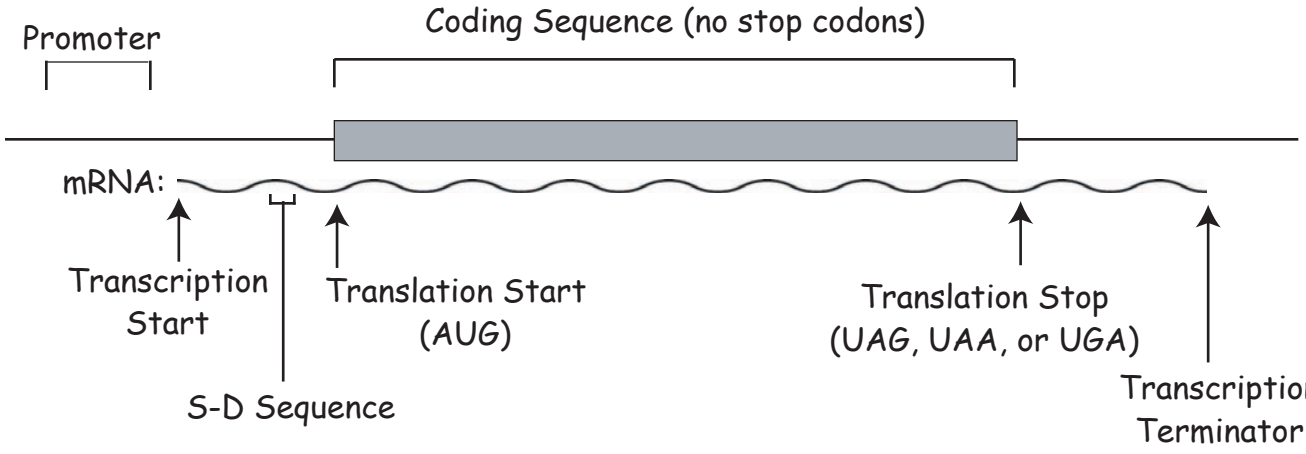
Lecture 11

Analysis of Gene Sequences

Anatomy of a bacterial gene:



Sequence Element	Function
Promoter	To target RNA polymerase to DNA and to start transcription of a mRNA copy of the gene sequence.
Transcription terminator	To instruct RNA polymerase to stop transcription.
Shine-Dalgarno sequence	S-D sequence in mRNA will load ribosomes to begin translation. Translation almost always begins at an AUG codon in the mRNA (an ATG in the DNA becomes an AUG in the mRNA copy). Synthesis of the protein thus begins with a methionine.
Coding Sequence	Once translation starts, the coding sequence is translated by the ribosome along with tRNAs which read three bases at a time in linear sequence. Amino acids will be incorporated into the growing polypeptide chain according to the genetic code.
Translation Stop	When one of the three stop codons [UAG (amber), UAA (ochre), or UGA is encountered during translation, the polypeptide will be released from the ribosome.

Example: A gene coding sequence that is 1,200 nucleotide base pairs in length (including the ATG but not including the stop codon) will specify the sequence of a protein 1200/3 = 400 amino acids long. Since the average molecular weight of an amino acid is 110 da, this gene encodes a protein of about 44 kd — the size of an average protein.

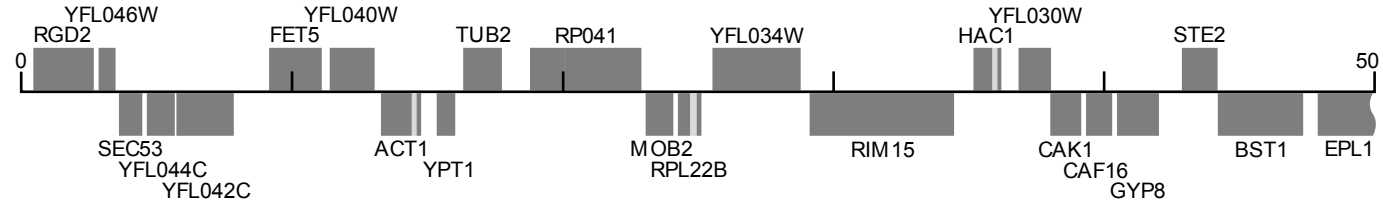
The Genetic Code

1st position (5′ end) ↓	2nd position U	2nd position C	2nd position A	2nd position G	3rd position (3′ end) ↓
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	STOP	STOP	A
	Leu	Ser	STOP	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

