Gene cloning

Methods of preparing large amounts of a gene of interest

• There are several methods of gene cloning, but all involve making recombinant DNA (A piece of DNA constructed by joining two pieces of DNA from different sources, usually different species)

Genomic library

A collection of DNA pieces from a species' chromosomes with each piece of DNA cloned into a cloning vector

• The genomic library contains the entire genome (all of the genes) of the species

Preparing a genomic library (a library that contains the entire genome)

(a) The chromosomal DNA is purified by breaking open cells and removing all lipids, carbohydrates, proteins, and other non-DNA molecules

(b) The chromosomes are digested with a restriction enzyme

- The restriction enzyme cuts each chromosome into many smaller fragments with sticky ends

(c) The genomic fragments are mixed with a cloning vector that has been cut with the same restriction enzyme

(d) DNA ligase enzyme is added to join each genomic DNA fragment to the cloning vector DNA

Fig 20.4

Restriction enzyme

An enzyme that cuts a piece of DNA at all sites that contain a specific sequence

- Restriction site = The DNA sequence that the enzyme recognizes and cuts

- There are many different restriction enzymes, each with a different restriction site

- Most restriction enzymes, when they cut DNA, produce sticky ends (short regions of single stranded DNA with complementary sequences)

- Example: The restriction enzyme *Eco RI* cuts DNA as shown below:

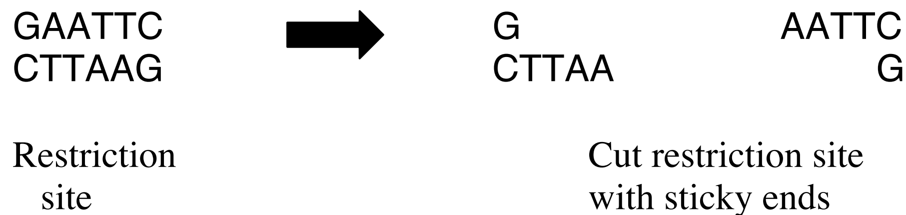

Fig 20.3

DNA ligase enzyme

An enzyme that will ligate (join together) two pieces of DNA into one single piece

- If the pieces have sticky ends, DNA ligase works much more efficiently

Reverse transcriptase

An enzyme that makes a complementary DNA (cDNA) to any RNA molecule

Cloning vector

A piece of DNA that can be used to make multiple copies of a gene of interest

- The gene is ligated to the cloning vector, then the recombinant vector is introduced (put inside) bacterial cells where it replicates

- Plasmids are the most common type of cloning vector

Plasmid

A small circular DNA piece that is found in bacteria

Fig 20.6

Probe

A small piece of DNA or RNA (usually radioactive) that is complementary to a gene of interest

- If mixed with several pieces of DNA, the probe will hybridize (bind) to only to the gene of interest

- The radioactivity will expose photographic film, which can be used to visualize exactly where the probe has bound

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(2) Screening the library (finding the cloned gene of interest)

Screening the library requires the researcher to have a probe for the gene

(a) The recombinant plasmids are introduced into bacterial cells

(b) The bacteria are spread on a plate containing solid growth media

(c) Each bacteria grows into a colony (a visible round clump containing millions of bacteria)

- All the cells in any one colony contain the same recombinant plasmid (because they all grew from a single cell) but each colony contains a different plasmid from the other colonies

(d) A filter (a piece of paper or nylon) is laid on top of the colonies

- Some of the plasmid DNA in each colony attaches to the filter

(e) The probe is hybridized to the DNA on the filter

(f) The filter is exposed to photographic film to visualize the location of the radioactive probe

- The probe will only hybridize to the location on the filter that contains the plasmid with the gene of interest

(g) By aligning the film to the colonies on the plate, the researcher can identify the colony that contains the gene of interest

- Unlimited amounts of the gene can be made by growing the bacteria in the colony

Fig 20.5

Polymerase Chain Reaction (PCR)

A method of rapidly amplifying (making millions of copies of) a gene of interest

The genomic DNA is mixed with…

- Primers (short pieces of DNA) complementary to the ends of the gene

- TAQ DNA polymerase (a heat-resistant DNA polymerase)

Step 1 of cycle: Denaturing the DNA:

- The DNA is heated to boiling temperature
- The heat denatures (separates the two complementary strands) of the genomic DNA.

Step 2 of cycle: Annealing the primers:

- The temperature is lowered slightly
- The lower temperature allows the primers to anneal (bind by complementary base pairing) to the ends of the gene

Step 3 of cycle: Extension of primers to form complimentary strands

- TAQ DNA Polymerase enzyme extends each primer (makes a complementary strand), which doubles the copies of the gene

Steps 1 – 3 are one "cycle". These three steps are repeated over and over. Each cycle doubles the number of copies of the gene.

