Recombinant DNA and Genetic Analysis

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Abstract:
The considerable advances made in microarray, sequencing technologies and bioinformatics analysis are now beginning to provide true insights into the development and maintenance of cells and tissues. Indeed areas of analysis such as metabolomics, transcriptomics and systems biology are now well established and allow analysis of vast numbers of samples simultaneously. This type of large-scale parallel analysis is now the main driving force of biological discovery and analysis. However, the techniques of molecular biology and genetic analysis have their foundations in methods developed a number of decades ago. One of the main cornerstones on which molecular biology analysis was developed was the discovery of restriction end nucleases in the early 1970s which not only led to the possibility of analyzing DNA more effectively but also provided the ability to cut different DNA molecules so that they could later be joined together to create new recombinant DNA fragments. The newly created DNA molecules heralded a new era in the manipulation, analysis and exploitation of biological molecules. This process, termed gene cloning, has enabled numerous discoveries and insights into gene structure, function and regulation.

Keywords: *Ligating DNA molecules, Gene Libraries, cDNA Libraries, Treatment of Blunt cDNA ends.*

1. Introduction

The isolation and purification of genomic DNA it is possible to specifically fragment it with enzymes termed restriction end nucleases. These enzymes are the key to molecular cloning because of the specificity they have for particular DNA sequences. It is important to note that every copy of a given DNA molecule from a specific organism will give the same set of fragments when digested with a particular enzyme. DNA from different organisms will, in general, give different sets of fragments when treated with the same enzyme. By digesting complex genomic DNA from an organism it is possible to reproducibly divide its genome into a large number of small fragments, each approximately the size of a single gene. Some enzymes cut straight across the DNA to give flush or blunt ends. Other restriction enzymes make staggered single-strand cuts, producing short single-stranded projections at each end of the digested DNA. These ends are not only identical, but complementary, and will base-pair with each other; they are therefore known as cohesive or sticky ends.

2. Ligating DNA Molecules

The DNA products resulting from restriction digestion to form sticky ends may be joined to any other DNA fragments treated with the same restriction enzyme. Thus, when the two sets of fragments are mixed; base-pairing between sticky ends will result in the annealing together of fragments that were derived from different starting DNA.

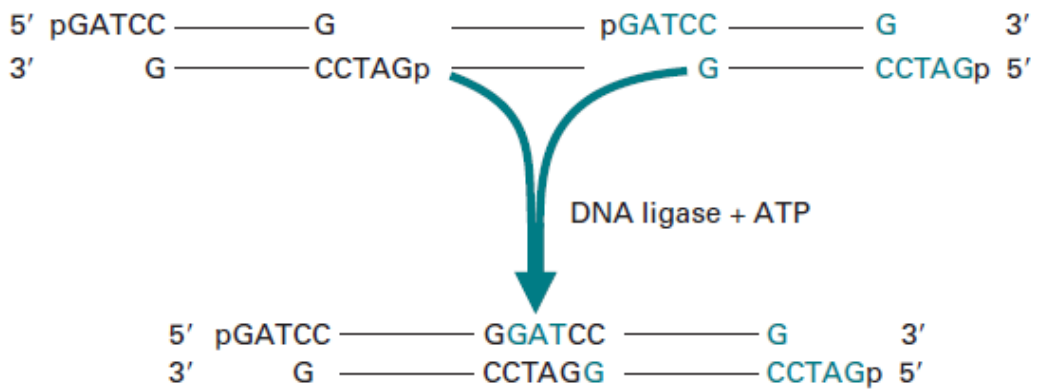

Figure: 1 Ligation molecules with cohesive ends

This enzyme, usually isolated from bacteriophage T4 and termed T4 DNA ligase, forms a covalent bond between the 50 phosphate at the end of one strand and the 30 hydroxyl of the adjacent strand (Fig.1). The reaction, which is ATP dependent, is often carried out at 10°C to lower the kinetic energy of molecules, and so reduce the chances of base-paired sticky ends parting before they have been stabilized by ligation. However, long reaction times are needed to compensate for the low activity of DNA ligase in the cold. It is also possible to join blunt ends of DNA molecules, although the efficiency of this reaction is much lower than sticky-ended ligations. Since ligation reconstructs the site of cleavage, recombinant molecules produced by ligation of sticky ends can be cleaved again at the 'joins', using the same restriction enzyme that was used to generate the fragments initially. In order to propagate digested DNA from an organism it is necessary to join or ligate that DNA with a specialized DNA carrier molecule termed a vector. Thus each DNA fragment is inserted by ligation into the vector DNA molecule, which allows the whole recombined DNA to then be replicated indefinitely within microbial cells (Fig. 2). In this way a DNA fragment can be cloned to provide sufficient material for further detailed analysis, or for further manipulation. Thus, all of the DNA extracted from an organism and digested with a restriction enzyme will result in a collection of clones. This collection of clones is known as a gene library.