Classically, genes are identified by their function. That is the existence of the gene is recognized because of mutations in the gene that give an observable phenotypic change. Historically, many genes have been discovered because of their effects on phenotype. Now, in the era of genomic sequencing, many genes of no known function can be detected by looking for patterns in DNA sequences. The simplest method which works for bacterial and phage genes (but not for most eukaryotic genes as we will see later) is to look for stretches of sequence that lack stop codons. These are known as "open reading frames" or **ORF**s. This works because a random sequence should contain an average of one stop codon in every 20 codons. Thus, the probability of a random occurrence of even a short open reading frame of say 100 codons without a stop codon is very small $(61/64)^{100} = 8.2 \times 10^{-3}$

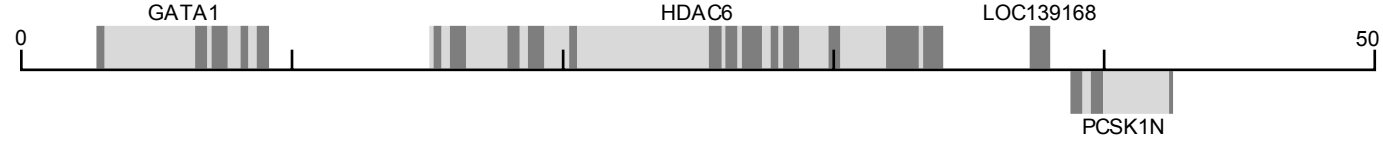
Identifying genes in DNA sequences from higher organisms is usally more difficult than in bacteria. This is because in humans, for example, gene coding sequences are separated by long sequences that do not code for proteins. Moreover, genes of higher eukaryotes are interrupted by **introns**, which are sequences that are spliced out of the RNA before translation. The presence of introns breaks up the open reading frames into short segments making them much harder to distinguish from non-coding sequences. The maps below show 50 kbp segments of DNA from yeast, Drosophila, and humans. The dark grey boxes represent coding sequences and the light grey boxes represent introns. The boxes above the line are transcribed to the right ant the boxes below are transcribed to the left. Names have been assigned to each of the identified genes. Although the yeast genes are much like those of bacteria (few introns and packed closely together), the Drosophila and human genes are spread apart and interrupted by many introns. Sophisticated computer algorithms were used to identify these dispersed gene sequences.

