tiny capillary tube and an electric current is applied. The DNA fragments, being strongly negatively charged, move through the gel toward the positive pole, with smaller fragments moving faster. A laser excites each fluorescent nucleotide as the fragments move past it, and a computer-connected reader determines which base the fragment ends with by the color of the fluorescence (Figure 8.11). Each succeeding fragment is one nucleotide longer than the one before it, and the pattern of fluorescence color and intensity allows the DNA "trace" (Figure 8.5) to be constructed. Sanger sequencing cannot read bases extremely close to the primer, as a fragment of some reasonable length is needed to resolve properly in its passage through the gel. High quality can typically be maintained for some 500–800 nucleotides from a single capillary tube, and 384 such tubes can be run simultaneously on a single instrument.

Shotgun Sequencing

In **directed sequencing**, a primer is used to obtain sequence from a particular template, and then a new primer can be synthesized to match the just-read bases from the end of that sequence and the process repeated. Thus, there is no ambiguity regarding what part of a long template has been sequenced, but the process is slow even if multiple templates are sequenced at the same time. **Shotgun sequencing** and computerized assembly revolutionized this process: A long DNA is fragmented by mechanical shearing or enzymatic digestion into many short pieces, each of which is joined to a cloning vector (plasmid). Because the vector sequence is known, the sequence from each end of each fragment can be obtained using primers that match vector sequences (**Figure 8.12**). When many random fragments have been sequenced, there should be overlapping sequences, allowing for computerized assembly. Using this technique, it is not necessary to wait for new primers to be synthesized: Fragmenting and cloning can go on at the same time as sequencing of already cloned fragments. The concept of shotgun sequencing is also used in all next-generation sequencing methods, but the need for the cloning step has been eliminated—for example, by direct analysis of uncloned fragments or PCR amplification of random DNA regions.

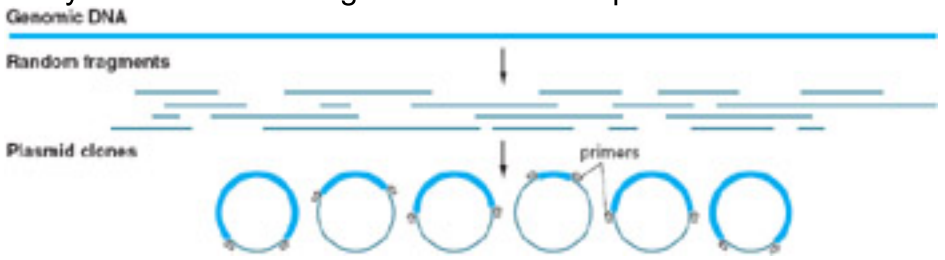

Figure 8.12: Shotgun sequencing: A large genomic DNA is broken into random fragments, which are cloned into plasmid vectors. Primers omplementary to the vector allow sequence to be obtained from both ends of the cloned fragments.

454 Sequencing

The first widely used next-generation sequencing method was developed by 454 Life Sciences (now owned by Roche) in 2004; this **pyrosequencing** method is popularly referred to as 454 sequencing. As with any shotgun sequencing method, the DNA to be sequenced must be fragmented, either chemically, mechanically, or by enzymatic digestion; fragments of 300–800 bp are suitable for 454 sequencing and must be made blunt (no single-stranded overhangs) on each end. Short oligonucleotide adapters of known sequence are then joined on to each end of the fragments; one adapter has a biotin molecule that can be reacted with a bead coated with streptavidin. Single-stranded DNA fragments with adapters thus become immobilized on the beads (**Figure 8.13**).

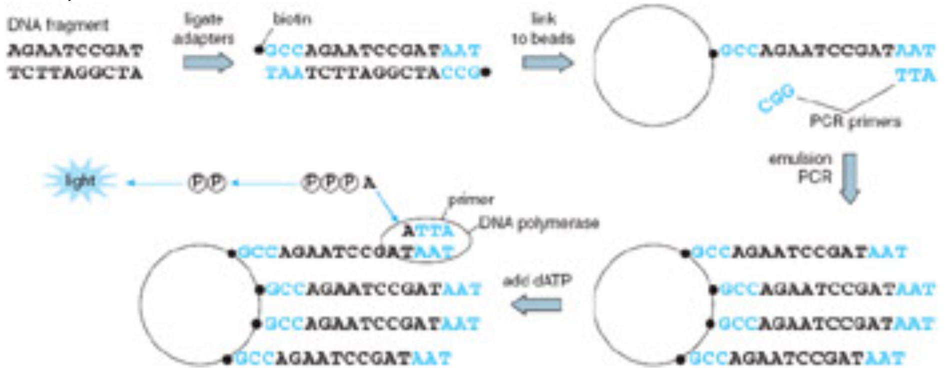

Figure 8.13: Sequencing DNA by the 454 (pyrosequencing) method: adaptors are ligated to DNA fragments, immobilized on beads, and amplified by PCR. Solutions of single nucleotides are added and light resulting from an enzymatic reaction involving the pyrophosphate cleaved from the nucleotide when it is added to the DNA chain is detected as evidence that a particular nucleotide was incorporated. Reactions can be done on 1.6 million beads in parallel

Next, the immobilized fragments are amplified. Beads are captured in individual oil droplets containing PCR reagents, and primers matching the adapters are used to generate some 10 million copies of the original fragments, all attached to a single bead. The beads are then transferred to individual wells, each holding only 75 pl of volume, of a PicoTiter plate capable of holding 1.6 million individual beads. Primers are then bound to the adapter sequences, and DNA polymerase can then add nucleotides complementary to the single-stranded template much as in Sanger sequencing (Figure 8.13). A solution containing a single nucleotide is "flowed" over the plate, and reagents bound to the beads react with the diphosphate (pyrophosphate) released from the polymerization reaction to produce a tiny emission of light. A camera monitors each well and detects the light, indicating that a particular nucleotide was successfully added to the growing chain (and was thus complementary to the template strand) in a particular well. The process then repeats with each of the other three nucleotides in turn and then the whole cycle of four nucleotides repeats. Recording which nucleotides are added in which order to the DNA in each well generates a sequence read, and the reads can then be assembled by computer to produce the complete sequence of the original DNA.

Illumina Sequencing

Solexa announced its high-throughput sequencing platform in 2006; this company was acquired by Illumina, and the technology is variously referred to as Solexa, Illumina, or SBS sequencing. As in 454 sequencing, adaptors are added to the ends of DNA fragments; they are then bound to primers that in this case are already attached to a slide, and PCR creates local clusters of a particular DNA molecule. Fluorescent nucleotides are then added, each nucleotide capable of fluorescing a distinct color, and DNA polymerase can incorporate a single nucleotide into a growing complementary DNA strand. A laser removes a blocking group from each nucleotide, allowing its fluorescence to be visualized and the identity of the last-added nucleotide in each cluster thus determined. A new batch of nucleotides is then added. As before, the sequence of a fragment is generated by monitoring the order in which the different colors (wavelengths) of fluorescence appear in each cluster.

SOLID Sequencing

Both the 454 and Illumina methods (and, in fact, the Sanger method) involve "sequencing by synthesis," with a polymerase enzyme adding detectable nucleotides sequentially to a new strand. SOLiD sequencing, developed by Applied Biosystems and available since 2008, relies instead on the ability of two-nucleotide fluorescent probes to hybridize with (bind) the DNA template and be ligated to a growing chain by DNA ligase. Fragments of the DNA to be sequenced are linked to adapters, joined to beads, and amplified by emulsion PCR much as in 454 sequencing. Each two-base pair emits fluorescence at a distinct wavelength. The primer determines the position at which probes can hybridize, and after several cycles of hybridization, ligation, and cleavage, a new probe is used that is one nucleotide shorter, requiring a different set of probes to bind the same sequence to increase accuracy. Sequence reads produced by the SOLiD platform are very short, only 50 nt long, increasing the dependence of this technology on accurate and efficient assembly algorithms and powerful computers.

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Chapter 3: Sequence Alignment: Investigating an Influenza Outbreak

Chapter Overview

This chapter focuses on algorithms for optimal alignment of DNA sequences. Students in both programming and nonprogramming courses will understand how dynamic programming techniques can be used to make the complex problem of gene alignment tractable and, through the use of Web-based tools, how the choice of alignment parameters can influence the biological relevance of the results. Students will also consider how a basic algorithm can be modified to answer different biological questions. Students in programming courses will develop their own solutions that implement these algorithms.

- **Biological problem**: Origin of new influenza virus strains
- **Bioinformatics skills**: Optimal global, semiglobal, and local alignments of DNA sequences; gap penalties and alignment parameters
- **Bioinformatics software**: EMBOSS implementations of pairwise alignment algorithms
- **Programming skills**: Two-dimensional arrays, dynamic programming, backtracking

Understanding the Problem: The 2009 H_1N_1 Influenza Pandemic

In March 2009, epidemiologists responsible for influenza surveillance at the Centers for Dis-ease Control and Prevention (CDC) and the World Health Organization (WHO) were surprised by an outbreak of influenza in Mexico City. Because influenza virus mutates rapidly, the strains that are circulating change from year to year, necessitating annual revaccination; CDC and WHO are charged with monitoring flu virus strains and determining which will be used for vaccine development. In addition, these agencies monitor both human and animal influenza cases to identify new strains, watching for the emergence of a ***pandemic*** **virus—one capable of causing a severe, multicontinent outbreak. Uppermost in the minds of these scientists is the desire to prevent a repeat of the 1918 influenza pandemic—the single deadliest infectious disease event in history, infecting half the world's population and killing at least 20 million people in 120 days (; see also References and Supplemental Reading at the end of the chapter).**

Figure 3.1: The rapid spread and severity of the 1918 influenza pandemic placed an enormous burden on healthcare workers and facilities. Depicted here is a demonstration at the Red Cross emergency ambulance station in Washington, D.C. Courtesy of Library of Congress, Prints & Photographs Division [reproduction number LC-USZ62-126995].

In addition to ordinary, seasonal human viruses, WHO and CDC had been keeping tabs for some years on an avian (bird) influenza virus strain known as H_5N_1 that at the time they believed posed the greatest risk of a new pandemic. This "bird flu" virus has caused severe infections in domestic fowl and in humans in direct contact with birds (such as poultry farmers) but has thus far remained incapable of efficient transmission from person to person. In reality, however, the next human pandemic resulted not from H_5N_1 but from a previously unknown strain of H_1N_1 that had escaped detection. When Mexican authorities reported a number of cases of influenza caused by this relative of the 1918 flu strain, public health officials were concerned about the possibility of widespread severe illness. Particularly alarming were reports of severe cases and deaths among the young and middle aged, as virulence for these age groups (seasonal flu has serious health consequences mostly for infants and the elderly) was a hallmark of the 1918 virus. Fortunately, it later became clear that this new H_1N_1 virus was no more dangerous than ordinary seasonal strains. Nonetheless, in the interval between identification of the new strain and development of a vaccine, it caused at least 8,000 deaths and a large number of precautionary school closings.

What exactly is a "new strain" of influenza virus, and how is a new strain identified? What makes one strain a dangerous pandemic virus and another a mild seasonal virus?

Why are some strains transmitted easily among humans, whereas others are largely confined to animals?

Bioinformatics Solutions: Sequence Alignment and Sequence Comparison

Alignment of the sequences of two genes or proteins refers to matching them up in what we hope is a biologically relevant way to determine how similar they are. Sequence alignment is possible when the sequences are evolutionarily related: Similar sequences are similar because they are descended from the same common ancestor, with the differences among them resulting from mutation (for more detail, see BioBackground). **Figure 3.2** shows an example in which many different oxygen-carrying proteins have similar sequences because they all have the same origin.

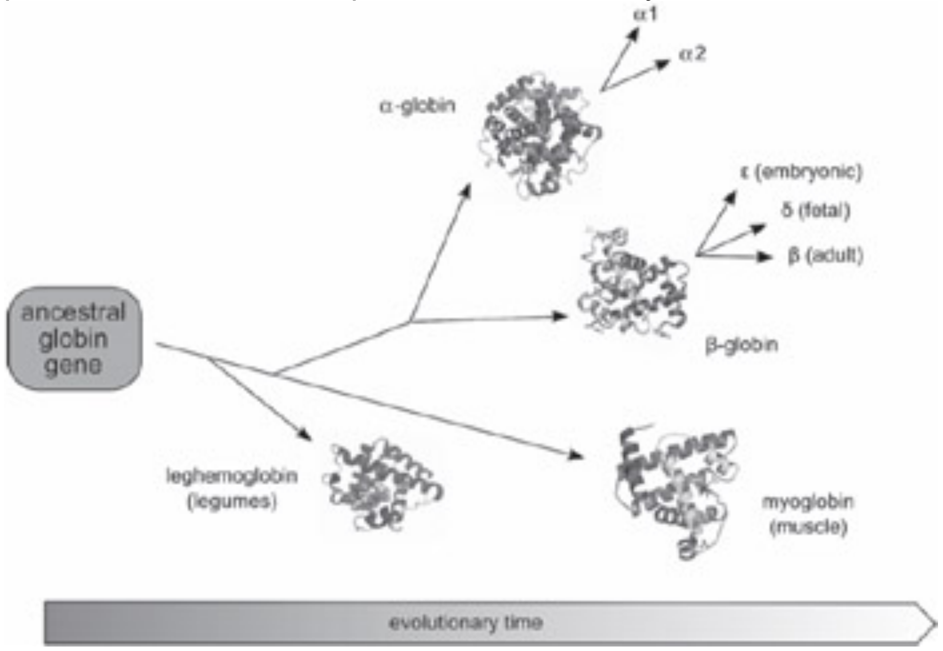
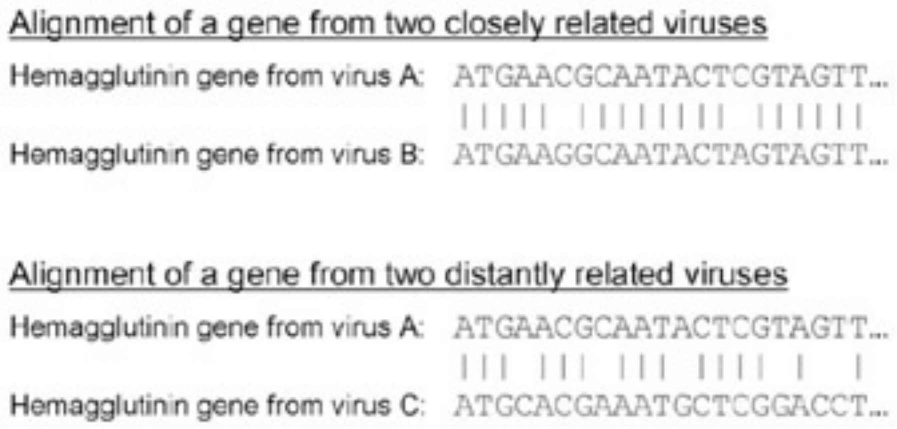


Figure 3.2: Alignment of DNA and protein sequences is possible because of evolutionary relationships. In this example, evolution from an ancestral globin gene is thought to have produced a variety of oxygen-carrying proteins—including the two subunits of hemoglobin found in human blood, myoglobin found in the muscles of mammals, and even leghemoglobin made by leguminous plants. Thus, all these different proteins would be encoded by genes with recognizably similar sequences. Structures from the RCSB PDB (www.pdb.org): leghemoglobin, PDB ID 2GDM (E. H. Harutyunyan et al. (1995) The structure of deoxy-and oxy-leghemoglobin from lupin. *J. Mol. Biol.* 251:104–115); alpha-globin and beta-globin, PDB ID 4HHB (G. Fermi and M. F. Perutz (1894) The crystal structure of human deoxyhaemoglobin at 1.74 A resolution. *J. Mol. Biol*. 175:159–174); myoglobin, PDB ID 1MBO (S. E. V. Phillips (1980) Structure and refinement of oxymyoglobin at 1.6 A resolution. *J. Mol. Biol*. 142:531–554).

The problem of alignment was introduced briefly in the last chapter, where sequence comparison was used to detect mutations. Sequence alignment is also used in developing phylogenetic trees based on molecular data, assembling genome sequences, predicting protein structure and function, and numerous other bioinformatics applications. Indeed, it would be fair to say that sequence alignment is *the* key technique in bioinformatics—and also a difficult computational problem because of the complexity of genomic information. This chapter presents an algorithm for identifying the best alignment of two sequences, with projects in which you will use this technique to investigate influenza virus strains and their virulence. Subsequent chapters will explore how variations of this basic algorithm may be extended to apply to many other important biological problems.

Despite their obviously different characteristics, St. Bernards and chihuahuas are members of the same species, *Canis familiaris*. Although we usually use the term "breed," we could think of them as different **strains** of dog: groups within a species that have distinct, inheritable genetic characteristics. Even in animals and plants, it can be very difficult to determine by simple observation whether two organisms belong to the same species; the problem is much more difficult for bacteria and viruses, where there are few if any visual distinctions among individuals. Comparison of DNA or protein sequences has become the new standard for classification (see BioBackground). Bioinformatic techniques provide a means of comparing genes and identifying species or strains. Each year, the genomes of many influenza viruses isolated from patients are sequenced, and it is the comparison of these sequences that allows agencies such as CDC to determine whether new viruses have arisen and whether they are minor variants of existing viruses (this is referred to as antigenic "drift") or are very different from circulating viruses (antigenic "shift" variants) and have pandemic potential (**Figure 3.3**). In addition, comparison of the genes of a new variant with known viruses that are highly virulent or more moderate in their effects allows experts to predict the potential severity of influenza outbreaks.



```
Alignment of a gene from two closely related viruses

Hemagglutinin gene from virus A:  ATGAACGCAATACTCGTAGTT...
                                  |||||  ||||||||  ||||||
Hemagglutinin gene from virus B:  ATGAAGGCAATACTAGTAGTT...


Alignment of a gene from two distantly related viruses

Hemagglutinin gene from virus A:  ATGAACGCAATACTCGTAGTT...
                                  |||  |||  |||  ||||  |   |
Hemagglutinin gene from virus C:  ATGCACGAAATGCTCGGACCT...
```

Figure 3.3: An example showing how sequence alignment can demonstrate similarity, and thus relatedness, of two DNA sequences.

BioConcept Questions

Computational techniques for gene alignment depend on understanding of the biological basis for gene comparison and the meaning of similarity and variation among the genes of different organisms. Use these questions to test your biological understanding; read the BioBackground box at the end of the chapter if you find that you need a better foundation.

1. How is similarity between genes related to the biological concept of descent from a common ancestor?
2. Given the sequences `ACGAT` and `CGATC`, why is the simplest alignment
   ```
   ACGAT
   CGATC
   ```
   not a very satisfactory one? What do we have to allow for in order to generate an alignment that appears more biologically relevant?
3. List all the possible ways to align the very short sequences `ACC` and `ACT`. Discuss why "brute-force" alignment (trying all the possible combinations to identify the best one) is not a practical method of aligning real genes.
4. Often, it is necessary to introduce gaps into one or both sequences to align them optimally. However, most alignment programs penalize gaps to keep them to a minimum. Why are gaps potentially problematic, particularly for sequences that represent coding regions?
5. The influenza virus mutates so rapidly that you would likely be able to identify at least a couple of mutations over the length of the complete virus genome even if you sequenced two viruses from two different patients within the same influenza outbreak. What might be some considerations in deciding whether two viruses with different genome sequences actually represent two different strains?

Understanding the Algorithm: Global Alignment

Learning Tools

Download From the *Exploring Bioinformatics* website, you can download a demonstration spreadsheet that shows visually how the Needleman-Wunsch algorithm aligns short sequences. Try the examples in the text or make up your own sequences to see how the algorithm deals with mutations, differences in length, and so on. Files are available for Excel and OpenOffice for Windows, Linux, and Mac OS.

The simple algorithms in the previous chapter that in essence align two genes to look for mutations are limited: One algorithm required the genes to be of the same length, whereas the other used an inefficient trial-and-error method. To be able to align *any* two sequences, we need a flexible algorithm that will match them up in a meaningful way, accounting for differences in length due to indels and recognizing that over evolutionary time mutation may have made similar genes look quite different. The algorithm needs a means of discriminating between better and worse alignments and also a scoring system to decide *how* similar the genes are.

Here, we discuss an algorithm for optimal, global alignment of pairs of genes published by Saul Needleman and Christian Wunsch in 1970 (see References and Supplemental Reading). This algorithm and modifications of it (discussed later in this chapter) are still widely used today, and the ideas they are based on are at the root of many other comparison algorithms as well. Indeed, it may interest you to know that when an Internet search engine such as Google asks, "Did you mean…," it is using an algorithm very similar to this one to match what you typed with common search words.

Optimal Alignment and Scoring

To compare two genes, such as the HA genes of two different influenza virus strains, we want to look for matches and mismatches along their entire lengths: a **global alignment**. (Reasons to compare only parts of genes are discussed later.) Global alignment is a technique used to compare sequences in their entirety; the Needleman-Wunsch algorithm is also a **pairwise**alignment algorithm, because it compares a sequence to only one other sequence at a time.

Sequences can be aligned in many different ways. For example, three ways to align the short sequences `ACGTACT` and `ACTACGT` are shown below:

```
ACGTACT   ACGTAC-T   ACGTACT----

ACTACGT   AC-TACGT   ----ACTACGT

**    *   ** *** *       ***
```

If we do not allow for insertions or deletions, there is only one way to align these sequences (left), but if we make the biologically reasonable assumption that indels could have occurred, we get many more possibilities. The hyphens used in the center and right alignments represent **gaps**, indicating that insertions in one sequence or deletions in the other occurred at these points.

Which alignment is best (**optimal**)? To decide, we need a scoring system. If we simply count nucleotides that match, then the introduction of one gap in each sequence (center) gives us a much better score (6) than simply aligning the ungapped sequences (3). However, indels pose a biological problem, because they can create frameshifts; thus, we should use them with caution. Intuitively, we recognize that the left alignment is far superior to the rather cumbersome right one, but both have three matches according to our simple scoring system. A more sophisticated scoring system (we could call this a **scoring metric**) would award a**match score** (or **match bonus**) for nucleotides that match, a **mismatch score** (or **mismatch penalty**) for nucleotides that do not, and a **gap penalty** where a gap was introduced. For example, if the match score is 1, the mismatch score is 0, and the gap penalty is -1, then the left alignment still scores 3, the center alignment scores 4, and the right one scores -5 (matching our judgment that this is likely to be a poor choice from a biological standpoint).

An obvious way to do a global alignment is simply to try every possibility and see which one gives the best score. However, even for these two short sequences, permitting gaps gives more than 40,000 possible alignments; that number quickly becomes staggering if we are working with real genes consisting of thousands of nucleotides. This is in fact an intractable problem even for a computer: A programmer would say that it is not bounded by polynomial time, meaning the time required to arrive at a solution increases so rapidly as sequence length increases as to become impractical.

The key element of Needleman and Wunsch's now-famous article was a solution based on**dynamic programming**. A dynamic programming algorithm divides a problem into a series of smaller subproblems, solves them, and then uses these solutions to build the solution to the original problem. Needleman and Wunsch solve the problem of a global, optimal alignment of large sequences by using a matrix of partial alignment scores and then backtracking along a path to the best possible alignment(s). This clever approach allows all optimal alignments to be found quickly, even for long sequences.

Needleman-Wunsch Algorithm

Let's see how the Needleman-Wunsch algorithm works to align two short sequences: `CGCA`and `CACGTAT`. We use a match score of 1, a mismatch score of 0, and a gap penalty of -1. First, construct an $N \times M$ matrix, where N is the length of the first sequence + 1 and M is the length of the second sequence + 1. Each position in the matrix represents a possible way to align part of the sequence. If two identical, equal-length sequences were being aligned, the matching nucleotides would line up right down the diagonal. In our example, however, we will obviously need at least two gaps, because one sequence is two nucleotides shorter than the other. Even when the two sequences are of equal length, gaps could be needed to obtain the optimal alignment in order to account for indels. These gaps move the matching nucleotides off the diagonal. We need to account for this as we initialize the matrix. We start with a zero in the first cell of the matrix and then initialize the first row and first column by adding the gap penalty (-1) to each successive cell, as shown in **Figure 3.4A**. These initial values show what happens if we have to introduce a gap at the beginning of one of the sequences. If a single gap was added to the beginning of the sequence, its maximum score would be reduced by one, two for a double gap, and so on.

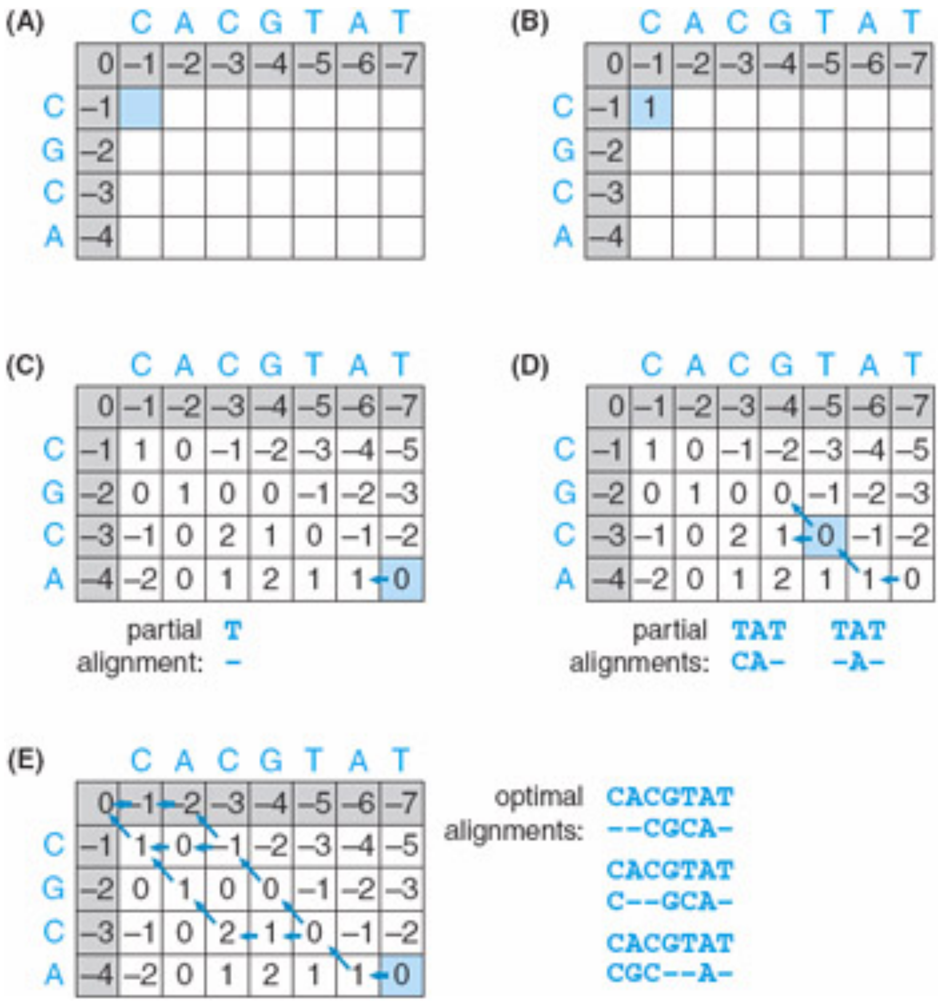


Figure 3.4: Using the Needleman-Wunsch algorithm to align two sequences: (A) Initializing the matrix using gap penalties; (B) Filling in the matrix using the best subscore; (C) The completed matrix with the optimal score (blue cell) and first backtracking step; (D) Backtracking through the matrix, with two possible paths shown; (E) The completed alignments.

Now we are ready to fill out the rest of the matrix, which we do by computing the optimum (maximum) score for each possible partial alignment. Each cell in the matrix represents a partial alignment: For example, the blue cell in Figure 3.4A represents the alignment of the C in the long sequence with the C in the short sequence. At each point, there are three choices:

1. If the two nucleotides match, their score is 1, but if they mismatch, they score zero. Add this match or mismatch score to the score diagonally above and to the left of the cell. This represents aligning nucleotides without leaving a gap. In our example, C matches C, so the score (representing the alignment of C with C) is 0 (from the cell on the diagonal) plus 1 for the match, or 1 total.
2. *Or*, we could introduce a gap in the short sequence, represented by moving horizontally rather than diagonally (moving to the next nucleotide along the top sequence but *not* making a corresponding move to the next nucleotide in the left

sequence). The gap penalty is -1, so in our example, we add -1 to the score in the cell to the left of the blue cell:-1+-1 = -2.

3. *Or*, we could introduce a gap in the long sequence, represented by adding the gap penalty to the score in the cell above the blue cell: -1 + -1 = -2. We want an optimal alignment in the end, so we should choose the *best* possible score for each partial alignment; in this case, the best of the three options is 1, so we put a 1 in the blue cell (**Figure 3.4B**).

This process now continues for the remaining cells of the matrix. In the cell to the right of the blue cell in Figure 3.4B, our choices are -1 (-1 on the diagonal ++0 for the A vs. C mismatch), 0 (for a gap in the short sequence), and -3 (for a gap in the long sequence), so 0, the best of the three, goes in this cell. Repeating this process for the remaining cells results in the matrix shown in **Figure 3.4C**.

Generating the Alignment

Remember that this is a *global* alignment, so we are comparing the two sequences along their entire lengths. That means the optimum score for the alignment as a whole is always represented by the number in the bottom-right cell of the matrix (at the end of both sequences, the blue cell in Figure 3.4C): in this case, 0.

Of course, we don't want just the score; we want to see *how* the sequences can be aligned optimally. To accomplish this, start from the bottom-right cell and work backward to determine how that subscore was obtained. In this case, the zero resulted from adding the gap penalty to the cell to its left, representing a gap in the short sequence, as indicated by the arrow in Figure 3.4C. So, the T in the long sequence is aligned with a gap in the short sequence (partial alignment at the bottom of Figure 3.4C).

Now, follow the path one cell to the left and consider the 1 there. It must have come from adding the match score to the cell diagonally above and left, so now you know that you can align the two A's and move diagonally (**Figure 3.4D**). Now we have an interesting situation. The zero in the next cell in the path (blue cell in Figure 3.4D) could have been generated*either* by adding the gap penalty to the cell on its left *or* by adding the mismatch score to the cell diagonally left. This means we have *two* possible paths from this point and thus two possible alignments that give equally good scores: one in which we add a gap to the short sequence and one in which we allow C and T to mismatch (arrows in Figure 3.4D). It is entirely possible for there to be more than one way to optimally align two sequences—and this is a great example of why real-world research requires the good judgment of scientists who understand both biology and computational algorithms.

We can now continue this way until we reach the upper-left cell of the matrix. Along the way, another point is reached at which two paths give the same score. Thus, there are three optimal ways to align these sequences, each giving an overall score of zero, as shown in**Figure 3.4E**.

We can change the scoring parameters (match and mismatch scores and gap penalty) based on the problem we are trying to solve. For example, to compare two protein coding genes, it makes sense to penalize gaps significantly because of the frameshift problem. But in genes for noncoding RNAs, a gap may be no worse than a mismatch, and we might set our gap penalty lower. Or, if we only wanted highly similar sequences to give good scores, we might penalize both gaps and mismatches.

The Needleman-Wunsch algorithm provides a straightforward way to find optimal, global alignments, and its use of dynamic programming (each cell in the matrix is the solution to a subproblem that is not computationally intensive to obtain) allows it to run efficiently even when long sequences are being compared. Furthermore, simple modifications of this basic algorithm allow different kinds of alignment that can provide additional information.

Test Your Understanding

1. How would the Needleman-Wunsch algorithm align the sequences `ACGTACT`and `ACTACGT`? Try them by hand or use the spreadsheet tool from the text website.
2. For a more challenging problem, find all the possible optimal alignments for the sequences `CTAG` and `CGCTAATC`. You should find 10 altogether; the score for each should be -1.
3. Now try aligning `CAG` with `TTTCAGCAGTTT`. What do you expect will happen? Are you surprised by what actually happens?

   Question 3 points out a problem with using global alignment to compare two sequences of very dissimilar lengths. There might in fact be a good match for the short sequence within the long sequence (e.g., perhaps the short sequence is one conserved domain of a larger protein), but the introduction of many gaps can prevent a global alignment algorithm from finding it. A solution is to use a**semiglobal** (sometimes called "glocal") alignment technique that does not penalize **terminal gaps**—those that occur at the beginning or end of the alignment.
4. How would you modify the Needleman-Wunsch algorithm to carry out a semiglobal alignment?

   Hint: Only two changes in how the matrix is used are required. Consider what parts of the matrix represent the terminal gaps.

Test Your Understanding

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   Hint: Only two changes in how the matrix is used are required. Consider what parts of the matrix represent the terminal gaps.

Learning Objectives

- Understand the value of aligning genes and some practical applications of this technique
- Gain familiarity with the use of Web-based alignment tools to explore sequence similarity and understand how to modify their parameters
- Know how the Needleman-Wunsch algorithm optimally aligns any two sequences
- Understand how the Needleman-Wunsch algorithm can be modified to yield other alignments

Suggestions for Using the Project

This project is designed to be used in courses that require programming skills as well as those that do not. Below are suggestions for modules of the project that instructors might choose to use in these two types of courses. Instructors should also feel free to ask questions of their own that use these same skills.

Programming courses:
- Web Exploration: Experiment with the Needleman-Wunsch algorithm and the effect of gap penalty parameters as well as the benefits of local alignment (Smith-Waterman algorithm). Parts I, II, and III can be used independently.
- Guided Programming Project: Implement the Needleman-Wunsch algorithm in a programming language of your choice.
- On Your Own Project: Modify the code for the Needleman-Wunsch program to implement a local alignment algorithm.

Nonprogramming courses:
- Web Exploration: Experiment with the Needleman-Wunsch algorithm and the effect of gap penalty parameters as well as the benefits of local alignment (Smith-Waterman algorithm). Parts I, II, and III can be used independently.

- On Your Own Project: Identify modifications to the Needleman-Wunsch algorithm that would convert it to a local alignment algorithm.

Web Exploration

Part I: Pairwise Global Alignment with the Needleman-Wunsch Algorithm

The genomes of influenza viruses are divided into eight segments, each representing essentially the coding information for a single protein. Segment 4 contains the gene for **hem-agglutinin** (**HA**), the viral surface protein essential for the initial interaction between the virus and its host cell. HA is one key determinant of which host(s) a particular virus can infect, because the virus cannot replicate or cause disease without being able to first bind to a host cell. The HAs of one of the major seasonal human viruses circulating before 2009, the 2009 H_1N_1 pandemic virus, and the 1918 human pandemic virus are all classified as the H_1 type, whereas recent outbreaks of severe avian flu are caused by a virus with HA classified as H_5. These classifications are based on binding of antibodies of known specificity, but sequence alignment provides much more detailed information about similarities and differences and where changes have occurred.

Download We can use the Needleman-Wunsch algorithm to compare influenza virus HA segments. To start with, let's see how the 2009 H_1N_1 virus—the reference strain is designated A/California/07/2009 (H_1N_1)—compares with the human seasonal H_1N_1 virus that was currently circulating at that time, A/Brisbane/59/2007 (H_1N_1). Download the DNA sequences of segment 4 for both viruses from the*Exploring Bioinformatics* website. We align the sequences using EMBOSS, a suite of alignment tools produced by the European Bioinformatics Institute (somewhat parallel to the U.S. NCBI). At the **EBI-EMBL's EMBOSS Web page** (not the page for the EMBOSS software itself), you should see a list of programs for pairwise sequence alignment. Under the heading `Global Alignment`, the program Needle is an implementation of the Needleman-Wunsch algorithm.

Link From the EMBOSS site, choose the version of Needle that compares nucleotide sequences, and then paste your two sequences into the designated text boxes. Notice that you can set some parameters for the comparison, most notably the gap penalty. Needle uses an **affine** gap penalty, which means it imposes a larger penalty when a new gap is added and a smaller penalty when that gap is extended (our earlier example used a **linear** gap penalty). Leave the parameters set to the defaults for now.

Run Needle to align your two sequences; your results should look similar to **Figure 3.5**. At the top, you will see parameters such as the gap penalty and two measures of similarity: the number and percentage of matching nucleotides (labeled "Identity") and an alignment score (based on the scoring matrix, in this case awarding a match bonus of 5). In the alignment itself, matching nucleotides are shown by a | character, mismatches by a dot (.), and gaps by a dash (-).

```
#=======================================
# Aligned sequences: 2
# 1: H5N1_NA # 2: 2009_H1N1_NA
# Matrix: EDNAFULL
# Gap penalty: 10.0
# Extend penalty:  0.5
#
# Length: 1417
# Identity:   1160/1417 (81.9%)
# Similarity: 1160/1417 (81.9%)
# Gaps:         70/1417 ( 4.9%)
# Score: 4944.0
#=======================================

H5N1 NA         1  ATGAATCCAAATCAAAAGATAATAACCATTGGGTCAATCTGTATGGTAAT   50
                  ||||||||||||•||||||||||•||••||||||||||||||||||••|||
2009_H1N1_NA    1  ATGAATCCAAACCAAAAGATAATAACCATTGGTTCGGTCTGTATGACAAT   50

H5N1_NA        51  TGGAATAGTTAGCTTAATGTTACAAATTGGGAACATGATCTCAATATGGG  100
                   ||||||•|•||•||||||•|||||||||||•|||||•||||||||||||•
2009_H1N1_NA   51  TGGAATGGCTAACTTAATATTACAAATTGGAAACATAATCTCAATATGGA  100

H5N1_NA       101  TCAGTCATTCAATTCAGAC-AGGGAATCAAAACCAAGTTGAGCCA-----  144
                   |•||•||•|||||||| || •|||||||||||•||••||||••||
2009_H1N1_NA  101  TTAGCCACTCAATTCA-ACTTGGGAATCAAAATCAGATTGAAACATGCAA  149

H5N1_NA       145  --------------------------------------------------  144

2009_H1N1_NA  150  TCAAAGCGTCATTACTTATGAAAACAACACTTGGGTAAATCAGACATATG  199

H5N1_NA       145  -----ATCAGCAATACTAATTTTCTTACTGAGAAAG-CTGTGGCTTCAGT  188
                        ||||||||•||•||•|||••|•||| ||•|| |•||||•|||•||
2009_H1N1_NA  200  TTAACATCAGCAACACCAACTTTGCTGCTG-GACAGTCAGTGGTTTCCGT  248
```

Figure 3.5: Sample output from the EMBOSS Needle program, showing scoring data and part of an alignment for two sequences. Matching nucleotides are represented in the alignment by a vertical line, mismatches by a dot, and gaps by a dash. Generated from: EMBOSS Needle/European Bioinformatics Institute.

Web Exploration Questions

1. How many matching nucleotides are there between your two sequences? What is the alignment score?
2. How many gaps were needed to align these sequences? Is there any particular pattern to where or how the gaps occur?
3. Can you suggest where the coding sequence might occur within this segment? What is your evidence?

Nearly all of segment 4 consists of coding sequence, so we would expect indels—especially one- or two-nucleotide indels—to be mutations with serious consequences for the HA protein. Considering this, perhaps it would be valuable to consider strongly penalizing gaps: Try setting the gap opening penalty to 50, rather than the default 10.

Web Exploration Questions

4. What is the logic behind the affine gap penalty, which imposes a large penalty for opening a new gap but a much smaller penalty for extending the size of an existing gap?
5. When you align the two HA sequences using a higher gap opening penalty, does the percent identity change significantly? How about the number of gaps and their placement or size?
6. Your alignments with higher and lower gap opening penalties are both optimal alignments (the best alignments given the parameters), and they give quite similar scores. Which alignment do you believe is "better," biologically, and what is your justification? (*Hint: What striking observation did you make when looking at the gaps in the second alignment?*)

Download The origins of the 1918 pandemic virus remain murky, but its H_1 HA gene is thought to be the source of the HA genes found in all modern human and swine H_1 viruses. Download the segment 4 sequence of the 1918 human pandemic virus from the *Exploring Bioinformatics* website and compare it with the others. Consider what gap penalty you would like to use for this alignment.

Web Exploration Questions

7. Discuss how closely the HA segments of the two modern viruses are related to each other and how closely they resemble the 1918 virus. Can you draw any conclusions from your data about the origin of HA in the 2009 pandemic virus?
8. If you were to use a different segment from the same viruses for your sequence comparisons, you might come up with different answers. How is this possible?

Part II: Local Alignment with the Smith-Waterman Algorithm

Download Another way to use sequence alignment is to find one sequence within another. The influenza virus M2 gene, for example, is another key player in the biology of the virus: Once the virus enters the cell, M2 is involved in the release of the virus genome subunits so they can travel to the nucleus and direct viral replication. Suppose we have sequenced segment 7 from the 2009 H_1N_1 pandemic virus but are uncertain what part of it represents the actual M2 coding region. To find out, we could align the well-characterized M2 coding sequence from the Brisbane strain with the full segment 7 sequence from the newly sequenced virus. Download the DNA sequence for segment 7 from A/California/7/2009 and the coding sequence for M2 from A/Brisbane/59/2007 from the *Exploring Bioinformatics*website and align them using Needle with the default gap opening penalty of 10.

Web Exploration Questions

9. How good are the score and the percentage of sequence identity for this comparison? Why don't these statistics tell the full story in this case?
10. Suppose we only looked at the portion of the 2009 segment that actually aligned with the M2 coding region of the Brisbane strain. How would this change the

percent identity? Is this degree of similarity as high as you would expect for these related viruses?

Considering what you know about the Needleman-Wunsch algorithm, you should see why it might not be the best choice for aligning sequences that are so drastically different in length. Because the need to make alignments of this kind arises frequently, in 1981 Smith and Waterman published a modification of the Needleman-Wunsch algorithm that allows for **local**alignments (see References and Supplemental Reading). A local alignment looks for optimal partial (subsequence) matches; how this works is discussed further in the On Your Own Project. EMBOSS includes an implementation of the Smith-Waterman algorithm, called Water. Choose the nucleotide version of the Water method and then set a gap open penalty of 10 and a gap extension penalty of 0.1 and align the sequences.

Web Exploration Questions

11. How does this alignment differ from the previous one? Is the percent identity, either for the whole alignment or just for the regions that actually match, significantly better than before?
12. There is an obvious difference in how the subsequences of the M2 coding region align with the 2009 segment 7 sequence in the local alignment. Can you suggest a hypothesis for *why* the sequences align this way? (*Hint: Remember that the M2 sequence is the protein coding sequence*.) Based on your hypothesis, is the local alignment superior to the global alignment in terms of its ability to help us understand the viruses *biologically*?

This alignment is very sensitive to the parameters used. If you want to demonstrate this, try changing the gap extension penalty (e.g., from 0.1 to 0.5). Although almost all bioinfor-matic programs come with default settings that are usable for many common purposes, this illustrates the importance of understanding the algorithm and the meaning of the parameters, as well as the value of considering what kind of alignment would be most appropriate for the sequences being aligned.

Part III: Using Alignment to Investigate Virulence
Influenza viruses have received a great deal of study, and the ability to compare many strains has led to significant advances in understanding what allows one strain to cause more severe disease than another. The H_5N_1 "bird flu" virus makes an interesting case in point. This virus causes severe influenza in birds and has become established in populations of domestic chickens and turkeys. Human cases occur sporadically, mostly in individuals heavily exposed to infected birds, such as poultry farmers, and H_5N_1 flu is severe for humans as well. Once a human case occurs, however, spread to another human is exceedingly rare, even among family members in close contact with the infected individual. A 2006 article by van Riel et al. (see References and Supplemental Reading) demonstrated that the avian H_5N_1 virus binds to a form of sialic acid receptor that in humans is found only far down in the lungs and lower respiratory system. Human viruses, in contrast, bind to a form common in the upper respiratory tract. Thus, it is difficult for H_5N_1 to infect humans because our respiratory defenses normally prevent viruses from reaching the lungs. However, a mutant strain in which HA was altered to

be able to bind to sialic acid in the upper respiratory tract could be a very dangerous strain indeed.

Download So far, no such H_5N_1 strains that infect humans efficiently have been observed. However, we might ask whether the strains that do make it into humans tend to have altered HA genes—if so, that would suggest that either adaptive mutations could be occurring within the human host or that the viruses that cause human infections are subpopulations that are already better adapted. There are many avian H_5N_1 sequences available and a number of sequences of H_5N_1 viruses isolated from infected humans, so we can use sequence alignment to see whether these have essentially the same HA or noticeable differences. Download sequences for segment 4 from two different avian H_5N_1 virus isolates and from a human H_5N_1 isolate from the *Exploring Bioinformatics* website and compare them using the Needleman-Wunsch algorithm.

Web Exploration Questions

13. What are the scores and sequence identities for a comparison of the two avian viruses? Are the differences between the human isolate and the avian isolates greater than the differences among avian isolates?
14. Based on your results (which of course are limited—it would be necessary to do many more comparisons in reality), do you believe there is evidence that human adaptation is occurring in H_5N_1 viruses that might merit concern about human-to-human transmission in the near future?

More to Explore

Link The sequences for all the influenza virus segments and genes used in this exercise come from the **Influenza Research Database**, which indexes a wealth of sequence information on influenza viruses of all types. If you are interested in exploring influenza virus sequences further, you can retrieve individual genes, segments, or whole genomes from this database using a flexible search interface.

Guided Programming Project: The Needleman-Wunsch Global Alignment Algorithm

In this project, you will gain an understanding of dynamic programming and how it can be used to tackle the difficult problem of sequence alignment by implementing the Needleman-Wunsch algorithm and using it to construct global, optimal alignments. You will then modify your program to implement a semiglobal alignment algorithm. (Local alignments are tackled in the On Your Own Project that follows.)

Download All the programming examples in this section are written in pseudocode: They are intended to show you the flow of program execution but do not represent the syntax of any particular language. Thus, you can implement them in any language you wish (we recommend Perl or Python). Depending on your programming experience, you may need a syntax guide for your language; some basic syntax related to the chapter projects can be found on the *Exploring Bioinformatics* website. Instructors can find complete programs in Perl or Python and solutions for the Putting Your Skills into Practice exercises and On Your Own Projects in the instructors' section of the *Exploring Bioinformatics* website.

Dynamic Programming and the Needleman-Wunsch Algorithm
The Needleman-Wunsch algorithm was one of the first to implement dynamic programming to solve an alignment problem. Dynamic programming is a problem-solving technique that breaks down a complex problem, such as the global alignment problem, into smaller overlapping subproblems. The solutions of the subproblems are then used to solve the original problem. Problems that can be solved with dynamic programming have a few common characteristics:

- There must be a way to divide the problem into smaller subproblems. (Each subproblem may then be broken down further.)
- The problem-solving process starts by solving these more manageable subproblems.
- Solutions to the smallest subproblems are then used in determining solutions to the next largest problems.
- The process repeats until the original (largest) problem is solved.

You learned earlier (review Understanding the Algorithm before continuing if needed) how the Needleman-Wunsch algorithm works. Building a scoring matrix divides the alignment problem into subproblems: The values in the matrix represent partial alignment scores or partial solutions to the overall problem. The bottom-right cell of the matrix always gives the optimal score, and backtracking through the matrix yields one or more "paths" that are interpreted as a series of aligned nucleotides or gaps that generate the corresponding optimal alignment(s).Figure 3.4E shows the matrix and paths for the sample sequences you have already seen.

