XXII – DNA cloning and sequencing

Outline

1) Deriving DNA for cloning

2) Vectors; forming recombinant DNA; cloning DNA; and screening for clones containing recombinant DNA [replica plating and autoradiography; lac Z gene (in vivo)]

3) Characterizing and sequencing DNA
   a) Southern Blotting
   b) Restriction mapping using $^{32}$P marker
   c) Chain termination sequencing using ddNTPs labeled with $^{32}$P
   d) Chain termination sequencing using ddNTPs labeled with fluorescent dyes
I. Purposes of DNA cloning:

   a) Artificial (in vitro) production of commercially important gene products.

   b) Increasing amounts of DNA that can be used for determining the nucleotide sequence of the gene of interest. Ex., PCR (polymerase chain reaction)

II. Two means by which DNA is derived for cloning:

   a) Use DNA of known character:

      purified mRNA→single stranded cDNA (reverse transcriptase)

      cDNA→ double stranded DNA (DNA polymerase I). Formation of cDNA library.

   b) Shot-gun approach:

      Use one of a variety of restriction endonucleases (ex. EcoRI) on a genome to generate specific DNA fragments.
Poly-A tail

3' AAAAA 5'

mRNA

Add poly-dT primer

AAAAA

TTTTT

Add reverse transcriptase

AAAAA

TTTTT

5' Double-stranded duplex 3' DNA synthesis continues

AAAAA

TTTTT

Digest RNA

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Digest RNA

Single-stranded cDNA

Add DNA polymerase I

Add $S_1$ nuclease to cleave hairpin loop

Double-stranded cDNA
III. Gene insertion and use of host cells for cloning:

a) A heterogenous group of DNA segments inserted into a plasmid (vector; ex. pUC18) using EcoRI (makes sticky ends) and DNA ligase. A vector joined to a desired DNA fragment is a recombinant DNA molecule. Plasmid and DNA fragment must be exposed to the same restriction enzyme.

b) Recombinant DNA molecule transferred to a host cell (bacterium). Host cell is heat shocked prior to transfer. Host cells produce many copies or clones (up to 500) of the recombinant DNA molecule during replication.

IV. Two screening processes:

a) Recombinants (with foreign gene of interest) formed from these are screened by replica plating and autoradiography ($^{32}$P - cDNA).

b) Bacteria containing recombinant molecules can be screened. DNA segment inserts disrupt lac Z gene in pUC18 causing blue to white color change of colonies grown on Xgal media. Other bacteria can also be excluded by use of ampicillin in media, as the pUC18 vector contains an ampicillin-resistance gene.
Host cell chromosome

Plasmid vector is removed from bacterial cell and cut with a restriction enzyme

The two DNAs are ligated to form a recombinant molecule

DNA to be cloned is cut with the same restriction enzyme

Introduction into host cell

Selection for cells carrying recombinant plasmids by plating on medium with antibiotic or color indicator
Phage λ

DNA molecule

Central gene cluster

Central region is removed by restriction-enzyme digestion

Phage λ arms

Insertion and ligation of foreign DNA

in vitro Packaging

A recombinant viral particle able to infect a bacterial host cell carries a cloned foreign insert and replicates to form plaques

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Recognition site

G-A-A-T-T-C

C-T-T-A-A-G

Treatment with EcoRI

Complementary tails

G

C-T-T-A-A

A-A-T-T-C

G
Cleavage with *EcoRI*

Fragments with complementary tails

Annealing allows recombinant DNA molecules to form by complementary base pairing. The two strands are not covalently bonded as indicated by shaded gaps.

DNA ligase

DNA ligase seals the gaps, covalently bonding the two strands.
V. Cloning without host cells:
   i) The PCR Reaction:

   **Denaturation:**
   1) The DNA to be amplified is denatured into single strands. This involves heating at 90 - 95°C.

   **Cycle (4-5 min.)**
   **Annealing:**
   2) Temperature is lowered to between 50 and 70°C. This allows DNA primers to bind to denatured (single strands of) DNA. DNA primers (15 - 30 nucleotides long; synthetic oligonucleotides) bind to sequences that flank the target segment.

   **Extension:**
   3) Heat-stable DNA Pol (Taq) synthesizes daughter strands complementary to target sequences from primers at 70 to 75°C. Taq adds nucleotides 5' → 3'.

   ii) Doubling of number of DNA strands after each full cycle. 25-30 cycles (≈ 3 hours) increases DNA by $1 \times 10^6$.

   iii) The amplified DNA is recovered, exposed to a variety of restriction endonucleases for multiple heterogenous cleaving, and its sequence analyzed by agarose or polyacrylamide gel electrophoresis (Southern Blotting).
Cycle 1

DNA to be amplified

Step 1
Denature DNA

Step 2
Anneal primers
Step 1 and 2
Denature and anneal new primers

Cycle 2

Step 3
Extend primers
(Product of first cycle is two new DNA molecules)

Step 2
Anneal primers
Cycle 2

Step 1 and 2
Denature and anneal new primers

Step 3
Extend primers

(Product of second cycle is four new DNA molecules)

25 cycles increase DNA copies by $>10^6$
VI. The cloned DNA is recovered (extracted) from host cells, purified, exposed to a variety of restriction endonucleases for multiple heterogenous cleaving, and its sequence analyzed by agarose or polyacrylamide gel electrophoresis (Southern Blotting).