Saccharomyces cerevisiae

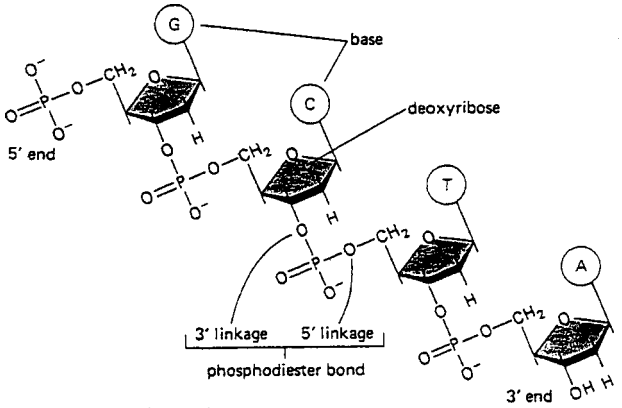


Drosophila melanogaster

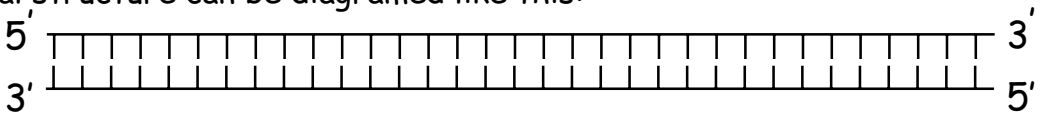
Human



To see how gene sequences are actually obtained, we will first need to consider some fundamentals of the chemical structure of DNA. Each strand of DNA is directional. The different ends are usually called the 5′ and 3′ ends; referring to different positions on the ribose sugar ring where the linking phosphate residues attach.



In a double stranded DNA molecule the two strands run anti-parallel to one another and the general structure can be diagramed like this:



• Note about representation of DNA sequences.

1) Single strands are always represented in direction of synthesis – 5′ to 3′

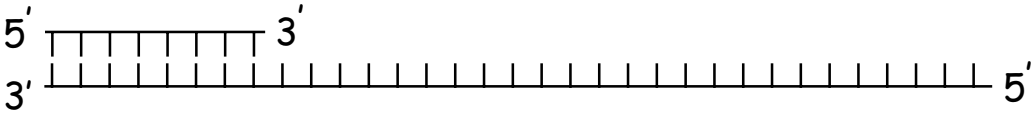
2) For double stranded DNA, usually one strand is represented in the 5′ to 3′ direction. For a gene, the strand represented would correspond to the sequence of the mRNA.

DNA polymersaes are the key players in the methods that we will be considering. The general reaction carried out by DNA polymerase is to synthesize a copy of a DNA template starting with the chemical precursors (nucleotides) dATP, dGTP, dCTP, and dTTP (dNTPs). All DNA polymerases have two fundamental properties in common.

(1) New DNA is synthesized only by elongation of an existing strand at its 3′ end.

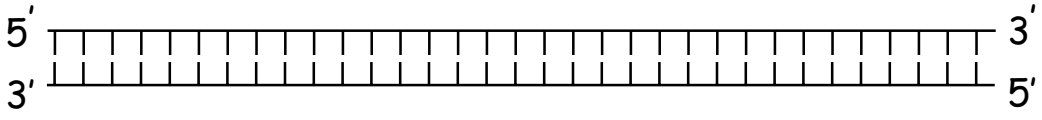
(2) Synthesis requires nucleotide precursors, a free 3′ OH end, and a template strand.

A general substrate for DNA polymerase looks like this:



Note that the template strand can be as short as 1 base or as long as several thousand bases.

After addition of DNA polymerase and nucleotide precursors this product will be readily synthesized:



DNA Sequencing

Consider a segment of DNA that is about 1000 base pairs long that we wish to sequence. (1) The two DNA strands are separated. Heating to 100°C to melt the base pairing hydrogen bonds that hold the strands together does this.

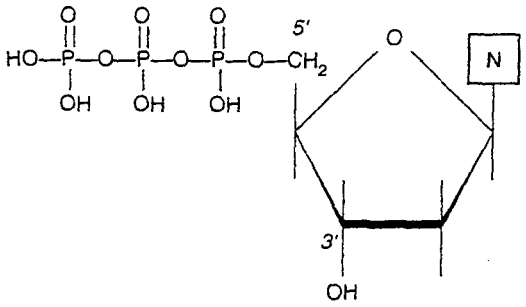
(2) A short oligonucleotide (ca. 18 bases) designed to be complimentary to the end of one of the strands is allowed to anneal to the single stranded DNA. The resulting DNA hybrid looks much like the general polymerase substrate shown previously.

(3) DNA polymerase is added along with the four nucleotide precursors (dATP, dGTP, dCTP, and dTTP). The mixture is then divided into four separate reactions and to each reaction a small quantity different dideoxy nucleotide precursor is added. Dideoxy nucleotide precursors are abbreviated ddATP, ddGTP, ddCTP, and ddTTP.