Implementing the Needleman-Wunsch Algorithm
To align sequences using the Needleman-Wunsch algorithm, a computer program must (1) build a scoring matrix, (2) find paths through the matrix, and (3) generate alignments from the paths. The scoring matrix should be relatively easy for you to implement. The matrix itself could be implemented as a two-dimensional array. The first row and first column are initialized the same way regardless of the sequences compared. Then, each cell in the matrix is filled using the optimal score from among three choices: match or mismatch, gap in the first sequence, or gap in the second sequence (see Understanding the Algorithm).

The more difficult problem is how to find the path(s) back through the matrix and convert them to actual alignments computationally. Recall that we start at the lower-right cell and then determine the direction to move based on which of the three bordering cells (above, left, or above-left diagonal) could have been used to arrive at the score in the current cell. The directional arrows in Figure 3.4E show how we moved from cell to cell, but computers cannot really deal with these arrows. So, we replace the arrows with directional strings, using "H" for a horizontal move, "V" for a vertical move, and "D" for a diagonal move.

Our example contains three possible paths, so the following three strings are created, following the path from the lower-right corner to the upper-left corner in each case: `HDHHDDD`,`HDDDHHD`, and `HDDDDHH`. Moving from left to right in the directional strings and right to left in the sequences (we start at the ends of the two sequences because the directional strings start with the lower-right cell), we create the alignments as follows:

1. If the directional character is a D, then align the two currently considered nucleotides and obtain new nucleotides to consider by moving to the left one position in each sequence.
2. If the directional character is an H, then align the current nucleotide in the second (top) sequence with a gap character. Obtain a new current nucleotide for sequence 2 (top) by moving to the left one position, but keep the same current nucleotide for sequence 1 (left).
3. If the directional character is a V, then align the nucleotide in the first sequence with a gap character and obtain a new current nucleotide by moving to the left one position in the first sequence but not the second.

This process continues until all nucleotides have been aligned. For our sample sequences, the result is as follows:

```
   Path 1:  HDHHDDD       Path 2: HDDDHHD      Path 3: HDDDDHH
Alignment:  CACGTAT    Alignment: CACGTAT   Alignment: CACGTAT
            CGC--A-               C--GCA-              --CGCA-
```

The memory usage required by this algorithm is bounded by the size of the two input sequences, because you need to keep an array of size *N* × *M* in memory at all times. The length of the sequences that can be aligned is limited to the memory size of the computer on which the program runs. In the pseudocode that follows, only one directional string is constructed; a function (subroutine) is used for this task to modularize the steps of the algorithm. Finding all possible strings is left as an exercise.

The pseudocode that follows will guide you in writing a Needleman-Wunsch program that prompts the user for sequences to align and for a scoring metric. The Putting Your Skills into Practice exercises that follow ask you to implement the program in whatever language your course is using and then provide suggestions for further exploration of the algorithm. Alternatively, your instructor may choose to provide the basic code (from the instructor section of the *Exploring Bioinformatics* website) for you to test and modify.

Algorithm

Needleman-Wunsch Algorithm

- **Goal:** Determine the optimal global alignment of two sequences.
- **Input:** Two sequences
- **Output:** Best, global alignment(s) of two input sequences

```
 // Initialization
```

```
Input the two sequences: s1 and s2
N = length of s1
M = length of s2 matrix = array of size [N+1, M+1]
gap = gap score
mismatch = mismatch score
match = match score

// STEP 1: Build Alignment Matrix
set matrix[0,0] to 0
for each i from 1 to N, inclusive
  matrix[i, 0] = matrix[i-1, 0] + gap
for each j from 1 to M, inclusive
  matrix[0, j] = matrix[0, j-1] + gap
for each i from 1 to N, inclusive
  for each j from 1 to M, inclusive
    if (s1[i-1] equals s2[j-1])
      score1 = matrix[i-1, j-1] + match
    else
      score1 = matrix[i-1, j-1] + mismatch
    score2 = matrix[i,j-1] + gap
    score3 = matrix[i-1, j] + gap
    matrix[i][j] = max(score1, score2, score3)

// STEP 2: Create Directional Strings
dstring = buildDirectionalString(matrix, N, M)

// STEP 3: Build Alignments Using Directional Strings
seq1pos = N-1 // position of last character in seq1
seq2pos = M-1 // position of last character in seq2
dirpos = 0

while (dirpos < length of directional string)
  if (dstring[dirpos] equals "D")
    align s1[seq1pos] and s2[seq2pos]
    subtract 1 from seq1pos and seq2pos
  else if (dstring[dirpos] equals "V")
    align s1[seq1pos] and a gap
    subtract 1 from seq1pos
  else // must be an H
    align s2[seq2pos] and a gap
    subtract 1 from seq2pos
  increment dirpos

// Function to create directional string
function buildDirectionalString(matrix, N, M)
dstring = ""
currentrow = N
```

```
currentcol = M
while (currentrow != 0 or currentcol != 0)
   if (currentrow is 0)
      add 'H' to dstring
      subtract 1 from currentcol
   else if (currentcol is 0)
      add 'V' to dstring
      subtract 1 from currentrow
   else if (matrix[currentrow][currentcol-1] +
      gap equals matrix[currentrow][currentcol])
      add 'H' to dstring
      subtract 1 from currentcol
   else if (matrix[currentrow-1][currentcol] +
      gap equals matrix[currentrow][currentcol])
      add 'V' to dstring
      subtract 1 from currentrow
   else
      add 'D' to dstring
      subtract 1 from currentcol
      subtract 1 from currentrow
return dstring
```

Putting Your Skills into Practice

1. **Download** Write a program in the language used in your course to implement the above pseudocode. Test your program by using the sample sequences above and the other short sequences you used in the Test Your Understanding exercises and verify that it finds the expected alignment (only *one* alignment,however: see question 4 for more about finding all possible alignments). Then try it on the influenza virus sequences you compared using Needle in the Web Exploration. (If your class skipped the Web Exploration section, download sequences for HA genes from various influenza virus strains from the *Exploring Bioinformatics* website.)
2. A user-friendly alignment program would format the output for readability, printing a specific number of characters on each line and then leaving a blank line between segments of the alignment. Numbering and a special character to indicate matches are also helpful (similar to the output you saw for EMBOSS). Modify your program to make it a more user-friendly solution.
3. Improve the program further by adding additional information beneficial to users: the alignment score and match percentage. You could also give the user the option to print the matrix and the path string for debugging purposes (which might also help you if your program is not doing exactly what you want it to).
4. The implementation of the Needleman-Wunsch algorithm shown previously finds only a single optimal alignment, but you can modify your program to find*all* possible optimal alignments. If you are familiar with the programming technique of recursion, you may want to consider a recursive solution, but this

problem can also be solved without using recursion. Test your modified program to see that it finds all optimal alignments of your short test sequences, then test your program with real influenza HA sequences. Are there multiple optimal alignments for these sequences? In general, would long sequences be more or less likely to lead to multiple optimal alignment paths?

Although global alignment algorithms are useful, they do not solve all alignment problems. An example mentioned earlier is the need to find the coding sequence for a gene within a longer DNA sequence, requiring alignment of a short sequence with a long one. The Needleman-Wunsch algorithm can perform a global alignment, but it will penalize not only internal gaps but also the many terminal gaps—gaps at the beginning and end of the alignment—needed to align the short sequence at its proper position within the large sequence. This idea is illustrated by three sample alignments of a pair of sequences:

```
CGCTATAG   CGCTATAG   CGCTATAG
--CTA---   C--TA---   --C--TA-
```

Using a global alignment, these alignments are all considered "optimal" (three different paths to the same optimal score, –2). However, it is clear that the first alignment would actually be the best, because it includes only terminal gaps used to "position" the short sequence. If you eliminated the gap penalty for terminal gaps, the scores for these three sequences would be 3, 1, and 1, with the best alignment getting the best score. This alignment, where terminal gaps are ignored, is called a semiglobal alignment.

Putting Your Skills into Practice

5. **Download** Modify the Needleman-Wunsch program so it implements a semiglobal alignment by eliminating the gap penalty for terminal gaps. (*Hint: This actually requires only a few minor changes in the code. Focus on what the outside rows and columns of the matrix represent and how they are used.*) Try your program on the short sequences above and then on the sequences shown in Test Your Understanding question 3. If it works correctly, try a real-world case by downloading the sequence of 2009 H_1N_1 pandemic influenza virus segment 7 and the coding sequence for the 2009 H_1N_1 virus M1 gene (do not use M2, because that requires a local alignment, discussed later in the chapter) from the *Exploring Bioinformatics* website. Align the sequences and see if your program can successfully pick out the M1 coding sequence within the segment 7 sequence.
6. If you try the M1 coding sequence versus segment 7 alignment just mentioned in the EMBOSS Needle program, you might not expect it to succeed. However, it does. Go back to the parameter page and look closely at how the default parameters are set and see if you can decide why it works.

On Your Own Project: A Local Alignment Algorithm

Understanding the Problem: Local Alignment

At this point, you should have a good understanding of how the Needleman-Wunsch algorithm constructs optimal, global alignments. You should have considered (in the Testing Your Understanding exercises) how this algorithm could be modified to produce a semiglobal alignment and perhaps actually programmed such a solution (see Putting Your Skills into Practice). Finally, you should have worked with the Water program from EMBOSS and have an idea why a local alignment would be useful.

Local alignments solve the problem of finding and aligning conserved regions in otherwise dissimilar sequences by looking for optimal partial or subsequence matches between the sequences. Consider the sequences `AAAGCTCCGATCTCG` and`TAAAGCAATTTTGGTTTTTTTTCCGA`. Two similar regions in these sequences, AAAGC and TCCGA, are separated by regions that are very different. A global or semiglobal alignment program should find the AAAGC alignment but will fail to correctly align the sequences so the TCCGA sequences also match up. To find subregions of similarity, large gaps must be expected and should not adversely affect the alignment score; this was the basis for Smith and Waterman's modification of the Needleman-Wunsch algorithm to produce a local alignment (see References and Supplemental Reading). Surprisingly, implementing the Smith-Waterman algorithm requires only a few changes to a semiglobal alignment algorithm.

Solving the Problem

A key element of a local alignment algorithm is the treatment of gaps. As with the semiglobal alignment, we should not penalize terminal gaps. But, for a local alignment, the Smith-Waterman algorithm also needs to consider how internal gaps are handled. For a global or semiglobal alignment, negative values can occur within the matrix, and they are useful because increasing negative values along an alignment path indicate a move away from similarity. However, for a local alignment, negative scores are no longer useful, because we do not necessarily expect the alignment to approximate an "ideal" diagonal path. Indeed, long gaps may be necessary to find optimally aligned subsequences, and these longer gaps should not be penalized so heavily as to negate good partial alignment scores. How might our system for placing a subscore in each cell of the matrix be modified to deal with this issue?

A second important modification involves the alignment score. Both the global and semiglobal alignment algorithms build the alignment path starting with the cell in the lower right of the matrix; this cell contained the optimal alignment score, because both algorithms considered the sequences in their entirety. However, a local alignment must consider subsequence matches, and high subsequence alignment scores could appear anywhere in the matrix, indicating the presence of a similar subsequence somewhere within the longer sequences. There could be many such similar subsequences within the longer sequences, and we want our local alignment algorithm to find all of them.

Finally, once a high score is found, continuing to follow the path until we reach the upper-left cell is not required: A highly conserved subregion may not extend all the way to the beginning of either sequence. Thus, the process of finding the path start and path end also requires modification.

Based on this information, describe a modified algorithm that would find local alignments given two sequences. Be sure to detail how the matrix is initialized, how the sub-scores are placed into each cell, and where the alignment path(s) should start and end.

Programming the Solution

Download If your course involves programming, your instructor may ask you to make the necessary modifications to your semiglobal alignment program and actually implement the local alignment algorithm you described. Test your program with the sample sequences shown previously and see if it can find both matches. Then, download the segment 7 sequence for the 2009 H_1N_1 pandemic influenza virus and the coding region of the M2 gene from the Brisbane seasonal strain from the *Exploring Bioinformatics* website and see if your program gives the same result as the EMBOSS implementation of the Smith-Waterman algorithm.

Connections: An Influenza Controversy

In early 2012, two different influenza virus research groups working on the H_5N_1 strain submitted papers to be considered for publication in prestigious scientific journals. Although their methods differed, the goal of both groups was to identify what mutations were necessary for the avian H_5N_1 flu virus to be transmitted readily among humans and whether the resulting virus would be as virulent as the current avian strains. Bioinformatics, including sequence alignment, played a major role in their research, but their work went beyond computational modeling to actually generate new virus strains whose virulence could be tested directly. The aim of this research was to better predict the future pandemic potential of H_5N_1 and thus better prepare medical researchers to deal with a human-transmissible version. Many scientists agreed that their research had significant merit and that the scientific and medical communities would benefit from publication. Others, however, expressed concern about the potential for accidental release of an engineered H_5N_1 virus that could itself become the next pandemic strain. Still others contended that publication of these results would essentially hand the "blueprint" for a bioweapon to any nation or terrorist organization interested in using it. Months of controversy ensued in an attempt to decide whether the work should be published, suppressed, or published with key techniques and details redacted. What do you believe should be done with this research?

BioBackground: The Influenza Virus and Molecular Evolution

Viruses sit at the interface between living and nonliving: Outside a host cell, they are metabolically inert, apparently nothing but nucleic acid in a protein shell, sometimes surrounded by a membrane-like envelope. Yet, every virus has some molecule on its surface capable of interacting with a receptor on the surface of a living cell. When the virus bumps into and attaches to a cell, this interaction results in entry of the virus into the cytoplasm, whereupon the viral genes are expressed and, pirate-like, the virus takes over the host cell machinery and subverts it to the manufacture of more viruses (**Figure 3.6**), ultimately destroying the cell. For the influenza virus, the preferred host cell is an epithelial cell of the upper respiratory system, and the cellular receptor is a sugar called sialic acid that binds the HA protein on the surface of the virus.

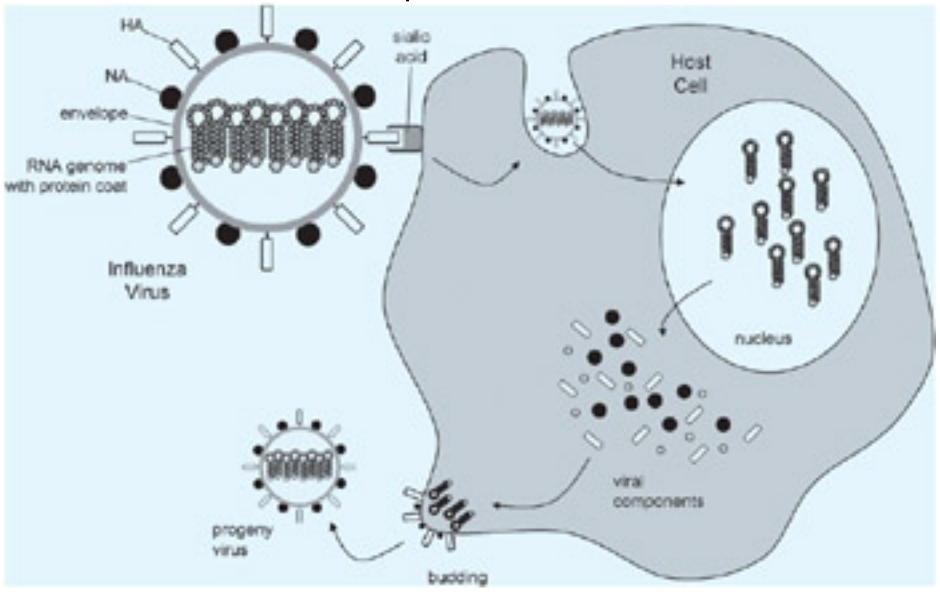

Figure 3.6: Schematic drawing of an influenza virus (greatly oversized) and a simplified overview of its replication cycle. After interacting with the sialic acid receptor, the virus enters the cell by endocytosis. The genome is released, moves to the nucleus, replicates, and directs synthesis of viral proteins. Assembly at the membrane is followed by budding to release new viruses.

An influenza virus can be classified based on the type of HA protein it carries, as well as a second protein, **neuraminidase** (**NA**) involved in releasing the viral progeny from the host. Several major types of HA (H_1, H_2, H_3) and NA (N_1, N_2, N_3) are known, so a virus can be denoted H_1N_1, H_3N_2, H_5N_1 and so on. However, mutations produce variation even within these types, so subtypes must be defined. For example, in 2009–2010, one major circulating seasonal flu virus was A/Brisbane/59/2007 (H_1N_1), a type A virus first identified in Brisbane in 2007, whereas in 2007–2008, A/Solomon Islands/3/2006 (H_1N_1) was common; both subtypes are different from the new pandemic virus discovered in 2009, A/California/7/2009 (H_1N_1), even though all three have the same H and N types.

The RNA genome of influenza virus is synthesized by a virus-encoded polymerase that does not "proofread" to remove errors; thus, mutations producing variant strains—new subtypes—occur frequently. Mutations in the HA and NA genes are particularly important because these are major molecules recognized by the host immune system: Variation here can allow a virus to escape immune detection and thus increase its opportunities to infect and spread. Such mutations would clearly be advantageous to the virus and selected for over time, allowing the new strain to become more prevalent in the population.

We would recognize the new strain as being evolutionarily related to the original one by the **similarity** of their genes: Two genes are similar if they have the same DNA sequence to a significant extent. This is determined by aligning genes from two strains (or, more broadly, from any two organisms), and we interpret significant similarity as evidence that these genes have a common origin. Differences between the sequences (**Figure 3.7**) are assumed to result from mutation, including substitutions of one base for another (resulting in mismatched bases in the alignment) as well as insertions or deletions (resulting in gaps in one of the aligned sequences). When a gene in one species or strain is very similar to a gene in a different species or strain, we say the genes are **orthologs** (**Figure 3.8**): Our conclusion is that the two species are descended from a common ancestor and that the genes have become modified by mutation over time in each of the daughter species. In fact, many or most genes in two evolutionarily related species should be orthologs. If we find two similar genes within the*same* species, we refer to these as **paralogs** and conclude that they arose by a gene duplication event followed by mutation.

```
Species 1:  T A A A G A C C A T A G G A A A T A A A G A T A A
Species 2:  T A A C G A C C A T - G G A A A C A A A G A T A A
```

Figure 3.7: Determining the similarity of two or more genes by aligning them so that their nucleotide sequences match up as well as possible. Differences resulting from mutation are highlighted; dashes represent the locations of insertion or deletion mutations (indels).

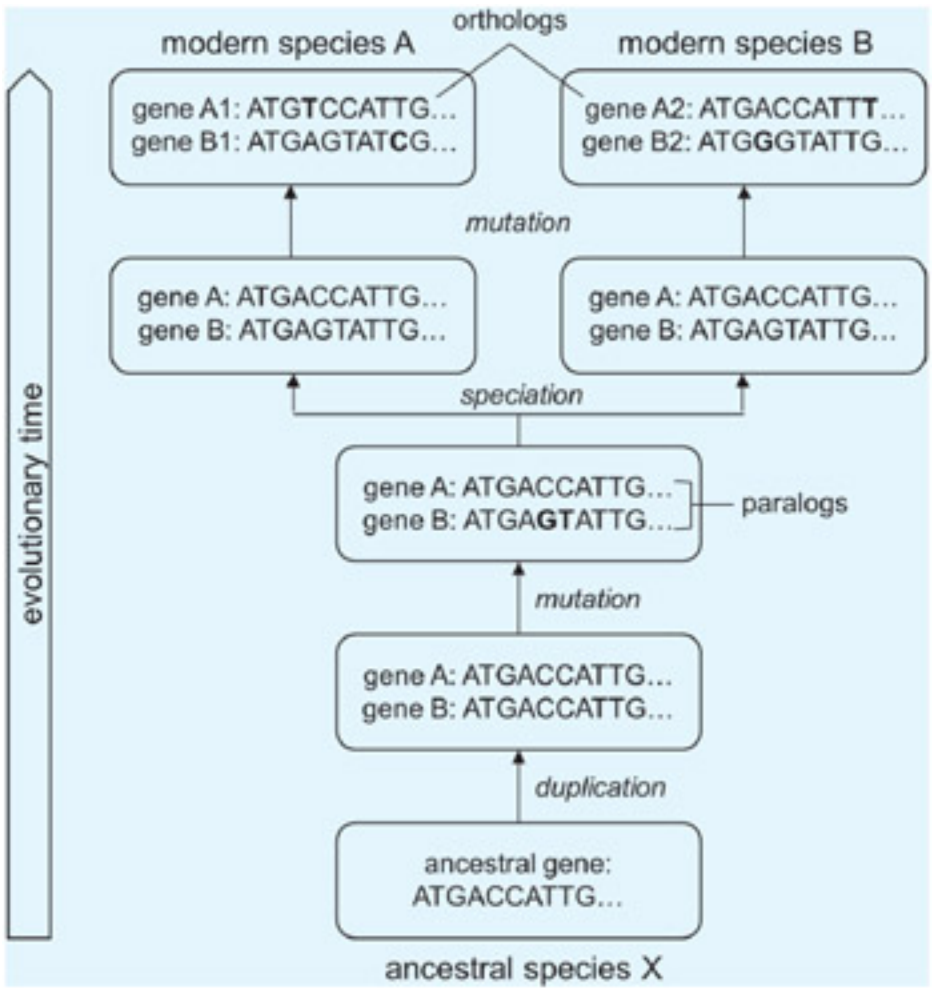

Figure 3.8: Sequences of genes or proteins reflect the pathways of change that have occurred in the evolutionary history of related species or strains.

Gradual evolution by mutation produces new influenza virus strains that have genome sequences closely related to their "parent" strain; aligning the sequence of, for example, the HA gene from a currently circulating virus with its ortholog from a suspected new variant demonstrates the similarity of the genes and reveals their differences. Differences in regions of the protein known to be bound by host antibodies suggest a new strain of potential medical importance that should be carefully tracked and perhaps included in the next season's vaccine formulation. In addition to mutation, however, influenza viruses can also change more suddenly by a recombination mechanism: If two viruses infect the same cell (this can often happen in pigs, which are susceptible to swine, avian, and human influenza viruses), the progeny of one virus can acquire a whole genome segment from the other. Sequence alignment is again the tool needed to establish that a more radically different virus has evolved.

Analysis of sequence comparisons (see References and Supplemental Reading) revealed that the 2009 pandemic H_1N_1 virus arose through this recombination mechanism: Its parent was a well-known "triple reassortant" strain common in swine that carries an HA gene descended from the 1918 pandemic virus along with other segments from avian and human viruses (**Figure 3.9**). This virus more recently acquired NA and M genes that originated in a Eurasian avian virus, generating a novel virus type that began circulating in the human population probably about a year before the first cases were recognized clinically. In addition to demonstrating origins and pathways of evolution, sequence alignment is a key tool in investigating the functions of genes and proteins. In the case of influenza virus, several specific variations have been associated with highly virulent viruses capable of causing severe disease: a mutation in HA allowing the protein to be processed by a more common protease, thus increasing host range; a mutation in the viral polymerase allowing higher activity at the lower temperature of the human respiratory tract; and so on. The virulence of a new influenza virus strain can thus also be characterized by aligning its genes with their orthologs to look for these specific changes.

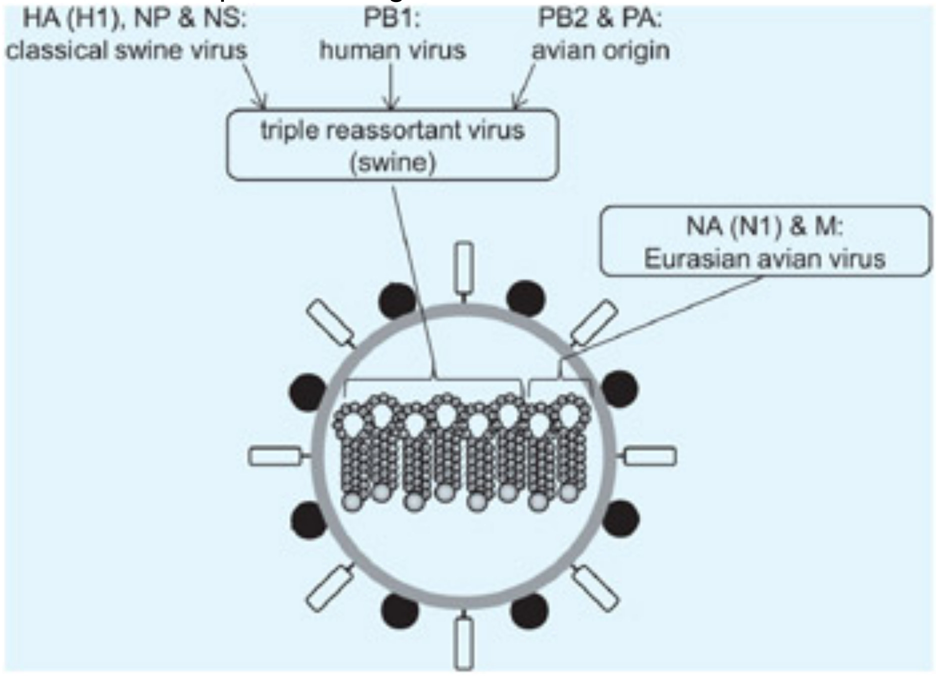
HA (H1), NP & NS: classical swine virus
PB1: human virus
PB2 & PA: avian origin
triple reassortant virus (swine)
NA (N1) & M: Eurasian avian virus

Figure 3.9: Origins of the genome segments of the 2009 pandemic H_1N_1 virus, as determined by sequence alignment.

References and Supplemental Reading

Variation in the Influenza Virus and Pandemic Influenza Virus Strains

Nicholls, H. 2006. Pandemic influenza: the inside story. *PLoS Biol.* **4**:e50.

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Eddy, S. R. 2004. What is dynamic programming? *Nat. Biotechnol.* **22**:909–910.

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H_5N_1 Influenza Virus Attachment

van
Riel, D.,V. J. Munster,E. de Wit,G. F. Rimmelzwaan,R. A. Fouchier,A. D.Osterhaus,and T. Kuiken. 2006. H_5N_1 virus attachment to lower respiratory tract. *Science***312**:399.

Chapter 4: Database Searching and Multiple Alignment: Investigating Antibiotic Resistance

Chapter Overview

This chapter develops skills in two very commonly used types of Web-based bioinformatics tools: searching sequence databases for high-scoring matches to a query sequence (using BLAST) and multiple sequence alignment (using ClustalW). No programming project is provided; however, the algorithms and parameters used by these programs, both of which use heuristic methods to speed up complex tasks, are discussed in some detail. This chapter focuses on algorithms for optimal alignment of DNA sequences. This chapter is recommended for both programming and non-programming courses because these techniques and those related to them are used extensively in real-world bioinformatics applications.

- **Biological problem**: Overuse of agricultural antibiotics and development of antibiotic resistance
- **Bioinformatics skills**: One-to-many sequence alignments and multiple sequence alignment
- **Bioinformatics software**: BLAST and ClustalW
- **Programming skills**: Heuristics

Understanding the Problem: Antibiotic Resistance

Fifty years ago, many people believed the newly discovered antibiotics—drugs that selectively kill bacteria without harming human hosts—would end infectious diseases caused by bacteria. Indeed, these "miracle drugs" have preserved the lives of millions. Today, however, tuberculosis, pneumonia, diarrheal disease, staph infections, and other bacterial diseases remain important— and in some cases increasing—causes of illness and death. One important reason is the dramatic rise of antibiotic-resistant bacteria no longer killed by commonly used antimicrobial drugs.

Resistance results from selection for mutants that can survive antibiotic treatment (see Bio-Background at the end of this chapter). As the use of an antibiotic becomes widespread, bacteria are increasingly exposed to it, escalating selective pressure and resulting in rapid evolution of strains that thrive when antibiotics kill their susceptible cousins. Thus, in an effort to curb resistance, physicians today are much more cautious than in the past, prescribing antibiotics only when the need is clear and holding those least prone to resistance in reserve.

The nontherapeutic use of antibiotics in agricultural animals and even on food crops (**Figure 4.1**) is at the center of a current controversy over resistance. Routine use of antibiotics in animal feed prevents disease and promotes growth, allowing more animals to be raised more cheaply in less space. But many believe these economic benefits come at a high cost: Are the 28 million tons of agricultural antibiotics used annually in the United States and Canada (far outweighing the 3 million tons for all human uses) promoting antibiotic resistance? Most scientists believe that antibiotic overuse is a major contributor to the development and spread of resistance, leading to bans on subtherapeutic agricultural use of antibiotics in Denmark in 1999 and in the European Union in 2006. No such legislation is yet in place in the United States, and those who oppose such laws argue that no causal link has been definitively established between agricultural antibiotics and antibiotic-resistant disease bacteria in humans. We can investigate this link using some more advanced sequence alignment techniques.

Figure 4.1: The extensive use of antibiotics in agricultural animals that are not sick has sparked controversy about the role of this practice in speeding the development of antibiotic-resistant bacteria. Courtesy of Scott Bauer/USDA ARS. Inset © AbleStock.

Bioinformatics Solutions—Advanced Sequence Comparison Algorithms

There is no question that intensive use of antibiotics in animals increases the prevalence of antibiotic-resistant bacteria—in animals. But how can a microbiologist determine experimentally whether these bacteria are an important source of resistance genes for bacteria that cause disease in *humans*? In 2001, Abigail Salyers and her colleagues used bioinformatics to look for evidence that bacteria inhabiting the human gut had been the recipients of antibiotic-resistance genes originating in bacteria found in domestic animals (see References and Supplemental Reading). Taking advantage of the many sequenced bacterial genomes and the huge collection of sequenced genes in public genome databases, they looked for *un*related animal and human bacteria that have closely related resistance genes.

New or altered genes, including those that allow a bacterial cell to resist an antibiotic, arise by random mutation, which is rare. However, once these genes exist in a bacterial community, they can be readily passed from one bacterium to another (usually on plasmids), a phenomenon known as horizontal gene transfer (HGT; see BioBackground), allowing resistance to spread rapidly in a bacterial community. If a "donor" bacterium gives a resistance gene to a "recipient" organism, the two should have the *same* gene—that is, one that encodes a protein with the same amino-acid sequence. Furthermore, if human pathogens have the same antibiotic-resistance genes as bacteria from domestic animals, it would suggest that HGT occurs between them, supporting the conclusion that increased resistance among agricultural bacteria is indeed dangerous to human health. Similarity, of course, can be measured by sequence alignment, so Salyers used alignment first to retrieve genes from GenBank that were similar to a particular resistance gene and then to ask how similar the genes from unrelated species were. Two resistance genes that were $\geq$ 95% identical were assumed to have resulted from an interspecies gene transfer event.

The pairwise comparison techniques we have used thus far are of limited value when many sequences must be compared efficiently. In the sections that follow, we explore tools that build on the alignment algorithms we have already seen to allow for the rapid comparison of one sequence to many or the simultaneous alignment of multiple sequences.

BioConcept Questions

To successfully complete this chapter's projects, you need to understand a little about antibiotic resistance, HGT, and how similarity measurement can help us decide whether HGT has occurred. Use these questions to test your biological understanding; read BioBackground at the end of the chapter if you need a better foundation.

1. What is the difference between vertical and horizontal gene transfer? Why are the terms "vertical" and "horizontal" used to describe these processes?
2. Any bacterium could become antibiotic resistant by means of mutation. Why is HGT considered so much more of a threat, at least in terms of medically important resistance?
3. How does the degree of similarity between two genes help us understand whether they descended vertically from a common ancestor (recent or distant) or whether they could have moved from one species to the other by HGT?
4. Suppose you have evidence that two genes in two different bacterial species have a single, common origin. Give two possible explanations for how this might have occurred.

Understanding the Algorithm—aDatabase Searching and Multiple Alignment

Blast: A Heuristic Approach to Database Searching

The Needleman-Wunsch algorithm is a relatively efficient algorithm for optimal, global pairwise sequence alignment. However, imagine that you wanted to align an antibiotic-resistance gene of interest with every other sequence in GenBank. The computational time required is the time to make one alignment (compute the matrix and alignment paths) times the number of sequences in the database—currently more than 100 million. We would say that the time required to solve this problem is O(N*S*) or on the order of N*S*, where *S* is the number of sequences. It gets large quickly: If one alignment took 1 second of computer time, the whole search would take more than 3 years.

Yet, **BLAST** (Basic Local Alignment Search Tool; see References and Supplemental Reading) can compare a query sequence to the entire database and return all matching sequences in a matter of seconds.

BLAST and its several variations are perhaps the most widely used of all bioinformatics software. As its name suggests, BLAST implements a local alignment algorithm similar in principle to the Smith-Waterman algorithm. However, it uses a **heuristic** or "shortcut" that makes it a practical and efficient solution to this complex problem.

To understand how BLAST works, we first need to clarify what we mean by a "matching" sequence. The point of comparing an antibiotic-resistance gene to GenBank is to identify similar sequences—generally, orthologs. Thus, a sequence matches the query if it shows statistically significant and/or biologically relevant similarity when aligned to the query. But how does BLAST make 100 million alignments so quickly? In fact, it does not make a full alignment for every sequence. Its first step is to break the query sequence into short "words" called *k*-tuples: subsequences *k* characters long. The default setting for *k* is 11 for DNA alignments and 3 for protein alignments. BLAST then scores close matches between these short sequences and each database sequence; this process is known as "seeding." Where it finds a good match, a local alignment algorithm finally comes into play, and the program tries to extend the alignment in both directions, comparing the resulting score with a threshold value. An alignment that can be extended to score above the threshold is referred to as a**high-scoring pair** (**HSP**).

Figure 4.2 shows an example of the BLAST algorithm using a famous quotation for which several variations can be found instead of a sequence (remember, alignment programs can compare any two strings). The alignments in the figure are scored with a simple match = 1, mismatch = 0, gap = -1 system. If the query sequence is broken down into three-letter words(*k*-tuples) and we focus on the 37th *k*-tuple, "ent," there is no match for any of the words in database sequence A, so this sequence can be discarded. Sequence B has an initial match, but attempting to extend the alignment does not increase the score above the threshold, so this sequence would not be reported as a significant alignment. Sequence C, however, has an alignment that exceeds the threshold score and would be reported as a match.

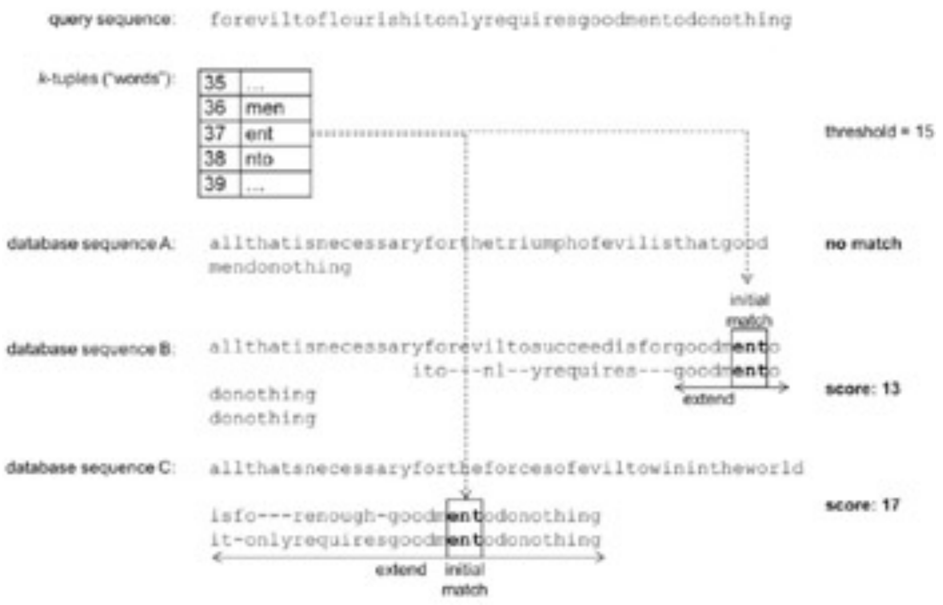

query sequence:
forevilltoflourishitonlyrequiresgoodmentodonothing
k-tuples ("words"):
35 ...
36 men
37 ent
38 nto
39 ...
threshold = 15
database sequence A:
allthatisnecessaryforthetriumphofevilisthatgood
mendonothing
no match
initial match
database sequence B:
allthatisnecessaryforeviltosucceedisforgoodmento
ito--i-nl--yrequires---goodmento
donothing
donothing
extend
score: 13
database sequence C:
allthatsnecessaryforttheforcesofeviltowinintheworld
isfo---renough-goodmentodonothing
it-onlyrequiresgoodmentodonothing
extend
initial match
score: 17

Figure 4.2: An example of how the BLAST algorithm finds an initial match between a short subsequence (*k*-tuple or "word") of the query and the target sequence, then extends the match to find a local alignment with scoring above a threshold value.

BLAST then calculates the statistical likelihood that a given score would occur based on mere chance alignment of unrelated sequences (the *e*-value) and orders the matching sequences according to this measure of statistical significance. As we will see in the next section, BLAST reports back to the user the name of the matching sequence, the score, the *e*-value, and the alignment itself. In addition to changing scoring parameters such as the gap penalty, BLAST allows the user to adjust the *k*-tuple value if desired. Although the default value typically works well, decreasing the word size allows the identification of sequences that match less well (useful when similarity of the query to other sequenced genes is weak) and is also needed if the sequence to be compared is very short (current implementations of BLAST do this automatically when a short query is entered).

You use heuristics all the time without realizing it. Consider, for example, how you decide which route to take when you have several alternatives. It is extremely difficult to calculate a truly optimal solution (accounting for traffic, construction, traffic lights, speed limits, school zones, and many more variables), so you apply a heuristic: You decide to take the route that is shortest in mileage or the one you believe has the least traffic. This allows you to choose rapidly but does not guarantee that you will in fact choose the fastest option. Similarly, BLAST's heuristic approach allows it to quickly discriminate possible matches from unrelated sequences. Although it may not find optimal alignments, it deals with large volumes of data extremely rapidly while finding solutions that are acceptably close to optimal.

ClustalW: Multiple Sequence Alignment

Although BLAST can quickly identify a large number of sequences similar to a query, it displays only individual alignments of the query with each matching sequence. However, we might instead want to see an alignment of a whole group of similar sequences at once (**Figure 4.3A**). For example, perhaps the sequences of genes similar to our query resistance gene fall into two or three distinctly identifiable groups. Or, we might want to identify a **consensus sequence**: the nucleotides or amino acids that appear the most frequently at each position in a given region of the sequence. Rather than a pairwise alignment, this requires a **multiple sequence alignment** algorithm.

The computational complexity problem for multiple sequence alignment is even greater than for database searching. Here, the order of adding sequences to the alignment matters. Suppose, for example, we have optimally aligned two sequences, GTCT and GGT as in **Figure 4.4A**. If we now want to align the sequence CT with the other two, we might get the alignment in **Figure 4.4B**. However, if we aligned GTCT with CT first, we might find the optimal alignment to be the one in **Figure 4.4C** instead. The dynamic programming approach of Needleman and Wunsch could deal with this problem by building a matrix of size $L \times M \times N$, each dimension one character longer than the length of one sequence. However, as more sequences are added, the matrix becomes four-, five-, six-dimensional, and so on and the computational time required becomes $O(N^S)$: the time required for one alignment raised to the *power* of the number of sequences, which obviously becomes impractical very fast.

Thus, multiple sequence alignment algorithms again use heuristics to manage the complexity of the problem. ClustalW (see References and Supplemental Reading) is one of the most popular multiple sequence alignment algorithms; it uses a **progressive alignment** algorithm in which the order of adding new sequences to the alignment is determined by first calculating a rough phylogenetic tree called a **guide tree** (**Figure 4.3B**). The guide tree is generated by first doing pairwise alignments and then using the score or percent similarity from those alignments to draw a tree showing which sequences are more and less closely related (we will have much more to say about the mechanics of generating a phylogenetic tree in subsequent chapters). Starting with the two most closely related sequences (in the example in Figure 4.3B, these are *Bacteroides xylanisolvens* and *B. fragilis*), ClustalW then does global, pairwise alignments to align each new sequence with those already aligned, in order of decreasing relatedness. Note that although this is an efficient way to produce a multiple alignment, the fact that it is based on global alignment means ClustalW may not correctly align sequences that share regions of similarity if the sequences are not very similar overall.

Test Your Understanding

1. Describe two features of the BLAST algorithm that enable it to complete a database search much faster than the Needleman-Wunsch algorithm would.

2. For the BLAST example in Figure 4.2, are there *k*-tuples within the query sequence that give a very different result? What might be an example of a query sequence that would yield an HSP for all three database sequences?
3. Describe briefly how the sequence differences you can see in Figure 4.3A relate to the lengths of the branches in Figure 4.3B.
4. In Figure 4.3, the sequence labeled CA_F7SDb01 is from an organism that has not yet been characterized sufficiently to give it a species name; all other sequences are from species within the genus *Bacteroides*. Based on the region of the multiple alignment shown in this figure, would you characterize CA_F7SDb01 as likely to belong to some *Bacteroides* species or likely to come from a different genus?
5. Write out a set of six short (seven or eight nucleotides) DNA sequences in which all six are related but there are two sets of three that are more closely related to each other than to the other set. Show how the guide tree might look for your sequences and then what the multiple sequence alignment might look like.

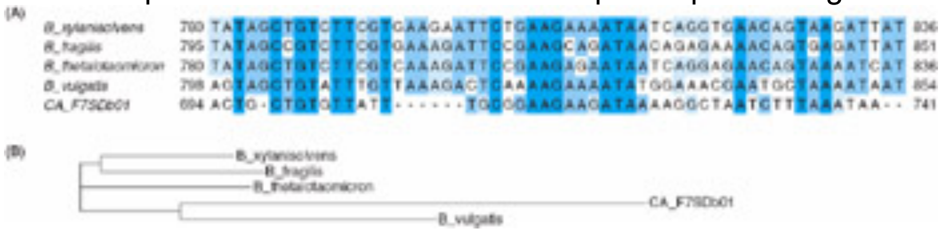


Figure 4.3: (A) Segment of a multiple sequence alignment for the coding region of a penicillin-resistance gene from five different species. Darker shading indicates nucleotides that are conserved among more of the five sequences. (B) Guide tree used by ClustalW to produce this multiple alignment. Data from—EBI ClustalW.

A	GTCT	**B**	GTCT	**C**	GTCT
	G-GT		G-GT		--CT
			CT--		-GGT

Figure 4.4: Multiple sequence alignment is complex because the order of adding sequences to the alignment can affect the alignment results.

Chapter Project—Horizontal Gene Transfer of Antibiotic Resistance

Salyers and her colleagues (see References and Supplemental Reading) used bioinformatics methods to look for evidence of horizontal transfer of antibiotic genes between bacteria found in animals routinely fed antibiotics and bacteria that might affect human health. Because of the enormous number of bacteria residing normally in the human large intestine, they hypothesized that these bacteria serve as a reservoir for HGT (**Figure 4.5**) and could easilyexchange genes with ingested bacteria, including antibiotic-resistant bacteria originating in agricultural animals. Thus, using alignment methods, Salyers focused on determining whether common intestinal bacteria might carry the *same* genes for antibiotic resistance as unrelated species that are not gut residents. Related species, of course, are likely to have highly similar genes, but a high degree of similarity between genes of otherwise *dissimilar* organisms strongly suggests horizontal transfer. Salyers used the criterion of ≥ 95% similarity to decide whether sequences from two organisms in fact represented the same gene. In this project, we will use BLAST to identify a set of resistance genes of interest and ClustalW to examine the similarity among them, enabling us to draw some conclusions about the impacts of subtherapeutic agricultural antibiotic use. We will focus on genes enabling bacteria to resist the antibiotic erythromycin, a drug commonly used in both therapeutic and agricultural applications.

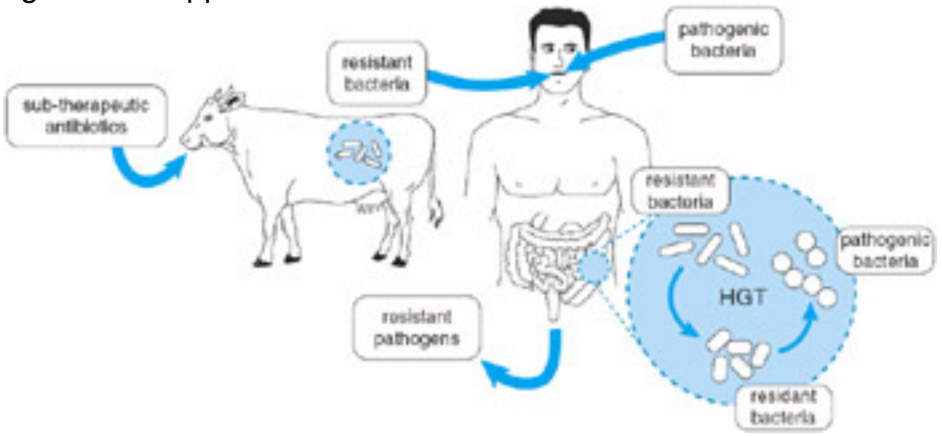


Figure 4.5: According to the "reservoir hypothesis" proposed by Salyers and others, resistant bacteria ingested in food that pass through the human large intestine have the opportunity to transfer resistance to any of the trillions of bacteria resident there, creating a reservoir of resistance, which could then lead to transfer to human pathogens.

Learning Objectives

- Understand the value of searching a database for sequences matching a query
- Gain experience with the use of BLAST in database searching and understand its parameters
- Appreciate the importance of a heuristic in processing large amounts of data rapidly
- Understand the use of multiple sequence alignment and know how to use ClustalW for this purpose

Suggestions for Using the Project

This project is designed to build skills in using two very important pieces of bioinformatics software: BLAST and ClustalW. Because of their wide use, familiarity with these tools is highly recommended for students in both programming and nonprogramming courses. The BLAST and ClustalW sections that follow can be used independently; instructors can down-load a set of *ermB* sequences from the *Exploring Bioinformatics* website if they would like their students to do the multiple alignment without first using BLAST to identify sequences of interest. Instructors could also ask students in programming courses to implement a BLAST-like algorithm based on the earlier discussion.

Searching for Erythromycin Resistance Genes with BLAST

Obtaining the ermB Sequence

Erythromycin is an antibiotic that halts bacterial growth by binding to the bacterial ribosome and blocking translation. Two different mechanisms of erythromycin resistance have been observed: Some resistant bacteria have acquired a gene whose product modifies the ribosome so erythromycin can no longer bind, whereas others have acquired a gene encoding a transport protein (called an efflux pump) that rapidly removes erythromycin from the cell. You already know how to find sequences in GenBank via a text search; however, a key word such as "erythromycin" will retrieve both kinds of genes and will fail to retrieve any resistance genes that were not annotated as such. Instead, using BLAST, we can search using a *sequence* as our query and retrieve all similar sequences, regardless of how they are annotated.