VII. Cloned DNA can also be transcribed, its mRNA translated, and the gene product isolated and used for research, or sold commercially.
Gel preparation and DNA fragment separation in horizontal electrophoresis.
Demonstration of the inverse relationship between DNA fragment size and migration distance toward the positive electrode in an electrophoretic gel.
Electrophoresis is a very useful tool to determine the sequence of DNA.

Materials used in restriction sequencing of DNA:

1. Single strand of DNA
2. Label the DNA at the 5' end
3. Divide the DNA into 4 separate tubes
4. Each one of the 4 tubes will be treated with either A, G, C, or T reagent to generate short fragments to sequence the DNA.

Steps in sequencing DNA:

1. Preparation of homogenous single strand of DNA
   
   5' ATTGACTTAGCC 3'

2. Label the 5' end with $^{32}$P

3. * ATTGACTTAGCC

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**ATTGACTTTAGCC**

G reaction
- ATTGACTTTAGCC
- ATTGACTTA
- ATT

A reaction
- ATTGACTTTAGCC
- ATTGACTT
- ATTG

T reaction
- ATTGACTTTAGCC
- ATTGACT
- AT
- A

C reaction
- ATTGACTTTAGCC
- ATTGACTTAGC
- ATTGACTTAG
- ATTGA
A DNA sequencing gel is given below. Starting at the 5' end of the gel, read off the DNA sequence. Using your genetic code table, indicate the amino acid sequence of the DNA segment.
Basic steps involved in the Southern Blot test are performed in the following order:

a) Digestion with restriction endonuclease enzymes
b) Electrophoresis
c) Use of radioactive probe
d) Autoradiography (x-ray film)
1. DNA samples cut with restriction enzymes are loaded on agarose gel for electrophoresis.

Lane 1: Radioactive size markers
Lane 2: DNA cut with restriction enzyme A
Lane 3: DNA with restriction enzyme B

2. DNA is separated by electrophoresis but invisible to the naked eye.

3. DNA-binding filter, paper towels and weight are placed on gel; buffer passes upward through sponge by capillary action transferring DNA fragments to filter.
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4. Filter is placed in heat-sealed bag with solution containing labeled probe.
4. Filter is placed in heat-sealed bag with solution containing labeled probe.

5. Filter is washed to remove unbound probe, dried; film is applied for autoradiography.

 Autoradiogram; all size markers show because they are radioactive; in lanes 2 and 3, only those bands that hybridize with probe show up.
Southern Blotting technique: A. DNA fragment separation by electrophoresis, B. Transfer of DNA fragments onto nitrocellulose filter, C. Hybridization of labeled probe with single-stranded DNA fragments, and D. Visualization of labeled double-stranded hybrid DNA on filter after washing.
1. Primer is bound to template strand

```
5' Primer 3'
GATCTGTGTA C
3' Template strand 5'
```

2. Reaction ingredients added

- DNA polymerase
- dATP
- dCTP
- dGTP
- dTTP

A small amount of ddATP dideoxynucleotide

3. Primer extension

```
CTA GATCTGTGTA C
```

4. Newly synthesized strands recovered, loaded on gel in "A" lane

```
CTAGA CTA
```

```
CTAGA CTA
```

```
CTAGACA CTA
```

Chain termination when a ddATP is inserted
1. Primer is bound to template strand

```
5' Primer 3'
```

```
G A T C T G T A C
```

3' 5'

Template strand

2. Reaction ingredients added

- DNA polymerase
- dATP
- dCTP
- dGTP
- dTTP
- small amount of ddATP dideoxynucleotide
3. Primer extension

Chain termination when a ddNTP is inserted

4. Newly synthesized strands recovered, loaded on gel in "A" lane
CHAIN TERMINATION SEQUENCING USING UNLABELED ddNTPs

Electrophoretic gel with four separate adjacent lanes, each corresponding to the four separate tubes containing their respective ddNTP - 1) ddGTP, 2) ddATP, 3) ddCTP, and 4) ddTTP

Sequence of the synthesized strand is: 5' CGTACTAGCC 3'
Polyacrylamide gel electrophoresis and autoradiography of the gel:

Sequence of nascent strand

Sequence of complementary template strand
CHAIN TERMINATION ELECTROPHORETIC GEL

Actual electrophoretic gel generated by chain termination sequencing showing the separation of DNA fragments in the four ddNTP (unlabeled) chain termination lanes. To obtain the $5' \rightarrow 3'$ base sequence of the DNA fragment, the gel is read from the bottom to the top, beginning with the lowest band in any lane. For example, the sequence of the DNA on this gel begins with CAGTCCGC.
1. Primer added
   5' Primer 3'

2. Reaction ingredients added
   DNA polymerase
dATP
dCTP
dGTP
dTTP
   small amount of ddNTPs with fluorochromes:
   ddATP —
ddCTP —
ddGTP —
ddTTP —

3. Primer extension
   Chain termination
   Product recovery

4. Electrophoresis, imaging, data analysis
1. Primer added

5' Primer 3'

GATCTGTAC

2. Reaction ingredients added

- DNA polymerase
- dATP
- dCTP
- dGTP
- dTTP
- Small amount of ddNTPs with fluorochromes:
  - ddATP
  - ddCTP
  - ddGTP
  - ddTTP
3. Primer extension
   Chain termination
   Product recovery

4. Electrophoresis, imaging, data analysis
Automated DNA sequencing using fluorescent dyes, one for each base. The separated bases are read in order along the axis from left to right.