CHAPTER 23--DNA SEQUENCING

Simple and reliable DNA sequencing methods have allowed for a relatively rapid accumulation of gene sequence data. Two methods for sequencing DNA are the dideoxy chain termination method developed by Sanger and the chemical cleavage method developed by Maxim and Gilbert. The dideoxy procedure is the less laborious and the most widespread. The chemical cleavage procedure is primarily used to sequence synthetic oligonucleotides.

DIDEOXY CHAIN TERMINATION

The dideoxy chain termination method is based upon the use of 2',3'-dideoxynucleotides (figure) as chain terminators. These ddNTPs will be randomly incorporated into the growing polynucleotide chain if DNA synthesis is carried out in a mixture of dNTPs and ddNTPs. Dideoxynucleotides lack the 3'-OH and, therefore, additional nucleotides cannot be added during template mediated DNA synthesis. Since the incorporation of the ddNTPs is random the results of the DNA synthesis will be a mixture of newly synthesized DNA fragments of various lengths with a dideoxy-nucleotide at the 3'-end. This mixture is then analyzed by gel electrophoresis under conditions which will resolve DNA fragments that differ in length by single nucleotides. The sequence is then determined from the dideoxy-nucleotide which is located at the 3'-end of progressively longer oligonucleotides.

Dideoxy chain termination requires ssDNA as a template. The ssDNA can either be isolated from M13, phagemids, etc., or be prepared by denaturing dsDNA with NaOH. The sequencing is more efficient and less template DNA is needed if ssDNA is used. However, it is more laborious to prepare and isolate ssDNA from filamentous phage, and therefore, plasmid DNA denatured with NaOH is the most widely used template. Following denaturation in NaOH, the ssDNA is precipitated with ethanol and redissolved in an appropriate buffer.

Original Method. The original dideoxy sequencing method is based on radiolabeling the product strand. The first step is to incubate the template ssDNA with a primer under conditions which promote hybridization. Primers are oligonucleotides complementary to the DNA to be sequenced. Common sequencing vectors have well characterized primer sequences that flank the MCS. The annealed primer/DNA is mixed with DNA polymerase, α-35S-dATP, dGTP, dCTP and dTTP and subject to a short labeling reaction. The nucleotide concentrations used in this labeling reaction are relatively low so that DNA synthesis proceeds slowly. This results in the synthesis of a short segment (15-30 bases) of radioactive DNA.

The labeled DNA is then divided into four tubes containing higher concentrations of dNTPs and either ddATP, ddGTP, ddCTP or ddTTP. This step is called the termination reaction. The increase in the dNTP concentration result in an increased rate of DNA synthesis. The ddNTPs are at relatively low concentrations and are incorporated at random in the growing DNA molecule. When a ddNTP is incorporated into the growing DNA strand no additional nucleotides can be added resulting in a chain termination. Since ddNTP incorporation is random, the result will be a mixture of nucleotides terminated at all possible positions. The ratio of ddNTP/dNTP is crucial for the success of DNA sequencing. If the ddNTP is too high then the DNA synthesis will terminate prematurely and only sequence close to the primer will be determined. If the ddNTP concentration is too low then insufficient oligonucleotide chains of appropriate lengths will be produced.

The newly synthesized DNA is denatured by heating and subjected to gel electrophoresis under conditions which separate DNA molecules that differ in size by only a single base. The standard conditions are polyacrylamide gels (6-8%) containing urea. The urea will prevent H-bonding and minimizes the formation of dsDNA and secondary structures. The four termination reactions are electrophoresed side-by-side and DNA strands are detected by autoradiography. The smaller oligonucleotides representing termination events that occurred near the primer will be at the bottom of the gel. Starting at the bottom of the gel the DNA sequence can be read directly from the autoradiograph by noting the lane (i.e., ddNTP termination reaction) of each progressively larger band.

Semi-automated Sequencing. Pouring, running and analyzing sequencing gels are laborious procedures. Semi-automated DNA sequencers based on fluorescent dyes are now the preferred method of sequencing DNA. In addition, to reducing the amount of work involved in DNA sequencing, automated sequencing yields significantly more bases of readable sequence. The basis of automated sequencing is similar to that of manual sequencing except that product strand is labeled with fluorescent dyes during the extension reaction and then detected during electrophoresis. Therefore, no preliminary labeling reaction is needed.

Sequencing reactions are carried out with a thermocycler in a single tube containing the template DNA, a primer, a heat stable DNA polymerase (eg., Taq), all four dNTPs and all four ddNTPs. The four ddNTPs that are conjugated with different fluorochromes (i.e.,