Download As our query sequence, we use an erythromycin-resistance gene called *ermB*from *Strep-tococcus agalactiae*, a Gram-positive bacterial species commonly associated with the udder of cows, where it can cause mastitis. This gene produces one of several known resistance proteins of the ribosome-modification type. Erythromycin resistance due to *ermB*has commonly been seen in the human pathogen *Streptococcus pneumonia*, the most common cause of bacterial pneumonia, so it will be interesting to determine whether HGT of this gene has occurred among diverse bacteria. Start by obtaining the DNA sequence for the*S. agalactiae ermB* coding region from GenBank in FASTA format by using a text search, by searching for the accession number DQ355148.1, or by downloading the file from the*Exploring Bioinformatics* website.

Understanding BLAST Results

BLAST results are shown in three sections. The top section is a graphical view (see sample of some representative BLAST results in **Figure 4.6A**), with a bar for each sequence that matches the query. The length of the bar shows the length(s) of the matching region(s), and its color represents the score for each segment. The middle section (**Figure 4.6B**) gives details about each match: the accession number and description for the gene matched and five parameters related to the quality of the match:

- **Max score**: the score of the best matching segment (remember, this is a local alignment, not a global one).
- **Total score**: the total scores of all matching segments found (same as max score if there is only one matching segment).
- **Query coverage**: the percentage of the query sequence that aligned to some part of the match.
- ***e*-Value**: a statistical measure evaluating how likely it is that a match this good would occur by chance. The lower the *e*-value, the more likely it is that the two sequences are truly similar and not just chance matches. Two identical sequences would have an *e*-value of zero.
- **Max ident**: the percentage of nucleotides that are identical between the query and target sequences within the matching regions.

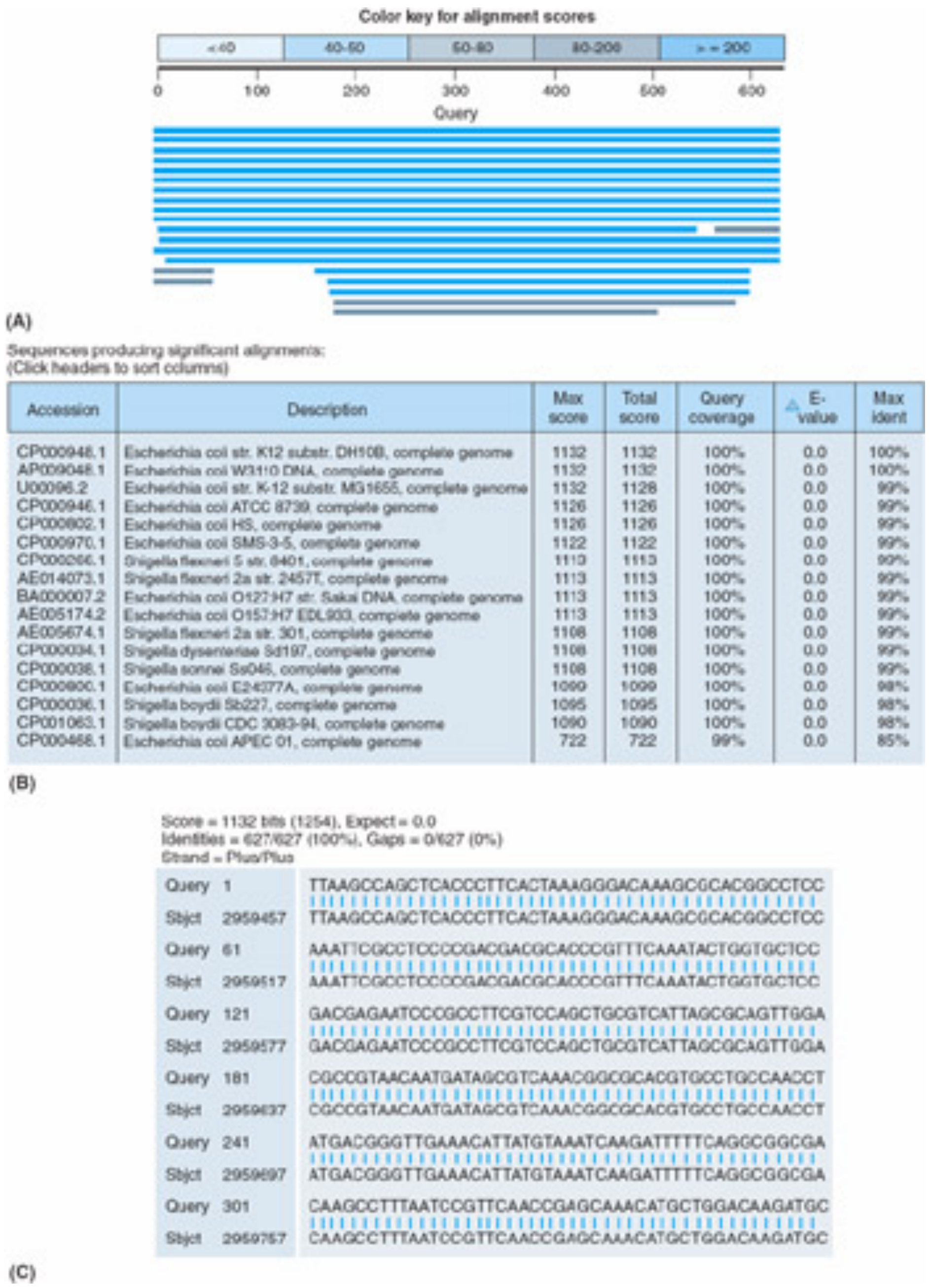
The third section (**Figure 4.6C**) shows the actual pairwise alignments between the query sequence and the top matching database sequences. Links in each section provide direct access to a variety of additional information about the matching sequences.

Identifying ermB Orthologs with BLAST

Link From the **NCBI BLAST home page**, you can see several ways to run BLAST, including both nucleotide and protein comparisons. For this exercise, we compare DNA sequences, so you should choose the nucleotide option. This should take you to a search form where you can either paste or upload your *S. agalactiae ermB* sequence.

Many options and parameters are available on this page. Notice the section labeled `Choose Search Set`, where you can specify the sequences to be searched. Importantly, the default set of sequences is the subset of GenBank containing human DNA sequences. This obviously will not work in our case, where we want to retrieve bacterial sequences. Change the database to `nucleotide collection (nr/nt)`, which will search all the unique ("nonredundant" or nr) sequences in GenBank. Furthermore, many sequences in GenBank are from bacteria that have been sequenced (using DNA harvested from an environmental sample) but never cultured; these are not useful to us because we do not know what species they come from, so check the box to exclude sequences from uncultured samples. To further refine the results, there is also an input box where you can limit your search to a particular organism or group of organisms; you could type `bacteria` here to exclude any nonbacterial sequences that might happen to match. Finally, there is a box where you can type an Entrez query to include or exclude specific kinds of sequences.

If you click `Algorithm parameters` near the bottom, you can set the parameters that BLAST uses for its comparison. These options should be starting to look familiar to you: For example, you can set a linear or affine gap penalty, change the match and mismatch scores, and alter the word size (*k*-tuple) for the initial match. Some of these parameters are set automatically when you make a choice from the `Program selection` section, where you choose the specific algorithm that will be used by selecting options such as `Highly similar sequences (megablast)` or `Somewhat similar sequences (blastn)`. With the parameters visible, try clicking each of these options and notice how the parameters change. For example, megablast has a default word size of 28, whereas blastn has a default of 11; how would this change the results? When you have finished exploring, choose `blastn`for now to see both very similar and less-similar sequences the program might identify. Click the `BLAST` button to start the search and compare your *ermB* sequence with the selected sequences. In a short time, you should get a page of results (see Figure 4.6 for an example of what this page would look like).



Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E-value	Max ident
CP000948.1	Escherichia coli str. K12 substr. DH10B, complete genome	1132	1132	100%	0.0	100%
AP009048.1	Escherichia coli W3110 DNA, complete genome	1132	1132	100%	0.0	100%
U00096.2	Escherichia coli str. K-12 substr. MG1655, complete genome	1132	1128	100%	0.0	99%
CP000946.1	Escherichia coli ATCC 8739, complete genome	1126	1126	100%	0.0	99%
CP000802.1	Escherichia coli HS, complete genome	1126	1126	100%	0.0	99%
CP000970.1	Escherichia coli SMS-3-5, complete genome	1122	1122	100%	0.0	99%
CP000266.1	Shigella flexneri 5 str. 8401, complete genome	1113	1113	100%	0.0	99%
AE014073.1	Shigella flexneri 2a str. 2457T, complete genome	1113	1113	100%	0.0	99%
BA000007.2	Escherichia coli O127:H7 str. Sakai DNA, complete genome	1113	1113	100%	0.0	99%
AE005174.2	Escherichia coli O157:H7 EDL933, complete genome	1113	1113	100%	0.0	99%
AE005674.1	Shigella flexneri 2a str. 301, complete genome	1108	1108	100%	0.0	99%
CP000034.1	Shigella dysenteriae Sd197, complete genome	1108	1108	100%	0.0	99%
CP000038.1	Shigella sonnei Ss046, complete genome	1108	1108	100%	0.0	99%
CP000800.1	Escherichia coli E24377A, complete genome	1099	1099	100%	0.0	98%
CP000036.1	Shigella boydii Sb227, complete genome	1095	1095	100%	0.0	98%
CP001063.1	Shigella boydii CDC 3083-94, complete genome	1090	1090	100%	0.0	98%
CP000468.1	Escherichia coli APEC 01, complete genome	722	722	99%	0.0	85%

(B)

```
Score = 1132 bits (1254), Expect = 0.0
Identities = 627/627 (100%), Gaps = 0/627 (0%)
Strand = Plus/Plus

Query  1        TTAAGCCAGCTCACCCTTCACTAAAGGGACAAAGCGCACGGCCTCC
                ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2959457  TTAAGCCAGCTCACCCTTCACTAAAGGGACAAAGCGCACGGCCTCC

Query  61       AAATTCGCCTCCCCGACGACGCACCCGTTTCAAATACTGGTGCTCC
                ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2959517  AAATTCGCCTCCCCGACGACGCACCCGTTTCAAATACTGGTGCTCC

Query  121      GACGAGAATCCCGCCTTCGTCCAGCTGCGTCATTAGCGCAGTTGGA
                ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2959577  GACGAGAATCCCGCCTTCGTCCAGCTGCGTCATTAGCGCAGTTGGA

Query  181      CGCCGTAACAATGATAGCGTCAAACGGCGCACGTGCCTGCCAACCT
                ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2959637  CGCCGTAACAATGATAGCGTCAAACGGCGCACGTGCCTGCCAACCT

Query  241      ATGACGGGTTGAAACATTATGTAAATCAAGATTTTTCAGGCGGCGA
                ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2959697  ATGACGGGTTGAAACATTATGTAAATCAAGATTTTTCAGGCGGCGA

Query  301      CAAGCCTTTAATCCGTTCAACCGAGCAAACATGCTGGACAAGATGC
                ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2959757  CAAGCCTTTAATCCGTTCAACCGAGCAAACATGCTGGACAAGATGC
```

(C)

Figure 4.6: Sample results of a BLAST search for database sequences matching a nucleotide query sequence—(A) graphical summary of results, (B) table of scores, and (C) alignments.

Web Exploration Questions

1. In their original survey, Salyers and colleagues used a cutoff of 95% identity for sequences considered similar enough to have been shared by HGT. You can get a quick measure of identity by using the `max ident` score in the BLAST results—however, you can also get a high max ident for a very small matched region, so also consider the query coverage. Looking at these parameters, are the matches that BLAST retrieved highly similar to your query or less similar? Do

your data suggest that all or most of them represent the same gene, transferred from organism to organism by HGT?

2. You may notice in your list that a number of the sequence matches come from cloning vectors— engineered DNA molecules used for laboratory manipulations. Construct an Entrez query to exclude these from your results and run your search again—but be careful not to exclude too much. Remember that unless you limit the field, the entire text of each entry will be searched for a match. What query did you use?
3. What evidence can you find among your BLAST results to support or refute the hypothesis that resistance genes are being shared between unrelated species—especially between agricultural species and human pathogens or human gut bacteria that might come into contact with pathogens? You will have to do some detective work to answer this question: For example, find a bacterial phylogenetic tree online to help you decide how closely related the different species in your list are, and then try to find out which ones might be found in domestic animals, which are residents of the human gut, and which are human pathogens.
4. There are so many sequences in GenBank today, including many whole genome sequences, that BLAST often fills up its list of top matching sequences without ever getting down to less related but potentially more interesting matches. In your initial BLAST results, for example, it is likely that most if not all sequences come from Gram-positive organisms, one major division of the bacteria. HGT to the more distantly related Gram-negative organisms would be very interesting but is hard to assess from this list. Construct a BLAST search that excludes Gram-positive matches. Or, another way to get interesting results might be to require matches to specific groups of Gram-negative organisms that you know live in the human gut, such as *Bacteroides* (the most common genus among human gut bacteria) or *Escherichia*. Be careful to exclude from consideration sequences that come from cloning vectors in this case—you only want sequences naturally found in these bacteria. Describe how you searched, the similarity of your results to the query, and whether the percent identity suggests that your results represent horizontally transferred genes or genes arising by mutation.
5. Based on your results thus far, would you say that you have evidence for (a) extensive HGT, (b) a mix of HGT and evolution by mutation, (c) evolution mostly by mutation with occasional HGT, or (d) a number of unrelated resistance genes? Support your answer with evidence.

Retrieving Sequences

In the next section, we will carry out a multiple alignment of some *ermB* genes from different species, which requires retrieving their sequences in FASTA format. The NCBI implementation of BLAST includes a number of useful tools for working with the sequences it finds, including a means of quickly retrieving the ones in which you are interested. Checkboxes next to the sequences BLAST aligned allow you to select interesting matches; chose some that are from different genera, from human pathogens or gut organisms, from Gram-negative organisms, and so on. Then, you should see a download link allowing you to retrieve the sequences in FASTA format. You can combine the results of several searches simply by downloading each set and then cutting and pasting in the resulting text files. Compile a file with several interesting sequences that you can go on to align with ClustalW.

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Multiple Sequence Alignment with ClustalW

Although you were able to get some information about the similarity of many sequences to your query sequence from your BLAST results, you undoubtedly noticed that BLAST still only made pairwise comparisons: It showed alignments between your query and one other sequence at a time. When comparing many sequences, it can be much easier to analyze the results when all alignments can be visualized at once. Furthermore, some questions might be better answered by aligning a group of sequences: for example, to ask if there are particular regions of the sequences that are more or less conserved. ClustalW is an example of a multiple sequence alignment program designed for this purpose; sample output is shown in Figure 4.3A.

Download For this part of the project, you will need a text file containing the sequences of at least six to eight sequences similar to *ermB* in FASTA format. You should have all your sequences in a single file, separated by their comment lines; be sure you have the coding regions only. If your class did not do the BLAST part of the project, your instructor can download a file with some interesting sequences from the *Exploring Bioinformatics* instructor website and make it available to you.

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When the results are returned, you will see the alignment in simple text format, with asterisks below the alignment wherever a particular nucleotide is found in all sequences. You can view the guide tree by clicking the appropriate tab, and the `Result Summary` tab shows the results of the individual pairwise alignments that were done. A more sophisticated presentation can be obtained by using Jalview, a Java-based viewer: click the `Result Summary` tab and then click `Start Jalview`. Here, you can see a consensus sequence representing the most conserved nucleotides at each position, and you can format and color the alignment in various ways. A convenient way to visualize differences among the sequences is by selecting `Percentage Identity` from the `Colour` menu; this gives a dark background for nucleotides conserved in all sequences and lighter colors for nucleotides conserved in fewer sequences.

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Web Exploration Questions

6. Which *ermB*-like genes are the most similar? Which are less similar? Are there particular regions of the gene that are highly conserved or less conserved?
7. What kinds of differences can you see among these genes? Do substitutions outnumber indels or vice versa? What do you notice about the indels that occur in the alignment?
8. Try running ClustalW again with a very low gap penalty. Do the alignments change significantly? Which alignment is more biologically relevant, and what is your evidence for this view?
9. Based on the criterion of closely related genes from unrelated organisms, do your results support the HGT hypothesis?

How would you summarize your findings and conclusions regarding the likelihood that agricultural use of antibiotics can result in resistant human gut residents and/or resistant human pathogens? Your instructor may ask you to write up your findings in the form of a short report.

BioBackground: Antibiotic Resistance and Gene Transfer

Bacteria can have **natural (intrinsic) resistance** to some antibiotics because of their cell structure. For example, Gram-negative bacteria (such as the common intestinal organism*Escherichia coli*) are resistant to penicillin simply because the cell wall that penicillin attacks is protected by an outer membrane that other bacteria lack. But the resistance that is really important medically is **acquired resistance**: when bacteria that were previously sensitive to (killed by) an antibiotic become resistant to it, making that antibiotic useless for treatment. Acquired resistance requires genetic change: Either a new gene or new variant of a gene arises by mutation or a cell acquires a preexisting gene by horizontal transfer.

Many people have the idea that using an antibiotic "makes" bacteria resistant. This is not true, however: Antibiotics do not *cause* resistance to occur (nor is it true that antibiotic use makes the *person* resistant to the antibiotic). However, antibiotic use can **select** for bacteria that have already become resistant, allowing them to become more prevalent in a population. As shown in **Figure 4.7**, if some bacteria in a population are more resistant to an antibiotic than others (due to mutation or to genes they have acquired), they will not be killed as easily when they encounter it. Thus, the antibiotic kills the most sensitive cells first and leaves the more resistant ones to pass their genes on. This can happen in your own body if you do not finish your antibiotic prescription: The most resistant cells remain alive and can then multiply and cause a relapse. The more we expose bacteria to antibiotics—whether in the body, in animals, or in the environment—the more we select for resistant organisms and thus the more prevalent the resistant bacteria become.

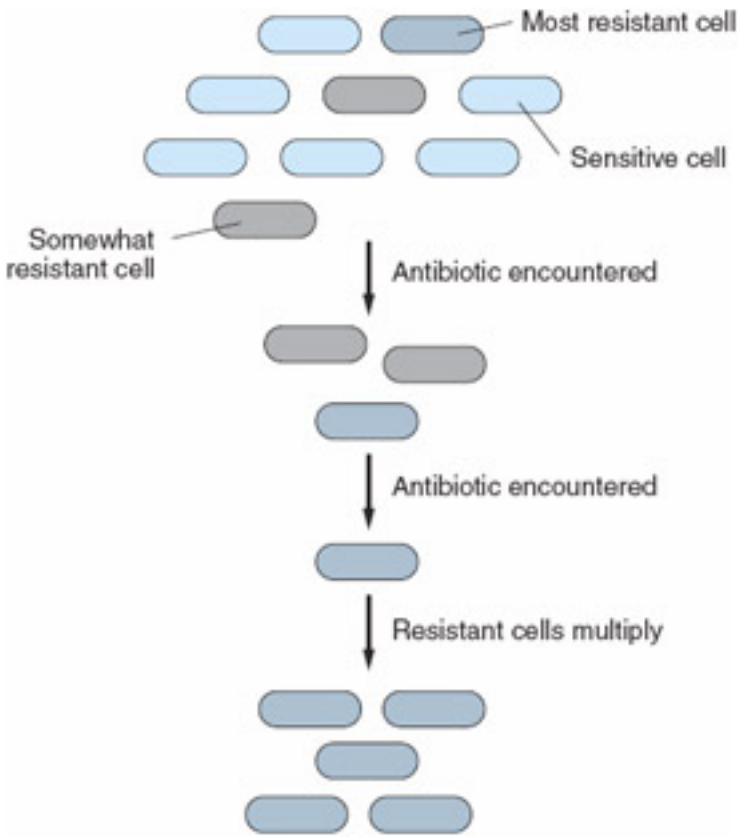

Figure 4.7: How exposure to antibiotics selects for the survival of resistant cells in a population of bacteria.

If a mutation gives a bacterial cell some advantage—and antibiotic resistance is just one of many possible examples—that cell's descendants inherit the altered gene. This is sometimes called **vertical gene transfer** (**Figure 4.8**, left panel) and could lead to increased resistance by selection if the population is challenged by an antibiotic. However, mutations are relatively rare, and resistance would develop slowly if bacteria had to rely on inheriting a rare mutation from their parents. A major reason for the rapid spread of resistance is that bacteria can also acquire genes by HGT. This refers to genetic material being transferred from one cell to another that is not its descendant (Figure 4.8, right panel). For example, many antibiotic resistance genes are carried on plasmids: small, circular, independent DNA molecules. A cell with a resistance plasmid can often transfer that plasmid to nonresistant cells around it, so that the resistance gene is passed not only to a cell's descendants but to its peers and to their descendants. Depending on the circumstances, this transfer could occur by cell-to-cell contact (conugation), by means of a bacterial virus (transduction), or by direct uptake of DNA released into the environment (transformation). Antibiotic resistance genes are also often found within transposons, semi-independent DNA sequences that can move within a genome, further promoting their mobility.

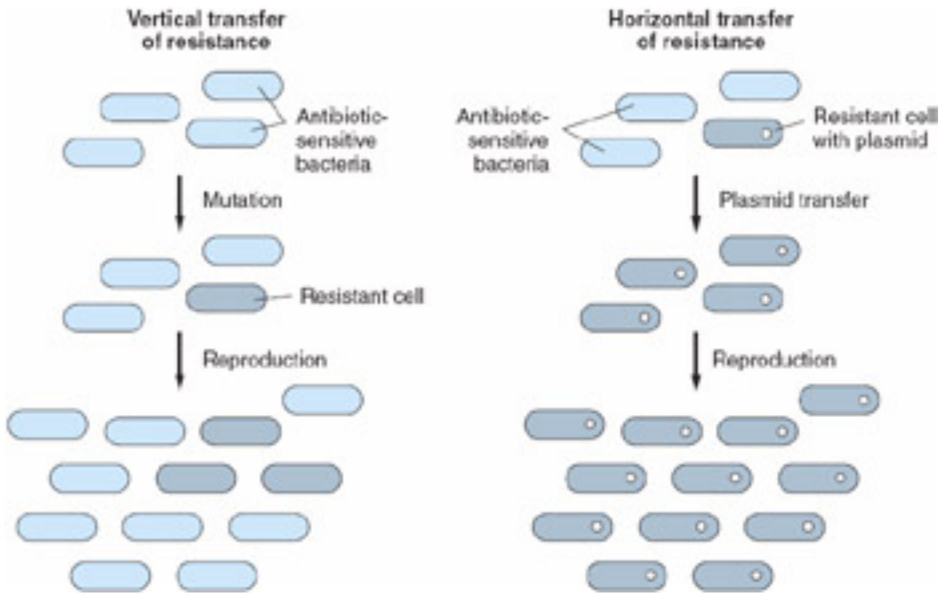
Vertical transfer of resistance
Antibiotic-sensitive bacteria
Mutation
Resistant cell
Reproduction
Horizontal transfer of resistance
Antibiotic-sensitive bacteria
Resistant cell with plasmid
Plasmid transfer
Reproduction

Figure 4.8: Vertical gene transfer occurs when a cell passes a resistance mutation to its offspring (left); horizontal transfer from cell to cell (right) allows much faster spread of resistance.

As discussed in the preceding chapter, when the sequences of two genes are similar, we conclude that they have a common origin; furthermore, we assume that highly similar genes diverged from that common origin only recently and have not had much time to evolve independently. Two very similar sequences found in dissimilar organisms—those that do not have a recent common ancestor—suggest that HGT has occurred: The gene evolved in one species but was then transferred intact to another relatively recently, so there has been limited opportunity for mutation.

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Chapter 7: Tree-Building in Molecular Phylogenetics: Three Domains of Life

Chapter Overview

Measuring evolutionary distance from a sequence alignment is only half the problem in phylogenetics. Given a complex dataset, a set of pairwise distance measurements can likely be compiled into any number of distinct trees. This chapter deals with the key problem of tree-building: how to use computational methods to obtain biologically relevant groupings of species in a phylogenetic tree. The value of a phylogenetic tree is in what we learn about evolution by observing groups (clades) with a common ancestor; we generate these groups computationally by means of what computer scientists refer to as clustering algorithms and/or by methods that search through possible trees to identify an optimal solution. The projects in this chapter will help students in both programming and nonprogramming courses understand how distance metrics we have already discussed are used by clustering algorithms to group related organisms. Through the use of Web-based tools, students will develop phylogenetic trees using both distance-based and character-based methods. Students in programming courses will develop their own solutions that implement two important distance-based algorithms.

Biological problem: Origins of genes in the bacteria, eukaryotes, and archaea

Bioinformatics skills: Agglomerative clustering, single linkage, UPGMA, neighbor joining, probabilistic methods in phylogenetics

Bioinformatics software: MUSCLE, Gblocks, BioNJ, PhyML, MrBayes (all at Phylogeny.fr), UPGMA

Programming skills: Hash table and nested hash table data structures

Understanding the Problem: Rooting the Tree

In 1977, Carl Woese initiated a revolution in how biologists think about the living world. As phylogenetic thinking came to dominate systematics and taxonomy, evolutionary relationships among living organisms became the paramount criterion for classification. By the late 1960s, the "five-kingdom" system came into popular use (and sadly is still taught in many high-school curricula today): Linnaeus' plant and animal kingdoms, which obviously contained unrelated organisms, were divided into five kingdoms: plants, animals, fungi, protists, and bacteria. However, biologists also recognized the fundamental distinction in cell structure between the prokaryotes (bacteria) and eukaryotes (everything else). The waters were further muddied by the recognition that some prokaryotes living in extreme environments had rather different structures. With the advent of DNA sequencing and molecular phylogeny based on the universal 16S rRNA genes, Woese was able to recognize that these prokaryotes were as evolutionarily distant from the bacteria as the bacteria are from the eukaryotes. He *proposed a higher level of classification, and we now recognize three* ***domains*** **of living things (Figure 7.1**

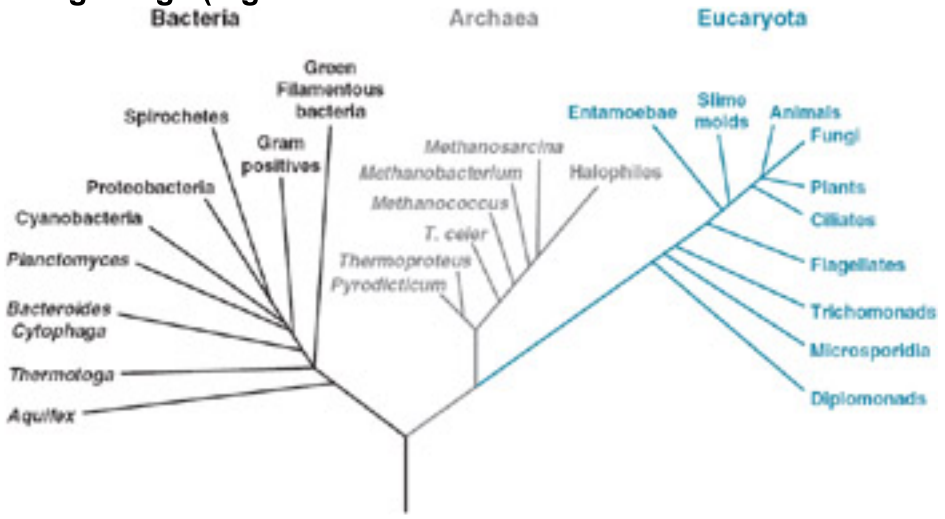

Figure 7.1: Phylogenetic tree for representatives of the three domains of life based on analysis of 16S rRNA sequences.

The impetus for Woese's phylogenetic look at the prokaryotic world came from the growing recognition that certain prokaryotes found in hot springs, acidic pools, salt marshes, and other harsh environments were structurally very different from the more familiar bacteria that inhabit more temperate realms as well as our own bodies. They have cell walls like other prokaryotes but lack peptidoglycan, the carbohydrate universally present in all previously known bacteria. Their DNA is wrapped around histone proteins like the DNA of eukaryotic cells. Furthermore, they have some unique features of their own, like double-ended lipids that span their membranes. Many microbiologists believed these prokaryotes could represent the modern remnants of the ancestors of all living things; they were therefore termed "arachaebacteria" and later renamed the archaea to emphasize that they are unlike the bacteria. Once they were established as a distinct group, researchers soon began finding archaea everywhere, even in the human gut, and recognized their importance in the environment and in the evolution of life on earth.

However, do the archaea really tell us what the original living cells were like? Some researchers still believe archaea represent the oldest evolutionary line, but that conclusion is far from clear. It is certain that the archaea have been around at least as long as the oldest bacteria, that their ability to survive in extreme environments suggests adaptations that would have been essential in the harsh conditions of the early earth, and that their genes clearly distinguish them from both bacteria and eukaryotes. Much remains to be learned about these organisms, and the application of bioinformatic methods to uncover their origins has led to some surprising results. We examine the relationships of the archaea to the other domains in this chapter's projects.

Bioinformatics Solutions: Tree-Building

Looking at the relationships of the three domains of life using bioinformatic methods will require us to align orthologous genes and produce phylogenetic trees based on that information. In Chapter 6, we introduced the idea of a molecular clock and the value of molecular and bioinformatic methods in investigating evolutionary relationships. We considered how sequence diversity can be related to evolutionary distance and how various distance metrics can be applied to sequence alignments to model the evolutionary pathways that led to the observed substitutions. Ideally, any gene shared by two groups could be used to determine the evolutionary distance between them, but in practice different sequences have different functional constraints, and we sometimes find evidence of unexpected behavior over evolutionary time.

If you completed the Web Exploration exercises in Chapter 6, you even used these distance measures to draw a phylogenetic tree to show relatedness among mammals. Tree-building, however, is more complicated than using distance measures to draw a phylogenetic tree to show relatedness among mammals—or the rapid production of an attractive tree by the suite of programs at Phylogeny.fr—might lead you to believe. Given only four species, we can draw three different unrooted trees to show the relationships among them (**Figure 7.2A**). Distance data might help us choose one of these three, but in each case we can draw five rooted trees (**Figure 7.2B**), each maintaining the species relationships found in the unrooted tree but showing a unique evolutionary pathway. This means there are 15 different possible trees altogether for the four species. For 10 species, there are more than 2 million possible trees, and by the time we get to 50 species, there are a stunning 10^{74} possible trees. Thus, what phylogeneticists call "tree space" is intractably complex unless the dataset is extremely small; exhaustively drawing each possible tree and comparing it with the data is impossibly computationally intensive. Given that our goal is to construct a tree that represents biological reality by reconstructing to the extent possible the actual pathway of evolution, algorithms that are both computationally efficient and able to select an appropriate tree according to meaningful criteria are essential.

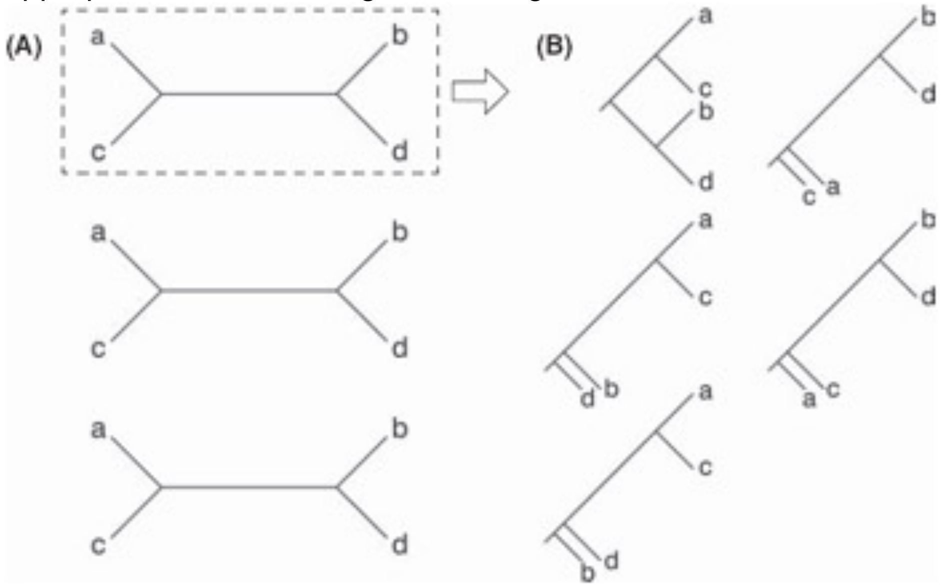

Figure 7.2: Possible phylogenetic trees for four species. (A) Three possible unrooted trees, showing relationships between species but not evolutionary pathways. (B) For the

top unrooted tree, five possible rooted trees that preserve branch lengths and show evolutionary pathways.

Link All tree-building methods depend on a multiple sequence alignment of the genes being considered. This is in itself a computationally difficult problem; Chapter 4 discussed heuristic methods by which ClustalW arrives at an alignment efficiently. It is then common for experienced researchers to examine the alignment by eye and make small adjustments, particularly to the positions of gaps. For example, the multiple alignment output might include a three-nucleotide gap in all the sequences, but that gap might be shifted left or right by a base or two in some sequences relative to others, when aligning the gaps would yield a better alignment overall. A multiple alignment editor such as **Jalview** (included in Phylogeny.fr's implementation of **MUSCLE** and the EBI implementation of **ClustalW**) or the desktop program **BioEdit** can be used for making these adjustments. Gapped positions can then be removed from the alignment using a program such as **Gblocks** (see Chapter 6). The result is a multiple alignment where every mismatched nucleotide or amino acid should represent (at least if our alignment algorithm is sufficiently good) the result of a substitution over evolutionary time.

There are two general ways in which bioinformatic programs can then attempt to select an optimal tree from the sequence data. **Distance-based** methods, as their name implies, apply a distance metric to the sequences and then use some form of **clustering algorithm** to decide how species should be grouped based on those distances. The UPGMA and neighbor-joining (NJ) algorithms are commonly used in distance-based methods; we explore those methods in detail in this chapter. **Character-based** methods are more probabilistic: They apply some model of evolution and then attempt to find the highest probability tree given that model and a particular dataset (alignment). For example, some models use parsimony: they apply the principle of Occam's razor ("the simplest explanation is the best one") and propose the evolutionary pathway that requires the fewest independent mutation events to generate the observed substitutions as the best one. Algorithms using Bayesian statistics to find an optimal tree are currently widely used in character-based methods. We do not specifically discuss character-based algorithms in this chapter (a comprehensive introduction to tree-building methods is beyond the scope of this text) but do use these methods in the Web Exploration.

BioConcept Questions

1. If all five rooted trees in Figure 7.2B are equivalent to the unrooted tree inFigure 7.2A, why is it so important to develop an algorithm for choosing among them? Describe in evolutionary terms in what important ways these trees are different.
2. Various distance metrics attempt to model what happens biologically as DNA mutates over evolutionary time. Yet, many researchers choose to use character-based tree-building methods that essentially ignore any calculation of distance. What limitations do you see in distance metrics that might keep us from accepting distance-based methods as the single best approach?
3. The distance metrics used in Chapter 6 apply specifically to nucleotide sequences. In this chapter's exercises, we use amino-acid sequence alignments

as the basis for tree-building, and you may notice that we do not explicitly discuss distance metrics. In what way is a distance metric implicit in the alignment of protein sequences?

4. Suppose you are studying a group of organisms that are genuinely descended from a common ancestor and have many orthologous genes. Given a relatively constant rate of mutation and a relatively even distribution of mutations across the genome, we would expect that *any* of the orthologous genes could be used to construct a phylogenetic tree and that whatever gene we picked would give essentially the same results. It turns out, however, that not all genes are equal in terms of phylogenetic analysis. What factors can you think of that might account for differences between genes?

Understanding the Algorithm: Clustering Algorithms

Learning Tools

Understanding clustering algorithms is one key idea in this chapter. To help with this, the*Exploring Bioinformatics* website has a link to a visual, interactive clustering simulation.

The goal of a phylogenetic tree is to reveal the evolutionary relationships among organisms, allowing us to classify (group) them according to genuine relatedness rather than superficial similarity. Thus, building a phylogenetic tree from a sequence alignment is in essence just grouping sequences according to their similarity as a means of inferring the evolutionary groupings of species. Whenever objects need to be grouped, computer scientists use clustering algorithms, which simply determine which objects are most similar and should be included in a group and which are less similar and should be excluded. Hierarchical clustering (**Figure 7.3**) is appropriate for a phylogenetic tree, because it places the most similar objects in groups and then relates those groups into larger clusters and then still larger ones—very much like the idea of common ancestors giving rise to broad groups of species that can then be subdivided into smaller groups with their own common ancestors. Specifically, we use a form of hierarchical clustering called agglomerative clustering that begins with individual objects (sequences representing species, in our case) and then merges the clusters until a single large group is formed.

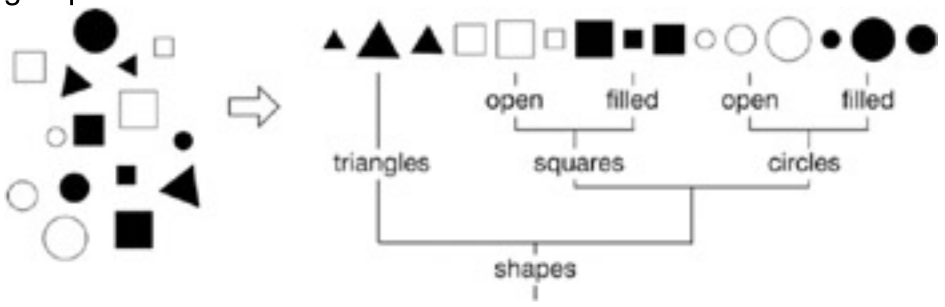

Figure 7.3: Example of hierarchical clustering. This is also agglomerative clustering if we start by grouping similar individual objects rather than by dividing the whole collection.

You know something about how to find the distances between individual sequences; clustering also requires a **linkage method**, which determines how the distance metric is applied when two *groups* are compared. After computing distances there is a **merge step**, in which those groups shown to be most closely related are brought together. The outcome of clustering is the information needed to draw the phylogenetic tree.

Let's use a small dataset as an example: Suppose we want to construct a tree for six species (A–F) that all diverged from a common ancestor. The most closely related species diverged from each other most recently and thus share a more recent common ancestor. After choosing an orthologous gene, aligning sequences, and applying a distance metric (remember that clustering is a distance-based method), we can construct the matrix shown in**Figure 7.4A** to show the distances between each pair of sequences. An agglomerative clustering algorithm works by sequentially merging the most closely related elements into**clusters** (or groups) until only one cluster remains. It starts with each individual element in its own cluster, and at each iteration the two closest clusters are determined and merged; for *n*elements, *n* - 1 iterations are required to complete the clustering. The key question we have not dealt with before is how to measure the distance between two clusters or between an individual element and a cluster. This is the linkage method, and we can choose from several linkage methods, depending on our assumptions about the data (**Figure 7.5**). **Single linkage**calculates the distances between each item in one cluster and each item in the other and chooses the smallest distance; it is suitable for elements that are not very tightly grouped.**Complete linkage** is the opposite: The largest individual distance value is chosen, which works best when the items are tightly grouped. **Centroid linkage** uses the distance between the centers of the clusters. The steps that follow show how the agglomerative clustering algorithm would produce a tree from the distances given in Figure 7.4, using the single linkage method.

A. Open table as spreadsheet

	A	**B**	**C**	**D**	**E**	**F**
A	0					
B	1	0				
C	3	2	0			
D	7	6	4	0		
E	17	16	14	10	0	
F	19	18	16	12	2	0

B. Open table as spreadsheet

	AB	**C**	**D**	**E**	**F**
AB	0				
C	2	0			
D	6	4	0		

	AB	C	D	E	F
E	16	14	10	0	
F	18	16	12	2	0

C. Open table as spreadsheet

	ABC	D	E	F
ABC	0			
D	4	0		
E	14	10	0	
F	16	12	2	

D. Open table as spreadsheet

	ABC	D	EF
ABC	0		
D	4	0	
EF	14	10	0

E. Open table as spreadsheet

	ABCD	EF
ABCD	0	
EF	10	0

Figure 7.4: Agglomerative clustering for six hypothetical species. (A) Distances between pairs of aligned sequences. (B–E) Successive iterations of the agglomerative clustering algorithm, merging the two closest clusters each time. Distances resulting from application of the single linkage method are shown in color.

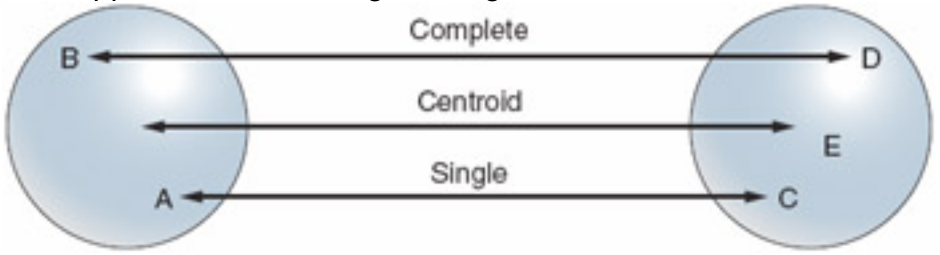

Figure 7.5: Three different linkage methods that could be used to compute the distance between two clusters.

Algorithm

Agglomerative Clustering Algorithm

1. Determine distances between sequences by alignment and a distance metric; for *n* sequences, create an *n* by *n* matrix of distance scores (Figure 7.4A).

Each row and column of the matrix is a cluster, and each cluster currently contains just one element.

2. Ignoring the diagonal, find the cell that contains the smallest distance (representing the closest elements, in this case A and B) and group those elements to form one cluster. There are now *n* – 1 clusters. This is the merge step.
3. Redraw the distance matrix with the merged cluster (**Figure 7.4B**). Use the linkage method to determine the distance between the cluster and the other sequences. The distance from A to C is 3, and the distance from B to C is 2, so using the single linkage method, we choose the smallest and say that the distance from the cluster (AB) to C is 2. This calculation is repeated for the distance from (AB) to D, E and F. The distances resulting from the linkage calculation are shown in color in the figure.
4. Repeat steps 2 and 3 until only one cluster remains. In Figure 7.4B, we can see that both (AB) to C and E to F have a distance of 2, so we have to arbitrarily choose one to merge. If we choose to merge (AB) with C and again recalculate distance with the single linkage method, we get the matrix shown in **Figure 7.4C**. The next merge gives the matrix in **Figure 7.4D** and then the one in**Figure 7.4E**. The last step is to merge the two remaining clusters.

Now, how does this process relate to a phylogenetic tree? We can see the relationship better if we represent the clustering process in a computer-friendly conventional format known as**Newick format**. We first merged A and B, so we represent them with `(A,B);`. This cluster then merged with C and then eventually with D, which can be represented by`(((A,B),C),D);`. E and F merged with each other but not with any of the rest, so the final outcome is `((((A,B),C),D),(E,F));`. This very condensed representation of the data can be used to draw the cladogram in **Figure 7.6**. Each cluster has a common ancestor: A and B have the common ancestor shown by the internal node at *y; x* represents the common ancestor of A, B, and C; and so on. Notice that E and F have a common ancestor, *z*, but share no common ancestry with any of the other species except at the root of the tree, *v*.

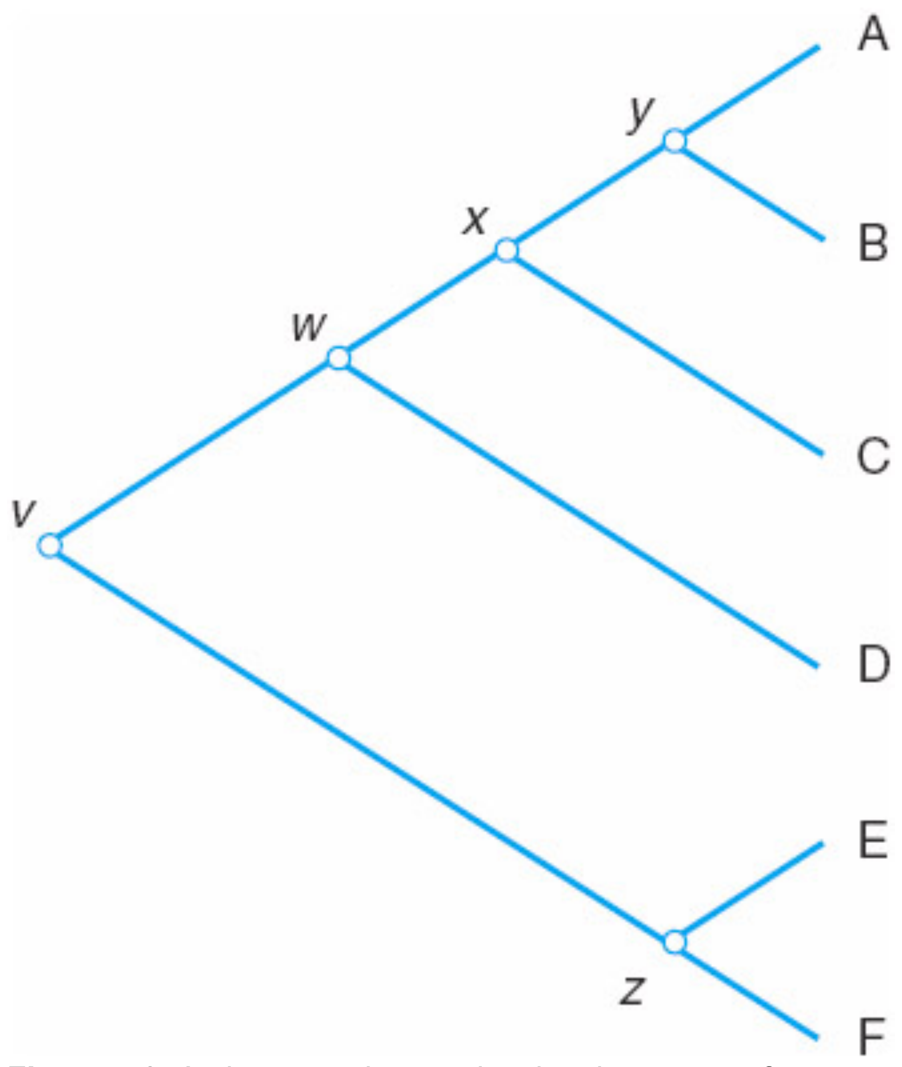

Figure 7.6: A phylogenetic tree showing the results of agglomerative clustering for six hypothetical species.

The agglomerative clustering algorithm is used in many distance-based methods for calculating phylogenetic groupings. One of the first widely used tree-building methods applied agglomerative clustering with a linkage method called **UPGMA** (Unweighted Pair-GroupMethod with Arithmetic Mean), which calculates the distance between two clusters by averaging the distances (arithmetic mean) between each species in the cluster and every species in the other cluster. (UPGMA is in practice much like the centroid linkage illustrated inFigure 7.5 as far as clustering of sequences is concerned.) This method assumes a constant rate of evolution, so each species in a cluster contributes equally to the new cluster value (unweighted). In the previous example, UPGMA would have given the distance from cluster (AB) to C as the average of the distances A–C (3) and B–C (2), or 2.5. More generally, if *x*and *y* are clusters containing *n* and *m* elements, respectively, and if x_i represents the *i*th element in cluster x and y_j represents the jth element in cluster y, the distance between the clusters is

$$d_{xy} = \frac{\sum_{i=1}^{n}\left(\sum_{j=1}^{m} d\left(x_i y_j\right)\right)}{n \times m}.$$

In the Web Exploration and the Guided Programming Project, we look at the use of agglomerative clustering with UPGMA to build a distance-based tree. The same basic algorithm is also the basis for the NJ method discussed in the Web Exploration and the On Your Own Project. In the Web Exploration, we also look at some character-based methods that employ probabilistic models to find optimal trees.