√ After n cycles, 2^n copies of the gene have been made.
For example, after 20 cycles, over a million copies the gene have been made.

Advantages of PCR

- Rapid (hours instead of days needed for traditional cloning)
- Can begin with small amounts of DNA/cells

Disadvantages of PCR

- It can't amplify long DNA segments well
- TAQ DNA Polymerase has a higher error rate than other DNA polymerase enzymes

DNA sequencing

Methods of determining the sequence of nucleotides (the order of the G, A, T, C) in a piece of DNA

• There are many different methods of "Next generation" DNA sequencing. Some methods can sequence an entire genome is a few days.

Fig 20.12

Agarose gel electrophoresis

A method of separating a mixture of DNA fragments by size

- The fragments are placed in an agarose gel
- Electricity is applied to the gel
- The nucleic acids migrate through the gel toward the positive electrode (because of their negative phosphate groups in the DNA backbone)
  - √ The smaller pieces migrate faster, which spreads the mixture out by size

Fig 20.8

Identification of a person by DNA fingerprinting

- There are several loci in the human genome that contain a form of repetitive DNA called short tandem repeats (STRs).
  - √ Example: The four nucleotide sequence AGAT is repeated many times in the human genome

- For many of these STR loci, the number of repeats is highly variable. These regions are called VNTRs (Variable Number of Tandem Repeats)
  - √ Example: At a certain VNTR locus in chromosome 3, there can be 2 – 5 repeats of the sequence AGAT

  - √ Each of the different numbers of repeats is a different allele of the VNTR locus.

    - In the above example, there are 4 different alleles of the locus: The 2-repeat allele, the 3-repeat allele, the 4-repeat allele, and the 5-repeat allele.

- Each person's genotype at a VNTR locus are the two alleles they have at that locus.
  - √ Example: Person A has the 2-repeat allele and the 3-repeat allele at a certain VNTR locus. Person B has two of the 5-repeat allele at a certain VNTR locus.

- The alleles a person has at any VNTR locus can be visualized by amplifying that VNTR locus using PCR followed by agarose gel electrophoresis
  - √ The sizes of the amplified bands indicate how many repeats the person has in each of their alleles.

Calculations relating to VNTR loci

(a) The number of possible genotypes (the number of 2 allele combinations) at a VNTR locus = N x (N +1)/2

(N = the total number of possible alleles at the locus)

- Example: At a certain VNTR locus there can be 2-5 repeats of a sequence. Therefore there are 4 alleles at this VNTR locus.

The number of possible genotypes at this VNTR locus is 10.

(b) The portion of the population that has a random match (the same genotype) as the genotype of a VNTR locus from a DNA sample is roughly equal to 1/number of genotypes at that locus.

- Example: The DNA from a crime scene has a certain genotype at VNTR locus A. There are 10 possible genotypes at this VNTR locus. Therefore roughly 1 out of 10 people will match the crime scene DNA at this locus.

(c) The portion of the population that has a random match (the same genotype) for more than one VNTR locus from a DNA sample is calculated by multiplying together the 1/number of genotypes for each locus.

- Example: The DNA from a crime scene has a certain genotype at a VNTR locus A, and there are 10 possible genotypes at VNTR locus A. The crime scene DNA also has a certain genotype at VNTR locus B, and there are 6 possible genotypes at VNTR locus B. Therefore roughly 1 out of 60 people will match the crime scene DNA at this locus.

- The FBI uses 13 VNTR loci (the "CODIS 13") to create a DNA fingerprint of any DNA sample or suspect

- Fewer than 1 out of a 100 million people match all 13 loci by random match

CAS-9

A bacterial enzyme that cuts DNA at any location where a specific sequence occurs in the DNA

• Guide RNA = An RNA strand that CAS-9 can bind to

√ CAS-9 cuts DNA at sequences that are complementary to the guide RNA sequence

Repair of DNA cut by CAS-9

(a) Non-homologous end joining (NHEJ) = When enzymes in the nucleus rejoin the cut DNA by inserting a random DNA sequence into the gap in the DNA

• NHEJ tends to inactivate ("knock out") a gene because it introduces a random sequence change into the gene's original sequence

(b) Homologous repair of ends (HRE) = When enzymes in the nucleus rejoin the cut DNA by inserting a piece of DNA into the gap in the DNA

• The inserted DNA piece can be a DNA piece created in a laboratory that has a chosen sequence

• Therefore HRE can be used to repair the sequence of a mutated gene or to give a gene a new function.

In trial studies, CAS-9/CRISPR has been used to cure previously uncurable genetic diseases

• A woman with sickle cell anemia was cured of the disease by using CAS-9/CRISPR to change sequences in her hemoglobin gene cluster