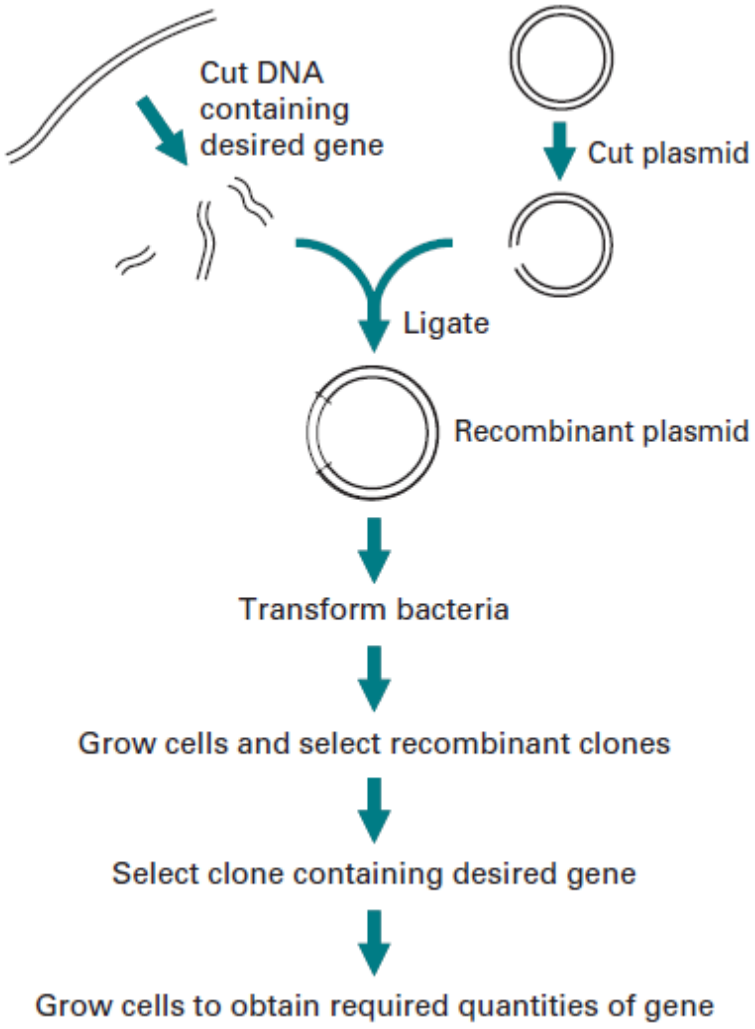

Figure: 2 Outline of gene cloning

3. Gene Libraries

There are two general types of gene library: a genomic library which consists of the total chromosomal DNA of an organism and a cDNA library which represents only the mRNA from a particular cell or tissue at a specific point in time (Fig.3). The choice of the particular type of gene library depends on a number of factors, the most important being the final application of any DNA fragment derived from the library. If the ultimate aim understands the control of protein production for a particular gene or the analysis of its architecture, then genomic libraries must be used. However, if the goal is the production of new or modified proteins, or the determination of the tissue-specific expression and timing patterns, cDNA libraries are more appropriate.