Test Your Understanding

1. Given aligned sequences for four species with distances W-X = 1.8, W-Y = 0.8, W-Z = 2.4, X-Y = 1.8, X-Z = 2.4 and Y-Z = 2.4, cluster the sequences using single linkage and show the result in Newick format.
2. Apply the clustering algorithm to the distance data that you calculated for whales and their relatives in Chapter 6 (Chapter 6 Web Exploration exercise 6, 7, or 8). Do you get the same groupings as in the tree you drew from those data (Chapter 6 Web Exploration exercise 9)?
3. Try the UPGMA linkage method instead of the single linkage method for our sample dataset presented previously in Understanding the Algorithm. Do you get the same groupings? The same distances?
4. In the sample dataset used in Understanding the Algorithm, at the second merge we had a choice of either merging cluster (AB) with C (which we chose to do) or merging clusters E and F (which we ignored); both choices had a distance value of 2. Use the clustering algorithm to determine how the tree would have come out if we had chosen E and F instead. Would it have been different? Would this always be the case? In other words, does the arbitrary choice of one grouping when there are two possibilities have the potential to affect our view of the evolutionary relationships?
5. The tree in Figure 7.6 is drawn as a cladogram, not a phylogram: that is, the branch lengths are not strictly proportional, although the evolutionary pathways are shown correctly. Try putting branch lengths onto the tree, using the data in Figure 7.4A. What problem do you encounter? How would you explain this difficulty, biologically? (*Hint: what assumption are we implicitly making when we calculate distances between clusters?*) In the On Your Own Project, you will see how the NJ algorithm deals with this important complication by changing the way the distances between clusters are calculated.

Chapter Project: Placing the Archaea in the Tree of Life

Learning Objectives

- Understand how groups of organisms are clustered to develop a phylogenetic tree
- Recognize the difficulty of choosing a "best" phylogenetic tree and various approaches to that problem, including distance- and character-based methods
- Gain experience using Web-based software to develop trees using different algorithms
- Understand how molecular phylogenetics can help unravel relationships among the three domains of living things
- Identify some potential pitfalls of molecular phylogeny

Suggestions for Using the Project

This project is designed to be used either in courses that require programming skills or in nonprogramming courses. Following are suggestions for modules of the project that instructors might choose to use in these two types of courses. Instructors should also feel free to ask questions of their own that use these same skills.

Programming courses:
- Web Exploration: Gain experience with Web-based tools to build phylogenetic trees from sequence data, compare various tree-building methods, and develop a set of sequences for use with the programming projects.
- Guided Programming Project: Implement a clustering algorithm and extend the solution to give a workable program to determine phylogenetic relationships using the UPGMA method.
- On Your Own Project: Implement the NJ method to deal with unequal rates of evolution, and compare the results with the UPGMA method.

Nonprogramming courses:
- Web Exploration: Gain experience with Web-based tools to build phylogenetic trees from sequence data, and compare various tree-building methods.
- On Your Own Project: Identify modifications to the clustering algorithm that would allow for unequal rates of evolution; compare trees built by UPGMA and by NJ.

Web Exploration: Molecular Clocks and the Archaea

As described previously, due to molecular phylogenetics we realized that the diverse species of archaea in fact represented a coherent clade and that the archaea as a group are as different from the bacteria as they are from the eukaryotes. Many questions remain unanswered, however, including what the archaea might tell us about the origins of life on earth. Their adaptation to extreme environments (like the harsh conditions of 4 billion years ago) and the finding that their structures are similar in some ways to bacteria but in others to modern eukaryotes has suggested to some researchers that the archaea might be the closest living relatives of the first living things.

However, interpretation of the molecular data is not always straightforward. In this project, we develop a phylogenetic tree using representatives of the three domains, examine the effect of different tree-building methods, and then look at what happens when different "clock" genes are used. The Phylogeny.fr site will be our primary tool for this exercise, because it provides a convenient and consistent framework for using several different phylogenetic tools.

Developing the Dataset

Download We need a sequence alignment to serve as the basis for our phylogenetic tree, and that means we need a molecular clock—in this case, a gene conserved across all three domains. It might surprise you to learn that humans and bacteria have recognizably similar proteins, but indeed they do. A good example is an accessory factor involved in the translation process that helps bring amino acid–carrying tRNA into the ribosome. This protein is called EF-1α in eukaryotes and EF-Tu in prokaryotes but is structurally and functionally similar in both: a good example of a protein conserved all the way from bacteria to humans and thus a suitable molecular clock for comparing species across all three domains of life. Because we are looking at such long time spans and because DNA sequences change faster than protein sequences, we use the EF-1α/EF-Tu *protein* rather than the DNA sequence of its gene.

Link Start with a file of representative sequences. For the eukaryotes, let's use human and yeast (*Saccharomyces cerevisiae*) EF-1α. Search the NCBI **Protein** and/or **Gene** databases for these proteins or download them from the *Exploring Bioinformatics* website. Save the sequences in FASTA format in a single text file, separated by the comment lines for each sequences (no blank lines). Change the comment line to something readable, like "Human_EF-1a," but remember it must be a single line and some programs do not like spaces. For bacteria, two rather different well-studied species would be *Escherichia coli* strain K-12 and *Bacillus subtilis*. Remember the protein is EF-Tu in prokaryotes. For the archaea,*Methanosarcina acetivorans* and *Haloarcula marismortui* represent two distinct groups.

A Distance-Based Tree Using UPGMA

Let's start by building a tree using UPGMA as an example of a straightforward distance-based linkage method. UPGMA is still commonly used by multiple sequence alignment programs but has become less common in tree-building programs. This method is not an option in the Phylogeny.fr suite of phylogenetic software, but we can use Phylogeny.fr to align sequences, calculate UPGMA distances with EMBOSS, and then return to Phylogeny.fr to benefit from the flexible tree rendering of TreeDyn.

Link Navigate to **Phylogeny.fr**, but this time choose `A la Carte` under `Phylogeny Analysis`. This option will give you more control over the steps of the analysis. Choose the programs you will use: MUSCLE for alignment, Gblocks for curation, ProtDist/FastDist + BioNJ (a distance-based method) for tree construction, and TreeDyn for tree visualization. Choose to run the workflow step by step and click `Create workflow`. You should now see an input box for your sequences; paste them there (or upload your file) and click Submit to run MUSCLE and produce a multiple sequence alignment.

The final tree will be based on the multiple alignment, so it is valuable to verify its quality at this stage. As you scroll through the sequence, can you find any specific evidence to suggest the sequences are aligned appropriately? For example, what does the alignment suggest about the similarity of the two representatives of each domain to each other versus their similarity to the other domains? After examining the alignment, click `Next step` to go on to curation. At this point, you can choose whether to hand-adjust the alignment; to do so, click`Edit stage input data` to see the multiple sequence alignment in an editable form. Press F2 to get a black editing cursor, and then press `delete` to remove a gap or `space` to add a gap where you believe you can improve on the alignment. Most likely these spots will be in areas where gaps have been added, especially if they have not been added in the same place across all the sequences.

When you have finished, click `Submit` to allow Gblocks to curate the sequences and then proceed to the phylogeny step. Notice the distance metrics (substitution matrices) available to you; some should sound familiar. Continue to the phylogeny results page. Below the tree (which we ignore for now), you should see several output options, one of which is a distance matrix in Phylip format. Click this link to see the distances between all possible pairs of sequences: We can use this to create a tree by the UPGMA method in an external program. Save this matrix to a text file. Keep your Phylogeny.fr window open; carry out the next step in a new browser window or tab.

Link An agglomerative clustering program to build trees using UPGMA can be found at**emboss.bioinformatics.nl**. Find `fneighbor` in the list at the left, under `Phylogenydistance matrix`. This program accepts a distance matrix in Phylip format as input; upload your distance matrix file. Change the tree to UPGMA; the other parameters can be left at their defaults. Run the program. On the output page, you should see the data for a tree in Newick format (notice that specific branch lengths can be incorporated within this format, as well). The TreeDyn program at Phylogeny.fr can use this as input, giving us a nicer, more configurable tree. Copy the Newick formatted data to the clipboard.

Back at Phylogeny.fr, click `Next step` to get to the Tree Rendering tab. Click Edit stage input data to feed TreeDyn the UPGMA tree data. Paste the UPGMA tree data into the input box and run the program to see your tree. As you examine the tree, consider it both qualitatively and quantitatively. Qualitatively, the hypothesized pathway of evolution is shown by the patterns of branching and grouping. You would expect the two members of each domain to cluster together (share a more recent common ancestor); do they? Which group branches off first? What does this tell you about the hypothesized relationship of the domains? Quantitatively, examine the branch lengths. Remember that this is a phylogram, so branch lengths are meaningful. What do they tell you about the evolutionary time between the branch points? Which branchings are more ancient and which more recent? What do the branch lengths tell you about the assumptions of the program? Notice that this tree has a root, but where the tree *should* be rooted is unclear—we do not really know what "the" ancestral organism was like, and we do not have an agreed-upon outgroup. Therefore, you may get a more realistic tree if it is unrooted; click one of the radio buttons labeled Radial to look at it this way. What would you conclude about these groups of organisms, based on this (admittedly very limited) analysis? Save or print the tree for later comparison.

Neighbor-Joining Algorithm
The On Your Own project discusses in some detail a variation of agglomerative clustering called the **neighbor-joining (NJ) algorithm**. NJ is still a distance-based method, but it models evolution differently. A strength of Phylogeny.fr is that it is easy to rerun a phylogenetic scenario with a different algorithm. Click the `Phylogeny` tab and choose either`BioNJ` or `Neighbor` (two implementations of the NJ algorithm). The same curated multiplealignment and even the same distance calculations will be used, but the NJ algorithm will be applied to build the tree. Again, examine the resulting tree both qualitatively and quantitatively and look for differences as compared with the UPGMA tree. Can you see the important difference in the program's assumptions?

Character-Based Algorithms
Character-based algorithms consider individual characters—nucleotides or amino acids—in building a tree. For example, if at a particular position in the alignment four of six sequences have `A`, it is probable that `A` represents the **ancestral state**, or the hypothesized sequence of the common ancestor of all the modern sequences. The default tree-building algorithm at Phylogeny.fr is PhyML, a character-based algorithm that uses **maximum likelihood**. Maximum likelihood applies some model of evolution (which might take into account transitions and transversions or other known biases in the data) and then identifies trees with the highest likelihood given the model. For example, in a coin flip, if your model is that the coin is normal, 50% heads would be a high-likelihood result and 100% heads would be an extremely low-likelihood result; if the model is a two-headed coin, the reverse would be true.

The likelihood model can be further extended to use **Bayesian statistics**. Bayes' theorem involves an initial prior probability leading to the computation (based on an evolutionary model) of a posterior distribution of trees with high likelihood given the dataset. There is often minimal *a priori* information, so the prior distribution may be merely the distribution of all trees; the algorithm can then iterate repetitively using the outcome of one computation as the prior distribution for the next. (See References and Supplemental Reading if you are interested in knowing more about these statistical methods.)

Using the same curated alignment as before, use the PhyML method to draw a tree at Phylogeny.fr. Again compare your tree qualitatively and quantitatively to the other trees you have drawn. Then, try MrBayes, an algorithm based on Bayesian statistics. Here, you need to set some limits or the computation can take a very long time. Limit the number of generations (iterations) to 1,000 and sampling to every 100 generations. Even with those limits, expect this analysis to take some time; you may wish to submit the job and request an email when it is done.

Web Exploration Questions

1. In what important way is a tree computed using the UPGMA algorithm different from a tree computed by the NJ algorithm? Which do you believe better models evolution, and why?
2. Summarize concisely what you learned about the relationships among the three domains from your trees. Were the trees you developed by different methods consistent in terms of branching orders and evolutionary pathways? How consistent were they in terms of branch length?
3. It would make sense that if one highly conserved protein works as a "molecular clock," then any other similarly conserved protein would give the same results. To test that assumption, generate a phylogeny with a different highly conserved protein, the heat-shock protein Hsp70 (also known as DnaK in bacteria). Download the amino-acid sequence of the Hsp70 protein for the same six organisms (NP_002145, AET14830, DNAK_ECOLI, DNAK_BACSU, YP_306886, DNAK_HALMA), align the sequences, examine and curate the alignment, and produce trees using NJ and maximum likelihood methods. Summarize the results of this analysis and discuss anomalies between the two molecular clocks. What did you learn about the reliability of evolutionary hypotheses based on molecular data from this exercise?

More to Explore: Generating Datasets

Thus far, you have looked at molecular phylogeny using small datasets built by looking up individual genes. Larger datasets increase reliability: In a small dataset, one or two sequences that contain sequencing errors or are for some reason far from typical, misidentified, or incomplete could readily lead to spurious conclusions. However, text searching is not the easiest way to assemble a larger dataset. Instead, BLAST could be used to search by similarity for sequences similar to one known sequence of interest that can then be used to build the dataset for phylogenetic analysis. Additional tools have been developed specifically to accomplish this kind of task, including BLAST Explorer, which is included in the Phylogeny.fr workspace. BLAST Explorer makes it easy to identify proteins similar to a query sequence and choose from among them the sequences to include in a phylogenetic analysis; this method allows the use of sequences that may not have been annotated as orthologs of your query. You could explore further (or an instructor could assign further exploration) by using BLAST Explorer to collect additional EF-1a or Hsp70 sequences.

Guided Programming Project: Phylogenetic Trees Using Agglomerative Clustering

The programming projects in this chapter implement distance-based algorithms. In the Guided Programming Project, you will develop a program to perform agglomerative clustering using the single linkage method. The skills exercises will ask you to expand your program by producing the final tree in Newick format, allowing a user to choose between single and UPGMA linkage, printing branch lengths, and allowing the program to handle sequence input data. The On Your Own project will lead you to modify the solution further by implementing the NJ method.

As you saw in Understanding the Algorithm, hierarchical clustering is a matter of determining distances between clusters using a linkage method, merging the two closest clusters, and iterating until all clusters have been merged. Initially, each sequence (species) is an individual cluster, with the distances between clusters calculated by alignment and the application of some distance metric. For this project, we assume that the input for our program is a set of calculated distances between sequences. You will read these data in from a Phylip-formatted input file. The discussion and pseudocode that follow use single linkage, paralleling the example given earlier, but this is easily modified to use UPGMA (see Putting Your Skills Into Practice).

Let's take a moment to consider the data structures we might need. In Understanding the Algorithm, a distance matrix was used to represent cluster distances. We could use a two-dimensional array to hold this matrix, but it might be more efficient to use a nested hash structure. What happens, however, when we want to merge two clusters? Assume we merge clusters A and B. We could remove these two elements from the hash table and replace them with a merged element whose key is AB. But we need the original distances between A, B, and the other clusters when we apply our linkage method. Therefore, we might want to hold the original distances in one nested hash structure and use another nested hash structure to represent the working cluster distances, which would change as we merge. At the start of the algorithm, the original distances could be stored in a nested hash structure similar to the following (only a partial set is shown; keys C, D, E, and F are not included):

Hash of Hash Table of Original Distances

```
key = A, value = {key = A, value = 0}
                 {key = B, value = 1}
                 {key = C, value = 3}
                 {key = D, value = 7}
                 {key = E, value = 17}
                 {key = F, value = 19}

key = B, value = {key = A, value = 1}
                 {key = B, value = 0}
                 {key = C, value = 2}
                 {key = D, value = 6}
                 {key = E, value = 16}
                 {key = F, value = 18}
```

This structure would not change during the program, so we can always reference original distances. A copy should be made of this structure and used to represent merging clusters, similar to those in Figure 7.4. After the first iteration, the structure representing merging clusters would look as follows, assuming we use single linkage and merge clusters A and B (only a partial set is shown; keys D, E, and F are not included):

Hash of Hash Table of Merging Clusters

```
key = AB, value = {key = AB, value = 0}
                  {key = C, value = 2}
                  {key = D, value = 6}
                  {key = E, value = 16}
                  {key = F, value = 18}
key = C, value = {key = AB, value = 2}
                 {key = C, value = 0}
                 {key = D, value = 4}
                 {key = E, value = 14}
                 {key = F, value = 16}
```

We would continue to work with this nested hash structure, reducing the size by one with each iteration. At the end, we would be left with two keys in our nested hash structure, which would represent the final two clusters to merge. The following pseudocode presents a solution to cluster a set of items using the approach just described. This implementation assumes the data file is a Phylip-formatted file and with each iteration the merging clusters are printed.

Algorithm

Agglomerative Clustering Algorithm to Determine Evolutionary Relatedness

- **Goal:** To cluster a set of data items
- **Input:** A set of sequence distances in a Phylip formatted file
- **Output:** Clusters merged at each step

```
// Initialization - Read in data and build nested hash structures
Open input file containing sequence distances: infile numSeq = read first
line of infile
clusterNames = array of size numSeq
distances = array of size numSeq
i = 0
for each line of data in infile

   clusterNames[i] = first value in line
   distances[i] = remaining data in line split using space as
   delimeter

// Build nested hash structure of original and cluster distances
originalDist = nested hash structure
clusterDist = nested hash structure
for each i from 0 to numSeq-1
   for each j from 0 to numSeq-1
      originalDist[clusterNames[i]][clusterNames[j]] =  distances[i][j]
      clusterDist[clusterNames[i]][clusterNames[j]] =   distances[i][j]

// STEP 1: Cluster
while numClusters > 2
```

```
   shortestD = shortest distance in clusterDist
   shortestI = outer key of shortest
   distance in clusterDist shortestJ = inner key of shortest distance in
   clusterDist

   // merge clusters I and J
   newClusterName = shortestI + shortestJ
   remove shortestI from clusterNames
   remove shortestJ from clusterNames
   remove shortestI keys and nested keys from clusterDist
   remove shortestJ keys and nested keys from clusterDist

   singleLinkage(clusterDist, newClusterName, originalDist, clusterNames)
      append newClusterName to clusterNames

      output "merging clusters" shortestI and shortestJ

      numClusters—

output remaining two clusters

// function to calculate distances between new cluster and all
// other clusters using single linkage
function singleLinkage(clusterdist, newClusterName, originalDist,
clusterNames)
   for each cluster in clusterNames
      smallestD = maximum integer
      for each c1 in cluster
         for each c2 in newClusterName
            if originalDist[c1][c2] < smallestD
               smallestD = originalDist[c1][c2]
      clusterDist[newClusterName][cluster] = smallestD
      clusterDist[cluster][newClusterName] = smallestD
```

Putting Your Skills Into Practice

1. **Download** Write a program in the language used in your course to implement the given pseudocode. Test your program using the sample data values for the six species (A–F) used as an example in Understanding the Algorithm. You can create your own distance matrix data file or **download Phylip-formatted data**(see Web Exploration) from the *Understanding Bioinformatics* website. Be sure your program correctly deals with the format of the data file. Ensure that the program merges the clusters as expected.
2. Although an implementation of this pseudocode shows which clusters are merged at each iteration, a representation of the final evolutionary tree in Newick format would be much more useful. Modify your program to output a tree in Newick format; as discussed earlier, for our sample data, the output should be `((((A,B), C), D),(E,F));`.
3. Modify your program so it allows the user to choose between the single linkage and the UPGMA linkage method.
4. As you may have observed when you obtained a UPGMA-based tree for input to TreeDyn in the Web Exploration, Newick format also allows for branch lengths to be explicitly specified. Adding branch lengths would not only convey additional

information to the user but would also allow your program to output data that could be used directly by TreeDyn or another tree-rendering program. Modify your program to calculate branch lengths and include them in the Newick format output. Remember that these agglomerative clustering methods assume constant rates of evolution, so at each node (for example, where A diverges from B), the distances (from A to the node and from B to the node) should be the same.

5. Currently, your program takes a distance matrix as input. A more flexible program would allow you to input sequence data, calculate distances, and then output the clustered data. To do this realistically would require a multiple sequence alignment algorithm, which is beyond the scope of this project. However, you already have programs that can do global alignment (Chapters 3and 5) and apply distance metrics to pairwise alignments (Chapter 6); you could incorporate distances calculated by these methods into your phylogenetic tree. This requires two modifications to the program. (1) Read in nucleotide sequences from a text file and store them as the hash *value* of each species. Use alphabetic characters to represent the *key* for each species or cluster. (2) Align sequences and calculate distances, using either a nucleotide alignment with a choice of distance metrics (start with the code from the On Your Own Project in Chapter 6) or a protein alignment with a substitution matrix.

On Your Own Project: The Neighbor-Joining Method

Understanding the Problem: Determining Branch Lengths

The agglomerative clustering algorithm discussed in Understanding the Algorithm, particularly when coupled with the UPGMA linkage method, was at one time widely used in constructing phylogenetic trees and is still used in many multiple sequence alignment algorithms. However, simple agglomerative clustering is rarely used in tree-building today because of its limitations, notably the fact that it is **ultrametric**: It assumes a constant rate of evolution or a molecular clock that "ticks" at a constant rate. In the phylogenetic tree shown in Figure 7.6, for example, note that the distance from A to the node at *y* is the same as from B to *y*. You should have observed similar results for the branch lengths when you constructed a tree using the UPGMA method in the Web Exploration. There is a biological basis for this assumption: Because the two modern species A and B have been evolving for the same amount of time since they diverged from their common ancestor (*y*), the distance (i.e., number of substitutions) should be the same along each branch.

Unfortunately, in reality, distances between sequences may not be ultrametric. As we saw in Understanding the Algorithm, our simple example tree fails when we attempt to label branch lengths. Our example resulted in the grouping ((A,B),C);, for instance, given the distances A–B = 1, A–C = 3, and B–C = 2. Assuming a constant rate of evolution, the distance from A to *y*and from B to *y* should be equal, 0.5 each. Then, the distances from A to C and from B to C should also be equal—but they are not! Therefore, although UPGMA is a convenient and easy-to-implement linkage method, it is not suitable for building phylogenetic trees under all conditions.

Solving the Problem

The **NJ method** is an alternative that does not require the assumption of a constant rate of evolution across all species. The NJ method is a variation of the agglomerative clustering technique and can be applied to a set of sequences for which distances have been calculated using any desired metric. As before, there is a merge step in which the two closest clusters are merged. The difference is in the linkage method: NJ calculates a **transformed distance**value when calculating the distances between the remaining clusters at each iteration. This allows the branch lengths to correspond to the observed distance between species, even when those branch lengths are not ultrametric, accounting for differences in the rate of evolution.

Using NJ, each iteration of the clustering algorithm thus begins by calculating an *r* value for each cluster, representing the corrected net distance between it and all other clusters. This is essentially the average distance between a given cluster, *x*, and each other cluster (*i*); if there are *n* total clusters, we can use the following formula:

$$r_x = \frac{\sum_{i=1\ldots x-1,x+1\ldots n} d_{ix}}{n-2}$$

The value d_{ix} is the distance between cluster *x* and cluster *i* as determined by the previous iteration (or the initial distance matrix, for the first iteration). This distance is determined for every cluster *i* other than *x* itself and summed.

These *r* values are then used to compute transition distances (*td*) to be used in determining which cluster to merge at the merge step. The following formula shows how this is done given clusters *x* and *y*, where *x* != y:

- $td_{xy} = d_{xy} - r_x - r_y$

The cluster pair that has the smallest transition distance is merged. Once the clusters are merged, new distances are calculated between the newly formed cluster (*K*) and all other clusters (the distances between unmerged clusters do not change). After a merge of clusters *i*and *j*, the distance from the new cluster to any cluster *x* is given by

$$d_{Kx} = \frac{d_{ix} + d_{jx} - d_{ij}}{2}$$

As before, this process repeats for additional clusters; we stop when only two clusters remain and join the last two based on calculated distance between them (see the next section). Finally, the branch lengths within the tree must be calculated; because the distances from anancestor to its descendants need not be the same, the distance from cluster *i* to *j* must be calculated as two branch lengths, from each of the clusters to their shared ancestor *K*:

$$d_{iK} = \frac{d_{ij} + r_i - r_j}{2}, d_{jK} = \frac{d_{ij} + r_j - r_i}{2}$$

Now that we have the formulas, let's see how they work with a simple example. Suppose we have sequences from five species with initial distances as shown in **Table 7.1**. The first step is to calculate transformed *r* values. For our first iteration, these are A = 13, B = 12, C = 11.34, D = 11, and E = 10. Using these values, we can compute transition distance values for our first iteration, resulting in the transition matrix in **Table 7.2A**. The transition matrix is used to determine which clusters to merge. Because the lowest value in the transition matrix is in the cell represented by clusters A and B, these two clusters are merged. The new distance matrix is then populated with the initial matrix distances, except for the distances between the newly created cluster, represented by AB, and the other clusters, which must be calculated. These distances are shown in **Table 7.2B**.

Table 7.1: Initial distances for the neighbor-joining example.

Open table as spreadsheet

	A	B	C	D	E
A	0				
B	5	0			
C	11	10	0		
D	12	11	7	0	
E	11	10	6	3	0

Table 7.2: First transition matrix (A) and recalculated distance matrix (B) for the neighbor-joining example.

A. Open table as spreadsheet

	A	B	C	D
B	-20			
C	-13.34	-13.34		
D	-12	-12	-15.34	
E	-12	-12	-15.34	-18

B. Open table as spreadsheet

	AB	C	D	E
AB	0			

C	8	0		
D	9	7	0	
E	8	6	3	0

Before moving on to the next iteration, let's look at the partial tree represented by the merge of clusters A and B. This merge implies these two species have a common ancestor (AB), and to obtain the branch length from the common ancestor to each species, we apply the branch length formula just given:

$$d_{A(AB)} = \frac{d_{AB} + r_A - r_B}{2} = 3, \; d_{B(AB)} = \frac{d_{AB} + r_B - r_A}{2} = 2$$

This partial tree can now be drawn as shown in **Figure 7.7A**. Notice that the two branch lengths are unequal, something that would not have been possible using the UPGMA method.

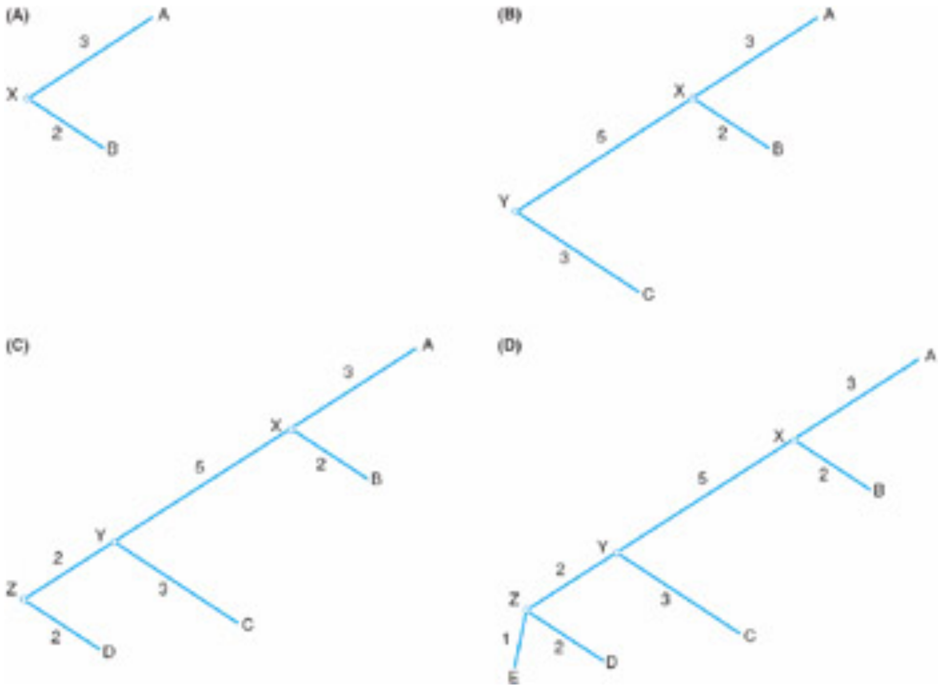

Figure 7.7: Merging of clusters to generate a phylogenetic tree from the data in the text using the neighbor-joining method. (A) Species A and B merge to form the first cluster, with a common ancestor designated by X. (B) Species C merges with the AB cluster, giving a common ancestor designated by Y. (C) Species D merges with the ABC cluster, giving a common ancestor designated by Z. (D) The finished tree after adding the branch to species E.

Our next iteration begins by recalculating transformed *r* values: AB=12.5, C =0.5, D=9.5, and E=8.5. **Table 7.3** shows the new transition matrix (A) and new distance matrix after the second merge (B). In this iteration, two cells contain the lowest value in the transition matrix. We can choose to merge cluster AB with C or cluster D with E; here, we arbitrarily choose to merge AB with C. The new distance matrix is populated with the previous iteration's distances, except for the distances between the newly created cluster, represented by ABC, and the other clusters. Calculating branch lengths and adding the results of this merge to our partial tree results in the tree shown in **Figure 7.7B**.

Table 7.3: Transition matrix (A) and recalculated distance matrix (B) after the second merge in the neighbor-joining example.

A. Open table as spreadsheet

	AB	C	D
C	-15		
D	-13	-13	
E	-13	-13	-15

B. Open table as spreadsheet

	ABC	D	E
ABC	0		
D	4	0	
E	3	3	0

With the next iteration, we obtain the transition matrix in **Table 7.4A**. According to this transition matrix, we could now merge any of the remaining clusters, because they have the same value. We choose to merge ABC and D, and again we recalculate distances (**Table 7.4B**) and branch lengths and then add our newly merged clusters to our partial tree (**Figure 7.7C**). Because we are now left with only two clusters, we can simply attach these two clusters using our distance information. In our example, notice that the final distance matrix (Table 7.4B) conveniently gives us the distance between species E and the cluster ABCD (or common ancestor of species A–D), and we get the final tree shown in **Figure 7.7D**. If we were merging two clusters at this point, the last distance we need for our tree would be the distance between two internal nodes (ancestral species), and we could calculate this by going back to the original distance matrix, finding the distance between a species in one cluster and a species in the other, and then subtracting the already calculated branch lengths to get the distance between the remaining internal nodes.

Table 7.4: Transition matrix (A) and recalculated distance matrix (B) after the last

merge in the neighbor-joining example.

A. Open table as spreadsheet

	ABC	D
D	-10	
E	-10	-10

B. Open table as spreadsheet

	ABCD	E
ABCD	0	
E	1	0

Notice that the NJ method has produced an unrooted tree, whereas UPGMA produced rooted trees. The NJ branch length formula allows for the calculation of unequal branch lengths. If you compare the distances in the final phylogenetic tree (Figure 7.7D) with our original set of distances (Table 7.1), you will see that the tree matches the original distances, demonstrating the additivity property of the NJ method.

Given the equations and example presented here, you should now be able to use the NJ algorithm to construct a phylogenetic tree with calculated branch lengths for the six sample species whose distance matrix is given previously in Understanding the Algorithm. How does the tree thus generated differ from the tree shown in Figure 7.6?

Download If your course involves programming, your instructor may ask you to implement the NJ algorithm as described next. If it does not, a completed program implementing NJ can be downloaded from the instructor section of the *Exploring Bioinformatics* website and used to complete the exercises at the end of the Programming the Solution section without programming.

Programming the Solution

Using your solutions to the Guided Programming Project exercises as a starting point, implement the NJ method in the programming language of your choice. Depending on the exercises your instructor chose previously, you may have a program to carry out agglomerative clustering given a distance matrix or a more comprehensive program to generate a Newick format tree from nucleotide or aminoacid sequence data. Any of these solutions can be readily modified to implement NJ or offer NJ as a choice of method for the user.

The initial steps (reading sequence or distance files, aligning sequences, calculating initial distances, etc.) will not change, but you will need to make changes to the decision process in the merge step and the calculation of intercluster distances thereafter, as well as a calculation of final branch lengths. Use the formulas given in the chapter to make these calculations. You will also notice some differences in the data that need to be stored. In the guided project, a nested hash table was used to hold cluster information. This was important, because we needed to keep track of each cluster element's value to determine distance. However, the NJ method recalculates distances at each iteration from the previous cluster distances. For troubleshooting purposes, you may wish to print out the clusters merged and the branch lengths as each merge occurs, but the final program should output results in Newick format, including branch lengths.

Run your program on the following test data set using a simple nucleotide count as your distance metric and NJ as the linkage method:

```
(A) TCAT, (B) TCCT, (C) TCCC, (D) GCGT, (E) GCTT
```

You should end up with the following tree: `((C:1,B:0):0.5,(A:0.5,(D:0.5, E:0.5):1));`, after merging D with E, DE with A, and C with B.

Then, try your program with the data from Understanding the Algorithm. You should get the same results as when you worked out the tree by hand. Compare your outcome with the results using UPGMA as a linkage method. Can you explain why there are differences? Finally, test your program on the eIF-1α and Hsp70 sequences from the Web Exploration. Which algorithm do you believe gives you the best picture of the actual evolutionary pathways?

Connections: What Is a Species?

Chapter 5 included the example of two salamander populations that became separated by California's Central Valley and had evolved into subspecies. Assuming continued separation, these subspecies may eventually become two distinct species. But just how do we define a species? One long-used biological definition is that two organisms are members of the same species if they are able to mate and have fertile offspring. Perhaps, however, you can already see problems with this definition. All domestic dogs, for example, are considered to be members of a single species—indeed, a single subspecies, *Canis lupus familiaris*—but it is obvious that successful mating between a St. Bernard and a chihuahua is unlikely.

Where we find similar but distinct kinds of birds, such as the readily distinguishable Eastern Bluebird and Mountain Bluebird, do we have one species or two? What do we do about the many kinds of organisms that have no sexual reproduction? What about plants, where in some cases two quite different plants can mate and yield a new type of plant with twice as many chromosomes? (This happened naturally at least twice in the history of our modern red wheat.) And perhaps most puzzling of all, what about bacteria and archaea, where we find enormous biochemical and metabolic diversity despite very limited visually distinguishable features and a complete lack of genuine sexual reproduction?

Bioinformatics and molecular evolution are central to research aimed at untangling difficulties in the concept of a species and in classifying organisms throughout the living world. Where morphology, ecology, physiology, and even biochemistry cannot resolve the question, bioinformatics can quantify differences in DNA and protein sequences and establish standards for how different two organisms need to be in order to be considered two species. Evolutionary journals are currently full of articles in which bioinformatic tools are used to investigate questions such as these, frequently resulting in splitting what was thought to be one species into two, or the reverse—sometimes producing heated debates. As more and more DNA sequences and complete genomes become available, we can anticipate ongoing progress in this area.

References and Supplemental Reading

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Chapter 9: Sequence-Based Gene Prediction: Annotation of a Resistance Plasmid

Chapter Overview

Assembling a genome sequence (Chapter 8) does not by itself reveal key information such as where the genes are within that sequence. This chapter and the next one focus on gene prediction: how to identify possible genes within a genome sequence. In this chapter, sequence-based methods suitable for gene prediction in prokaryotes are explored and their value and limitations in eukaryotic gene discovery examined; the next chapter will take up the more complex gene prediction methods needed for eukaryotic genome annotation. Students in both programming and nonprogramming courses will be introduced to algorithms for gene prediction. Using a variety of Web-based tools, students will be able to use sequence-based methods for gene prediction in prokaryotes. Students in programming courses will implement sequence-based algorithms for gene prediction in prokaryotes. The On Your Own Project will then examine the extent to which these algorithms can be applied to eukaryotes.

- **Biological problem**: Prediction and annotation of genes in a resistance plasmid sequence
- **Bioinformatics skills**: Sequence-based ORF finding and promoter prediction
- **Bioinformatics software**: NCBI ORF Finder, NEBcutter, EasyGene
- **Programming skills**: Pattern-matching algorithms, modularization, functions

Understanding the Problem: Gene Discovery

We have come a long way since the preantibiotic days when the risk of infection made surgery often more dangerous than the condition it was intended to cure. However, despite our many medical advances and modern methods of controlling infectious agents, in the United States approximately 1.7 million individuals per year acquire infections while hospitalized. Of these hospital-acquired, or ***nosocomial****, infections, some 99,000 cause or contribute to the death of the patient. Control of nosocomial infections is difficult because of the high concentration of infectious agents in the hospital environment, the already compromised or immunodeficient state of the patients, and the close contact of medical personnel with many patients per day. Furthermore, the use of invasive measures such as surgical procedures, catheters, and intravenous tubes may grant pathogens access to areas of the body that are normally well protected. Among the most common agents of nosocomial infection are* Enterococcus *species* (**Figure 9.1**), *normally harmless residents of the human colon that can seize an opportunity to enter other parts of the body where they can be highly pathogenic. To make matters worse, many* Enterococcus *isolates are highly antibiotic resistant—even to "last resort" drugs such as vancomycin—and capable of transferring multiple resistance genes horizontally on large plasmids. Sequencing of plasmid DNA from these resistant strains is one way to learn more about the nature of the resistant organisms and their potential to spread resistance*.

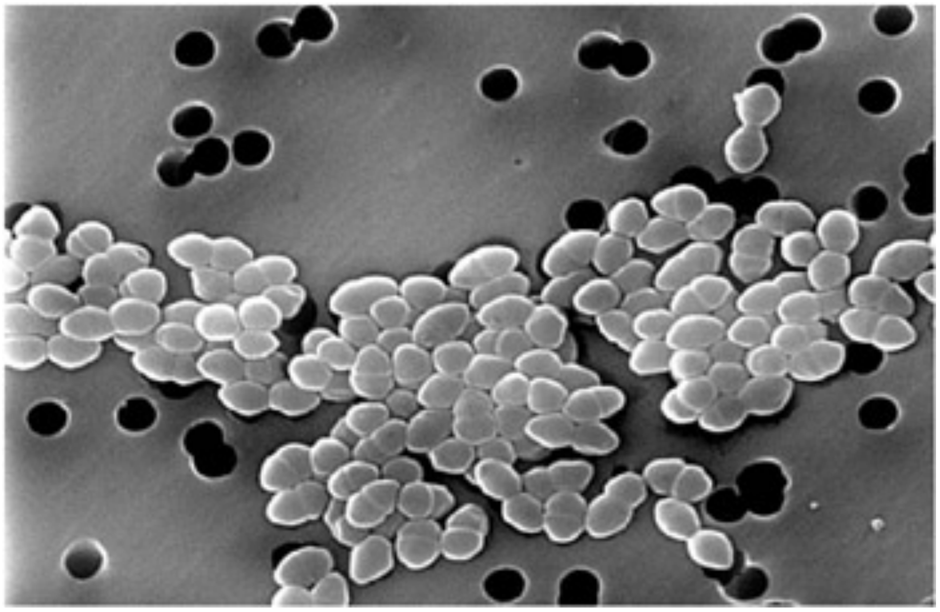


Figure 9.1: Scanning electron micrograph of a group of vancomycin-resistant Enterococcus cells. Courtesy of Janice Haney Carr/CDC.

Once a genome, chromosome, plasmid, or other large piece of DNA has been sequenced, the processes of **gene discovery** (also called **gene prediction**) and **genome annotation**begin. By itself, a DNA sequence is just a bunch of As, Cs, Gs, and Ts with no obvious meaning; to use that sequence to cure a genetic disease or understand how a specialized cell type develops, we have to find the genes within that sequence and understand their functions. Many people are surprised that we still cannot say exactly how many genes there are in the human genome, let alone identify all their functions. The presence of introns, the existence of surprisingly short or long genes, and the difficulty of definitively identifying promoters and translational start sites are among the complexities involved. Furthermore, although we tend to focus on protein coding genes, genomes also include protein binding sites, genes for noncoding RNAs, regions important to chromatin structure, methylation sites, and more.

Gene discovery is one of the major applications of bioinformatics to genomics. Although we often think of gene discovery as it applies to the analysis of major genome sequencing projects, it is also important on a smaller scale. Consider, for example, the major medical problems created by the horizontal transfer of antibiotic resistance. Often, resistance is due to large multi-drug resistance plasmids that by horizontal transfer can make another cell simultaneously resistant to many antibiotics—in some cases, even to *all* the classes of antibiotics in current use, including such "last resort" drugs as vancomycin.

In this chapter, we apply gene prediction methods to a large plasmid isolated from a highly resistant *Enterococcus faecium*, a bacterium that is naturally resistant to some antibiotics, including the penicillin family, and can readily acquire additional resistance. The plasmid we examine was isolated from a patient with a life-threatening postsurgical abdominal infection. Using gene prediction methods, we can identify potential resistance genes within this plasmid sequence and annotate them by looking for conserved sequences, thus determining what resistances the bacteria have and potentially how best to treat infection.

Bioinformatics Solutions: Gene Prediction

Back in Chapter 2, we considered a gene to be a coding sequence within an mRNA (or cDNA) sequence; an AUG start codon and a UAG, UGA, or UAA stop codon identified this sequence, and the genetic code table allowed us to find the amino acid encoded by each three-nucleotide codon in between. This coding sequence is called an **open reading frame**(**ORF**). However, finding a gene is not as simple as finding an ORF. An ORF-like sequence could occur accidentally in noncoding DNA. Therefore, long ORFs are usually considered more likely to be real genes—but we also do not want to miss short but genuine genes that encode short proteins (sarcolipin, the shortest known protein in mice, is only 31 amino acids long). Additionally, genes for untranslated functional RNAs (tRNAs, rRNAs, snRNAs, and others) have no coding sequence. Predicting which sequences serve as promoters can help us recognize actual genes, but this is in itself complex, especially in eukaryotes. **Introns**introduce a huge amount of difficulty in eukaryotic genomes: An average protein coding sequence in the human genome is only about 1,500 base pairs long, but an average complete gene (typically including four to five introns) is nearly 10 times that long.

No method exists yet that can comprehensively and unambiguously identify all the genes in a DNA sequence; indeed, the problem is usually approached from multiple directions by applying a variety of methods. Commonly used computational approaches to this problem fall into several categories: algorithms based on alignment, sequence, content, or probability.

Alignment-based algorithms. If a region of a newly sequenced genome is orthologous to a previously identified gene in a well-studied organism such as mice, zebrafish, fruit flies, nematodes, or even bacteria or yeast, that would be good evidence that it is a gene. Indeed, even if no specific orthologous gene has yet been identified, strong conservation of a genome region over evolutionary time is strongly suggestive of its functional importance. **Alignment-based** algorithms look for genes based on conserved sequences; the alignment tracks in the UCSC genome browser (Chapter 1) gave you some idea of the value of this kind of comparison.

Sequence-based algorithms. Searching for ORFs is an example of a **sequence-based method** of gene prediction: A simple ORF-finder program would look for the sequence AUG (the start codon) followed by some amino-acid codons and a UAG, UGA, or UAA stop codon. More complex variations would take into account additional sequence clues such as promoter sequences and intron–exon boundaries. These functional regions of DNA would be identified based on the development of **consensus** sequences (see BioBackground at the end of this chapter) that can then be computationally identified in a genome. Sequence-based methods do not require similarity to other organisms, but they can only find genes that include sequences matching known patterns, and they have difficulty with sequence patterns that are relatively loose, like the sequences at the boundaries of exons and introns. Sequence-based methods are the focus of this chapter.

Content-based algorithms. **Content-based methods** do not look for specific sequences but rather for patterns such as nucleotide or codon frequency that are characteristic of coding sequences in a particular organism. These methods can identify novel genes and find coding regions that would be missed by sequence-based methods. One tool used in the Web Exploration in this chapter includes a content-based method (codon frequency); content-based methods will be discussed in more detail in the next chapter.

Probabilistic algorithms. More sophisticated gene discovery methods may combine elements of both sequence-based and content-based gene prediction in algorithms that model the probability that a given sequence is part of a gene. Hidden Markov models and neural network algorithms are two major examples of probabilistic solutions; these will be discussed in the next chapter

In this chapter's Web Exploration and Guided Programming Project, we see how sequence-based methods work and use them to identify genes involved in antibiotic resistance and virulence within the sequence of a large bacterial plasmid. In the On Your Own Project, we apply similar methods to eukaryotes and explore their limitations. A good understanding of gene structure is essential to the development and use of computational methods for gene discovery. The BioBackground section in Chapter 2 introduced the structure of genes, and that introduction is extended in this chapter's BioBackground section, along with an introduction to how the sequences of promoters and other functional sites are identified.

BioConcept Questions

1. Why are long ORFs sometimes considered to be the same as genes? In what ways is this definition insufficient?
2. How does RNA polymerase find the transcriptional start site of a gene in prokaryotes? How can we use this information in a gene prediction algorithm?
3. How does RNA polymerase find the transcriptional start site of a gene in eukaryotes? Why is it more difficult to develop an algorithm to find a eukaryotic promoter than a prokaryotic promoter?
4. How does a prokaryotic ribosome find the correct start codon within an mRNA? How can we use this information in distinguishing which ORFs are genes?
5. Why can't we use a similar strategy to distinguish which ORFs are genes in eukaryotes?
6. A simple ORF-finding program would do a very poor job of predicting the amino-acid sequences of the proteins encoded in the human genome. Discuss why this is the case.
7. How might you identify a gene encoding a functional RNA (that does not encode a protein)? How does the discovery of key functions for very small RNA molecules complicate the issue?

Understanding the Algorithm: Pattern Matching in Sequence-Based Gene Prediction

Learning Tools

Download If you want to better understand how a consensus sequence for a promoter or other element is developed and why identification of these sequences is not as clear-cut as it sounds, you can download an exercise from the *Exploring Bioinformatics* website that will take you through the generation of a prokaryotic promoter consensus sequence using data from sequenced genomes.

Sequence-based methods of gene prediction examine DNA sequences for patterns (often called **motifs**) that provide clues about the existence of transcriptional or translational units. Sequence-based prediction methods rely on **pattern-matching algorithms**: Given a string to search (such as a plasmid or genome sequence) and a pattern to be matched (such as AUG), they can identify whether, how often, and where the pattern occurs. Indeed, content-based and probabilistic methods usually include elements of pattern matching as well.

An ORF-finding program is a good example of pattern matching in gene prediction. This program could begin by **traversing** the searched text—that is, searching through the nucleotide string from beginning to end—examining each group of three nucleotides for the pattern ATG to find a potential start codon. Then, it would have to find an in-frame stop codon. The process of testing three-nucleotide groups for a match to the pattern would stay the same, so a single algorithm could be provided with different **parameters**. Parameters are values set when an algorithm starts that allow it to solve variations of a problem using the same main steps; in this case, our parameters would be the searched text, the pattern, start and stop locations, an increment value, and a threshold value. When looking for the start codon, the start location is the first nucleotide, the stop location is three nucleotides from the end (no point in looking at the last two), the increment value is one in order to search in all three possible reading frames (in the sequence `CCATGGAC`, look first at `CCA`, then `CAT`, then`ATG`, etc.), and the threshold value is 100%, because we need a perfect match to ATG. Once a start codon is found, we would change the increment value to three (after finding `ATG`, look at `GAC` but *not* `TGG` or `GGA`) and the pattern to TAG, TGA or TAA, again requiring a perfect match.