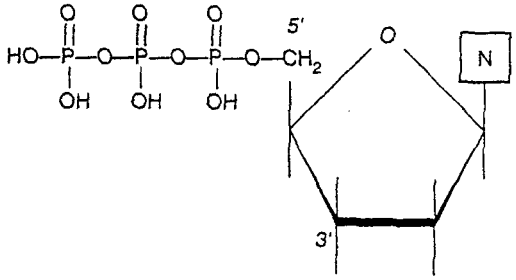
(4) The polymerase reactions are allowed to proceed and, using one of a variety of methods, radiolabel is incorporated into the newly synthesized DNA.

(5) After the DNA polymerase reactions are complete, the samples are melted and run on a gel system that allows DNA strands of different lengths to be resolved. The DNA sequence can be read from the gel by noting the positions of the radiolabeled fragments.

The crucial element of the sequencing reactions is the added dideoxynuclotides. These molecules are identical to the normal nucleotide precursors in all respects except that they lack a hydroxyl group at their 3' position (3' OH).



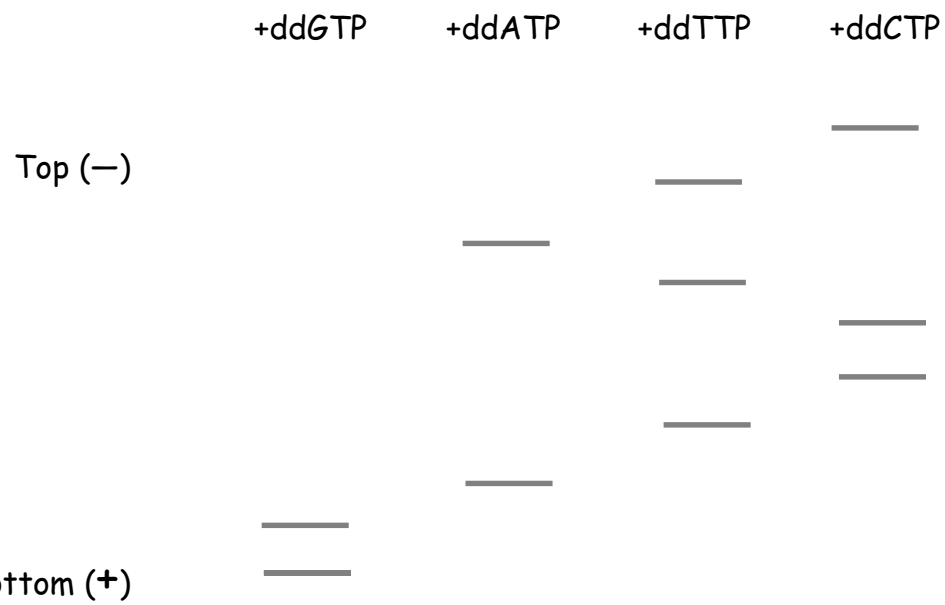
dNTP



ddNTP

Thus dideoxynuclotides can be incorporated into DNA, but once a dideoxynuclotide has been incorporated further elongation stops because the resulting DNA will no longer have a free 3' OH end. Each of the four reactions contains one of the dideoxynuclotides added at about 1% the concentration of the normal nucleotide precursors. Thus, for example, in the reaction with added ddATP about 1% of the elongated chains will terminate at the position of each A in the sequence. Once all of the elongating chains have been terminated there will be a population of labeled chains that have terminated at the position of each A in the sequence.

A part of the final gel will look like this:



(Note that larger molecules migrate more slowly to the cathode on these gels)

The deduced DNA sequence obtained from this gel is: 5′ GGATCCTATC 3′

Polymerase Chain Reaction

Now let's consider how to obtain DNA segments that are suitable for sequencing. At first, DNA sequences were obtained from cloned DNA segments (we will discuss some methods to clone new genes in a subsequent lecture). Presently the entire DNA sequence for *E. coli*, as well as a variety of other bacterial species, has been determined. If we want to find the sequence of a new mutant allele of a known gene we need an easy way to obtain a quantity of this DNA from a culture of bacterial cells. The best way to do this is to use a method known as PCR or polymerase chain reaction that was developed by Kary Mullis in the mid-1980's. The steps in a PCR reaction are as follows.