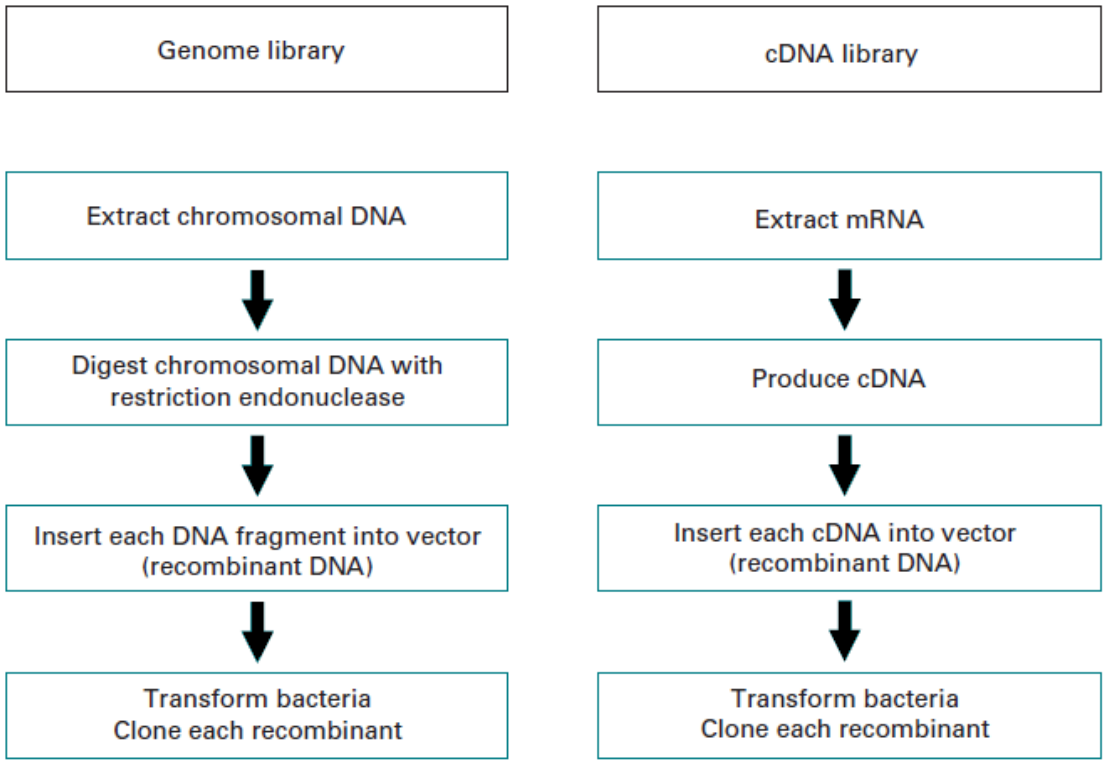

Figure: 3 Comparison of the general steps involved in the construction of genomic and complementary DNA (cDNA) libraries

There are a variety of cloning vectors available, many based on naturally occurring molecules such as bacterial plasmids or bacteria-infecting viruses. The choice of vector depends on whether a genomic library or cDNA library is constructed.

4. cDNA Libraries

There may be several thousand different proteins being produced in a cell at any one time, all of which have associated mRNA molecules. To identify any one of those mRNA molecules the clones of each individual mRNA have to be synthesized. Libraries that represent the mRNA in a particular cell or tissue are termed cDNA libraries. mRNA cannot be used directly in cloning since it is too unstable. However it is possible to synthesize complementary DNA molecules (cDNAs) to all the mRNAs from the selected tissue. The cDNA may be inserted into vectors and then cloned. The production of cDNA (complementary DNA) is carried out using an enzyme termed reverse transcriptase which is isolated from RNA-containing retroviruses. Reverse transcriptase is an RNA-dependent DNA polymerase, and will synthesize a first-strand DNA complementary to an mRNA template, using a mixture of the four dNTPs. There is also a requirement (as with all polymerase enzymes) for a short oligonucleotide primer to be present (Fig. 4). With eukaryotic mRNA bearing a poly (A) tail, a complementary oligo (dT) primer may be used. Alternatively random hexamers may be used which randomly anneal to the mRNAs in the complex.

Figure: 4 Strategies for producing first-strand cDNA from mRNA

5. Treatment of Blunt $_C$DNA Ends

Ligation of blunt-ended DNA fragments is not as efficient as ligation of sticky ends, therefore with cDNA molecules additional procedures are undertaken before ligation with cloning vectors. One approach is to add small double-stranded molecules with one internal site for a restriction end nuclease, termed nucleic acid linkers, to the cDNA. Numerous linkers are commercially available with internal restriction sites for many of the most commonly used restriction enzymes. Linkers are blunt-end ligated to the cDNA but since they are added much in excess of the cDNA the ligation process is reasonably successful. Subsequently the linkers are digested with the appropriate restriction enzyme which provides the sticky ends for efficient ligation to a vector digested with the same enzyme. This process may be made easier by the addition of adaptors rather than linkers which are identical except that the sticky ends are preformed and so there is no need for restriction digestion following ligation (Fig. 5).