Algorithm

Pattern-Matching Algorithm

1. Initialize parameters of algorithm:
   - `pattern` = search pattern
   - `searchedText` = text that will be searched for pattern
   - `start` = start location of search (assumes first character is position 1)
   - `stop` = stop location of search (this represents last location to search from)

- `increment` = incrementing value (negative number for upstream search, positive number for downstream search)
- `threshold` = minimum percentage match required

2. Compare pattern to characters of searchedText starting at position start. If percentage of matching characters is >= threshold, output start position and end algorithm. If not, add increment to start and continue to step 3.
3. If increment is positive and start is <= stop, repeat step 2. If increment is negative and start is >= stop, repeat step 2. If neither statement is true, pattern was not found, end algorithm.

You can quickly see, however, that this straightforward algorithm will not make a great ORF finder. ATG is not *just* a start codon but is used every time the amino acid methionine occurs in a protein. That means the simple algorithm would find apparent ORFs that are actually within other ORFs. Furthermore, ORF-like sequences could occur by chance in noncoding DNA: The pattern `ATGGGGTGA` would occur at random once every 4^9 nucleotides, or about 19 times in the *E. coli* genome, but is clearly not an ORF. Thus, ORF-finding programs commonly allow the user to limit results to ORFs of a certain length, perhaps 100 codons. This would only require setting a start location 300 nucleotides downstream to start looking for the stop codon after finding an ATG—but this modification also brings with it the danger of overlooking small but genuine genes. If you will not be completing the programming projects in this chapter, you may wish to download the sequence of the *Enterococcus* plasmid from the *Exploring Bioinformatics* website and look by hand for some potential ORFs to get an idea of how these parameters would affect the process.

Download Despite these adjustments, a simple ORF-finding algorithm will not be a very reliable method of gene prediction: Even a reasonably long ORF might not really be a gene, and a short ORF possibly could be a gene. To help distinguish real genes, we can also look for regulatory sequences: In bacteria, genes are preceded by promoter sequences (-10 and -35 sequences) and the start codon is preceded by a Shine-Dalgarno sequence (see **Figure 9.2** and BioBackground). Unfortunately, finding these patterns is less straightforward. In *E. coli*, the Shine-Dalgarno consensus sequence is `AGGAGG`, but the match to this pattern can be imperfect. The end of this sequence should be approximately five nucleotides upstream of the start codon, give or take two positions (so, -5 ± 2 relative to the ATG). Promoters can also be inexact matches to the consensus -10 (`TATAAT`) and -35 (`TTGACA`) sequences; these sequences should be 17 ± 2 nucleotides apart but can occur anywhere from 50 to 500 nucleotides upstream of a start codon. (If you are familiar with prokaryotic molecular biology, you know that even this is a simplified view given the frequent use of operons and alternative sigma factors.) The pattern-matching algorithm can find these sequences given appropriate parameters, such as start and stop locations and threshold values.

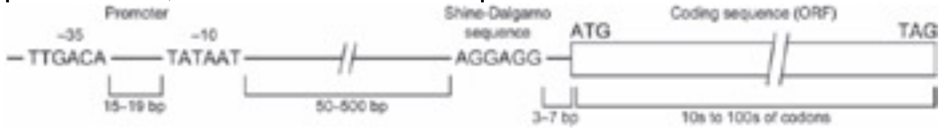


Figure 9.2: Elements of a prokaryotic gene that can be searched by a sequence-based

algorithm include the coding sequence or ORF, the Shine-Dalgarno sequence, and the promoter sequence.

Test Your Understanding

1. DNA is double stranded, and one strand may serve as the template (copied) strand for one gene (in one region) but the nontemplate (mRNA-like) strand for another (in another region). The algorithm given could find an ATG start codon in one of three reading frames by reading a sequence entered in the 5′ to 3′direction, but really we should consider all *six* possible reading frames: three from the DNA as it was entered and three more on the complementary strand. What would we need to do to find ORFs in all six possible reading frames?
2. As noted, the pattern-matching algorithm might find an ORF within another ORF, because within a gene there could be multiple ATG codons. How could your algorithm filter out these undesirable matches?
3. Identify parameters that could be used in the pattern-matching algorithm to search for a Shine-Dalgarno sequence once an ATG is found. Assume an exact match to the consensus sequence.
4. Identify parameters that could be used in the pattern-matching algorithm to search for a promoter once an ATG is found. Assume that five of the six bases in the -10 and -35 sequences must match their consensus.

Chapter Project: Gene Discovery in a Resistance Plasmid

This chapter's project focuses on sequence-based methods of finding genes within DNA sequence data. We consider only prokaryotic genes in the Web Exploration and Guided Programming Project, because the lack of introns and more clearly defined expression signals makes them easier from a practical standpoint. In the On Your Own Project, we consider how these principles apply to eukaryotes. Specifically, we look for genes within the sequence of a plasmid isolated from antibiotic-resistant *Enterococcus* and, in the Web Exploration, annotate those genes by looking for clues to function.

Learning Objectives

- Understand the structure of a gene and which features are useful in developing computational methods for identifying genes
- Appreciate the strengths and limitations of sequence-based methods for gene discovery
- Use Web-based gene discovery tools to annotate a plasmid
- Understand how pattern matching can be used in sequence-based computational solutions
- Apply sequence-based algorithms to the more complex problem of gene discovery in eukaryotes

Suggestions for Using the Project

This project provides an introduction to pattern matching in gene discovery for both programming and nonprogramming courses. The Web Exploration in this project guides students to predict and annotate genes in a plasmid sequence; the Guided Programming Project allows them to implement a pattern-matching algorithm that can be applied to the same problem. The On Your Own Project asks students to implement (in programming courses) or examine (in nonprogramming courses) the application of pattern matching to eukaryotic gene prediction clues. All tools described here could be applied equally well to any other question that the instructor wished to explore.

Programming courses:

- Web Exploration: Use Web-based tools to identify likely genes within a plasmid sequence; complete either Part I or Part II (or both parts, in teams) and Part III. Optionally, annotate genes with BLAST.
- Guided Programming Project: Implement a pattern-matching algorithm and compare its output with the Web-based tools.
- On Your Own Project: Extend the pattern-matching algorithm to eukaryotic gene prediction.

Nonprogramming courses:

- Web Exploration: Use Web-based tools to identify likely genes within a plasmid sequence and annotate the genes with BLAST. Complete either Part I or Part II (or both parts, in teams) and Part III.
- On Your Own Project: Consider how a pattern-matching algorithm could be used to identify sequence-based clues to eukaryotic genes.

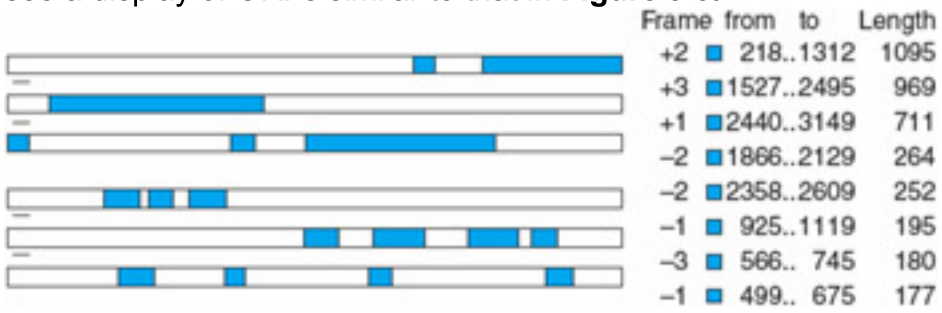
Web Exploration: Prokaryotic Gene Prediction and Annotation

In this part of the project, we use Web tools to find genes within an *Enterococcus* resistance plasmid sequence. Sequence-based methods for gene prediction work well for prokaryotes, because they lack exons and have more easily predictable patterns for regulatory elements(see BioBackground). Parts I and II use two different simple ORF finders to accomplish the same task. It is suggested that pairs of students work on these exercises together: Each can use one of the tools and then the results can be compared. Alternatively, an instructor may choose to assign only Part I or Part II. Part II uses a more advanced tool to search for Shine-Dalgarno sequences to better identify actual genes. BLAST can be used to annotate genes with putative functions and potentially to further explore the nature of resistance and the evolution of resistance plasmids (see More to Explore later in the chapter); instructors may skip this part of the exercise if they wish.

Part I: Sequence-Based ORF Identification Using the NCBI ORF Finder

The simplest gene discovery program would simply look for an ORF as described in Understanding the Algorithm: a start codon followed by a coding sequence longer than some length specified by the user and terminating with a stop codon. The ORF could occur in any of the six possible reading frames (three on each strand). Such a program would actually be fairly effective in finding genes in a prokaryotic genome, given the absence of introns. There are many such programs; we use NCBI's ORF Finder to identify ORFs in the *Enterococcus*resistance plasmid.

Download Start by downloading the sequence of the ***Enterococcus faecium* resistance plasmid** from the *Exploring Bioinformatics* website. Open NCBI's **ORF Finder** and paste the sequence into the input box. There are not a lot of parameters available; note that you could search only a portion of the sequence if desired, or you could change the genetic code used if you were working with something like mitochondrial DNA where a few codons are different. Run the program; you should see a display of ORFs similar to that in **Figure 9.3**.



```
Frame from  to    Length
 +2  ■  218..1312  1095
 +3  ■1527..2495    969
 +1  ■2440..3149    711
 –2  ■1866..2129    264
 –2  ■2358..2609    252
 –1  ■  925..1119   195
 –3  ■  566..  745  180
 –1  ■  499..  675  177
```

Figure 9.3: Sample output from the NCBI ORF Finder. Colored regions of bars represent ORFs, listed on the right. Only a portion of the ORF list is shown here. Generated from the NCBI ORF Finder.

You may be surprised by the number of ORFs found by this program. How long is the DNA sequence? Click on `View` to find out. Does it seem reasonable to have this many genes in a sequence this long? How are the genes distributed on the two strands of DNA? Notice that the ORFs are listed by size and that some of them are pretty short. By default, ORF Finder shows any ORF longer than 100 nucleotides, or about 33 amino acids. Change the drop-down to view only ORFs that have at least 100 amino acids (300 nucleotides) and see how this changes the list.

Gene prediction is more valuable if we can also annotate the genes with putative functions based on sequence comparison. Click on one of the ORFs either in the list or the graphical view to see its nucleotide and amino-acid sequences. Notice that you can then directly submit the sequence of just this ORF for either a protein (blastp) or nucleotide BLAST search. Try a protein search and try to find a putative function for each ORF. Some should match known antibiotic-resistance genes; for these, find out what antibiotic the gene confers resistance to and try to find the mechanism of action for the resistance protein (for example, does it inactivate the antibiotic, modify the cellular target of the antibiotic, or perhaps pump the antibiotic out of the cell?). For those that do not appear to be antibiotic-resistance genes, do they have functions that make sense in the context of this resistance plasmid? Remember that some of the ORFs may not be real genes at all. In addition to annotating the genes, notice that this BLAST search step effectively adds an alignment-based gene discovery method to increase the accuracy of our sequence-based predictions.

When you are satisfied with what you have learned about an ORF, use the `Back` button on your browser to return to the ORF Finder view of the gene. If you are convinced that the ORF is a genuine gene, click `Accept` and notice that the program changes the color of the gene in the graphical view and of its symbol in the list. This will help you keep track of the genes you have identified.

Part II: Sequence-Based OFR Identification Using NEBcutter

Download Another program that is useful for finding and annotating ORFs is New England Biolabs' **NEBcutter**. The primary goal of this program is to identify restriction endonuclease cut sites (useful, for example, in gene cloning; see References and Supplemental Reading), but it also identifies ORFs and places them on a map of the DNA in relation to the restriction sites. Find the NEBcutter page and paste the ***Enterococcus* plasmid sequence** into the input box. This is the complete sequence of a plasmid, so choose the option to show a circular DNA molecule (plasmids are always circular). Notice that you can change the minimum length of the ORF displayed from this page and set other options (you can even customize the colors of the output if you wish). Submit the sequence; you should get a result similar to the sample data shown in **Figure 9.4**.

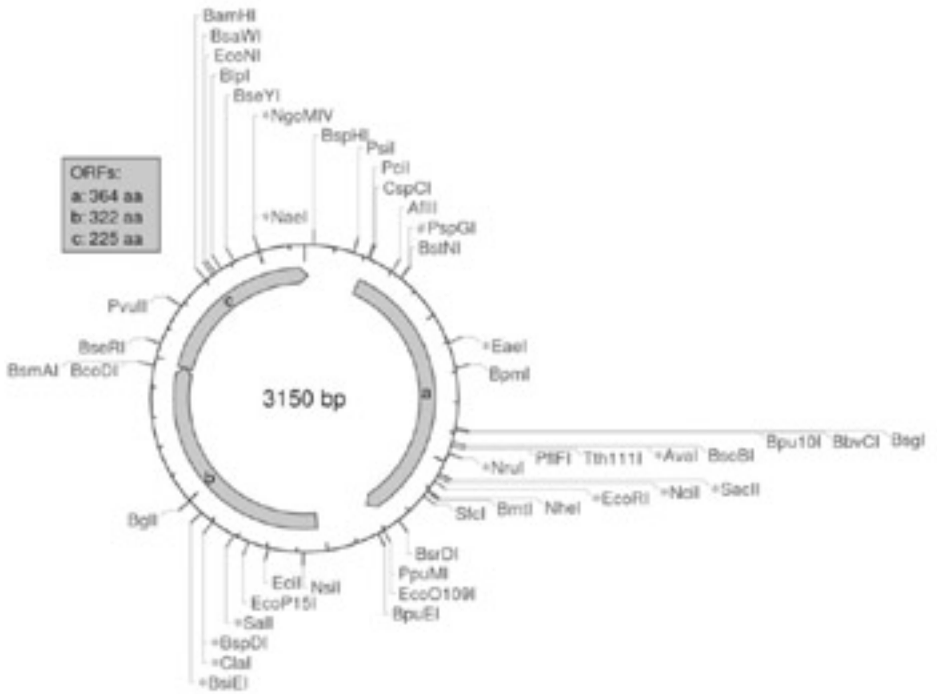


Figure 9.4: Sample output from NEBcutter showing a plasmid map with restriction sites; ORFs are represented by the gray arrows and listed by size in the box at left. Generated from NEBcutter; Vincze, T., Posfai, J. and Roberts, R. J. "NEBcutter: a program to cleave DNA with restriction enzymes." *Nucleic Acids Res*. 31: 3688–3691. (2003).

Link Like NCBI's ORF Finder, NEBcutter shows the number and size of ORFs that met the specified criteria graphically and in a list. The NEBcutter display, however, does not separate the ORFs by the strand or reading frame in which they were found; notice that this might help you decide which genes might be grouped into operons. You can choose options to see a list of the ORFs with more detailed information. As with ORF Finder, you can change the minimum length of the ORF displayed based on your expectations.

To annotate genes in the plasmid, click on an ORF either in the list or graphical view to see its amino-acid sequence and find a link for a protein BLAST search. Use the BLAST results to find a putative function for each ORF. Some should match known antibiotic-resistance genes; for these, find out what antibiotic the gene confers resistance to and try to find the mechanism of action for the resistance protein (for example, does it inactivate the antibiotic, modify the cellular target of the antibiotic, or perhaps pump the antibiotic out of the cell?). For those that do not appear to be antibiotic-resistance genes, do they have functions that make sense in the context of this resistance plasmid? Remember that some of the ORFs may not be real genes at all. In addition to annotating the genes, notice this BLAST search step effectively adds an alignment-based gene discovery method to increase the accuracy of our sequence-based predictions.

Close the BLAST window when you are satisfied with your investigation of the ORF. If you are convinced it is a real gene, you can click `Edit` to name the gene (you might give it the same name as its orthologs: for example, β-lactamase proteins involved in penicillin resistance are named *bla* in many organisms) and describe its protein product. These data will then show up in the ORF list and in the description when you click on the ORF. When you have finished annotating genes, you can use the `Print` option to save your map to a PDF or GIF file.

Part III: Shine-Dalgarno Prediction and Codon Usage Analysis with EasyGene
Using ORF Finder or NEBcutter, we got a long list of potential genes we had to narrow down by hand. We were able to eliminate many ORFs from the list by requiring that the ORFs be at least 100 amino acids long. NEBcutter also ignores overlapping ORFs in its main display. However, we might have missed some genes this way: What if some of the short ORFs also encode functional genes? What if two genes do overlap (rare in eukaryotes but not infrequent in bacteria and common in viruses)? We could improve our ability to find authentic genes by determining whether the start codon is preceded by a Shine-Dalgarno sequence (a sequence similar to `5′ AGGAGG` located 5 ± 2 nucleotides before the start codon). This is still a sequence-based method of gene prediction, because we are still looking for a match to a specific sequence pattern; however, to use it effectively, we have to relax the stringency of the search to allow for imperfect matches.

We can use EasyGene (see References and Supplemental Reading) to add this element of sophistication to our prokaryotic gene prediction. EasyGene looks for ORFs and examines the region just before the putative start codon for a possible Shine-Dalgarno sequence. It also adds a content-based method of gene prediction: It asks whether the codons used in the ORF match the typical codon usage for the organism of interest. For example, six different codons all specify the amino acid leucine, but CTG is the codon actually used in *E. coli* genes more than 50% of the time. **Table 9.1** shows the codon usage frequencies for *E. coli*. EasyGene compares the codons in each ORF to a training set taken from whichever prokaryotic genome the user selects and calculates a significance score representing the likelihood that it is a genuine gene. Only ORFs scoring above a selected threshold are displayed.

Table 9.1: Codon usage table for *Escherichia coli.*

Open table as spreadsheet

Codon (aa)	Freq.[1]	Codon (aa)	Freq.	Codon (aa)	Freq.	Codon (aa)	Freq.
UUU (F)	19.7	UCU (S)	5.7	UAU (Y)	16.8	UGU (C)	5.9
UUC (F)	15	UCC (S)	5.5	UAC (Y)	14.6	UGC (C)	8
UUA (L)	15.2	UCA (S)	7.8	UAA (*)	1.8	UGA (*)	1
UUG (L)	11.9	UCG (S)	8	UAG (*)	0	UGG (W)	10.7
CUU (L)	11.9	CCU (P)	8.4	CAU (H)	15.8	CGU (R)	21.1
CUC (L)	10.5	CCC (P)	6.4	CAC (H)	13.1	CGC (R)	26
CUA (L)	5.3	CCA (P)	6.6	CAA (Q)	12.1	CGA (R)	4.3
CUG (L)	46.9	CCG (P)	26.7	CAG (Q)	27.7	CGG (R)	4.1
AUU (I)	30.5	ACU (T)	8	AAU (N)	21.9	AGU (S)	7.2
AUC (I)	18.2	ACC (T)	22.8	AAC (N)	24.4	AGC (S)	16.6
AUA (I)	3.7	ACA (T)	6.4	AAA (K)	33.2	AGA (R)	1.4
AUG (M)	24.8	ACG (T)	11.5	AAG (K)	12.1	AGG (R)	1.6
GUU (V)	16.8	GCU (A)	10.7	GAU (D)	37.9	GGU (G)	21.3
GUC (V)	11.7	GCC (A)	31.6	GAC (D)	20.5	GGC (G)	33.4
GUA (V)	11.5	GCA (A)	21.1	GAA (E)	43.7	GGA (G)	9.2
GUG (V)	26.4	GCG (A)	38.5	GAG (E)	18.4	GGG (G)	8.6

Data from: Codon Usage Database.

[1]Frequency of codon per 1,000 codons

Link Navigate to the **EasyGene** site. You will want to compare your EasyGene results with what you found with ORF Finder and/or NEB Cutter, so you may want to open this site in a new window or tab. Paste your *Enterococcus* plasmid sequence into the input box. From the list of organisms, choose the most closely related available species; this organism is used to determine what Shine-Dalgarno sequence to search for as well as the codon usage pattern to use for comparison. Note the lack of an option to limit the size of ORFs; given the additional features of EasyGene, it is preferable to limit results by the significance score rather than an arbitrary size cutoff.

Examine the EasyGene results and compare them with your results from ORF Finder. We might expect EasyGene to ignore ORFs that lack Shine-Dalgarno sequences or that do not match codon usage data; does this appear to be the case? How does EasyGene's list compare with ORF Finder's when ORF Finder is limited to 100-amino-acid ORFs? What if ORF Finder is allowed to find 30-amino-acid ORFs? Does EasyGene identify any of the short ORFs excluded by the length limit as actual genes? Does EasyGene fail to identify any genes that you annotated as genuine based on your ORF analysis and BLAST searches? (If so, does lowering the significance score cut-off allow it to find these genes?)

Looking at the EasyGene results, you should see a column showing the initiation codon for each gene it found; do you see any surprises here? In fact, ATG is not the start codon for every gene: tRNA carrying methionine can in some cases bind to a bacterial ribosome positioned at a GTG or TTG codon. Take a look at the ORFs EasyGene identified as having an alternative start codon, and then find the same ORF in ORF Finder. How long was the ORF that ORF Finder identified? What happens if you click `Alternative Initiation Codons`? Why is this result better, biologically? Why did EasyGene's algorithm, even though it is still sequence based, find the longer ORF instead of the shorter one with the more obvious start codon? Does BLAST confirm that this is a better result?

Web Exploration Questions

Report your findings regarding antibiotic resistance in the *E. faecium* strain isolated from the abdominal infection. Discuss whether this strain is multidrug resistant and to what antibiotics it is resistant. Then, provide an annotated list of genes on the plasmid for which you have solid evidence: Name them if possible (refer to them by the starting position of the ORF where you cannot find a suitable name), give their start and stop positions and their length in amino acids, and list their functions briefly but specifically.

More to Explore: Evolution of Antibiotic Resistance and a Resistance Plasmid

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If you would like to use your gene prediction data to dig deeper into the nature and evolution of this resistance plasmid, try answering the following questions:

1. Multidrug-resistant bacteria are often capable of transferring resistance to multiple antibiotics on a single plasmid. Such resistance plasmids have frequently evolved when resistance genes become associated with transposons, mobile pieces of DNA able to move from place to place within a genome. If a

transposon carries a resistance gene from the chromosome to a plasmid, that gene can now be more easily passed to another strain. As resistance plasmids are passed around among bacteria, there is a good chance they will eventually be in a cell carrying a different transposon-associated resistance gene, so that the resistance plasmid can "collect" additional genes over time. Transposons have repeated sequences at their ends and transposase genes that carry out the reaction of "cutting and pasting" the transposon DNA. Is there evidence to suggest that this resistance plasmid evolved in this manner?

2. Vancomycin is considered a "last resort" antibiotic for infections caused by gram-positive bacteria such as *Enterococcus*. Resistance to this drug is known, but it has developed more slowly than other antibiotic resistances, and most bacteria can still be killed by vancomycin. Physicians therefore do not use it unless it is the only drug that will work in a given situation, so that further spread of resistance is not encouraged. Based on what you have been able to learn about the genes in this resistance plasmid, can you suggest why it is more difficult for bacteria to develop resistance to this antibiotic than to others?

Guided Programming Project: Pattern Matching for Sequence-Based Gene Prediction

Sequence-based gene discovery methods are really quite simple in concept: As you saw previously in the Understanding the Algorithm, they simply search a string (DNA sequence) for a match to a pattern (start codon, stop codon, Shine-Dalgarno sequence, etc.). We can use parameters to limit the range of the search and whether to consider imperfect matches. In this guided project, you are asked to write the code to implement the ORF finder algorithm. We again limit our scope to prokaryotic gene prediction, where we can use sequence-based methods most effectively.

In Understanding the Algorithm and Web Exploration, you saw that a good gene prediction program must be able to search for multiple patterns—for example, to find a start codon and then look upstream in the same sequence for a Shine-Dalgarno sequence. The pattern-matching algorithm described previously can be used repetitively by changing its parameters, so a good programming approach is to modularize your code by implementing a subroutine or function to search the sequence for the pattern. For this chapter's exercise, the focus is on reusing the function to find different kinds of patterns. Therefore, let's review how this might work. To write a function, we need to know the main task of the function, the parameters we need to pass to the function, and the information the function needs to return to the calling routine:

- **Main task**: The main task of our pattern-matching function is to traverse an input sequence searching for a pattern.
- **Parameters passed in**: For a flexible and reusable function, we should use parameters to pass in the distinctive information for a particular search: the pattern, the searched text, the start and stop locations, the increment value, and the threshold value.
- **Information returned**: For a gene prediction program, we need to know the location where the pattern was found. If we use 0 to represent the location of the

first character in the sequence, then -1 is an invalid location and we can use this value to represent a failed search. The calling program can determine whether the function returned a positive number (location of a successful match) or a negative number (pattern not found). In some situations, we might also need to return additional information such as the number of matched nucleotides or the number of matches.

The following pseudocode shows a solution for our function.

Algorithm

Pattern-Matching Function

Goal: A function that can be used to find a pattern within a search text.

Parameters: pattern, searched text, start location of search (assumes 0 is the first position in the search text), stop location of search, increment, threshold

Return Value: The location of the pattern in the search text (assumes first character represented by location 0) or -1 if pattern not found.

```
//  Function findPattern
findPattern(pattern, searchText, startLoc, stopLoc, increment,
threshold)
   textLen = length of searchText
   patternLen = length of pattern
   for each i from startLoc to stopLoc by increment
      ctr = 0
      j = i
      // count number of matching characters
      for each k from 0 to patternLen
         if searchText[j] == pattern[k]
            ctr ++
         j++
      // compare number of matched characters to threshold
      if ctr/patternLen >= threshold
         return i
   return -1
```

Notice in this example that a `return` statement appears within the loop, so that the loop terminates as soon as a match is found. Some programmers prefer to exit a loop only when the conditional statement of the loop fails, a technique that improves readability in long, complex programs. In this short function, however, the return will not detract from readability and saves unnecessary looping as well as an additional flag variable. If the loop ends (reaches the end of the sequence without finding the pattern), the search has failed and -1 is returned. The function just given can be used to find any of the patterns necessary for gene prediction in prokaryotes and can be called multiple times within a complete gene prediction program. Your main program should carry out the following steps, calling the pattern-matching subroutine to look for each pattern:

1. Search for a start codon. If found, continue; otherwise, end.
2. Search for a stop codon in the same reading frame as you found the start codon. Determine if the number of codons between the start and stop codons is >= a user-defined minimum value. If a large enough ORF is found, continue; otherwise, end.
3. Search for a Shine-Dalgarno sequence no less than three and no more than seven bases upstream of the start codon. The sequence should match at least five nucleotides of the six-nucleotide consensus. If found, the ORF is a possible gene: continue; otherwise, end.
4. Search the 500 nucleotides upstream of the Shine-Dalgarno sequence for a promoter sequence: `TTGACA` located 15–19 nucleotides upstream of `TATAAT`, allowing at most one mismatch in each sequence.

Putting Your Skills Into Practice

1. Implement the pattern-matching algorithm within a complete prokaryotic gene prediction program as described earlier. You may wish to review the Test Your Understanding questions, where you should have already considered parameters that would allow your algorithm to search for these elements. Generate a short test sequence with clearly defined promoter and Shine-Dalgarno sequences to ensure your program works as expected.
2. Modify your program to allow the user to choose the match threshold for the Shine-Dalgarno and promoter sequences. Test the program using the*Enterococcus* plasmid sequence. Because this is a large sequence, you might want to start by testing only the ORF-finding routine on a segment of the plasmid sequence. Use your ORF Finder results for comparison. Then add the Shine-Dalgarno and promoter prediction and compare your results with those obtained using EasyGene. Can the program find the genes and promoters EasyGene found? What happens if the thresholds for the consensus sequences are relaxed?
3. Modify your program so it searches all six reading frames. Did you modify your function or the calling routine?
4. On any sizeable piece of DNA, there will probably be more than one ORF; however, the previous steps stopped searching after any step failed. Modify your program so it continues to search until all possibilities are exhausted.
5. Modify your program to discard an ORF if it has the same stop codon as an ORF already found and is shorter.
6. In prokaryotes, ORFs that are part of operons (see BioBackground) may not be directly preceded by promoters: One promoter is used for the entire operon. However, each ORF will still be preceded by a Shine-Dalgarno sequence. Modify your program to take this information into consideration: For example, you might check for upstream ORFs oriented in the same direction and then look for promoters, or you might look farther upstream for a promoter first.

7. How do the genes identified by your program in the *Enterococcus* sequence compare with those found by EasyGene? Your program uses a sequence-based search for promoters, whereas EasyGene uses a content-based analysis of codon usage; which mechanism seems to have worked best in terms of identifying the genes you classified as genuine in the Web Exploration?

On Your Own Project: Sequence-Based Gene Discovery in Eukaryotes

Download In the Web Exploration, you used—and in the Guided Programming Project developed— programs to find genes in prokaryotes using ORFs and sequence clues like promoter and Shine-Dalgarno sequences. In this project, you will apply these skills to predicting genes in eukaryotic genome sequences such as the human genome. If you are taking a nonprogramming course, there are exercises dealing with how sequence-based methods can be applied to eukaryotes, and your instructor can make a **completed gene prediction program** available for you from the *Exploring Bioinformatics* website.

Understanding the Problem: Sequence-Based Pattern Matching in Eukaryotes

Clearly, our gene prediction program from the guided project does not care whether the input sequence is from a prokaryote or a eukaryote; it can just as well find eukaryotic patterns. Unfortunately, it is more difficult to determine what patterns to search for in eukaryotes. Although the start and stop codons are identical, there is no Shine-Dalgarno sequence to identify the correct start codon, nor is there a single, clear promoter sequence (see Bio-Background). Worse, the ORFs are usually interrupted by introns, so we cannot start with simple ORF finding. However, we do have some options.

In eukaryotes, the start codon is almost always the *first* AUG from the 5′ end of the mRNA and thus the first one after the transcriptional start site. Furthermore, in about 75% of cases, the transcriptional start can be identified by the presence of a **core promoter** pattern. Thus, you should be able to modify your solution to the Guided Programing Project to look for a start codon (not an entire ORF, because of the intron problem) preceded by the core promoter pattern. Subsequent analysis could then identify the ORF by looking for splice sites and predicting where the exons are (much more on this topic in Chapter 10).

The core promoter can be recognized by a consensus sequence called the **TATA box**, a sequence similar to `5´ TATA(A or T)A(A or T)` followed by three additional nucleotides that are rarely cytosine or guanine. The TATA box is usually found within about 150 nucleotides upstream of the start codon and at about the -25 position relative to the +1 nucleotide (first nucleotide transcribed into mRNA). The transcriptional start site itself (+±1) commonly lies within an additional consensus sequence, the **initiator sequence** (***Inr***). *Inr*consists of six nucleotides: The first two are usually `C` or `T`, the last two are usually `G` or `A`, and the middle two are `CA`, where the `C` is usually the +1 nucleotide. We can write this sequence more easily by using code letters to represent combinations of nucleotides (so-called**ambiguous** nucleotides): Y (for pYrimidine) to represent C or T and R (for puRine) to represent G or A. The *Inr* sequence is then YYCARR. Similarly, in the TATA sequence, W is used to represent A or T, and the sequence is written TATAWAW. **Table 9.2** shows the complete set of ambiguous nucleotide codes.

Table 9.2: One-letter code for ambiguous nucleotides.
Open table as spreadsheet

Code	Meaning
N	A, T, C or G (aNy base)
R	A or G (puRine)
Y	C or T (pYrimidine)
W	T or A (Weak)
M	C or A (aMino)
K	T or G (Keto)
S	C or G (Strong)
B	C, T, or G ("not A")
D	A, T, or G ("not C")
H	A, C, or T ("not G")
V	A, C, or G ("not T")

We can think of the core promoter as the minimal requirement for eukaryotic transcription. Unlike prokaryotic RNA polymerase, which binds directly to the -10 and -35 promoter sequences, eukaryotic RNA polymerase II (the form of RNA polymerase that transcribes mRNA) binds to **transcription factors**: proteins that in turn bind to the DNA sequences. The transcription factors that bind the core promoter (e.g., TFIID, which binds the TATA box) direct RNA polymerase to the correct location for transcription, but a gene with *only* these promoter elements is only very weakly transcribed. Higher-level transcription requires additional transcription factors bound to additional sequences. Some transcription factors bind to sequences common at many different promoters, such as the CAT box (5´ CAAT) and the GC box (5´ GGGCGG), both of which usually occur within about 100 bp of the +1 site. Other transcription factors promote the transcription of genes only in a specific cell type or in response to some particular condition; their binding sites may be hundreds or even thousands of bp upstream. Examples include the estrogen response element (ERE; 5´ AGGTCANNNTGACCT) bound by the estrogen receptor in response to the hormone estrogen, the NF-κB site (5´ GGGRNNYYCC) used to activate growth and genes of the immune system, and the heat-shock element (5´ AGAAN repeats) activated in response to elevated temperature. Finding binding sites like these in a putative promoter region not only strengthens the case that a transcribed region has been identified but also provides clues about how the gene is regulated.

Solving the Problem
The questions in this section should help students in programming courses develop their implementation of a eukaryotic gene prediction program. Students in nonprogramming courses may wish to use these questions as exercises to test their understanding of the algorithms involved in sequence-based gene prediction.

The pattern-matching algorithm discussed earlier uses a threshold parameter to decide how closely a sequence must match the pattern. How is this different from matching ambiguous nucleotides? If the eukaryotic gene prediction algorithm can match ambiguous nucleotides, does it still need the threshold parameter? Which of the sequence patterns discussed previously would you want to require a program to find to identify a gene, and which would be optional or perhaps user-selected?

ATG codons used as start sites occur most commonly within a consensus sequence known as the Kozac sequence: 5′ `gccRccATGG`. In this sequence, capital letters represent highly conserved bases and lower case letters represent bases that are common but not as highly conserved. How could the algorithm be modified to account for the Kozac sequence? A short distance past the stop codon, eukaryotic genes have a polyadenylation site where the mRNA is cleaved and the poly(A) tail added. Although this sequence, 5′ `AAUAAA`, is known, why would it probably not be worthwhile to search for this sequence as a marker for the end of a predicted gene?

Programming the Solution
Your eukaryotic gene prediction program should search for start codons preceded by a core promoter sequence and allow users the opportunity to select other regulatory patterns from a list or read them in from a file (for example, one user might want to find estrogen-regulated genes but someone else might be interested in heat-shock genes).

Your program will need to recognize the codes for ambiguous nucleotides such as Y and W. Suppose you are searching for `YYCARR` (the *Inr* sequence). One approach is to search for the unambiguous bases CA and then search backward and forward for valid nucleotides. Or, you could create a list of all possible values (`CCCAGG`, `CTCAGG`, `TCCAGG`, `TTCAGG`, `CCCAGG`,`CTCAGG`, etc.) and then search for an exact match with any one of those values. Regular expressions or character classes could be used to help with this search if appropriate for your language. Your program will also need to allow for some variation from the consensus sequence.

Running the Program
Download Create some short sample sequences to test your program; include ATGs that are and are not preceded by core promoter sequences or other promoter elements. Once you have a working version of your program, download a **test sequence** containing a eukaryotic chromosome region with one predicted gene from the *Exploring Bioinformatics* website. How does your program fare with this complex sequence? After completing the Web Exploration inChapter 10, you may wish to compare your program's output to that of a program with more sophisticated prediction methods.

More to Explore: Transcription Factor Binding Sites

Link Although most currently popular eukaryotic gene prediction programs incorporate content-based or probabilistic methods (Chapter 10), sequence-based methods remain important for exploring how predicted genes might be regulated by identifying binding sites for known transcription factors. If you would like to explore this idea further, you may want to look at the **`Jaspar`** database of transcription factor binding sites or the **`TFSEARCH`** or **`MAST`** search tools that can look for binding sites in a sequence you provide.

Connections: Ongoing Need for Gene Discovery

With the human genome "finished" since 2003, you might wonder if the need for gene discovery is fading. On the contrary, gene prediction remains a thriving part of bioinformatics for a number of reasons. Next-generation sequencing offers more sequences faster and cheaper than ever before, and new genomes—from viruses and bacteria to vertebrates—are being sequenced at the rate of dozens per month. Although there are often related genomes that allow annotation by alignment, each genome is unique and has genes never previously sequenced. Sequencing of metagenomes (seeChapter 8) of completely unknown organisms is resulting in the identification of many genes unlike anything in the databases. Even within sequenced genomes, gene discovery is an ongoing process; as discussed in Chapter 10, no one yet knows with certainty the actual number of genes in the human genome—let alone how many total proteins (considering alternative splicing and other complications) they encode.

The study of small RNAs has become a key area of molecular genetics within the past few years, with the increasing recognition that short functional RNA molecules play important roles in the lives of cells. In addition to tRNAs, small RNAs are found as components of the ribosome, the spliceosome, and some enzymes ("ribozymes" such as telomerase, the enzyme that constructs the ends of chromosomes). Genes encoding the extremely small (20–25 nucleotide) short-interfering RNAs (siRNAs) and micro RNAs (miRNAs) recently shown to regulate gene expression and contribute to antiviral defenses are especially difficult to predict, and some estimates suggest there may be tens of thousands of such genes in the human genome. It is certain that the need for gene discovery will not soon disappear. New kinds of genes require the development of new computational algorithms and bioinformatic techniques, and similarity and structure analyses will continue to be needed to uncover the functions of newly discovered genes.

BioBackground: ORFs, Consensus Sequences, and Gene Structure

There are many ways to define a gene. One that covers most bases is that a gene is a**transcription unit**: a segment of DNA that can be transcribed into RNA. Although we most often think about genes encoding proteins, this definition also covers genes that encode functional RNAs, such as tRNAs and rRNAs used in the process of translation, as well as small regulatory RNAs and components of various enzymes. A transcription unit must have a **promoter**: DNA sequences allowing RNA polymerase to identify and transcribe the gene. If it is a protein coding gene, then within the transcribed region, there must be an **open reading frame**: a start codon (ATG, or AUG in RNA), a set of codons encoding various amino acids, and a stop codon (TGA, TAG or TAA).

For a protein coding gene, the eukaryotic ribosome begins translating at the first start codon of an mRNA. Thus, the eukaryotic transcription unit can contain only a single ORF. However, this ORF may occur in segments called **exons** broken up by noncoding regions called **introns**. In a prokaryotic cell, the ribosome finds the correct start codon by binding to a sequence known as the **Shine-Dalgarno sequence** or **ribosome binding site** that precedes the start codon by a few bases. Thus, a prokaryotic transcriptional unit may contain multiple ORFs, each encoding a distinct protein and each preceded by a Shine-Dalgarno sequence. A transcription unit containing two or more ORFs is known as an **operon**, and the proteins encoded by genes in an operon often function together in some cellular process. **Figure 9.5** compares eukaryotic (A) and prokaryotic (B) transcription units.

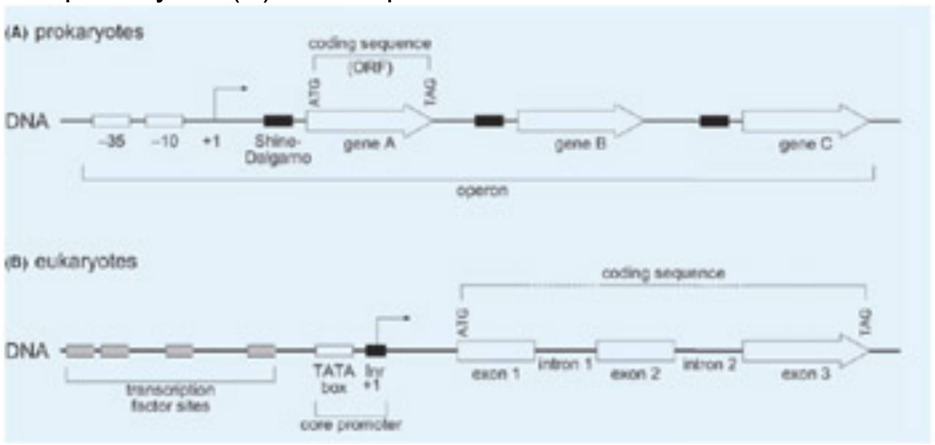

Figure 9.5: Comparison of (A) a prokaryotic transcription unit, showing a three-gene operon with a single promoter and individual Shine-Dalgarno sequences marking the start codon for each ORF; and (B) a eukaryotic transcription unit, showing a single gene interrupted by introns and preceded by a core promoter region and additional transcription factor binding sites.

Because prokaryotes lack introns, we can readily identify unbroken ORFs by looking for start and stop codons; the amino acids encoded by the codons between the two can be identified by reading the nontemplate (mRNA-like) strand and applying the genetic code (Chapter 2). Certainly, long ORFs are likely to be genes, but it is harder to tell if a short ORF might encode a short protein. An ORF preceded by a Shine-Dalgarno sequence and (farther upstream) a promoter sequence can be identified as a gene with more confidence, although the possibility that an ORF may be separated from its promoter by one or more other genes in the same operon must be considered.

Eukaryotic DNA lacks Shine-Dalgarno sequences to conveniently mark start codons, and an intron-interrupted ORF may be spread over tens or hundred of thousands of nucleotides. Promoter regions still serve as useful clues, but whereas prokaryotes have clear consensus sequences for promoters, eukaryotic RNA polymerase looks not for a specific sequence but rather for an assembly of transcription factors bound to sites that may be near the transcriptional start site or hundreds of base pairs away. Some transcription factors bind most promoters, whereas others are specific to a particular cell type or condition. Furthermore, in both prokaryotes and eukaryotes, variation among species can be seen in the binding proteins and thus the sequences they bind. Promoters are also used to initiate transcription of genes for noncoding RNAs, but in eukaryotes, there are three distinct RNA polymerases (I, II, and III) that transcribe different kinds of genes (rRNA, mRNA, and tRNA/small RNAs, respectively), each with its own distinct promoter structure.

Table 9.3 shows some DNA sequences that are important in gene expression in prokaryotes and eukaryotes. These are referred to as **consensus sequences**, because they are not as precise as might be imagined. The prokaryotic promoter, for example, is defined by two six-nucleotide sequences. One, the 2**10 sequence**, is centered at about 10 bp upstream of the transcriptional start site and is similar to 5′ TATAAT. The other, the 2**35 sequence** is centered at about 35 bp upstream of the start site and is similar to 5′ TTGACA. However, few if any natural promoters contain exactly these two sequences. Genes expressed at a high level tend to have closely matching promoter sequences, whereas weaker promoters are farther from the consensus sequence, but even strongly expressed promoters typically vary from these "canonical" sequences by a nucleotide or two. The consensus sequences were developed by sequencing and aligning the promoter regions (determined by biochemical and molecular experiments) of multiple genes and looking for the sequences that are conserved among them (**Figure 9.6A**). The nucleotides in the consensus are those that occur most frequently; ambiguous nucleotide codes (**Table 9.2**) are used when two or more occur with nearly equal frequency. A graphical representation called a **sequence logo** (**Figure 9.6B**) gives a better idea of the relative occurrences of the four nucleotides at each position. The sequences given in this chapter for the Shine-Dalgarno site, TATA box, *Inr* site, transcription factor binding sites, and so on are all consensus sequences derived from studying the sequences found in many genes.

Table 9.3: Consensus sequences for gene expression in prokaryotes and eukaryotes.

Open table as spreadsheet

Sequence	Consensus (5' → 3′)	Function
	Prokaryotes	
-10 sequence	TATAAT	RNA polymerase binds to start transcription
-35 sequence	TTGACA 17±2 from -10	RNA polymerase binds to start transcription
Shine-Dalgarno	AGGAGG 5±2 from ATG	Ribosome binds to find start codon
	Eukaryotes	
TATA box	TATAWAW	Core promoter; binds TFIID
Inr sequence	YYCARR	Core promoter; contains +1 sequence (C)
GC box	GGGCGG	Transcription factor binding site
CAT box	CAAT	Transcription factor binding site
Kozak consensus	gccRccATGG	Context of start codon
5' splice site	MAG | GTragt	Bound by spliceosome to remove introns
3' splice site	cAG | G	Bound by spliceosome to remove introns
intron branch site	CTRAY	3' end of intron binds to mark for degradation
polyadenylation site	AAUAAA	Cleavage of mRNA for poly(A) tail

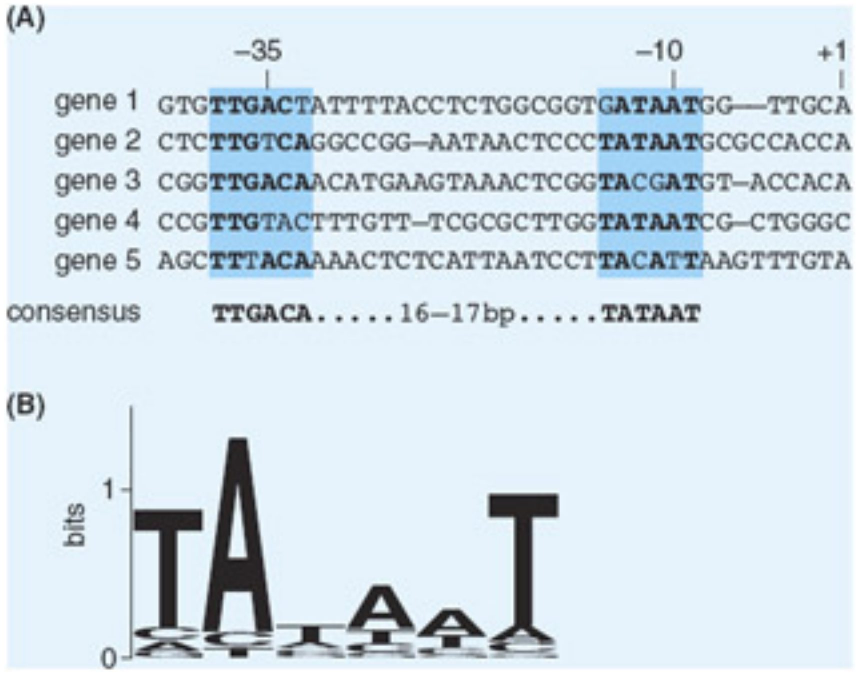
(A)
−35
−10
+1
gene 1 GTGTTGACTATTTTACCTCTGGCGGTGATAATGG—TTGCA
gene 2 CTCTTGTCAGGCCGG–AATAACTCCCTATAATGCGCCACCA
gene 3 CGGTTGACAACATGAAGTAAACTCGGTACGATGT–ACCACA
gene 4 CCGTTGTACTTTGTT–TCGCGCTTGGTATAATCG–CTGGGC
gene 5 AGCTTTACAAAACTCTCATTAATCCTTACATTAAGTTTGTA
consensus TTGACA.....16–17bp.....TATAAT
(B)
bits
1
0

Figure 9.6: Generation of a consensus sequence. (A) The prokaryotic promoter consensus sequence derived from sequences of individual promoters. Conserved regions are shaded, with individual nucleotides that match the consensus in bold. (B) Sequence logo showing the occurrence of the four nucleotides at each position in the -10 promoter consensus, generated with WebLogo from a subset of the data published by Harley and Reynolds (see References and Supplemental Reading). Sequence logo generated from WebLogo: Crooks et al., *Genome Res.* 14:1188 (2004).