(1) A crude preparation of chromosomal DNA is extracted from the bacterial strain of interest.

(2) Two short oligo nucleotide primers (each about 18 bases long) are added to the DNA. The primers are designed from the known genomic sequence to be complimentary to opposite strands of DNA and to flank the chromosomal segment of interest.

(3) The double stranded DNA is melted by heating to 100°C and then the mixture is cooled to allow the primers to anneal to the template DNA.

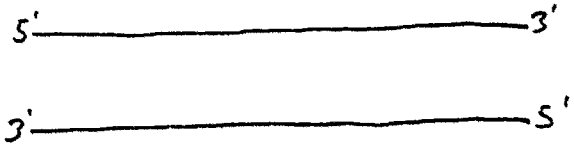
(4) DNA polymerase and the four nucleotide precursors are added and the reaction is incubated at 37°C for a period of time to allow a copy of the segment to be synthesized.

(5) Steps 3 and 4 are repeated multiple times. To avoid the inconvenience of having to add new DNA polymerase in each cycle a special DNA polymerase that can withstand heating to 100°C is used.

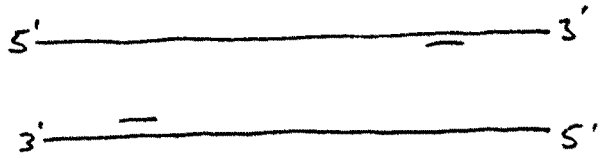
The idea is that in each cycle of melting, annealing and DNA synthesis the amount of the DNA segment is doubled. This gives an exponential increase in the amount of the specific DNA as the cycles proceed. After 10 cycles the DNA is amplified 10^3 fold and after 20 cycles the DNA will be amplified 10^6 fold. Usually amplification is continued until all of the nucleotide precursors are incorporated into synthesized DNA.

A PCR Reaction

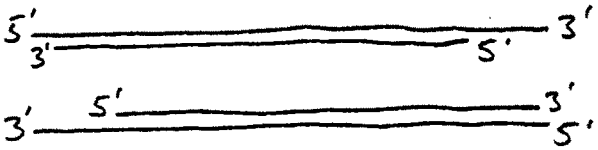
Separate DNA strands



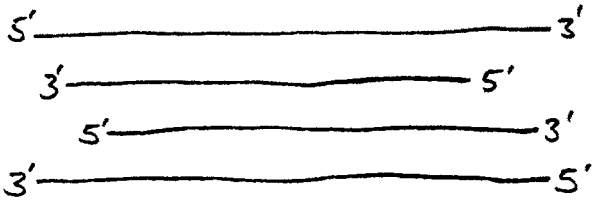
Anneal primers



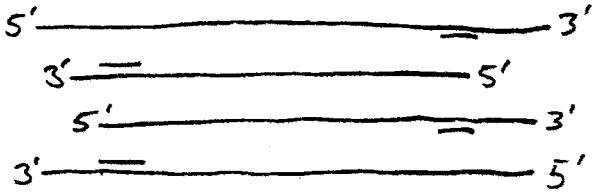
DNA synthesis



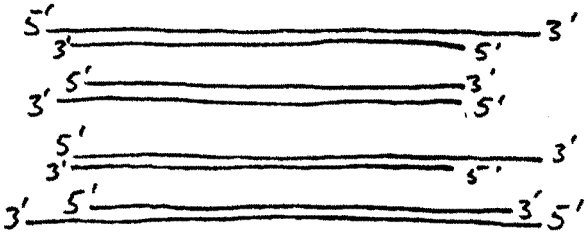
Separate DNA strands



Anneal primers



DNA synthesis



Repeat cycle 20 times