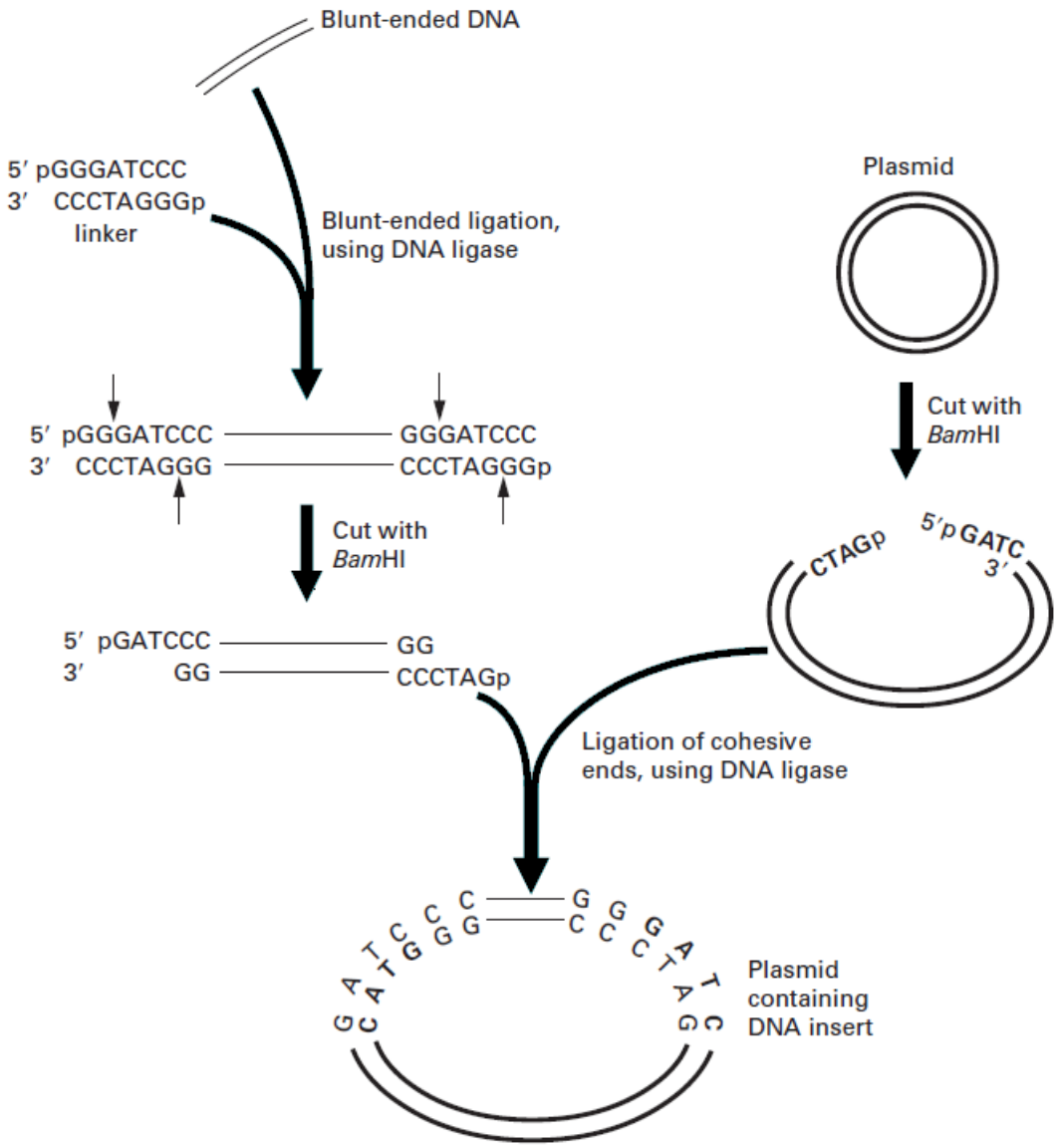

Figure: 5 in this example, blunt-ended DNA is inserted into a specific restriction site on a plasmid, after ligation to a linker containing the same restriction site

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