References and Supplemental Reading

Introduction to Gene Prediction Methods

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Chapter 10: Advanced Gene Prediction: Identification of an Influenza Resistance

Chapter Overview

This chapter builds on the sequence-based gene prediction methods discussed in Chapter 9and examines content-based and probabilistic methods of gene discovery. These methods are of particular importance in eukaryotic gene prediction: The division of eukaryotic coding sequences into multiple exons separated by variable-length introns with poor consensus sequences at their boundaries greatly increases the difficulty of identifying coding sequences computationally. Codon usage and CpG island identification are introduced as content-based algorithms contributing to gene prediction, and neural networks and hidden Markov models are presented as examples of probabilistic gene prediction. The Web Exploration gives students the opportunity to use some of these prediction methods, whereas the Guided Programming Project enables programming students to experiment with prediction of CpG islands. In the On Your Own Project, students explore the design of a gene prediction method based on a hidden Markov model.

- **Biological problem**: Identification of an influenza-resistance gene
- **Bioinformatics skills**: Exon–intron prediction, neural networks, hidden Markov models
- **Bioinformatics software**: GENSCAN, AUGUSTUS, Sequence Manipulation Suite (CpG island prediction), Neural Network Promoter Prediction
- **Programming skills**: Frequency matching and sliding windows, hidden Markov modeling

Understanding the Problem: Exon Prediction

Among the priorities for influenza research laid out by the World Health Organization in 2009 is the investigation of genetic factors affecting susceptibility of individuals to influenza virus infection. Understanding how individual genetic variation might result in either increased susceptibility to influenza or increased resistance to the disease could lead to new preventative or therapeutic measures, either conventional or genetic. To be useful, however, recognition of heritable factors altering resistance must be followed by identification of specific genes and alleles. Methods such as genomewide association studies (GWAS; see Chapter 1) can identify general areas of the genome connected to a phenotype, but gene prediction methods may be needed to identify specific genes located in the identified region.

One of the surprises in the "rough draft" of the human genome announced in June 2000 was the small number of protein coding genes: Whereas many researchers had predicted 80,000 to 100,000 genes in the human genome, the actual number appeared to be less than 30,000. Indeed, by the time a "finished" genome sequence was announced in April 2003, the estimate of protein coding genes had been further revised downward to between 20,000 and 25,000. Even today, the exact number of genes in the genome remains uncertain. Annotation of the genome, the identification of genome elements and their functions (**Figure 10.1**), is an ongoing effort. A 2012 report from the ENCODE consortium, whose goal is to definitively catalog the human genomes, identified 20,687 protein coding genes, but further studies are likely to change that number. Gene discovery software plays an importantrole in this continuing process.

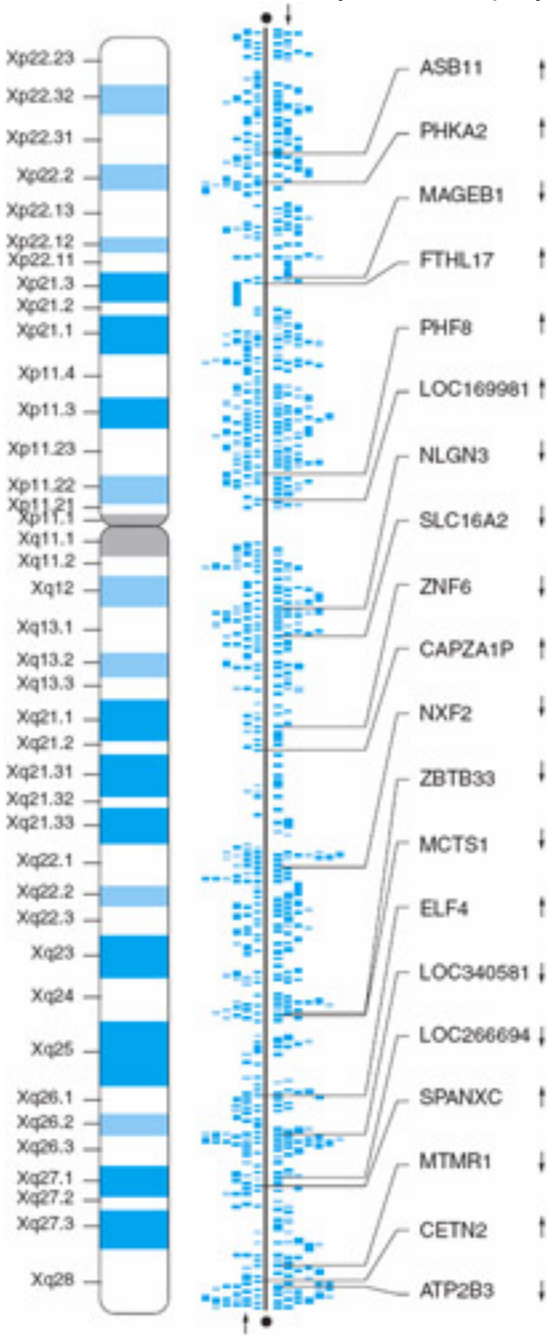


Figure 10.1: A map of the human X-chromosome, showing locations and identities of some of its genes.

As discussed in Chapter 9, sequence-based methods of gene prediction are the most straight-forward and are quite reliable in prokaryotes. In eukaryotes, however, a number of problems arise. First, there is no Shine-Dalgarno sequence to mark the start codon;

eukaryotic translation begins at the *first* start codon in the mRNA, and unambiguous identification of the transcriptional start site is difficult. Second, eukaryotic promoters are a collection of transcription factor binding sites rather than the more consistent -10 and -35 sequences of prokaryotes; many include the TATA box and *Inr* sequences of the core promoter, but this is not universally the case. Third, most importantly, there are very few unbroken ORFs: Nearly all genes in eukaryotes, especially higher eukaryotes, are split into multiple exons separated by introns. Finally, the sequence patterns at the intron–exon boundaries lack the clarity needed for reliable sequence-based prediction; it is clear from the sequence logos for the 5′ (or splice donor; **Figure 10.2A**) and 3′ (or splice acceptor; **Figure 10.2B**) sites that only a dinucleotide is truly conserved at each boundary, surrounded by a weak consensus. Thus, we need to consider additional methods of gene discovery in annotation of eukaryotic genomes.

Gene prediction is used to identify genes within a newly sequenced genome but is also valuable in identifying genes when a particular genome region has been associated with a disease or phenotype of interest. In this chapter, we see how gene discovery algorithms designed to distinguish exons from introns can lead to the identification of a potential influenza resistance gene within a large DNA region correlated with inherited resistance. The gene examined in this chapter is known to interact with the influenza virus and has been suggested by Wolff et al. as a possible resistance gene (see References and Supplemental Reading); however, the identification of its chromosomal region with resistance is hypothetical.

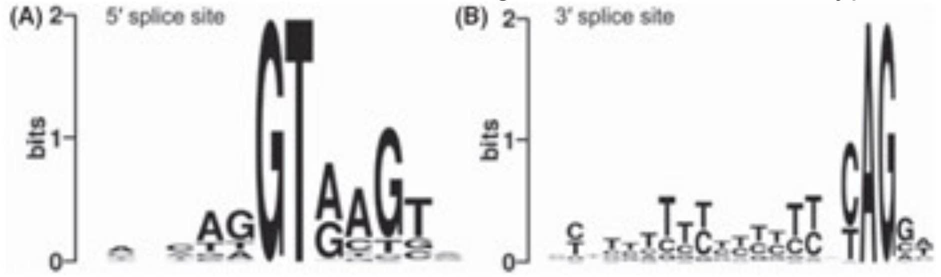


Figure 10.2: Sequence logos showing the poor consensus sequences found at the (A) 5' (splice-donor) and (B) 3'(splice-acceptor) sites between introns and exons. Sequence logo generated from WebLogo: Crooks et al.,.*Genome Res*. 14:1188 (2004).

Bioinformatics Solutions: Content- and Probability-Based Gene Prediction

If we cannot rely on ORFs and consensus binding sites to clearly define the set of genes in a eukaryotic genome, how else can we approach this problem? In Chapter 9's Web Exploration, we used one method that did not depend on identifying particular sequence patterns: EasyGene combines sequence-based searches for ORFs and Shine-Dalgarno sequences with an examination of codon-usage patterns. Codon usage is an example of a content-based method of gene prediction: A putative sequence is examined to see if the frequency of usage of different codons matches that observed for the organism as a whole. In reality, there could be reasons why some genes have a different codon bias than others (for example, some genuine genes may have been acquired by horizontal gene transfer), but, in general, authentic genes all show similar codon usage within one organism. This technique can also be applied to prediction of introns and exons within a presumed transcription unit: Where codon usage changes noticeably from the norm, a boundary between an exon and an intron has probably been crossed. Another content-based method is looking for **CpG islands** (see BioBackground), structures associated with transcribed regions.

A problem with content-based methods is that they are not very precise. We may be able to find regions where codon usage matches the expected frequency well or poorly, for example, but this is unlikely to tell us exactly where an exon–intron boundary lies. Combining two methods, such as looking for a consensus exon–intron boundary sequence within the region where codon usage changes, can yield a better prediction than either the sequence- or content-based method alone.

Better predictions still can be achieved by probabilistic methods such as **hidden Markov models** (**HMM**s). These are not truly distinct methods, but rather they use sequence and content data to calculate probabilities, such as the probability that any given nucleotide lies within an exon. Points where that probability declines sharply are likely to mark the boundaries between exon and intron, whereas points where it increases sharply mark intron–exon boundaries. This chapter considers some content- and probability-based methods to see how they are used to identify the segments of a coding sequence within a larger sequence. In the Guided Programming Project, you will experiment with how to identify CpG islands, and in the On Your Own Project, you will try your hand at designing an HMM to identify introns and exons.

BioConcept Questions

1. Why is codon usage a poor predictor of the point where an exon and intron are joined? Why is the 5′ splice site consensus also a poor predictor?
2. How much of a typical human gene is usually coding sequence, versus intron sequences that are spliced out (you may wish to recall the gene displays you saw in the UCSC Genome Browser in Chapter 1)? How does this pattern affect the difficulty of predicting introns and exons?
3. Why are CpG islands considered valuable for gene prediction? Where would you expect to find one with respect to a eukaryotic transcription unit? What other elements might you look for in connection with the CpG island to increase the strength of a gene prediction?
4. How could alignment of a sequence with orthologous sequences contribute to the prediction of exons and introns? How could expression data (e.g., cDNA sequences) contribute?

Understanding the Algorithm: Codon Usage, Frequency Matching, HMMs, and Neural Networks

Learning Tools

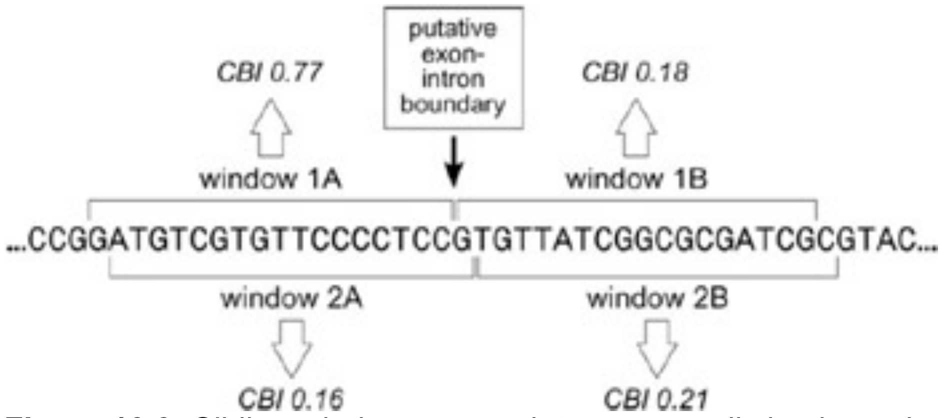
Link The *Exploring Bioinformatics* website has a link for an online hidden Markov model demo that you can use to get a better idea of how this model chooses the most likely hidden states given a set of probabilities.

This section briefly considers algorithms for two content-based methods of gene prediction: codon usage and identification of CpG islands. We then spend some time on understanding hidden Markov models and briefly cover neural networks, two probabilistic methods that can be applied to gene prediction but are also used in many other areas of bioinformatics, including protein structure prediction and sequence alignment.

Using Codon Frequencies in Gene Prediction

In a protein coding sequence, codons are not used with equal frequency. Some amino acids are much more common in proteins than others: Serine is the most common amino acid in vertebrate protein sequences (about 8% of all amino acids), whereas tryptophan is the least common (only 1%). Additionally, the genetic code is redundant, and where there are multiple synonymous codons for one amino acid, they are not used with equal frequency. This idea was discussed briefly in Chapter 9, with the codon frequency table for *E. coli* given in Table 9.1.

How might we apply the idea of codon frequency to predicting which sequences are exons and which are introns? An exon–intron boundary would be expected to separate a region where the codon frequency closely matches the expected frequency for the organism from a region where the frequency matches poorly, and an intron–exon boundary would do the reverse. As shown in **Figure 10.3**, we could examine a range or "window" of nucleotides, perhaps 75 nt (illustrated with a short sequence as window 1A in Figure 10.3), break it into codons (25 codons, in this case), and measure codon usage. Several codon usage measuresare in common use; one is the **codon bias index** (**CBI**) proposed by Bennetzen and Hall (see References and Supplemental Reading) that compares the usage of "preferred" (most common codons) to the random occurrence of those codons, giving a number between 0 (random codon usage) and 1 (exclusive usage of preferred codons). The same procedure is then repeated for the 75 nucleotides immediately downstream (window 1B in Figure 10.3) and the difference between the two is determined. The two windows are then shifted by one nucleotide (windows 2A and 2B in Figure 10.3), and the difference in CBI is computed again; note that the codons examined here are in a different reading frame.


CBI 0.77
putative exon-intron boundary
CBI 0.18
window 1A
window 1B
...CCGGATGTCGTGTTCCCCTCCGTGTTATCGGCGCGATCGCGTAC...
window 2A
window 2B
CBI 0.16
CBI 0.21
Figure 10.3: Sliding-window approach to exon prediction by codon-usage bias. Codon usage is compared for two adjacent same-length sequence windows (1A and 1B); a large difference suggests an exon–intron boundary. The windows slide along the sequence (2A and 2B) to identify potential boundaries in different reading frames along the length of the sequence.

Continuing through the sequence with this **sliding window** approach, we expect to find points at which the boundary between the "A" and "B" windows corresponds to a drop in CBI to near zero (exon–intron boundary) or a sudden increase in CBI from near zero to a larger number (intron–exon boundary). Additional constraints can be added to the algorithm based on our understanding of gene structure. For example, the putative boundaries can be rejected if the conserved GT and AG pairs are not present. Additionally, the first exon must start with ATG and should not be preceded by a splice consensus, and the last exon ends with a stop codon and is not followed by a splice consensus.

Prediction of CpG Islands

Given the difficulty of unambiguously recognizing a eukaryotic promoter region based on consensus sequences, identification of CpG islands (see BioBackground) adds valuable corroboration and can be used in combination with sequence-based methods and exon prediction techniques to help identify the first exon of a gene. We can find CpG islands with a**frequency matching** algorithm. This algorithm uses a sliding window approach like the one just discussed (except that only one sliding window is needed) combined with elements of a pattern-matching algorithm (Chapter 9), counting up CG pairs within each window and computing a CpG ratio. The steps of this algorithm are outlined next. Notice that the CpG ratio is really an odds ratio: The result is 1.0 if the number of CpG pairs found in a window is the same as the number that would be expected by chance. **Figure 10.4** shows the result of graphing the CpG ratio as the window slides through a DNA sequence.

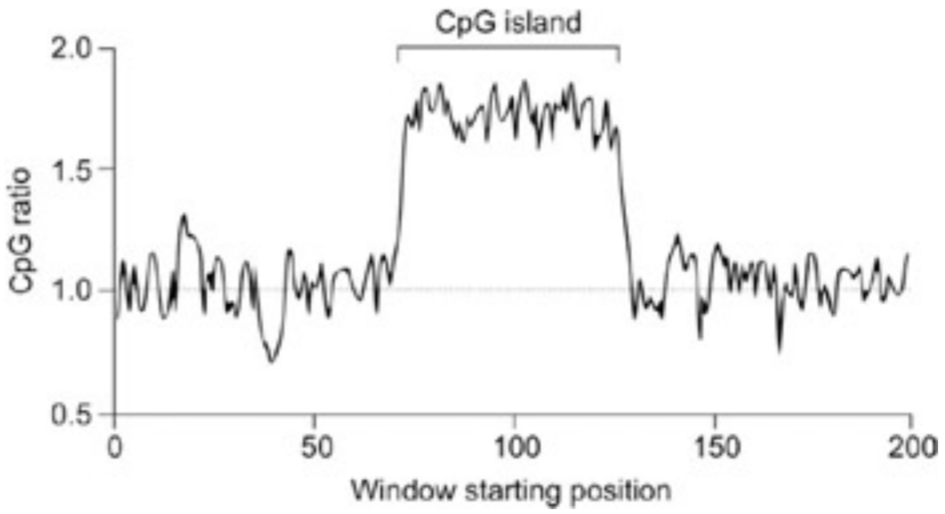


Figure 10.4: Sample of graphical output from a CpG island prediction program, with the CpG ratio (1.0 if the CpG frequency is the same as expected by chance) measured for each window as a sliding window moves across a sequence. A region of consistently high CpG ratio values represents a CpG island.

Algorithm

Frequency-Matching Algorithm

1. Determine window size and set start position to the first nucleotide in the sequence.
2. Count the number of CG pairs, C nucleotides, and G nucleotides in the window.
3. Calculate the ratio of observed to expected CpGs for the window:

$$\frac{observed\,CG\,pairs}{C\,nucleotides \times G\,nucleotides\ /\ total\,nucleotides}$$

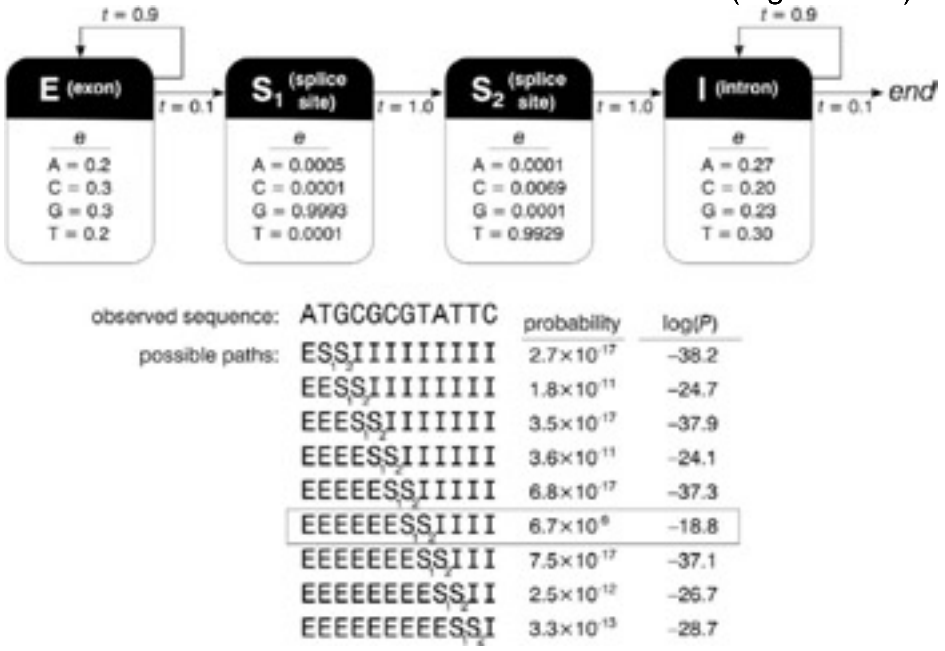
4. Increment the start position by 1. If the window is not longer than the remaining sequence, repeat step 3; otherwise, continue.
5. Examine the CpG ratios for all the windows and identify areas of CpG islands where the ratio is higher than a threshold.

HMMs for Gene Prediction

The difficulties with eukaryotic exon prediction discussed previously in combination with the explosion of genomic information available (especially with the advent of faster, cheaper next-generation sequencing) have driven the development of gene discovery algorithms to be more powerful even than combinations of sequence- and content-based methods. Many popular gene prediction programs are now based on implementations of hidden Markov modeling, probability-based algorithms that use sequence and content data to inform a calculation of the likelihood that a given sequence is part of an intron or exon.

Simply put, an HMM seeks to draw a conclusion about something that cannot be directly observed ("hidden") based on a set of observations and a set of known probabilities. A commonly given example is someone who wants to determine the weather in a certain city based on an observation such as umbrella sales or the activities a friend chooses. Given these observations and some basic data, such as the overall frequency of sunny and rainy days in that city, an HMM can compute the highest probability for the actual weather, which is the hidden state.

Applying an HMM to gene prediction, the nucleotides of a DNA sequence would represent the input observations. In a simple model for an exon–intron boundary (**Figure 10.5**), the nucleotides could exist in one of three hidden states: exon (E), intron (I), or splice site (S). For each state, we have an alphabet of possible symbols that could be output. A position in an exon, for example, could be any nucleotide from the alphabet A, C, G, and T. We then use the data we have about genes in the organism being studied to determine **emission probabilities** (*e*): the likelihood of each output. For example, we might assume that As, Cs, Gs, and Ts occur with equal frequency within an exon and assign each one an emission probability of 0.25. Given more information, however, we could refine the probabilities further: It turns out that in human exons, codon bias and other factors increase the likelihood of a G or C (see References and Supplemental Reading), so a better set of emission probabilities for the exon or E state would be 0.3 for G and C and 0.2 for A and T (Figure 10.5).



observed sequence:	ATGCGCGTATTC	probability	log(P)
possible paths:	$ES_1S_2IIIIIIIII$	2.7×10^{-17}	–38.2
	$EES_1S_2IIIIIIII$	1.8×10^{-11}	–24.7
	$EEES_1S_2IIIIIII$	3.5×10^{-17}	–37.9
	$EEEES_1S_2IIIIII$	3.6×10^{-11}	–24.1
	$EEEEES_1S_2IIIII$	6.8×10^{-17}	–37.3
	$EEEEEES_1S_2IIII$	6.7×10^{-9}	–18.8
	$EEEEEEES_1S_2III$	7.5×10^{-17}	–37.1
	$EEEEEEEES_1S_2II$	2.5×10^{-12}	–26.7
	$EEEEEEEEES_1S_2I$	3.3×10^{-13}	–28.7

Figure 10.5: A hidden Markov model for the transition between an exon and an intron through a splice site (defined as the two nucleotides at the 5′ end of the intron). Black boxes show the four possible states in this model, with emission probabilities (*e*) in the white boxes below each state and transition probabilities (*t*) shown by the arrows between states. Below the model are the nine possible paths for a short DNA sequence and the probability of each; the highest probability is boxed and corresponds to a GT splice site.

We also know there is a nucleotide bias at the splice-donor site (Figure 10.2A). A more realistic HMM could take into account all the data depicted in this sequence logo, but to keep our example simple, let's only use the data for the first two nucleotides of the intron, which are almost always G and T. Knowing the frequencies with which each nucleotide is found at these two positions (**Table 10.1**), we can construct a list of emission probabilities for each nucleotide of a two-nucleotide splice-donor site (the S_1 and S_2 states in Figure 10.5). Finally, we need emission probabilities for the intron or I state. Human introns tend to be slightly AT rich, with T (0.3) favored over A (0.27) and G (0.23) favored over C (0.2).

Table 10.1: Nucleotide frequencies for the first two intron positions.
Open table as spreadsheet

Nucleotide	Position 1	Position 2
A	0.0005	0.0001
C	0.0001	0.0069
G	0.9993	0.0001
T	0.0001	0.9929

The last parameters needed for our model are the **transition probabilities** (*t*): the likelihood of changing from one state to the next versus the likelihood of remaining in the same state. A genuine splice-donor site is always followed by an intron, never an exon or another splice site, so we can assign $S_2 \rightarrow I$ a transition probability of 1.0. The probabilities for $S_2 \rightarrow E$ and $S_2 \rightarrow S_1$ are zero and therefore not shown in Figure 10.5. Similarly, we require a two-nucleotide splice-donor site, so $S_1 \rightarrow S_2$ would also have a transition probability of 1.0. For this example, we set the transition probability for $E \rightarrow S_1$ at 0.1, with $E \rightarrow E$ (remaining in the exon state) at 0.9. E cannot go to I without going to S_1 first, so $E \rightarrow I$ is zero. Finally, we set the probability of continuing in an intron, $I \rightarrow I$ at 0.9 as well, with the probability of ending the intron at 0.1. These transition probabilities are shown as arrows in Figure 10.5. Notice that the entire model can be easily represented with a picture; many authors have commented that the ability to make a statistical model for anything you can represent visually is a strength of hidden Markov modeling.

Now, our HMM can examine all possible states for each nucleotide in our input nucleotide sequence and then determine the overall probability of each outcome, or path through the states. Suppose we have a sequence that represents a two-codon exon followed by a GT splice site and four more intron nucleotides: `ATGCGCGTATTC`. In our simple model, because we have to start in an exon, end in an intron, and the splice site is a dinucleotide pattern, there are nine possible paths for this short sequence, as shown in Figure 10.5. For each, we can determine the probability at each position and then multiply to get the total probability. For example, for $ES_1S_2IIIIIIIII$, the emission probability of A in an exon position is 0.2, and the transition probability for $E'S_1$ is 0.1. Then, the emission probability for T as the first nucleotide of a splice-donor site is 0.0001, the transition probability for $S_1'S_2$ is 1.0, and the emission probability for G at S_2 is 0.0001. Next, the transition probability from $S_2 \rightarrow I$ is 1.0, the emission probability for C in an intron position is 0.2, the transition probability for $I \rightarrow I$ is 0.9 and so on. The total probability, *P*, is the product of all these individual probabilities: $0.2 \times 0.1 \times 0.001 \times 1.0 \times 0.001 \times 1.0 \times 0.2 \times 0.9\ldots$, which works out to 2.7×10^{-17}. Taking the natural log of *P* gives a log probability value of -38.2.

After computing the probability for each of the nine possibilities (see Figure 10.5), it is easy to determine which probability is the greatest (largest log *P*). In this example, the result matches the design of our test data, with a splice site following the two-codon exon.

In our simple model, we are not considering what happens downstream of the intron. In reality, there would be another transition to another splice site and then to another exon—which we could similarly model by adding additional states with corresponding transition and emission probabilities. We also used somewhat arbitrary transition probabilities; a better model would base these on the typical length of exons and introns in the organism. We also have not yet accounted for the fact that the first exon begins with an ATG and is not preceded by an intron, whereas the last exon ends with a stop codon and is not followed by an intron. We can further strengthen the model by explicitly including the probabilities of other nucleotides surrounding the two splice sites. Additional sophistication could be built into the model in many ways: The CG bias in the promoter region could also be taken into account; for example, our codon bias data could be calculated into the exon emission probabilities. Some HMMs even include advanced statistical methods such as Bayesian statistics to calculate the emission and transition probabilities at each step. You will use existing HMM-based gene prediction software in this chapter's Web Exploration, and the On Your Own Project will give you an opportunity to design an HMM that is a little more complex than our initial example.

Neural Network Modeling

The **neural network** (**NN**) algorithm is one more important gene prediction method that we touch on briefly here. It takes its name from the network of neurons in the brain, which clearly recognizes patterns better and faster than a computer can. You immediately recognize a friend's face regardless of its setting, for example, whereas face-recognition software can readily be fooled by a hat or sunglasses. Although no one knows exactly how neural processing works, we know that each of your neurons is connected to many other neurons and fires when the sum of its many inputs, positive and negative, exceeds some threshold. It is this behavior that neural network algorithms attempt to mimic.

The decision-making process illustrated in **Figure 10.6** is a simple example of a neural network: We decide whether to go to a movie based on the sum of four inputs. Each input is given a different weight, and the sum must exceed a threshold (2) to make the choice to see the movie. Similarly, inputs for a neural network to predict exons might include codon bias, CG content, consensus sequences, length, and so on.

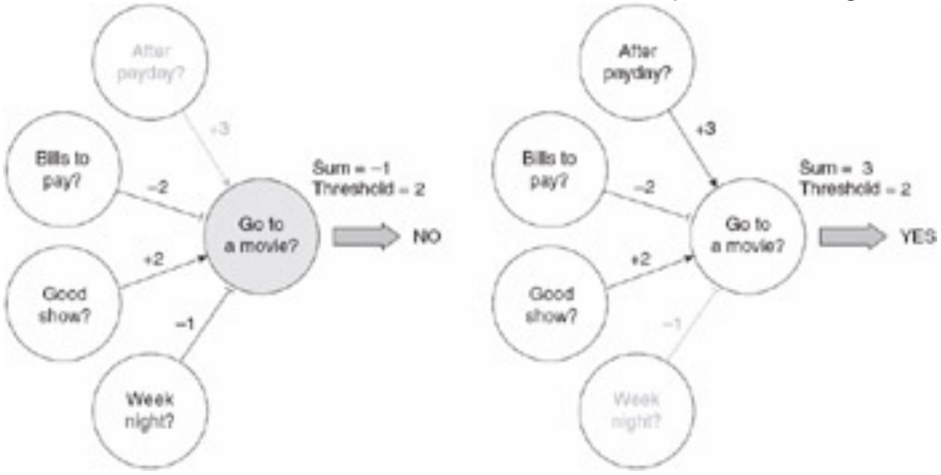


Figure 10.6: Decision making with a neural network. Four inputs, each weighted differently, contribute to deciding whether or not to see a movie. The sum of the inputs must exceed 2 in order to see the movie; this is not true in the left diagram but is true in the right diagram.

The hardest part of developing a neural network algorithm is deciding how to weight the inputs and set the threshold. Often, this is accomplished by adding a machine learning algorithm. An initial model is developed and used to classify a training set of known sequences as intron or exon sequences; the algorithm "learns" by adjusting weights and threshold until it can classify the training set with minimal errors. You will use a neural network algorithm in this chapter's Web Exploration.

Test Your Understanding

1. Suppose you use the sliding window algorithm described to analyze codon bias. At several points in a DNA sequence, you see a high score in your first window and a low score in your second window. But, when you slide the window by one or two nucleotides, you get low scores in both windows. How would you explain this pattern? How might you want to account for it in deciding where your exon–intron boundaries are?
2. Explain why the codon-usage method is likely to be imprecise in defining exon–intron boundaries.
3. CpG island prediction algorithms generally require not only a higher-than-expected frequency of CG pairs but also that the region under examination has an overall higher percentage of G+C than the average in the genome. What is the value of this constraint?
4. CpG islands are associated with promoter regions. How can this help with exon prediction?
5. Draw an HMM that requires an ATG followed by some exon nucleotides, a splice-donor site, and then some intron nucleotides.
6. How might the first exon be distinguished from internal exons in an HMM?

7. Suggest some qualities of a DNA sequence that you would weight positively and some that you would weight negatively in developing a neural network model to identify an exon.

Chapter Project: Identifying an Influenza Resistance Gene

Often, the study of a genetic disease or another genetic trait leads to a general region of the genome but does not immediately identify a particular gene. Chapter 1 dealt with how SNPs can be identified in GWAS experiments; as you saw in that chapter, the extensive human genome data now available often allows us to simply browse a genome region to look for genes of potential interest. But what happens when there is less information with which to work? This chapter's projects focus on a hypothetical but realistic scenario involving a chromosome region suspected of including an influenza resistance gene.

Learning Objectives

- Understand how eukaryotic genes introduce additional complexity into the problem of gene prediction and recognize the limitations of sequence-based methods
- Know some content-based methods of gene prediction and appreciate their strengths and limitations
- Be able to combine content-based and probabilistic methods of gene discovery to identify the most probable locations of introns and exons in a eukaryotic DNA sequence
- Know how to design an HMM to integrate sequence and content data for a more precise and accurate determination of exon–intron boundaries

Suggestions for Using the Project

In the Web Exploration for this project, students analyze a large DNA sequence to look for potential genes using several different gene prediction techniques. The different methods have different strengths, and the value of combining multiple methods will be recognized. If time is limited, the first part of the Web Exploration gives the most comprehensive look at gene prediction. In the Guided Programming Project, students implement a sliding window algorithm for a content-based gene prediction method, identifying CpG islands. In the On Your Own Project, students design (and, in programming courses, implement) an HMM that builds on the discussion in Understanding the Algorithm and includes a splice-acceptor site.

Programming courses:
- Web Exploration: Use existing tools including CpG island prediction, HMMs, and neural networks to identify exons, introns, and transcriptional units within a 90-kb segment of human DNA sequence. Part I could be used alone if needed.
- Guided Programming Project: Implement an algorithm to identify CpG islands using a sliding window algorithm.

- On Your Own Project: Design an HMM that incorporates both splice-donor and splice-acceptor sites and implement the HMM in a desired programming language. Optionally, increase the sophistication of the model by incorporating start codons and the potential for multiple exons.

Nonprogramming courses:

- Web Exploration: Use existing tools including CpG island prediction, HMMs, and neural networks to identify exons, introns, and transcriptional units within a 90-kb segment of human DNA sequence. Part I could be used alone if needed.
- On Your Own Project: Design an HMM that incorporates both splice-donor and splice-acceptor sites and then increase the sophistication of the model by incorporating start codons and the potential for multiple exons.

Web Exploration: Finding Genes in a Eukaryotic Genome Sequence

As an influenza researcher, you have become interested in a small number of individuals you know were unvaccinated and repeatedly exposed to the 2009 H_1N_1 influenza virus but did not become ill. When immunological testing showed they were not actually immune to the virus, you began to seek a genetic link that might explain their resistance to this disease. Using next-generation sequencing, you were able to identify common transcripts from respiratory epithelial cells that are missing in your resistant patients. This leads you to sequence a particular genome region in one patient, some 90,000 bp (90 kb) from the 1q25.3 region of chromosome 1. You would now like to analyze that genome fragment to identify genes within it that might be involved in susceptibility or resistance to influenza.

Part I: Gene Prediction with Genscan and Augustus

The number of available tools for gene prediction is somewhat mind-boggling. Several popular gene prediction programs are comprehensive in nature, bringing together several kinds of analysis in one piece of software; these would be a good place to start the analysis of a genome sequence or segment. We initially work with two such gene prediction programs, GENSCAN and AUGUSTUS.

Download GENSCAN (see References and Supplemental Reading) combines HMM-based models for coding-region and splice-site prediction with models that attempt to account for additional factors that affect splice-site choice as well as observed changes in splice sites and gene density in low-GC versus high-GC regions of human DNA. GENSCAN claims to correctly identify 70–80% of known exons. This comprehensive program produces clear and compact graphical output, making it easy to compare other programs' results.

Link Start by downloading **1q25.txt**, containing 90 kb of DNA sequence from human chromosome 1, from the *Exploring Bioinformatics* website. Navigate to a Web-based implementation of **GENSCAN** (there are several available) and input or upload your sequence in FASTA format. Choose a training set appropriate to analyzing human DNA from the drop-down menu: the GENSCAN implementation at the Pasteur Institute provides`HumanIso`, suitable for humans and other vertebrates and *Drosophila* (click the help icon to see this information), whereas other implementations provide a vertebrate training set. The Pasteur implementation includes additional options for `Verbose output`, providing some additional information in the output file and to `Create Postscript output`, giving a graphical representation of the results; set these options to get the most useful output. Other parameters can be left at their defaults for now; if needed, they could be set to reduce the stringency of the criteria for exons or to scale the output. Run the program.

When the results appear, you will see a window containing text output (make this window full screen to make it easier to see). The output includes the specific locations of the predicted introns and exons, information on reading frames and splice sites, and translations for the putative coding regions. There is also useful information about the reliability of the predictions. You may want to save this output to a text file (from which you could copy protein sequences for later alignment, for example) and/or print it for later reference.

There will also be a window for graphical output. If you are using a Macintosh, you can simply right-click the small visible region of the graphical output and choose `Open with Preview`. PCs unfortunately lack built-in software to deal with Post-Script files; alternatives include uploading the file to Google Drive, downloading the free Ghostscript viewer, opening with a graphics program such as Inkscape or Photoshop, installing a utility that makes PostScript files viewable with Adobe Reader, or finding an online conversion program. Choose one of these options as appropriate to view your graphical results. **Figure 10.7** shows an example of the kind of output expected from GENSCAN.

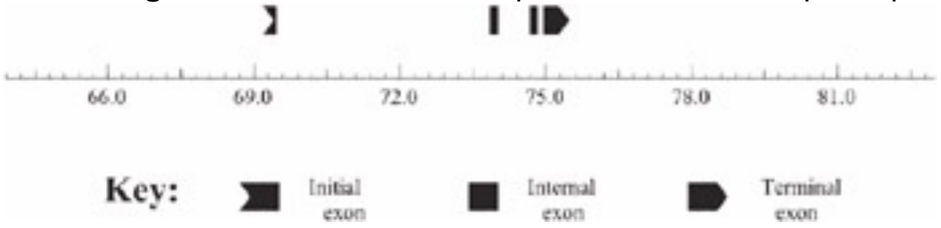


Figure 10.7: Sample output from GENSCAN, showing a single gene with four exons. Graphical output produced by GENSCAN. *J. Mol. Biol*. 268:78, 1997.

Web Exploration Questions

1. List the genes that GENSCAN found within the sequenced region, along with their lengths and the approximate length of the processed mRNAs. Why do the gene arrows point in different directions?
2. What is the difference between an exon marked Init and an exon marked Intr (in the text output)? Why is this difference significant in predicting genes?
3. Look at how the predicted proteins begin. Does this information strengthen or weaken the case for any of the genes?
4. What other features did GENSCAN identify (look in the text output)? Do these provide additional support for any of the predicted genes?

Unfortunately, there is no perfect gene prediction algorithm. Not only will most prediction programs return some potential genes that aren't "real," but they may place introns and exons at different positions. However, we might imagine that "real" genes should be detected by a variety of algorithms while false positives might tend to be more program specific. So, it is useful to run other prediction programs on the same sequence and see how their results compare.

Link AUGUSTUS is another popular gene prediction program that combines multiple kinds of prediction into a single piece of software (see References and Supplemental Reading). The core of AUGUSTUS is an *ab initio* prediction algorithm that uses HMMs to find the most likely sequence of hidden states (i.e., exon or intron for each nucleotide) that accounts for the sequence as a whole. The program can be "trained" by uploading sets of data (e.g., known genes from the organism being studied) and can incorporate user-defined information (such as locations of known expressed sequences) to improve its accuracy.

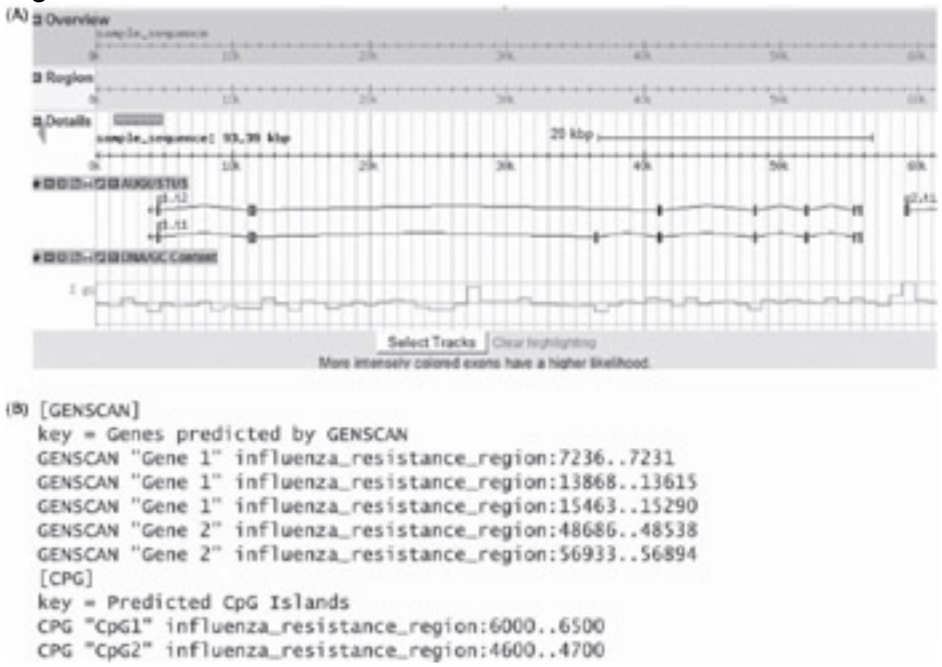
Download Navigate to the Web-based implementation of **AUGUSTUS**. Choose the Web interface, then upload the sequence from **1q25.txt**. Choose the correct organism from the drop-down menu; this will change the dataset used to "train" AUGUSTUS, so the training set should match the organism from which the sequence being analyzed originates. Note that you have some options for where AUGUSTUS will look for genes, as well as some "expert" options you can leave alone for now. Run the program to look for genes in your sequence.

AUGUSTUS will initially show text output that is quite similar to the output from GENSCAN: lists of predicted initial, internal, and terminal exons and translations of the predicted genes. Use the link provided to get to a list of available files containing graphical and text output, then choose `graphical browsable results`, which will show the results in a genome browser format similar to the UCSC Genome Browser (see **Figure 10.8A**). Exons are shown in color, with darker colors representing greater confidence in the predictions. Hovering over or clicking on regions of predicted genes will display details such as the coding sequence or predicted amino-acid sequence.

Will two different gene prediction programs give the same results? By now, you should realize there are no perfect criteria to identify exons, so you can probably guess that different programs using different algorithms will not necessarily identify the same sequences as exons. Indeed, if an exon is identified as such by more than one method, it would strengthen the evidence that it's a genuine exon. Thus, it's useful to compare the results of GENSCAN and AUGUSTUS. You could do this by examining the text output (importing it into a spreadsheet could make it easier to line up the exons identified by each program) or by using the graphical output. One approach would be to print the graphical output of one of the two programs and draw in the exons found by the other.

AUGUSTUS has a feature that makes this comparison easy: because its output is in genome browser format, custom tracks can be added. For example, you could add a track listing exons found by GENSCAN and compare them side by side with the AUGUSTUS results.**Figure 10.8B** shows the format of a text file listing a series of exons that could be added to AUGUSTUS as a track; you can easily manipulate your

GENSCAN text output into this format. Unfortunately, the Web implementation of AUGUSTUS does not support the addition of custom tracks, so to use it would require that you install AUGUSTUS locally. If you choose this option, you could also upload tracks with your data on CpG islands or predicted promoters (see part II) as shown in Figure 10.8B.



```
[GENSCAN]
key = Genes predicted by GENSCAN
GENSCAN "Gene 1" influenza_resistance_region:7236..7231
GENSCAN "Gene 1" influenza_resistance_region:13868..13615
GENSCAN "Gene 1" influenza_resistance_region:15463..15290
GENSCAN "Gene 2" influenza_resistance_region:48686..48538
GENSCAN "Gene 2" influenza_resistance_region:56933..56894
[CPG]
key = Predicted CpG Islands
CPG "CpG1" influenza_resistance_region:6000..6500
CPG "CpG2" influenza_resistance_region:4600..4700
```

Figure 10.8: (A) Sample output from AUGUSTUS. Exons (rectangles) and introns (thin lines connecting rectangles) are shown in a format similar to a typical genome browser. Potential splice variants are identified, and the overall G+C content of the DNA is shown in the bottom track. (B) Format of a text file in Feature File Format (FFF) to add two custom tracks to AUGUSTUS. Bracketed text is the name of the track; each line requires the name of the track, gene, or feature name and its location in the sequence. Graphical output produced by AUGUSTUS (Bioinformatics 19S2:215, 2003).

After comparing the two programs' output (by any method), you should be able to identify one major gene on which the two programs agree to a significant degree (though not perfectly). This would represent a gene on which further efforts to understand influenza resistance should be concentrated.

Web Exploration Questions

5. How does the number of genes predicted by AUGUSTUS compare to the results from GENSCAN?
6. How does the structure (i.e., length, number of introns and exons, position in the DNA) of the genes predicted by AUGUSTUS compare to GENSCAN?
7. How do the predicted proteins compare? Clearly, they're not identical, but do they appear related? For example, are they basically the same protein with perhaps some different splicing choices, or do they come from entirely different reading frames or even regions of the DNA? (You can of course use EMBOSS or BLAST to directly compare the proteins or their exons if you wish.)

8. Describe the gene that you conclude may be important in influenza resistance: total length, number of exons, processed length, number of amino acids, etc.

Part II: Evidence of Gene Expression

GENSCAN and AUGUSTUS served to identify at least a candidate gene of interest that might be responsible for the observed resistance to influenza infection. Clearly, however, the matter is not settled. At this point, the investigator might turn to less comprehensive programs to look for some specific features that might support the existence of a gene in this region and hopefully clarify its specific location. Indeed, we do not yet know for sure whether any gene expression occurs in this region: The putative coding sequence could turn out to be a pseudogene. Therefore, let's look for evidence that something could be expressed from this region of interest.

Link CpG islands are commonly found in the promoter regions of expressed genes, so let's start with a content-based method to see if there are CpG islands within the sequenced fragment. **The Sequence Manipulation Suite** includes a simple CpG island prediction program. Navigate there and paste or upload your sequence and submit it. At first, the resulting long list of CpG islands may seem daunting. However, notice that many of the results overlap: As discussed earlier in the chapter, CpG prediction uses a sliding window, and SMS shows results for *each* 200-bp window that meets the criteria. Therefore, consider how many nonoverlapping islands the program found. Given a set of overlapping sequences, one island would extend from the first nucleotide of the first sequence found to the last nucleotide of the last sequence in that set. It may also be useful to apply more stringent criteria; although the definition of a CpG island is operational, islands at least 500 bp in length with an overall GC content of at least 55% and a ratio of observed to expected CpG pairs exceeding 0.65 are considered most likely to genuinely function in gene expression.

Link Next, we might look at whether programs specifically designed to identify promoters would find any transcriptional signals in reasonable locations relative to the putative genes in our sequenced region. **Neural Network Promoter Prediction (NNPP)** looks for core promoter features using a neural network algorithm based on training sets containing known promoters. Promoter prediction, however, often returns too many putative promoters to be useful from any large region of DNA. It is thus desirable to cut down the size of the DNA sequence to be examined. Using your GENSCAN and/or AUGUSTUS map, decide how much sequence to use. Include the first exon and all upstream sequences for the putative gene on which you are focusing. To avoid having to count nucleotides, use the `Group DNA`option in the Sequence Manipulation Suite to number the sequence. Then cut the numbered sequence down to the nucleotides you decided on and use the `Filter DNA` option to get rid of the numbers again. Save your cut-down DNA, now the potential promoter region, to a new file.

Finally, submit your potential promoter region to NNPP for processing and view the results. Remember to consider whether you need to look at both strands or can focus on just one. You may be surprised by the number of potential promoters predicted; this should give you some insight into the complexity of eukaryotic genome data.

Link Again, we can increase our confidence in the results by comparing them with the results from other programs using different algorithms. **TSSG** claims to be the most accurate mammalian promoter prediction program; it uses a combination of sequence motifs and nucleotide composition analysis to identify promoters. Submit your putative promoter region to this program for analysis. You may wish to print the results for easy comparison with NNPP. If you have time and are interested, you may also wish to try analyzing your sequence with **TSSW**, which is very similar to TSSG but is based on a different database of protein sequence motifs.

Web Exploration Questions

9. Link Do the CpG islands within the sequenced region support your hypothesis about the genes that are found here? Do they provide any information that might help distinguish between the GENSCAN and AUGUSTUS results?
10. Link Higher scores in the NNPP results mean putative promoters that better match the criteria. Note on your map where the strongest predicted promoters are. The large letters represent the predicted transcriptional start sites. Can you see good matches to the consensus TATA box sequence (tATAWAW) upstream of potential translational starts?
11. How does the number of promoters returned by TSSG compare with the NNPP results? What else is different about the TSSG results, and how might this difference be useful?
12. Higher scores from TSSG again represent better promoter predictions. Do any of the high-scoring promoters match up (at least approximately) with high-scoring promoters from NNPP?
13. Does your expression analysis help to reconcile the differences between the GENSCAN and AUGUSTUS predictions?
14. Choose the gene you believe is founded on the most solid evidence, obtain its coding sequence, and use BLAST and OMIM to find out what is known about this gene. Have you actually identified a gene that makes sense in the context of influenza resistance?

More to Explore: Further Analysis

You could further pursue the discrepancies in identification of introns and exons between GENSCAN and AUGUSTUS by using additional analyses. Two programs in common use that focus more specifically on splice site identification are **HMMgene** and the neural network-based **NetGene2**. NetGene2 integrates a variety of rules that affect identification of exons, including nucleotide and codon bias, splice site consensus sequences, reading frame predictions, and lengths of introns and exons. This program claims to detect 95% of donor and acceptors sites with less than 0.4% false positives. HMMgene, as its name suggests, uses an HMM algorithm to predict gene structure. It only finds splice sites that make sense in the context of a whole gene, leading to fewer predicted genes but better predictions.

Once a putative gene has been identified and we have a hypothesis about the locations of its exons, promoter, and other features, we still need confirming data, which usually come from "wet lab" experiments. We might, for example, obtain complementary DNA from cells of interest and carry out a microarray or deep sequencing experiment to identify all the expressed genes and determine whether any match our putative gene. Given the wealth of available information about the human genome, we can also take advantage of experiments done by others. One way to find out if our putative gene is actually expressed is to compare it with the Expressed Sequence Tag (EST) database to see if a unique expressed sequence has been identified within our gene. Another method is to use a BLAST search with output limited to sequences that include "mRNA" in their titles to look for DNAs from this region and compare them with our predicted exons.

Guided Programming Project: Predicting CpG Islands

Rather than searching DNA for a particular site or sequence, content-based gene prediction methods look at the DNA sequence more broadly for clues to which sequences are genes (or, more precisely, which are within exons). Here, we work with one specific example of a content-based algorithm to search sequences for CpG islands (see BioBackground) that may indicate a nearby promoter. An increase in the frequency of CG pairs has been observed between nucleotides -1,500 and +500 relative to a transcriptional start site; finding such a CpG island appropriately positioned upstream of a putative gene would strengthen the case that it is an actual gene.

In a random DNA sequence, we would expect CG dinucleotides to occur once in every 16 nucleotides (1 of every 4 nucleotides should be a C, and the next nucleotide will be a G one-fourth of the time). To identify CpG islands, we will not merely search for the sequence pattern (CG) but will also need to determine how frequently it occurs. As described in Understanding the Algorithm, a frequency-matching algorithm is a variation on the pattern-matching algorithm (Chapter 9) that can accomplish this. We use a sliding window to traverse our sequence, counting up CG pairs within each window and looking for higher than average CpG ratios. The following pseudocode shows how this could be done. In this example, all CpG ratios are stored and displayed; however, if a CpG ratio is >1.5 (strong indicator), stars (***) print next to the value to highlight the ratio. Of course, another alternative is to only print the windows where the ratio is >1.5. In the skills exercises, we explore other options.

Algorithm

CpG Island Prediction Algorithm

- **Goal:** To identify regions of CpG islands
- **Input:** A FASTA formatted input file containing a sequence
- **Output:** Window start positions, CpG ratios, and text indicating high ratios.

```
// Initialization—Read in sequence data
Open input file containing sequence: infile
Input window size from user: window
read and discard first line (fasta comment) from infile
for each remaining line of data in infile
   seq = seq + line
```

```
// Step 1: Determine CpG ratios
lenSeq = length of seq ratios = array of size lenSeq-window+1 (holds CpG
ratio of each window)
for each i from 0 to lenSeq-window+1
   cCtr = gCtr = cgCtr = 0
   for each j from 0 to window-1
      if seq[j+i] == 'C'
         cCtr++
         if seq[j+i+1] == 'G'
            cgCtr++
      else if seq[j+i] == 'G'
          gCtr++
   if cCtr*gCtr != 0
      ratios[i] = cgCtr/((cCtr*gCtr)/window)
   else
      ratios[i] = 0

// Step 2: Print window start position and CpG ratios
for each i from 0 to length of ratios
        if ratios[i] > 1.5
                output i+1, ratios[i], '***'
        else
                output i+1, ratios[i]
```

Putting Your Skills Into Practice

1. **Download** Write a program to implement the CpG island prediction algorithm in the language of your choice as outlined in the given pseudocode. You should read in a sequence from a file and produce a tabular list of high-CpG regions with their scores. Devise some simple test sequences to test your program, and then try it on the long sequence (**1q25.txt**) used in the Web Exploration.
2. **Link** Compare the output of your program with the output of the CpG island prediction program from the Sequence Manipulation Suite. How similar are the predictions of the two programs? Can you suggest an explanation for any discrepancies? You may also want to look for additional CpG island prediction programs for comparison, such as **CpGProD**.
3. The initial program as described here has the same problem we saw when we used the CpG island prediction program from the Sequence Manipulation Suite (Web Exploration, earlier): because it shows each window where the CpG ratio exceeds a threshold value, it produces a long list of overlapping CpG islands. Make the output of your program more user-friendly by merging overlapping CpG islands into single entries in the results table.
4. To make your program even more effective, you might apply additional criteria. CpG islands associated with actual promoters are usually at least 500 bp in length and have an overall G+C content greater than 55% and a ratio of observed to expected CpG pairs exceeding 65%. Implement these additional criteria as part of your program.

On Your Own Project: Hidden Markov Modeling in Gene Prediction

Understanding the Algorithm introduced HMMs as a very flexible means of identifying coding segments by calculating the most probable match between an observed sequence and an exon–intron pattern based on our understanding of content and sequence cues. A fairly simple model accounting only for an exon–intron junction was presented there (Figure 10.5). This On Your Own Project asks you to design (and, for programming courses, implement) an HMM that also considers the 3′ splice-acceptor site.

Understanding the Problem

Our original HMM example included four states: exon nucleotides, a two-nucleotide splice site (the GT nucleotide pair occurring at nearly all 5′ intron boundaries), and intron nucleotides. We determined emission probabilities based on observed nucleotide frequencies in human introns and exons and established the probability of a transition from exon to splice site at 10%. Clearly, there are many more parameters that should be considered for a program to accurately identify exons and introns.

Solving the Problem

Although an HMM could become very complex indeed, let's add only a moderate level of complexity to our model. First, let's consider the difference between the first exon and an internal exon. The first exon begins with the ATG start codon, and in eukaryotes this is essentially the only possible start codon. Therefore, we could require an invariant ATG as the states of the first nucleotides of our model. The next states could be exon nucleotides, a splice-donor GT site, and intron nucleotides as described in Understanding the Algorithm.

The splice-acceptor site can be defined for the purposes of this model as a near invariant AG occurring as the last two nucleotides of the intron. To determine the emission frequencies, use the following data: A occurs with a frequency of 99.98% at the first position, with all other nucleotides occurring at equal frequency. G occurs with 99.93% frequency at the second position, C with 0.05% frequency, and A or T with equal frequency. This leaves the transition probabilities to be considered. For this exercise, allow an intron to transition to a splice-acceptor site with a 10% probability, similar to the original model. The splice-acceptor site always transitions to an exon—but not to the start codon, which is only in the first exon. Exons should have a 10% probability of transitioning to a splice-donor site but also a 10% probability of being the last exon and terminating the gene. Based on these parameters, design an HMM using a diagram similar to Figure 10.5 that will find a multiple-exon gene.

Programming the Solution

Once you have developed an appropriate design for your HMM, it should be relatively easy to implement in a programming language, if you are in a programming course. The first task is to generate the list of possible paths for the observed sequence. A recursive approach is appropriate because a state may be able to transition to any number of possible states, including itself. You should consider how you will deal with the start codon, because it is not expected to be the first three nucleotides of the input sequence.

The end of the gene is also a problem. For this project, we assume any exon could be the last exon, and thus we need to assign a low transition probability from E→end, such as 0.001.

Then, for each path, the emission and transition probabilities are calculated for each nucleotide and multiplied to give an overall probability, *P*. The natural log of *P* is then stored for each possibility, and the maximum value for log(*P*) is chosen as the best way to classify the observed sequence into exons, splice sites, and introns.

You certainly do not want to turn your program loose on the entire 90-kb sequence from the Web Exploration without testing it carefully first. Develop some short test sequences with obvious start codons and splice sites (similar to the very short sequence used as the example in Figure 10.5) to test the program. Then, test it with longer sequences—perhaps a single gene as predicted by GENSCAN or AUGUSTUS. If your program proves capable of handling these longer sequences, you may then want to try it on the full-length sequence and compare its results with those of the programs you used in the Web Exploration.

More to Explore

To make your HMM even more realistic, you could incorporate the observed frequencies of nucleotides at other positions within the splice site (Figure 10.2). If you would like to try this,**Table 10.2** gives the nucleotide frequencies for the dataset used to make the sequence logos.

Table 10.2: Nucleotide frequencies for the 5′ and 3′ splice sites.

Open table as spreadsheet

	Splice-donor (5′) Site				Splice-acceptor (3′) Site			
Position	**A**	**C**	**G**	**T**	**A**	**C**	**G**	**T**
-21					0.22	0.31	0.10	0.37
-20					0.28	0.15	0.25	0.32
-19					0.13	0.37	0.29	0.21
-18					0.08	0.44	0.11	0.37
-17					0.16	0.22	0.22	0.40
-16					0.08	0.26	0.16	0.50
-15					0.08	0.31	0.20	0.41
-14					0.16	0.20	0.11	0.53
-13					0.03	0.24	0.13	0.60
-12					0.07	0.26	0.12	0.55
-11	0.30	0.25	0.27	0.17	0.04	0.41	0.09	0.46
-10	0.36	0.27	0.28	0.08	0.05	0.37	0.20	0.38

Table 10.2: Nucleotide frequencies for the 5′ and 3′ splice sites.

Open table as spreadsheet

	Splice-donor (5′) Site				Splice-acceptor (3′) Site			
Position	**A**	**C**	**G**	**T**	**A**	**C**	**G**	**T**
-9	0.16	0.23	0.29	0.31	0.11	0.32	0.11	0.46
-8	0.16	0.31	0.36	0.16	0.04	0.35	0.17	0.44
-7	0.34	0.23	0.25	0.17	0.08	0.36	0.15	0.41
-6	0.30	0.22	0.22	0.25	0.03	0.31	0.08	0.58
-5	0.45	0.23	0.13	0.18	0.07	0.36	0.04	0.53
-4	0.29	0.28	0.25	0.17	0.27	0.20	0.17	0.36
-3	0.22	0.45	0.11	0.21	0.04	0.69	0.00	0.27
-2	0.61	0.09	0.10	0.20	0.98	0.00	0.02	0.00
-1	0.17	0.05	0.60	0.18	0.00	0.02	0.98	0.00
+1	0.0005	0.0001	0.9993	0.0001	0.16	0.21	0.56	0.07
+2	0.0001	0.0069	0.0001	0.9929	0.38	0.19	0.07	0.36
+3	0.59	0.02	0.38	0.01	0.32	0.15	0.25	0.28
+4	0.68	0.18	0.06	0.08	0.18	0.25	0.20	0.37
+5	0.02	0.04	0.83	0.11	0.14	0.29	0.29	0.28
+6	0.03	0.15	0.19	0.63	0.15	0.22	0.37	0.26
+7	0.31	0.31	0.27	0.10	0.17	0.28	0.23	0.32
+8	0.25	0.24	0.30	0.20	0.33	0.17	0.30	0.20
+9	0.16	0.34	0.23	0.26	+0.19	0.40	0.13	0.28
+10					0.09	0.21	0.45	0.25
+11					0.32	0.34	0.18	0.16
+12					0.20	0.36	0.26	0.18
+13					0.27	0.30	0.14	0.29
+14					0.21	0.14	0.42	0.23
+15					0.14	0.35	0.33	0.18
+16					0.25	0.20	0.30	0.25
+17					0.34	0.20	0.23	0.23
+18					0.18	0.43	0.26	0.13
+19					0.22	0.22	0.36	0.19

BioBackground: Splicing and CpG Islands

mRNA Splicing in Eukaryotes

When a gene is expressed, it is transcribed in the nucleus to make a single-stranded RNA complementary to the entire template strand of the DNA for that gene: the **pre-mRNA**. A methylated G nucleotide is added to the 5′ end of the mRNA by an unusual 5′-to-5′ linkage; this **5′ cap** is the structure by which a ribosome recognizes the mRNA. At the 3′ end, cleavage occurs at a **polyadenylation site** (consensus sequence 5′AAUAAA), and a **poly(A) tail** of 200–300 A nucleotides is added to protect the mRNA from rapid degradation.

Splicing is carried out by the **spliceosome**, a large complex made up of several **small nuclear ribonucleoproteins** (snRNPs, pronounced "snurps"): functional units composed of both RNA and protein. The snRNPs direct the binding of the spliceosome to sites at the beginning and end of an intron to cut an mRNA, remove the intron, and rejoin the ends (see **Figure 10.9**). At the 5′ end of an intron (5′ splice site), the exon usually ends with a consensus sequence close to MAG, and the intron almost invariably begins with GU, usually followed by RAGU. On the other end, the 3′ splice site is defined by an AG sequence, most often CAG, at the end of the intron, with G as the first base of the next exon. Within the intron itself is a **branch site** with the consensus sequence CURAY 20–50 bases from the 3′ end of the intron; after cutting the mRNA, the 3′ end of the intron is joined to this site, forming a "lariat" structure that marks the intron for degradation rather than transport to the cytoplasm. The exons are joined together, and when splicing is complete, the **mature mRNA** moves to the cytoplasm for translation.

CpG Islands

Although each species has a characteristic ratio of G and C nucleotides in its DNA to A and T nucleotides, the frequencies of these nucleotides are not constant across the genome. A pattern noted in the study of genomes is that the promoter regions of known genes tend to be higher in G and C nucleotides than A and T nucleotides. Furthermore, the dinucleotide CG—which molecular biologists call **CpG**, with the letter *p* representing the phosphate in the sugar-phosphate DNA backbone—occurs in these regions much more frequently than would be expected by chance. Because the C in a CG pair is a target for methylating enzymes, the concentration of methylated nucleotides is higher in promoter regions that overlap CpG islands, altering gene expression patterns. The identification of CpG islands is therefore one marker for a promoter region. Remember that the promoter region for a eukaryotic gene can be long, and it is not precisely defined with regard to the translational start site. Similarly, CpG island(s) are not precisely aligned with a particular promoter element but can occur anywhere within the broadly defined promoter region. However, CpG-rich regions in an area where there is other evidence of gene expression can add credibility to the prediction of a promoter and a downstream first exon.

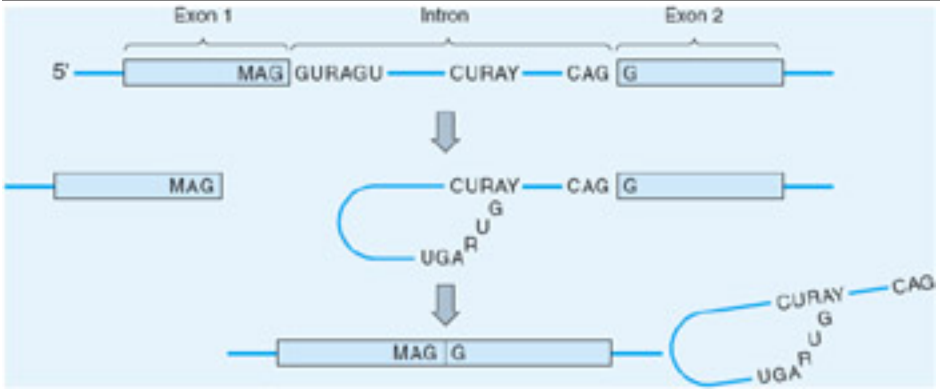


Figure 10.9: The process of mRNA splicing in a eukaryotic cell, showing the consensus sequences occurring at the two splice junctions and the internal branch site.

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Chapter 11: Protein Structure Prediction and Analysis: Rational Drug Design

Chapter Overview

Thus far, we have worked with the *sequences* of proteins: we have viewed them as simple chains of amino acids. But, a protein is actually a folded, three-dimensional structure (see BioBackground at the end of the chapter), and this structure is crucial to the protein's function. In this chapter, we use Web-based software to model protein structure and see how such molecular modeling can aid in drug design. We learn to "align" protein structures and observe that even when sequence similarity is limited, proteins can be very similar in structure and thus function. In the Web Exploration we also examine how a protein's structure might be predicted from its sequence, and in the Guided Programming Project and On Your Own Project, we implement one algorithmic solution to this complex problem.

- **Biological problem**: Designing an HIV protease inhibitor
- **Bioinformatics skills**: Protein structure modeling and structural comparison, structure prediction
- **Bioinformatics software**: Jmol, SWISS-MODEL, PDBeFold, PSIPRED
- **Programming skills**: Chou-Fasman algorithm, sliding windows, hash tables

Understanding the Problem: Structure Prediction

When HIV-1, the virus that causes AIDS, was discovered in 1984, it was commonly assumed a vaccine, effective antiviral drugs, or both would be found within a few years. However, 2012 marked the 25th World AIDS Day, and the pandemic is still going strong, with an estimated 34 million living with HIV or AIDS worldwide and nearly 2 million annual deaths (**Figure 11.1**)*. Despite two and a half decades of intensive research, we still have no vaccine and no drugs that can cure the infection. Perhaps this is less surprising when we realize no antiviral drug exists that can cure any viral disease, and indeed there are few effective antivirals on the market. Part of the reason for this is that unlike bacteria, viruses replicate within our own cells and use our own cellular machinery to copy their genomes and synthesize their proteins, leaving us few virus-specific targets to attack with pharmaceuticals.*

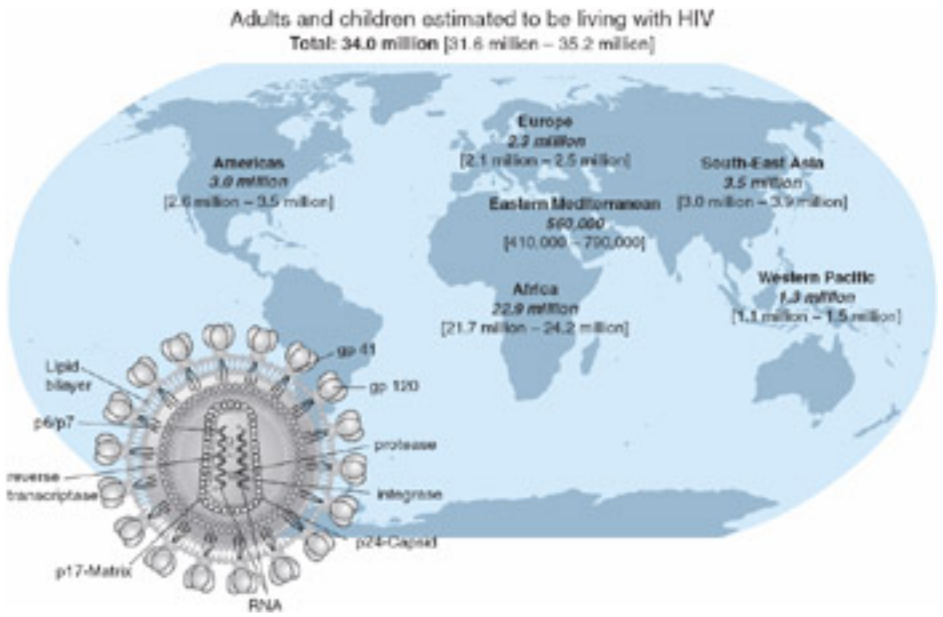


Figure 11.1: World Health Organization (WHO) data on the global HIV pandemic as of the end of 2010, with a drawing showing the structure of the HIV virus. Data from: WHO.

A detailed understanding of the three-dimensional structure of virus proteins may be one route to new breakthroughs in antiviral research. The two key goals of any antimicrobial drug are (1) to be effective against the disease-causing agent and (2) to be **selectively toxic**: able to kill or inhibit the microbe without causing harm to the patient. Viruses have no metabolism outside host cells and few proteins of their own; this makes it difficult to identify effective and selective antiviral drugs by the standard approach of testing libraries of potentially bioactive molecules. **Rational drug design** provides an alternative: By examining the three-dimensional structure of a viral protein, one should be able to design a molecule to precisely fit some part of that protein and block its function. Two of the first examples of commercially available antiviral agents designed this way are anti-HIV drugs: raltegravir (Isentress), an inhibitor of the HIV integrase enzyme, and enfuvirtide (Fuzeon), which blocks entry of HIV into cells.

Unfortunately, rational drug design poses its own difficulties. Determination of the detailed three-dimensional structure of a protein requires crystallizing that protein and then measuring how the crystal scatters x-rays, a process called **x-ray crystallography**. Many proteins are difficult to crystallize, particularly if they have hydrophobic regions that insert into membranes, and this process is slow and labor intensive. Once a crystal structure is known, there remains the problem of accurately determining the shape of a molecule that fits into some part of the structure, synthesizing that molecule, and then testing it to see if it has the desired biological effect. Furthermore, although our skills in these areas are improving, it still remains difficult to predict potential toxicity of a prospective therapeutic molecule as well as how quickly it will be metabolized by the patient and lose its effect. Fortunately, today's bioinformatic techniques are improving our ability to predict and model protein structure.

In addition to its application to drug development, we can use protein structure in many other ways. For example, a key functional region of a protein may actually be made up of amino acids scattered throughout its primary sequence but brought together by folding and thus not recognized in ordinary alignments. Furthermore, we are becoming increasingly aware that changes in macromolecular structure are important components of many diseases, both genetic and infectious: For example, the F508 mutation causes cystic fibrosis (Chapter 2) by interfering with the folding of the CFTR protein, and prion diseases such as "mad cow disease" result from "contagious" misfolding of a specific protein (see References and Supplemental Reading for more on protein folding in human disease).

Bioinformatics Solutions: Predicting and Modeling Protein Structure

Molecular biology and bioinformatics have worked together to make great strides in sequencing genes and even entire genomes, identifying genes within genomes, predicting amino-acid sequences of proteins, and comparing sequences to obtain clues to function and evolutionary relatedness. However, determining the nucleotide sequence of a gene allows us to predict only the **primary structure** (amino-acid sequence; see BioBackground) of the protein it encodes. An actual cell is a three-dimensional arena where molecules with specific structures interact, and the three-dimensional structure of a protein (**Figure 11.2**) determines what interactions it can have with other molecules. An enzyme must have the correct shape to bind a specific substrate and exclude nonsubstrate molecules, for example, whereas a transport protein on the surface of a cell must have a specific structure to selectively allow specific molecules to enter or exit.

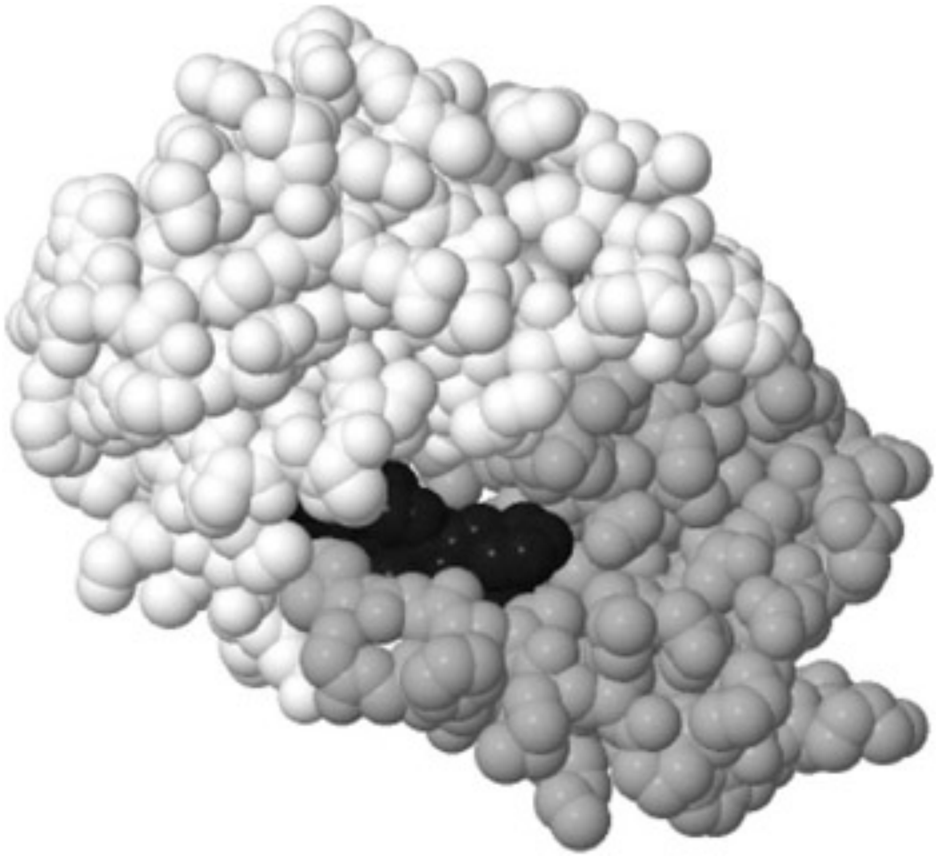


Figure 11.2: Three-dimensional structure of the HIV protease, showing its two folded protein chains (gray and white) and a protease inhibitor in its active site (black). Structure from the RCSB PDB (www.pdb.org): PDB ID 1AID E. Rutenber et al. (1993) Structure of a non-peptide inhibitor complexed with HIV-1 protease: Developing a cycle of structure-based drug design. *J. Biol. Chem.* 268:15343–15346.

To date, no experimental methods for determining the structure of either proteins or nucleic acids can keep up with the tremendous rate at which their primary sequences are being determined. Although we have successfully determined tens of thousands of protein structures, genome sequencing projects have given us tens of *millions* of primary sequences of nucleic acids and proteins. One goal of computational structural biology is to solve this problem by predicting the structure of a protein given only its primary sequence. The possible conformations any protein can assume are determined by its amino-acid sequence, and its final, folded state is thus determined to a large degree by its primary structure (see BioBackground). Thus, given sufficient understanding of individual amino acids and the conditions under which they are folding, this should be possible. However, it is a big problem: We might know that a particular amino acid has an –OH group that can form a hydrogen bond with an amino group on another amino acid, but how do we know which two amino acids to pair up in a protein hundreds or thousands of amino acids long?

The number of possible folded structures for a protein is enormous, so algorithms that predict folding from sequence rely on structural rules to arrive at a likely folded structure. Many of these rules originated with Linus Pauling's pioneering work on protein structure (see References and Supplemental Readings), which defined the nature of the chemical bonds between amino acids and how bond angles, rotation of atoms, and flexibility of chains limit the structures that can be formed. Pauling predicted the structure of the a-**helix** (see BioBackground) as a major component of folded proteins, later confirmed by x-ray crystallography. In an α-helix, the C=O group of one amino acid must be able to form a hydrogen bond with the amino group of an amino acid located four residues farther down the chain. However, not just any amino acid can be included in the helix; proline, for example, introduces a turn into the protein backbone and disrupts helical structure. Similar rules can be worked out for amino acids likely to form β-sheets (see BioBackground) and other elements of protein secondary structure. Anfinsen (see Refer-ences and Supplemental Reading) and others then went a step further, explaining that the thermodynamics of the cellular environment determines how these structures fold into a three-dimensional tertiary structure. Bioinformatic algorithms use secondary structure rules and thermodynamic optimization algorithms to predict how a protein folds into an overall stable structure.

Our ability to effectively predict tertiary structure from sequence alone (**ab initio** or **de novo prediction**) is unfortunately quite limited at present. However, the combination of increasing numbers of experimentally determined protein crystal structures with the enormous explosion in genomic data has given rise to two additional bioinformatic techniques that are very important in modeling protein structure. **Homology modeling** (**Figure 11.3A**) is used to find the structure of a protein when an ortholog or paralogs with a known structure can be identified. To construct a homology model, the protein of interest is aligned with the sequence of a similar **template** protein, and the alignment is used to map its amino acids onto a structural model based on the template structure. If there is no closely related protein with a known structure, **threading** (**Figure 11.3B** and **C**) can be used instead. Threading takes advantage of the observation that most proteins whose structures are known are built on a limited number of basic folded units. For example, the immunoglobulin fold shown in Figure 11.3B is a basic structural unit found one or more times in dozens of different proteins; although many of these proteins function in the immune system, their molecular functions are very diverse. As shown in Figure 11.3C, new protein sequences can be "threaded" onto common structural units, allowing at least a partial structural model to be constructed.

This chapter's projects explore protein structure prediction and modeling in the context of rational drug design. In the Web Exploration, we use modeling software to examine the structure of the HIV protease, examine how its structure relates to function, and then construct a homology model of a drug-resistant protease mutant. In the Guided Programming Project, we examine de novo structure prediction and compare predicted secondary structure with experimentally verified protein conformation, implementing a more complete solution in the On Your Own Project.

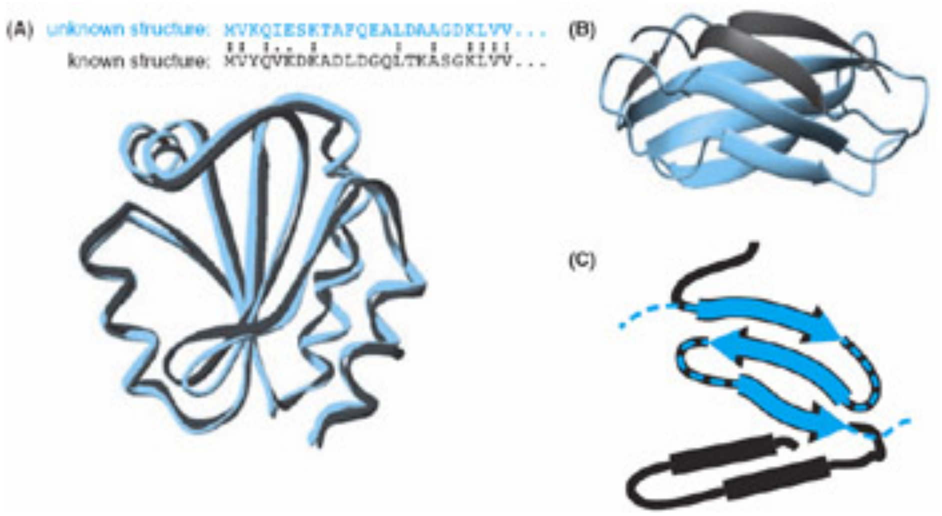

(A) unknown structure: MVKQIESKTAFQEALDAAGDKLVV...
known structure: MVYQVEDKADLDGQLTKASGKLVV...
(B)
(C)

Figure 11.3: Predicting protein structure based on similarity to known structures. (A) Homology modeling: protein of unknown structure (blue) is an ortholog or paralog of a protein of known structure (black), allowing structure to be modeled from a sequence alignment. Courtesy of Tim Vickers. (B) The immunoglobulin fold, a common protein structural domain. (C) Threading: sequence comparison allows part of a protein of unknown structure (blue) to be threaded onto a protein of known structure (black), showing that it contains an immunoglobulin domain. Structures created from MOLMOL.

BioConcept Questions

1. Why is it valuable to know the three-dimensional structure of a protein?
2. Both secondary and tertiary structures of proteins are three-dimensional structures; what is the difference between the two?
3. What characteristics of amino acids help determine how they will participate in the folding of the protein?
4. Sickle-cell anemia results from changing a single hydrophilic amino acid (glycine) found on the surface of the folded protein to a hydrophobic amino acid (valine). Discuss how the hydrophobicity of the amino acid could be so important in this disease.
5. The amino-acid sequence of a protein clearly must determine what folded structures are possible for that protein. What other factors contribute to the structure that is actually chosen? What complications arise in trying to predict a folded structure from an amino-acid sequence?

Understanding the Algorithm: The Chou-Fasman Algorithm for Secondary Structure Prediction

Learning Tools

Link The Protein Data Bank (PDB), managed by the Research Collaboratory for Structural Bioinformatics, is the major repository for proteins whose structures have been determined experimentally. The PDB's long-standing "Molecule of the Month" series is an excellent way to improve your understanding of the relationship between protein structure and function. Every month, a protein important to some key biological process is discussed from a structural perspective and illustrated by molecular models made from structures available in the PDB; the site's archives now include hundreds of proteins.

The ab initio prediction of the three-dimensional (tertiary) folded structure of a polypeptide structure from its amino-acid sequence is a "holy grail" of structural biology. Because of the enormous complexity of proteins and the many factors that could affect amino-acid interactions, this is a very difficult problem to solve. Indeed, even accurately predicting the folding of the amino-acid chain into the secondary structures (e.g., α-helices and β-sheets) that underlie tertiary structure remains an open problem in bioinformatics.

Many of our ideas about secondary structure prediction stem from an algorithm proposed by Peter Chou and Gerald Fasman in 1974 (see References and Supplemental Reading). At that time, a handful of protein crystal structures were known, and Chou and Fasman developed the idea of examining these known structures to determine which specific amino acids within the proteins contributed to each secondary structure. Using this information, they developed**propensity values** (the likelihood that an amino acid would appear within a particular secondary structure) and **frequency values** (the frequency with which an amino acid is found in a hairpin turn) for each amino acid (**Table 11.1**). These values were updated in 1978 (see References and Supplemental Reading) using new training data and became known as the Chou-Fasman parameters.

Table 11.1: The Chou-Fasman parameters.

Open table as spreadsheet

Amino Acid	***P(a)***	***P(b)***	***P*(turn)**	***f*(i)**	***f*(i + 1)**	***f*(i + 2)**	***f*(i + 3)**
Alanine	142	83	66	0.060	0.076	0.035	0.058
Arginine	98	93	95	0.070	0.106	0.099	0.085
Asparagine	67	89	156	0.161	0.083	0.191	0.091
Aspartic acid	101	54	146	0.147	0.110	0.179	0.081
Cysteine	70	119	119	0.149	0.050	0.117	0.128
Glutamic acid	151	37	74	0.056	0.060	0.077	0.064
Glutamine	111	110	98	0.074	0.098	0.037	0.098
Glycine	57	75	156	0.102	0.085	0.190	0.152
Histidine	100	87	95	0.140	0.047	0.093	0.054
Isoleucine	108	160	47	0.043	0.034	0.013	0.056
Leucine	121	130	59	0.061	0.025	0.036	0.070

Table 11.1: The Chou-Fasman parameters.
Open table as spreadsheet

Amino Acid	*P(a)*	*P(b)*	*P*(turn)	*f*(i)	*f*(i + 1)	*f*(i + 2)	*f*(i + 3)
Lysine	114	74	101	0.055	0.115	0.072	0.095
Methionine	145	105	60	0.068	0.082	0.014	0.055
Phenylalanine	113	138	60	0.059	0.041	0.065	0.065
Proline	57	55	152	0.102	0.301	0.034	0.068
Serine	77	75	143	0.120	0.139	0.125	0.106
Threonine	83	119	96	0.086	0.108	0.065	0.079
Tryptophan	108	137	96	0.077	0.013	0.064	0.167
Tyrosine	69	147	114	0.082	0.065	0.114	0.125
Valine	106	170	50	0.062	0.048	0.028	0.053
Data from: Chou & Fasman, Adv. Enzymol. Relat. Areas Mol. Biol. 47:45-148 (1978).							

Chou and Fasman calculated three different propensity (*P*) values for each amino acid: *P*(a),*P*(b), and *P*(turn), representing the likelihood of finding the amino acid within an α-helix, β-strand, and β-turn, respectively. These values are log-odds ratios, where *P* > 1.0 indicates the amino acid has a greater than average chance of contributing to that particular structure, *P* < 1.0 means it has a less than average chance, and *P* = 1.0 means it is no more likely to contribute to that structure than any randomly chosen amino acid. Each amino acid also has four frequency (*f*) values: *f*(i), *f*(i + 1), *f*(i + 2), and *f*(i + 3), the frequencies with which it is found at each of the four positions of a hairpin turn (β-turn). From these parameters, Chou and Fasman developed rules to predict the locations of α-helices, β-strands, and β-turns. Different implementations of this algorithm vary in the threshold values for the parameters or the criteria for designating a region an α-helix or a β-sheet. One imple-mentation is presented here.

Algorithm

Chou-Fasman Algorithm

1. Identify α-helices
    a. Find a region of six contiguous residues where at least four have*P*(a) > 103.
    b. Extend the region until a set of four contiguous residues with *P*(a) < 100 is found.
    c. If the region's average *P*(a) > 103 and Σ*P*(a) > Σ*P*(b) for the region, then that region is predicted to be an α-helix.
2. Identify β-strands

   a. Find a region of five contiguous residues where at least three have *P*(b) > 105.
   b. Extend the region until a set of four contiguous residues with *P*(b) < 100 is found.
   c. If the region's average *P*(b) > 105 and Σ*P*(b) > Σ*P*(a) for the region, then that region is predicted to be a β-strand.
3. Determine β-turns
   a. For each residue *j*, determine the turn propensity or *P*(t) for *j* as follows:

      $P(\mathrm{t})_j = f(\mathrm{i})_j \times f(\mathrm{i}+1)_{j+1} \times f(\mathrm{i}+2)_{j+2} \times f(\mathrm{i}+3)_{j+3}$
   b. A turn is predicted at position *j* if *P*(t) > 0.000075, and the average*P*(turn) for residues *j* to *j* + 3 > 100, and Σ*P*(a) < Σ*P*(turn) > Σ*P*(b).
4. Handling overlaps

   If an α-helix region overlaps with a β-sheet region, the region's summed values for *P*(a) and *P*(b) are used to determine the overlapping region's most likely structure. If Σ*P*(a) > Σ*P*(b) for the overlapping region, then it is considered an α-helix. If Σ*P*(b) > Σ*P*(a), then the overlapping region is considered a β-sheet, and if Σ*P*(b) = Σ*P*(a), then no valid determination can be made.

Neural network methods (see Chapter 10) are common in secondary structure prediction programs such as PSIPRED, which we will use in the Web Exploration Project. However, although the Chou-Fasman algorithm is sometimes denigrated for its accuracy of only 50–60%, the ideas behind it underlie many of these newer methods. Indeed, some methods in current use are much more complicated yet only slightly more accurate. The Chou-Fasman algorithm remains very valuable for understanding the principles of protein structure prediction.

Test Your Understanding

1. Find an α-helix in the short sequence `N-MDGPDFWEAMKRISTQTYSNGHKMPS-C` using the Chou-Fasman rules.
2. Examine the Chou-Fasman rules carefully, and look at the *P*(a) and *P*(b) values for various amino acids in **Table 11.1**. What can you see that might reduce the ability of this algorithm to clearly distinguish between α-helices and β-sheets?
3. How do we define a β-turn in a protein structure? Given this definition, can you think of a simple rule you could add to the algorithm for identification of β-turns that might increase its accuracy?
4. Would it improve the predictive ability of the algorithm to specify that a region should be identified as a β-strand only if it is either preceded or followed by a β-turn? Why or why not?

5. Proteins that are part of the cell membrane or an organelle membrane typically have one or several α-helical domains about 20 amino acids long that pass through the membrane. These membrane-spanning helices consist almost entirely of very hydrophobic amino acids such as L, I, V, F, and W and are anchored in place by hydrophilic amino acids on their two ends. If you applied the Chou-Fasman algorithm to a membrane protein, why would it likely fail to predict the membrane-spanning helices?

Chapter Project: Protein Structure Prediction

This chapter's projects address the problem of identifying potential anti-HIV drugs that block the action of the viral protease and of overcoming the rapid development of drug resistance. We examine both ab initio and homology-based methods of predicting protein structure and examine how changes to the structure of a protein may affect its function.

Learning Objectives

- Understand how protein structure and function are related and why structure prediction is important
- Know how to use available tools to examine the experimentally determined structures of proteins and visualize structural and functional features
- Use homology-based tools to compare a novel protein sequence with a well-studied one and identify potentially significant differences
- Appreciate the value and limitations of ab initio approaches to protein structure prediction
- Understand how protein structure prediction and analysis can inform drug design

Suggestions for Using the Project

In the Web Exploration for this chapter, students start by using Web-based structure visualization tools to explore protein structure and understand the value of different ways of showing protein structure. They then use homology-based methods to compare an HIV protease mutant to the unmutated protein and see how mutation can affect drug effectiveness. They then experiment with ab initio structure prediction, comparing these results with the known structure of the protein. In the Guided Programming Project, they develop a solution for part of the Chou-Fasman algorithm and then completely implement this algorithm in the On Your Own Project.

Programming courses:

- Web Exploration: Use Web-based tools to become familiar with protein structure, model a mutant protein, and test ab initio structure prediction. If time is limiting, we recommend completing at least Part I to become familiar with protein structure and Part III to generate comparison data for the programming projects.

- Guided Programming Project: Implement the Chou-Fasman algorithm to find α-helices in an amino-acid sequence and compare results with known sequences and predictions from other ab initio tools.
- On Your Own Project: Fully implement the Chou-Fasman algorithm to find α-helices, β-strands, and β-sheets in an amino-acid sequence and compare results.

Nonprogramming courses:

- Web Exploration: Use Web-based tools to explore protein structure, homology modeling to examine the structure of a mutant protein, and ab initio methods to predict secondary structure from amino-acid sequence. Parts I, II, and III are independent enough to be used separately to match the focus of a particular course.
- On Your Own Project: Download an implementation of the Chou-Fasman algorithm for ab initio secondary structure prediction. Compare its results with those of prediction programs used in Part III of the Web Exploration and to experimentally determined structures.

Web Exploration: Protein Structure Modeling and Drug Design

Traditionally, new drugs have been discovered by performing initial testing of a huge number of molecules that might possibly affect some process of interest (for example, inhibiting bacterial growth, blocking pain receptors, or halting allergic responses). Pharmaceutical companies maintain large libraries of potentially useful chemicals for this reason; once a candidate molecule is found, it can then be chemically modified to increase its activity, reduce its toxicity, and so on. In many cases, the new drug needs to interact with an enzyme or other protein, and this is where rational drug design could drastically improve the selectivity and effectiveness of our pharmaceuticals and the speed with which we can identify new candidate drugs. If we were able to easily and quickly determine the structure of the protein and connect structural domains with protein functions, we could design a drug to "fit" precisely in an appropriate spot.

HIV and AIDS have been a major focus of pharmaceutical discovery for more than 25 years, and indeed we have developed an unprecedented number of new antivirals, some of which resulted from the study of protein structure and rational design. In this project, we focus on the HIV **protease**. When HIV infects a cell (**Figure 11.4**), one of the earliest steps is to make a DNA copy of the virus' RNA genome, a process called reverse transcription that does not occur in uninfected cells. To accomplish this, the virus must carry the enzyme reverse transcriptase (also a target of drug therapy). The HIV integrase protein then inserts the DNA into one of the host cell's chromosomes, where the viral genome behaves just like any ordinary gene. There is only one promoter within the HIV genome, so a single mRNA is made by transcription (although it can be spliced in more than one way to produce a few different mature mRNAs for translation). Because eukaryotic ribosomes begin translation with the *first*AUG on an mRNA, only one protein can be made from any particular mRNA, so to produce all the proteins HIV needs, the **polyprotein** product of translation is cleaved by the HIV protease into individual functional protein units (see References and Supple-mental Reading). For example, it cleaves a single polypeptide to become the functional reverse transcriptase, integrase, and protease proteins required for viral replication. Blocking the function of the HIV protease therefore inhibits the replication of the virus. The first protease inhibitor was approved for use in treating HIV and AIDS in 1996, and today 10 such drugs are on the market.

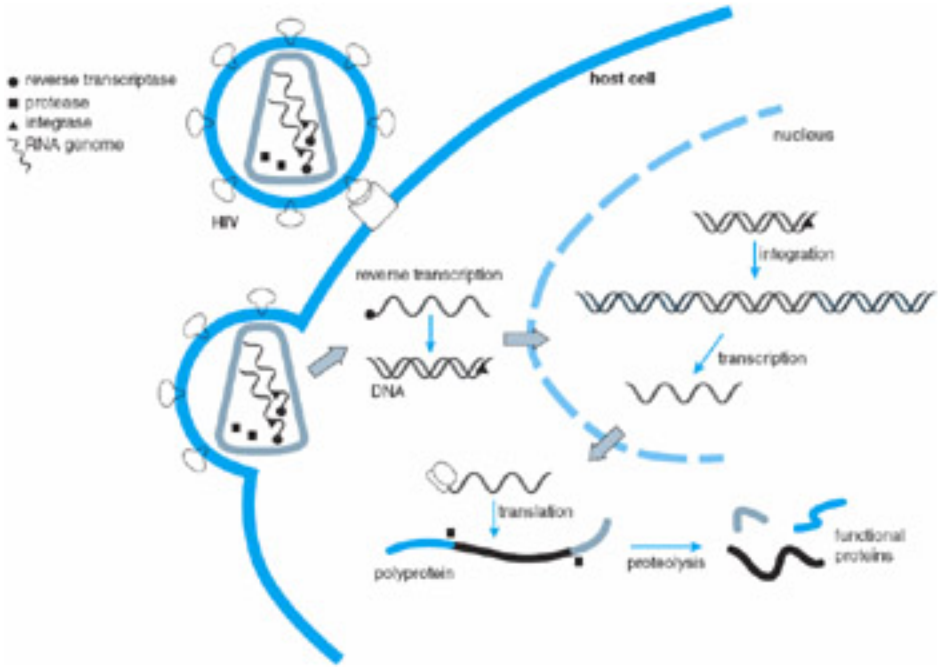
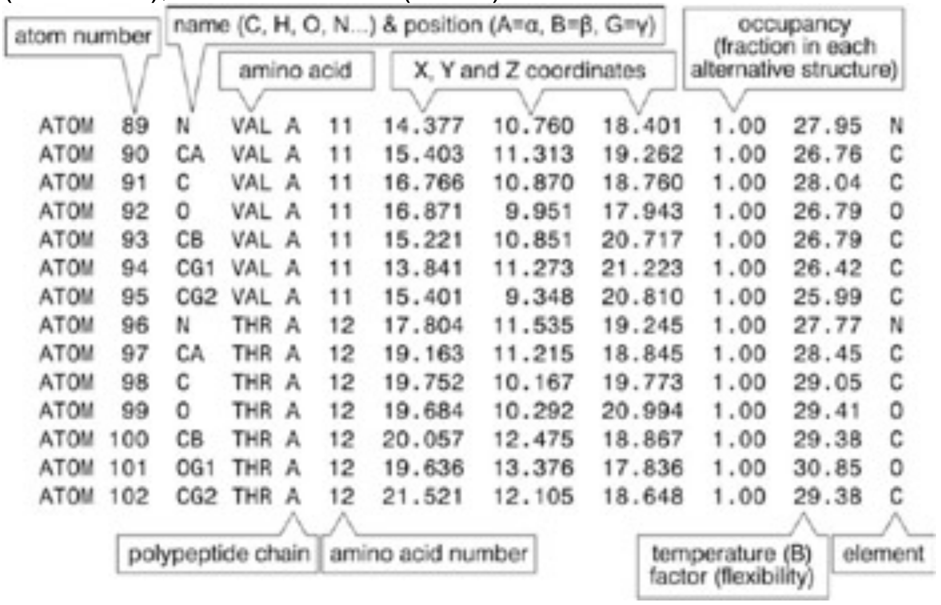


Figure 11.4: Replication of the HIV virus: After interacting with a cellular receptor, the virus fuses with the host cell membrane and RNA is reverse transcribed to DNA. The viral DNA integrates into the host chromosome and is then transcribed and translated to produce polyproteins. Viral protease cleaves the polyproteins to yield functional virus proteins.

Part I: Exploring the Structure of the HIV Protease

Link When the structure of a protein is "solved," we know where the atoms that make up its amino acids are found in space, allowing us to generate representations that show the locations of the various amino-acid side chains and how they interact to form secondary andtertiary structures. X-ray crystallography is the current gold standard for protein structure and can under the best conditions distinguish the positions of atoms less than 1 Å (10^{-10} m) apart. More flexible proteins may form less perfect crystals and generate structures with lower resolutions of 3 Å or more. Other techniques, such as nuclear magnetic resonance (NMR), can also be used to determine the structures of proteins; they typically generate lower-resolution structures but may have other advantages. NMR, for example, can be applied to uncrystallized proteins in solution. Structural data are deposited in public data-bases, most notably the **Protein Data Bank (PDB)**, in a standardized format that can be read by various kinds of software to visualize and work with the structure.

A text search of PDB for the HIV-1 protease (note that HIV-1 is the proper name of HIV and will return the best search results) will return a large number of results, mostly variations in which the protein is bound to various inhibitors. We want to see the protease interacting with more natural substrates, so search instead for a specific accession number, `1KJF`, to see a structure where a peptide substrate is used. You may wish to explore some of the features of the PDB entry for a protein; like many of the DNA and protein databases, many resources are brought together at this site, and you can find the sequence of the protein, information about the methods used to produce the structure, biochemical information about the enzyme, references, and more. On the right side of the page, you can see a graphical representation of the protein structure (discussed in more detail later in the chapter). However, the actual PDB data are not graphical at all: Take a look at what is actually stored in the PDB database by using the `Display Files` drop-down menu to examine the `PDB file` for the protease. As you can see, this is purely a text file. If you scroll down, you will realize that the heart of the file is simply a list of atoms, the amino acids to which they belong, and coordinates describing their spatial position (**Figure 11.5**). This is all the information required to minimally describe the protein's structure. Additional information in the file includes the amino-acid sequence of each polypeptide chain (look for SEQRES), locations of secondary structures (HELIX, SHEET, etc.), comments (REMARK), and references (JRNL).


atom number
name (C, H, O, N...) & position (A=α, B=β, G=γ)
amino acid
X, Y and Z coordinates
occupancy (fraction in each alternative structure)
polypeptide chain
amino acid number
temperature (B) factor (flexibility)
element

```
ATOM  89  N   VAL A  11  14.377  10.760  18.401  1.00  27.95  N
ATOM  90  CA  VAL A  11  15.403  11.313  19.262  1.00  26.76  C
ATOM  91  C   VAL A  11  16.766  10.870  18.760  1.00  28.04  C
ATOM  92  O   VAL A  11  16.871   9.951  17.943  1.00  26.79  O
ATOM  93  CB  VAL A  11  15.221  10.851  20.717  1.00  26.79  C
ATOM  94  CG1 VAL A  11  13.841  11.273  21.223  1.00  26.42  C
ATOM  95  CG2 VAL A  11  15.401   9.348  20.810  1.00  25.99  C
ATOM  96  N   THR A  12  17.804  11.535  19.245  1.00  27.77  N
ATOM  97  CA  THR A  12  19.163  11.215  18.845  1.00  28.45  C
ATOM  98  C   THR A  12  19.752  10.167  19.773  1.00  29.05  C
ATOM  99  O   THR A  12  19.684  10.292  20.994  1.00  29.41  O
ATOM 100  CB  THR A  12  20.057  12.475  18.867  1.00  29.38  C
ATOM 101  OG1 THR A  12  19.636  13.376  17.836  1.00  30.85  O
ATOM 102  CG2 THR A  12  21.521  12.105  18.648  1.00  29.38  C
```

Figure 11.5: A segment of the PDB file for the HIV protease describing the locations of the atoms in the protein. Data from: PDB.

Many programs can produce interactive three-dimensional visualizations based on PDB files. Web-based software is usually based on Jmol (see References and Supplemental Reading), a scriptable open-source viewer that runs within a browser as a Java applet. Indeed, a Jmol viewer can be invoked directly from the PDB entry page by clicking on the `View in 3D` link. For this exercise, we use FirstGlance in Jmol, which includes both a full-featured Jmol viewer and scripts to facilitate viewing of key structural features. Alternatively, you may wish to use one of the more powerful viewers listed in **Table 11.2**, which can be downloaded to run from a desktop computer; the activities in this section could equally well be completed with one of these programs.

Link From the **FirstGlance in Jmol** start page, enter `1KJF` to see the HIV protease model you found at PDB. When the applet loads, you should see the protease structure in a"cartoon" view similar to **Figure 11.6**, where α-helices are shown by spiral ribbons (arrows point toward the C-terminus of the protein) and β-sheets by parallel flat ribbons. Unstructured (**random coil**) areas of the protein look like thin ropes. When the program starts, the protein is rotating to show you the three-dimensional view; click on the menu at left to halt it. Notice that three different colors are used. The HIV protease functions as a **homodimer**, that is, the functional protease is composed of two identical polypeptides (quaternary structure). You should see that two colors represent two polypeptides with the same structure joined together. The third color shows a short peptide that represents a segment of a protein substrate in the active site of the enzyme.

Table 11.2: Desktop software for protein structure visualization.

Open table as spreadsheet

Program	Description
Cn3D	NCBI's protein structure viewer; structures can be downloaded from NCBI databases in Cn3D format. Free.
DeepView	Viewer comparable to Cn3D maintained by the Swiss Institute of Bioinformatics. Free.
PyMOL	Powerful Python-based visualization tool known for creation of publication-quality images. Source code and a limited prebuilt educational version are free; fully supported prebuilt versions require a paid subscription.
Chimera	Developed by a molecular visualization group at the University of California San Francisco. Free for academic and nonprofit use.
RasMol	One of the first popular visualization tools. Requires use of command-line commands. Free open-source and user-supported versions available.

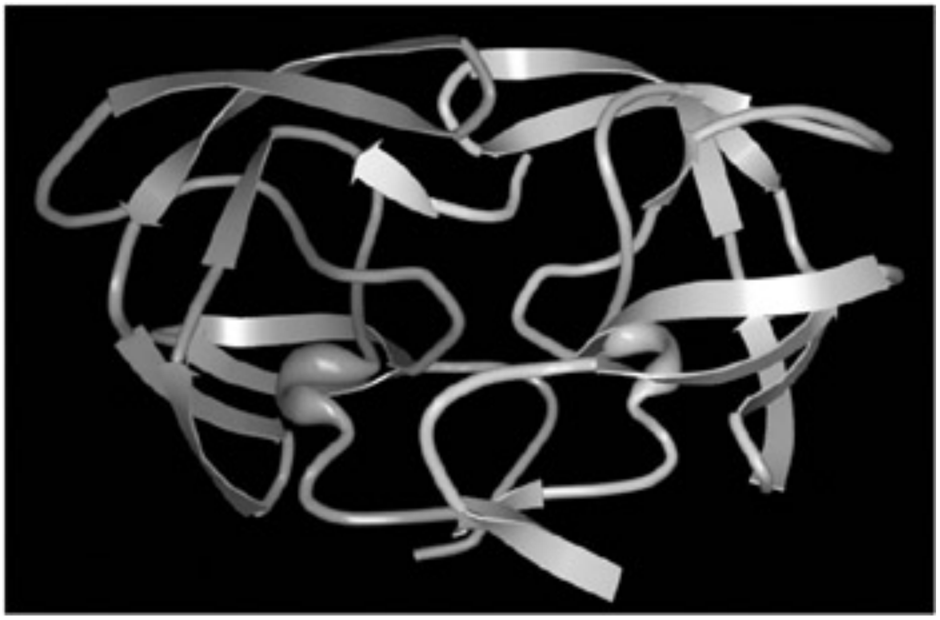


Figure 11.6: Cartoon structure of the HIV protease (monomers shown in dark and light gray) with a short peptide ligand (white) in its active site. Structure from the RCSB PDB (www.pdb.org): PDB ID 1KJF: M. Prabu-Jeyabalan et al., Substrate shape determines specificity of recognition for HIV-1 protease: analysis of crystal structures of six substrate complexes. *Structure* 10:369–381 (2002).

Jmol is an interactive program that allows the user to control how the protein is visualized. Notice that by clicking and dragging on the structure, you can rotate it to any desired position. Try rotating the molecule so you get a clear view of the substrate peptide. Can you see the distinct cleft where the substrate binds? This is where the active site of the enzyme is located. You can zoom in and out by clicking on the molecule and rotating the scroll wheel on your mouse or by holding shift while you click and drag. Holding shift also constrains the rotation of the molecule so it moves around a fixed point instead of in three dimensions. You can identify any amino acid in the protein by hovering over it.

Notice the links on the menu at the left. These run preset scripts to show you the kinds of information a typical user would want. Start by clicking on `Secondary Structure` to change the color scheme. Now, the α-helices, β-sheets, and random coils have distinct colors. Likewise, `Hydrophobic/Polar` allows you to see the hydrophobicity of the amino acids that make up the protein (you can click on Water to see where water molecules have access to the protein) and `Charge` lets you see amino acids colored by their charge. Notice these last two options change the view of the molecule to a **space-filling** model, which helps demonstrate that the protein is really not just a ribbon of amino acids but a three-dimensional structure. However, now it is hard to see the two chains and the substrate. Click on`Contacts` to see these highlighted in color again; does this change your understanding of how the peptide fits in the active-site cleft?

In addition to these preset shortcut links (unique to FirstGlance), there are two other ways to interact with Jmol (in any implementation): by menu or by using a command-line console. Right-click on the structure window to access the menus. Suppose, for example, you want to see only the peptide backbone. Open the menu and choose `Style | Structures | Backbone` (if nothing happens, choose `Select | All` and try again). But now you cannot see the individual chains, so choose `Color | Structures | Backbone | By Scheme | Chain` to change this. Many options here will allow you to look even at individual atoms and amino acids. For example, choose `Style | Scheme | CPK Spacefill` to show the space-filling model and `Color | Atoms | By Scheme | Chain` to highlight the individual chains again. Now, click on some of the atoms that seem like they are in close contact with the substrate and watch the display at the bottom to see which amino acids you have chosen and where they are on which chain.

The HIV protease is a member of the aspartyl protease family: The catalytic mechanism for these proteases involves an aspartate in the active site that can be recognized by the three-amino-acid motif Asp-Thr-Gly. Normally, HIV protease contains this motif, but to obtain a crystal structure with a peptide in the active site, a mutation changing the Asp to structurally similar asparagine (Asn) was used for the 1KJF structure. This mutation does not change the structure of the protein but prevents it from cleaving the substrate. Use `Select | Protein | By Residue Name` followed by `Color | Structures| Cartoon` (if you are in cartoon mode) or `Color | Atoms` (if you are in spacefill mode) to highlight asparagines. Then explore the adjacent amino acids by mousing over them (this is easier in cartoon or backbone view) or by selecting and coloring them and see if you can identify the Asn-Thr-Gly combination at the 1KJF active site.

It might be easier to see how the protease and substrate interact if we could get one of the chains out of the way. It is tricky to select a whole chain from the menus but easy from the command line. Show your protein in spacefill mode and choose `Console` from the menu to open the command-line interface. The two protein subunits and the peptide sub-strate are labeled A, B, and P, respectively (you could find this out by looking at the first few lines of the PDB file). Select all the atoms in the A subunit and color them blue by typing `select *:A; color atoms blue`. Then, color the B subunit red and the substrate yellow. Now hide the A subunit by simply typing `hide *:A` and rotate the molecule to get a good view of how the substrate fits in the cleft. Select and color your three active-site amino acids with commands similar to `select 10:B; color atoms white` and see how they interact with the substrate; hide the substrate if needed to see them better.

Web Exploration Questions

1. The HIV protease functions as a dimer. Some enzymes that form dimers then have two active sites. Is this the case for the HIV protease? Briefly describe the relationship of the active site and peptide-binding cleft to the subunits of the enzyme.

2. What kinds of amino acids do you find in the areas of the protein exposed to the water around it (e.g., when the protein is in solution in the cytoplasm)?
3. If you were to design an inhibitor of the HIV protease, where would you want it to bind? What kind of molecule might you use as the prototype to develop the structure of a good inhibitor?
4. Using the cartoon or ribbon view, you should be able to identify where a long β-strand on each subunit of the protease makes a hairpin turn, forming flexible flaps that cover the active site cleft. These flaps control access of the substrate to the active site. Which amino acids form the flaps (just give the range of numbers)? Although this region is very important to protease function, why are the flaps not likely to make a good target for rational drug design?
5. What are the numbers of the amino acids on each chain that form the Asp-Thr-Gly (Asn-Thr-Gly in this mutant) aspartate protease motif in 1KJF?

Part III: Predicting Secondary Structure from Amino-Acid Sequence
One of the major obstacles to pharmaceutical control of HIV is the virus' rapid rate of mutation. The DNA polymerases that replicate DNA in our cells "proofread" during synthesis, reducing their error rate to about one nucleotide in a billion. Reverse transcriptase, however, does not proofread and in addition appears to be much less accurate than other nonproofreading polymerases, producing one mutation for approximately every 10,000 nucleotides of DNA it synthesizes. Combined with its long-term residence in a single host and rapid rate of replication (up to 10^{10} new viruses per infected patient per day), this gives HIV extraordinary genetic variability and many strains can be in competition within a single patient, leading to the rapid evolution of variants that can escape from immune system controls as well as drug-resistant strains. Current drug therapies combine three or more individual antivirals in an attempt to stave off resistance, but even so, patients must be closely monitored and their drug regimens altered in response to the inevitable rise of resistance.

Download How do changes in HIV proteins lead to drug resistance? From the *Exploring Bio-informatics* website, you can download the **amino-acid sequence of a drug-resistant mutant HIV protease**. Because this protease variant has not been crystallized, its exact structure is not known. We expect, however, that its structure will vary only in specific locations and probably in minor ways (especially because this variant does function as a protease) from the protease we have already examined. Homology modeling is therefore an appropriate method of structure prediction: The sequence of the mutant can be aligned with the original sequence (**template**) and a structure generated that follows the template wherever the amino acids are identical. Where the two sequences are different, the program attempts to predict the effect of the substituted amino acids on the structure based on their properties.

Link SWISS-MODEL is a Web-based homology modeling program suitable for analysis of the mutant protease; its automated mode provides an easy way to model a protein expected to closely match the template. From the **SWISS-MODEL home page**, choose `Automated`Mode and enter the mutant protease sequence. Although the program can search the entire PDB to find a suitable template by similarity, in this case we know the identity of our protein. A suitable template would be an HIV protease structure that also does not include a substrate (because there is no substrate in our mutant sequence); we can use PDB structure 1ODW. Enter this accession number at the bottom of the page to be used as the template; you can enter either chain A or B, because both are the same. You can wait for the results (usually only a few minutes) or provide an email address to be notified when the analysis is complete.

Link The output of SWISS-MODEL is a PDB file for the mutant protein—a model structure, because it is based not on crystallography but on homology. This structure can be visualized with a Jmol-based viewer, such as the basic AstexViewer linked on the results page (go ahead and try this; the result should look very familiar). However, it would be more instructive to directly compare the mutant structure with the unmutated protease. Download the PDB model for the mutant using the appropriate link and save it as a local file. Then navigate to**PDBeFold**, a Web interface to a program capable of constructing a pairwise *structure*alignment.

Use the mutant protease PDB file you just downloaded as the query sequence (choose`Coordinate file` from the drop-down menu to upload it) and enter the 1KJF accession number as the target. `Chains` can be set to `*(all)`. Uncheck `match individual chains`—because our two chains are identical, there is no point in doing an A versus B and B versus A comparison. Leave the rest of the options at their defaults. Submit the alignment for processing. A single match should be returned; click on its number to see a detail page. Scroll down the page to see how the two proteins' amino acids matched up: In red are query amino acids matched with the same amino acid in the template, whereas blue shows those that aligned with a different amino acid. Back near the top of the page, you should see a button to superpose the two structures (there are two; use the top one); be sure `superpose whole entries` is checked (so we see both chains) and click on the button to see the structures in a Jmol viewer.

The default view is in cartoon format, with the two chains of the unmutated protease shown in cyan and the two chains of the mutant shown in gray. Set `Screen` to 80% or 90% to see the molecule better and then explore the structure. As you rotate the model, in most places the two structures are so similar that you see a single ribbon or rope, but you should be able to recognize some places where they are quite distinct. Let's focus on how the mutations affect the area of the active site. PDBeFold has essentially produced a composite PDB file in which the two chains of the mutant protease are A and B and the two chains of the original protease are D and E, with the substrate as chain F. To make it easier to see the overall outline of the structure, set `Rendering` to Backbone. Now let's use the console to highlight a couple of specific areas near the active site: Try `select 48-53:A; color backbone blue`,`select 48-53:B; color backbone blue` and `select 76-83:B; color backbone blue` to highlight regions of the mutant protein in blue, and then color amino acids 48–53 red on chains D and E and 76–83 red on chain E to show the unmutated protein. Explore the model to see how these regions relate to the location of the substrate; how might the mutations affect the fit of an inhibitory drug in the active site? To make this clearer, try`select *:F; spacefill` to make only the substrate chain spacefilling and `color` `atoms white` to make the colors less distracting. Of course, you are free to explore further with different views and color schemes.

Web Exploration Questions

6. How many mutations are there in the mutant protease sequence, as compared with the sequence of the protease you examined in Part I? Use pairwise alignment to find out.
7. In the regions you highlighted, how would you characterize the effect of the mutations on the structure of the protein, in general?
8. How would these structural changes affect the binding of a small inhibitor molecule to the protease active site? Why would they have less effect on the binding of the natural substrate?
9. If you wanted to design a drug that would inhibit this mutant protease, what characteristics would you want it to have?
10. Change the colors of your model so that everything is white except the three amino acids of the aspartyl protease motif (make the substrate gray for contrast). Make these three amino acids blue on the mutant chains, and then see what happens when you color them red on the nonmutant chains. Does their position change in the mutant relative to the unmutated protein? Is this what you expected? Certainly changes in the sequence or structure at these positions could lead to drug resistance; why then do we not observe them among drug-resistant HIV isolates?

More to Explore: Binding of the Mutant Protease to Inhibitors

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The previous exercise allowed you to formulate a hypothesis about why this mutant protease is drug resistant. As you saw, PDB has many examples of protease structures with various inhibitors bound to the protease. You could use PDBeFold to make alignments of the mutant protease with some of these structures to see the structural changes in the mutant relative to actual inhibitor binding.

Part III: Predicting Secondary Structure from Amino-Acid Sequence

Link Finally, let's look at the ability of bioinformatic software to predict secondary structure ab initio—from an amino-acid sequence unassisted by a known structure. Because we know the crystal structure of the HIV protease, we can try predicting secondary structures using its sequence and then compare the results with the known locations of α-helices and β-sheets; use the 1KJF sequence, which you can download from its PDB page. For the structure prediction, we use **PSIPRED** to look for regions of the protein likely to form α-helices, β-sheets, or random coils. PSIPRED uses a neural network algorithm and integrates both a Chou-Fasman–like prediction algorithm and comparative data obtained by searching for orthologous sequences with PSI-BLAST (see References and Supplemental Reading).

From the PSIPRED page, choose `Predict Secondary Structure`. (Notice that the same server offers two other structure prediction options.) Enter the protease sequence and your email and submit your request. You should get an email within half an hour or less indicating the job is complete. You can examine the results either in text form in the email or graphically by clicking the emailed link. Either way, you should see that each amino acid in the protein has been assigned a letter indicating whether it is predicted to be in an alpha (H)elix, a strand of a beta sh(E)et, or a random (C)oil. Each also has a number indicating the statistical level of confidence in the prediction (nine is highest). In the graphical version (**Figure 11.7**; the PDF file provides the nicest view), the confidence value is replaced by a bar whose height shows the level of confidence, and the α-helices and β-strands are shown graphically with cylinders and arrows, respectively. Save or print your results for easy comparison.

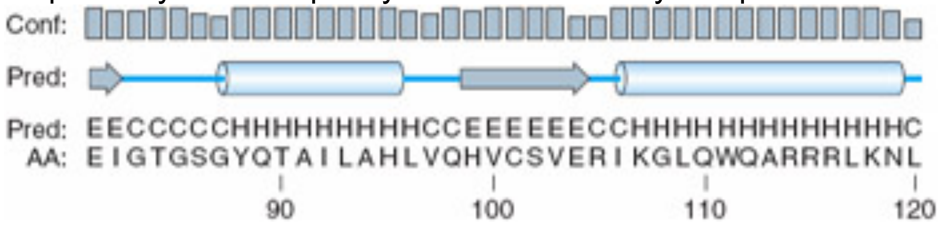
Conf:
Pred:
Pred: EECCCCCHHHHHHHHHCCEEEEEEECCHHHHHHHHHHHHHC
AA: EIGTGSGYQTAILAHLVQHVCSVERIKGLQWQARRRLKNL
90
100
110
120

Figure 11.7: Sample output from the PSIPRED server. The bars at the top represent the confidence level of each prediction. Arrows and cylinders in the next line represent predicted β-strands and α-helices, respectively, followed by text showing whether each amino acid is within a predicted β-strand (E), α-helix (H), or random coil (C). Data from PSIPRED server: McGuffin et al., *Bioinformatics* 16:404 (2000).

Now, return to FirstGlance in Jmol to visualize the HIV protease structure 1KJF. Color the structure by secondary structure so you can see the α-helices and β-strands clearly. You may want to hide one of the chains and the substrate for convenience. Now, identify the start and end points of the α-helices and β-strands in the crystal structure and note them on the PSIPRED results. How does PSIPRED's prediction compare with the actual structure?

Web Exploration Questions

11. How well did PSIPRED predict the secondary structures in the HIV protease? Give specific examples of structures predicted accurately by PSIPRED, predicted structures not found in the actual structure, and actual structures that were not predicted.
12. PSIPRED uses a prediction algorithm not unlike the Chou-Fasman algorithm we will use in the Guided Programming Project. However, instead of applying its algorithm directly to your input sequence, it first does a PSI-BLAST search to get a collection of sequences related to your input. It then applies its prediction algorithm to the results. Why might this method be advantageous in improving the program's ability to identify genuine secondary structure?

More to Explore: More Structure Tools

We have barely scratched the surface of protein structure prediction and analysis tools. **Table 11.3** lists a number of additional tools you may wish to apply to these or to other protein structure questions.

Table 11.3: Additional recommended protein structure analysis software.
Open table as spreadsheet

Program	Description
Ab Initio Protein Structure Prediction	
Jpred3	Secondary structure prediction, multiple neural network methods
PEP-FOLD	Tertiary structure prediction based on hidden Markov modeling
ROBETTA	Tertiary structure prediction: structure generation for short fragments followed by energy minimization
Membrane Protein Prediction	
MEMSTAT	Neural network-based prediction of transmembrane domains
HMMTOP	Hidden Markov model-based prediction of transmembrane domains
Homology Modeling	
ESyPred3D	Alignment and model generation; uses MODELLER algorithm to examine a probability density function for each atom
FoldX	Homology modeling and prediction of effects of mutations; useful to design protein variants with desired effects on structure
Threading	
GenTHREADER	Threading based on secondary structure prediction
HHpred	Based on multiple sequence alignment of related sequences identified by PSI-BLAST

Guided Programming Project: Structure Prediction with the Chou-Fasman Algorithm

As described in Understanding the Algorithm, the Chou-Fasman algorithm looks at the likelihood that each amino acid in a protein sequence occurs within an α-helix, β-strand, or β-turn. In this project, you will develop a program that implements the first step of this algorithm: finding α-helices. The complete Chou-Fasman algorithm will be implemented in the On Your Own Project.

Before you begin to write code, think about the data structures you need to store the Chou-Fasman parameters. You may want to consider hash table structures for easy and quick access using amino-acid names as keys. The following pseudocode presents a solution for finding α-helices.

Algorithm

Chou-Fasman Algorithm for Predicting Protein Structure

- **Goal:** To predict the location of α-helices.
- **Input:** An amino-acid sequence in FASTA format
- **Output:** The location of α-helices.

```
// Step 1: Initialization and Read in Sequence
open input file 1: infile1
aminoSeq = ""
read and ignore first line of data in infile1

for each line of data in infile1
        concatenate line of data to aminoSeq

// Step 2: Find Alpha Helices
// find region of six (step 1a)
lenSeq = length of aminoSeq
window = 6
pScore = 103
minWindow = 4
paHash = map of all amino acids to P(a) values
pbHash = map of all amino acids to P(b) values

for each i from 0 to (lenSeq—window)
        ctr = paSum = pbSum = 0
        // find possible alpha helices
        for each j from 0 to window-1
                paSum = paSum + paHash[aminoSeq[i+j]]
                pbSum = pbSum + pbHash[aminoSeq[i+j]]
                if paHash[aminoSeq[i+j]] > pScore
                        ctr++
        if ctr >= minWindow
                output "Possible alpha helix region found at" + (i+1)

                // extend region left (step 1b)
                extend = i-1
                done = false
                while extend >= 0 and !done
                        if extend >= 3
                                …… paHash[aminoSeq[extend]] < 100
                                …… paHash[aminoSeq[extend-1]] < 100
                                …… paHash[aminoSeq[extend-2]] < 100
                                …… paHash[aminoSeq[extend-3]] < 100
                                        done = true
                        else
                                paSum = paSum + paHash[aminoSeq[extend]]
                                pbSum = pbSum + pbHash[aminoSeq[extend]]
                                extend—
                left = extend + 1
                // extend region right (step 1b continued)
                extend = i + window
                done = false
                while extend < lenSeq and !done
                        if extend <= lenSeq - 3
                                ...... paHash[aminoSeq[extend]] < 100
                                ...... paHash[aminoSeq[extend+1]] < 100
                                ...... paHash[aminoSeq[extend+2]] < 100
                                ...... paHash[aminoSeq[extend+3]] < 100
                                done = true
                else
                        paSum = paSum + paHash[aminoSeq[extend]]
                        pbSum = pbSum + pbHash[aminoSeq[extend]]
                        extend++
right = extend - 1
// see if step 1c fulfilled
lenRegion = right - left
if paSum/lenRegion > pScore and paSum > pbSum
        output "Alpha Region:" + (leftStart+1) + "to"
                + (rightStart+1)
```

Putting Your Skills Into Practice

1. Download Write a program to implement the given pseudocode in the programming language used in your course. Short amino-acid sequences can be downloaded from the *Exploring Bioinformatics* website and used to test your program.
2. The PSIPRED secondary structure prediction program gives text output showing the predicted secondary structure for each position in the amino-acid sequence (Figure 11.7). Modify your program to produce output similar to PSIPRED, using H to represent helices and a dash (–) to indicate amino acids that are not in an α-helix.
3. Each chain of the HIV protease contains one α-helix. Identify the amino acids in one chain of the 1KJF structure that are within the α-helix, and then run your program on this sequence and compare its prediction with the actual crystal structure and to the PSIPRED prediction.

On Your Own Project: A Complete Chou-Fasman Program

Download In this project, you will complete the implementation of the Chou-Fasman algorithm that you started in the Guided Programming Project. If your course does not involve program-ming, you can download a **completed Chou-Fasman program** from the *Exploring Bio-informatics* website and use it to answer the questions that follow.

Understanding the Problem

The Guided Programming Project showed how to implement step 1 of the Chou-Fasman algorithm, finding all possible α-helices. Understanding the Algorithm introduced the remaining steps of the algorithm: predicting β-strands and β-turns, as well as dealing with overlaps where the same amino acid is within two structures. Amino acids not within any of these structures are considered to be within random coils.

Solving the Problem

A straightforward approach to code the entire algorithm is to traverse the sequence three times, each time searching for a particular structure (steps 1–3). You could then compare the results to handle overlaps (step 4). However, storing all the information from steps 1–3 before tackling step 4 may not be the most efficient approach, because many overlap-ping areas would require more storage than necessary. Additionally, making a separate pass through the sequence to find each structure adds unnecessary complexity.

Alternatively, your program could find all possible α-helices and then look for β-sheets, checking for overlaps as each is found before continuing. It could then continue on to find β-turns. To accomplish this, you would need to change your guided project solution so that each α-helix is stored rather than simply printed. Think carefully about what data you need to store as you find each α-helix.

Programming the Solution

Extend your solution to incorporate steps 2–4 of the Chou-Fasman algorithm. Your program should display text output similar to that of PSIPRED (**Figure 11.7**), showing the predicted structure for each amino acid: H for α-helices, E for β-strands, T for β-turns, and C for random coil.

Download Test your program with the **short test sequence** you can download from the*Exploring Bioinformatics* website. Then, run it on the 1KJF protease sequence and see if it finds the known locations of the α-helix and the β-strands.

1. How did your Chou-Fasman prediction compare with the actual structure of the HIV protease?
2. How did your prediction compare with that of PSIPRED? PSIPRED is a much more sophisticated program; does it give significantly better results?
3. You may also want to test your program on other proteins to better evaluate its capabilities. Try, for example, the HIV reverse transcriptase or the HIV capsid protein. For a bigger challenge, try it on the HIV envelope protein, which is a transmembrane protein.
4. It is possible that where Chou-Fasman fails to make an accurate prediction, it may be making the wrong choice between α-helix and β-strand in overlap regions. If you are in a programming course, you could modify your program so it reports overlaps and shows the decision it made, allowing you to see if the opposite decision would have led to a better prediction.

Connections: Distributed Computing to Improve Ab Initio Protein Structure Prediction

By now you have an appreciation for the complexity of protein folding and how hard it is to predict the final three-dimensional conformation of a protein based on its primary structure. Even our best computational algorithms for predicting secondary structure can do so with only moderate confidence. The enormous number of possible ways in which these secondary structures might fold into a tertiary structure compounds the problem. Furthermore, folding occurs differently in different environments—such as for a membrane protein, which is typically inserted into the membrane as it is being synthesized. Computational power is one limiting factor in coping with this complexity: Protein folding algorithms can be refined by comparing predicted structures with the increasing number of known protein structures, but a great deal of computer time is necessary to process the huge numbers of possible models.

Link **Distributed computing** offers an intriguing approach to this problem. At least two current projects, **Folding@home** and **Rosetta@home**, use software that can be downloaded freely by anyone and used like a screensaver, working on folding models when the computer is idle. A central server parcels out pieces of the problem to individual computers that process data and return the results to the server, thus harnessing the unused capacity of hundreds of thousands of individu-al computers. This yields total computing power much greater than any single computer and at very low cost. Both projects focus on structures important to understanding human disease, particularly diseases such as Huntington disease, Alzheimer disease, and prion diseases, which involve misfolded proteins.

BioBackground: Protein Structure

A protein's function depends on both its amino-acid sequence and its conformation, or folded structure. The three-dimensional shape of a protein determines the interactions it can have with other molecules. For example, a DNA-binding protein such as a transcription factor (**Figure 11.8A**) needs structural regions (**domains**), allowing it to fit into the grooves of a DNA molecule. In these binding domains, positively charged amino acids are needed to interact with the negatively charged DNA backbone, and additional amino acids interact with specific DNA bases to determine the DNA sequence to which the transcription factor binds. A channel protein (**Figure 11.8B**) has long helices that pass through the membrane; the exterior of these helices consist of amino acids with hydrophobic side chains to interact with the hydrophobic membrane lipids, but the interior contains hydrophilic amino acids that can interact with some molecule to be transported across the membrane.

How a protein can fold depends on its amino-acid sequence, known as its **primary** (18)**structure** (**Figure 11.9A**). Folding results from the interaction of amino-acid side chains, mostly weak noncovalent interactions such as hydrogen bonds (the attraction of a hydrogen attached to an oxygen or nitrogen atom for a nearby oxygen or nitrogen), ionic bonds (attraction between positively and negatively charged side chains), or hydrophobic interactions. Where two cysteine amino acids are close together, a covalent disulfide bond can be formed, as well. Thus, we can think of protein conformation as being "encoded" in its gene in some sense, but folding is also influenced by the environment in which the protein folds (such as the cytoplasm or endoplasmic reticulum) and in some cases by interactions with other proteins.

Folding begins while the protein is still being synthesized, as soon as the amino-acid chain begins to emerge from the ribosome. Local interactions among amino acids, often driven by the instability of hydrophobic amino acids exposed to the surrounding watery environment, result in the formation of **secondary (2**8**) structures** (**Figure 11.9B**). The two most common forms of secondary structures are α-helices and β-sheets. In an α-helix, hydrogen bonds between amino acids spaced along a contiguous region form a regular, relatively rigid spiral-shaped structure. A β-sheet is formed by hydrogen bonds among extended, uncoiled stretches called β-strands; β-sheets create relatively flat surfaces in the folded protein. The β-sheet may form from β-strands that follow each other in the primary structure—if so, the strands are separated by hairpin b-**turns**—or may result from β-strands from different parts of the primary structure coming together. Stretches of amino acids with no particular secondary structure are referred to simply as**random coil** regions (Figure 11.9B).

As protein synthesis proceeds, secondary structures can interact with each other, folding the protein into an overall three-dimensional shape called its **tertiary (3**8**) structure**(**Figure 11.9C**). Most proteins fold into a shape that is roughly spherical (globular), but some form long fibers or other configurations appropriate to their function. Within the tertiary structure of an enzyme, there is a binding pocket called the **active site** where the enzyme's substrate fits selectively, and there may also be binding pockets or clefts for other molecules that interact with the protein. Although any long amino-acid chain is commonly referred to as a protein, technically an amino-acid chain is a **polypeptide** and a protein as strictly defined is a *functional* unit. Some proteins, such as the CFTR protein, are composed of only a single polypeptide. However, some proteins require the association of multiple polypeptide subunits to function (**Figure 11.9D**); this is referred to as **quarternary (4**8**) structure**. The HIV protease, for example, is a **dimer**, composed of two identical polypeptide subunits. Hemoglobin, on the other hand, functions as a**tetramer** composed of two identical α-globin subunits and two identical β-globin subunits, four polypeptides in total.

When the structure of a folded protein is known, it can be represented in a variety of ways to quickly convey its major features to a viewer. A **ribbon** diagram (**Figure 11.10**) is a conventional way to represent the structure of a protein: Flat ribbons represent β-strands and coiled ribbons represent α-helices. Arrows point toward the protein's C-terminal end. A **cartoon** representation is very similar; here, the helices are shown as cylinders.

Proteins generally fold to reach their lowest energy state or most stable structure. Generally, hydrophobic amino acids fold into the interior of the protein, leaving hydrophilic ones on the outside to interact with the watery environment of the cytoplasm. Likewise, two negatively charged side chains fold to avoid each other and preferably interact with positively charged side chains. In practice, however, this process is constrained by factors such as the order of amino acids: If the first region of the protein folds as soon as it is synthesized to bring hydrophobic amino acids together, those amino acids are no longer available to interact with the next hydrophobic stretch. This reduces the number of possible folded structures for the real protein but tends to make computational prediction more difficult. Remember, too, that the interactions holding the folded structure together are generally weak and can be broken by increasing the temperature or changing the pH: We say this **denatures** the protein. We take advantage of this when we fry an egg, denaturing the watery, protein-rich goo into a more palatable form, or "perm" hair by chemically denaturing hair protein.

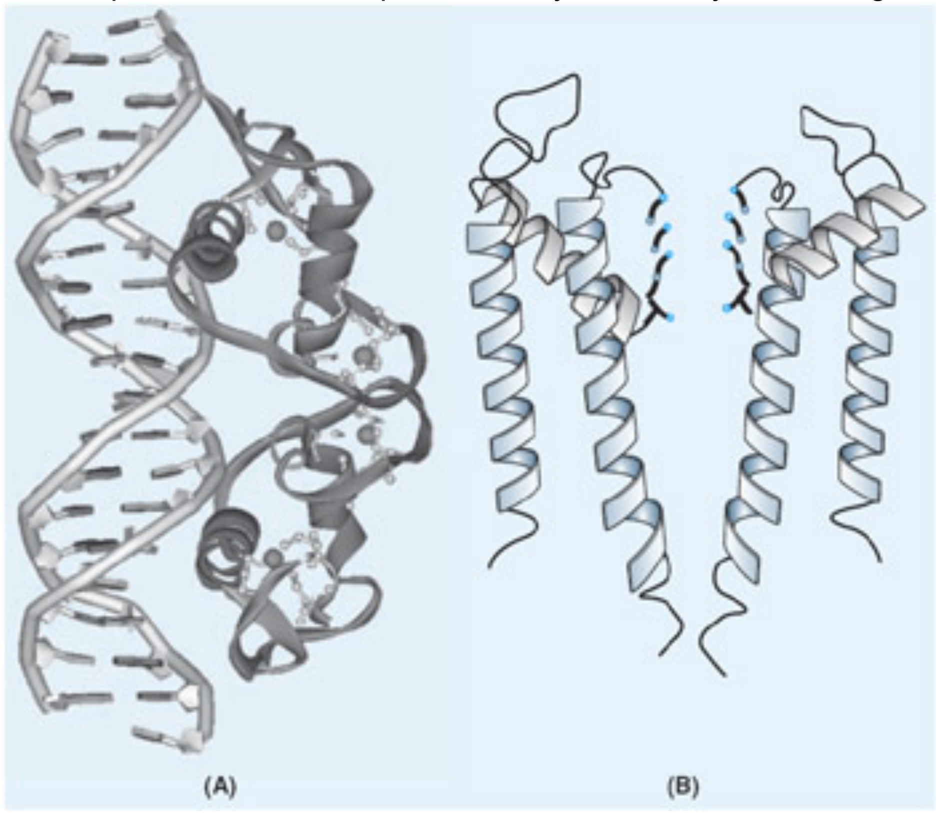

Figure 11.8: Examples of protein structure: (A) a DNA-binding protein interacting with DNA by means of two α-helices; (B) a channel protein that is anchored into a membrane by long helices creating a pore through which some transported molecule can pass. Part (A) structures from the RCSB PDB: PDB ID 1R4R (B. J. Luisi et al (1991) Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature. 352:497-505).

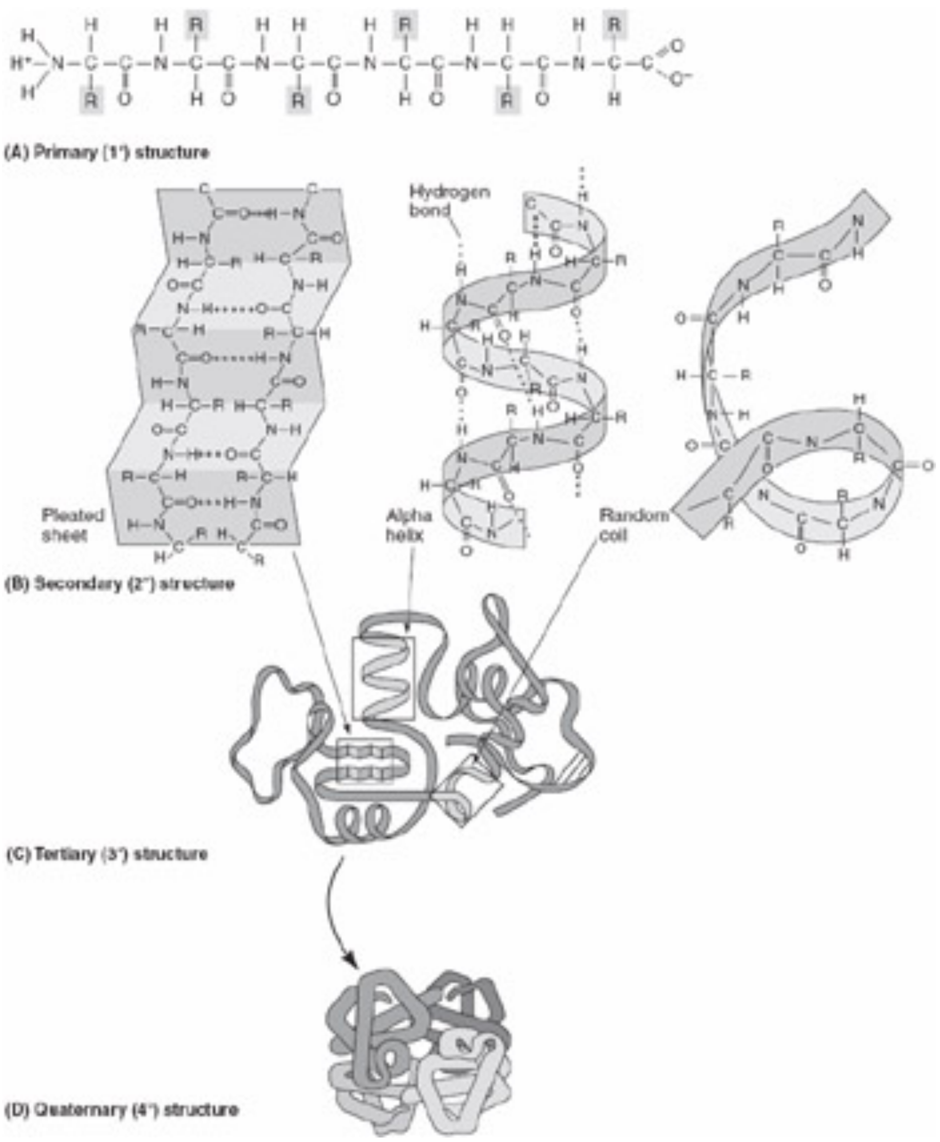
(A) Primary (1°) structure
Hydrogen bond
Pleated sheet
Alpha helix
Random coil
(B) Secondary (2°) structure
(C) Tertiary (3°) structure
(D) Quaternary (4°) structure

Figure 11.9: Folding of a protein: (A) primary structure, or the amino-acid sequence of the protein; (B) secondary structures formed by local interactions among amino acids: the β-sheet (or β-pleated sheet) and the α-helix; (C) tertiary structure, or the overall three-dimensional shape of the protein; (D) quaternary structure, or the association of two or more polypeptides to form a functional unit, necessary to the function of certain proteins.

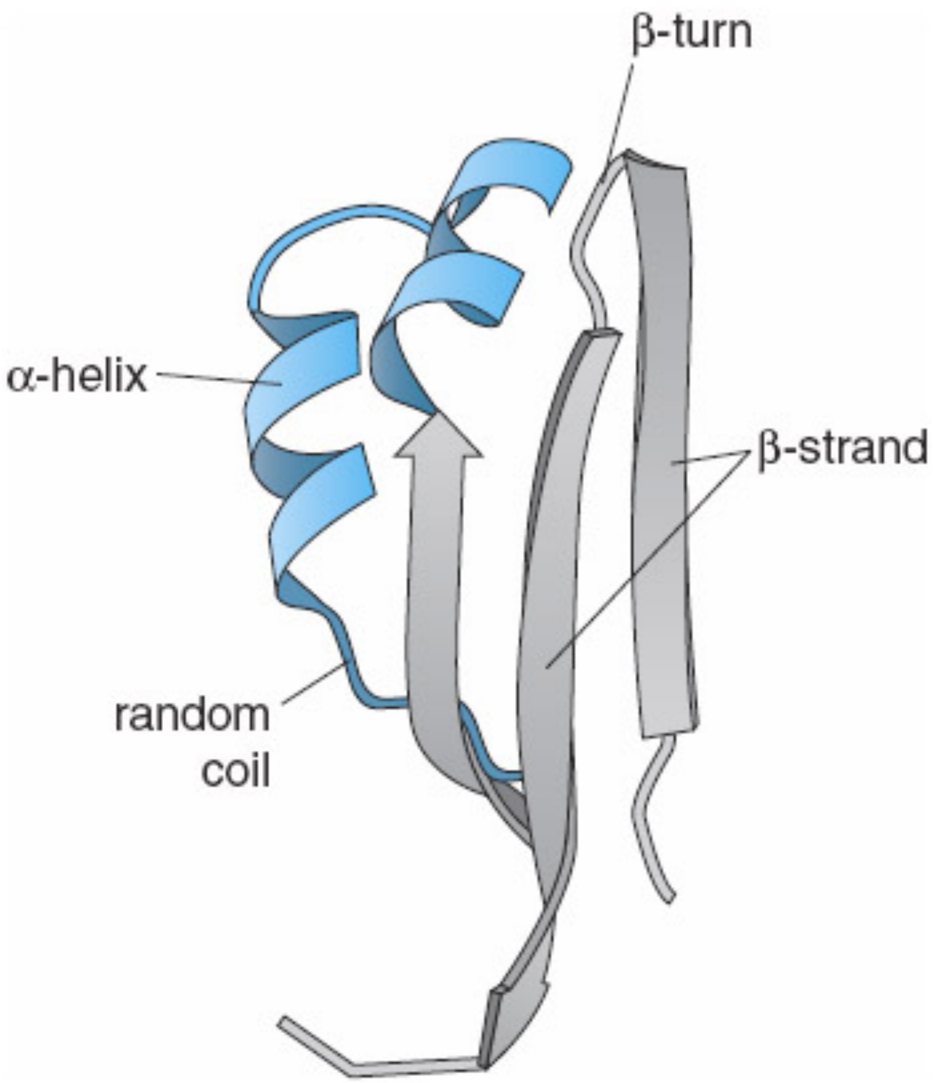

Figure 11.10: Ribbon diagram representation of the three-dimensional structure of a protein. Spiral ribbons represent α-helices, and flat ribbons represent β-strands. Ropes represent regions of random coil.

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