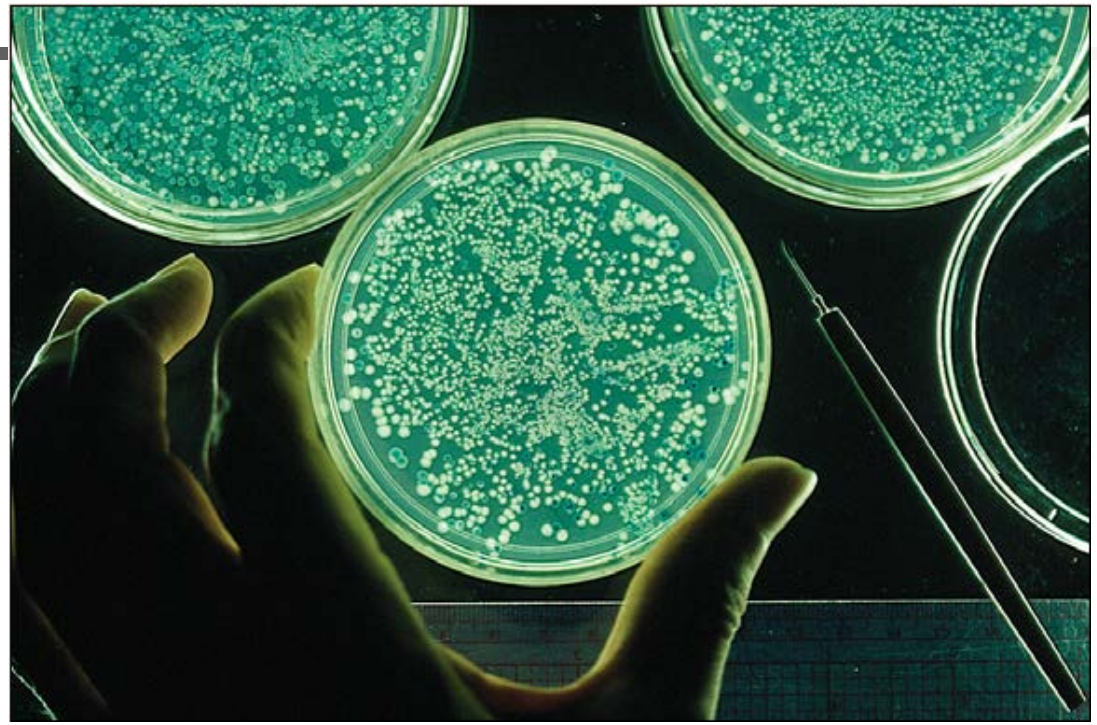
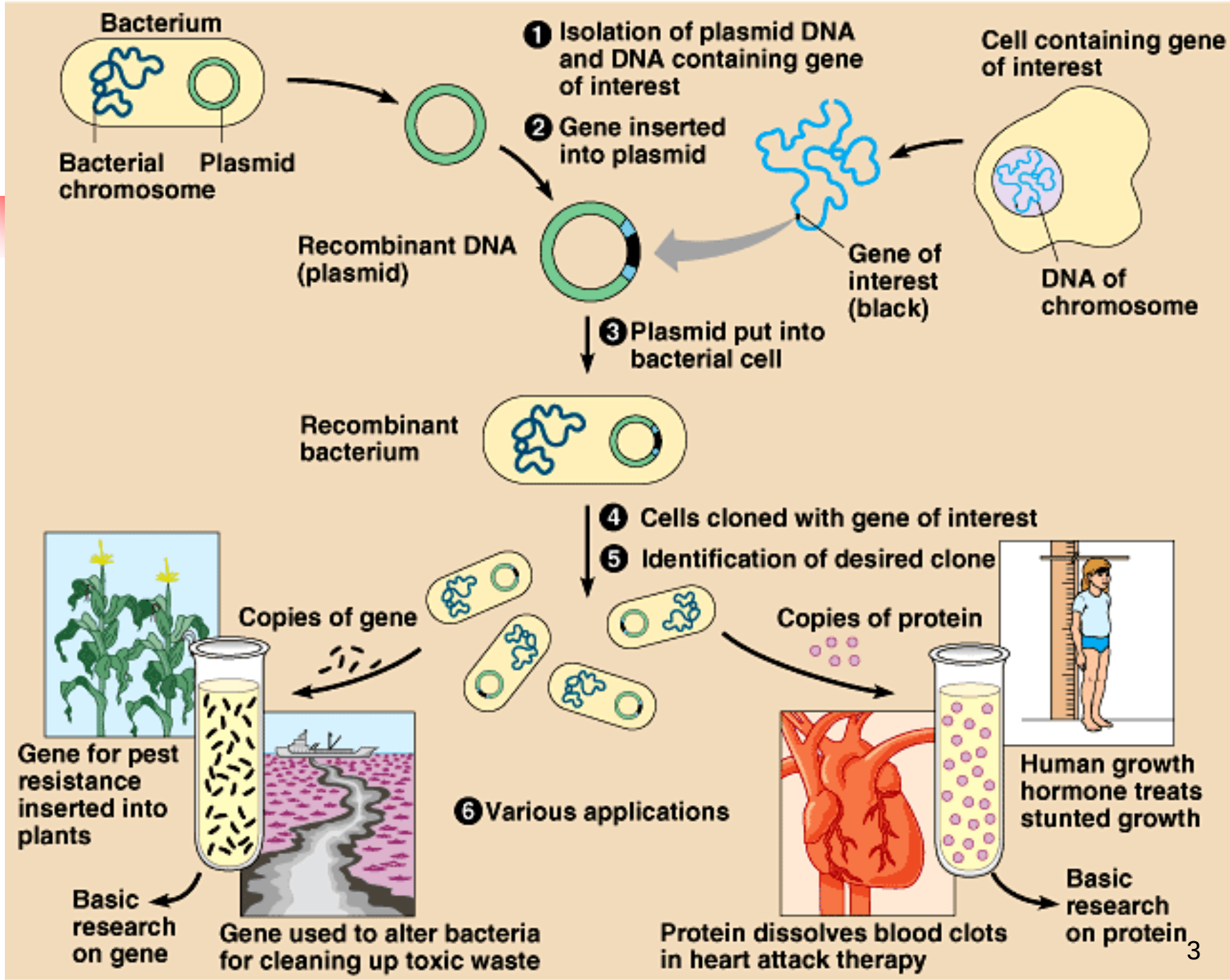
第七章　基 因 工 程
Gene Engineering



General process of gene engineering


Bacterium
Bacterial chromosome
Plasmid
1 Isolation of plasmid DNA and DNA containing gene of interest
2 Gene inserted into plasmid
Cell containing gene of interest
Recombinant DNA (plasmid)
Gene of interest (black)
DNA of chromosome
3 Plasmid put into bacterial cell
Recombinant bacterium
4 Cells cloned with gene of interest
5 Identification of desired clone
Copies of gene
Copies of protein
Gene for pest resistance inserted into plants
Human growth hormone treats stunted growth
6 Various applications
Basic research on gene
Gene used to alter bacteria for cleaning up toxic waste
Protein dissolves blood clots in heart attack therapy
Basic research on protein

第一节　重组DNA分子的构建

Construction of recombinant DNA molecules

I. Fragmenting Complex Genomes into Bite-Size Pieces for Analysis

Restriction Enzymes Fragment the Genome at Specific Sites

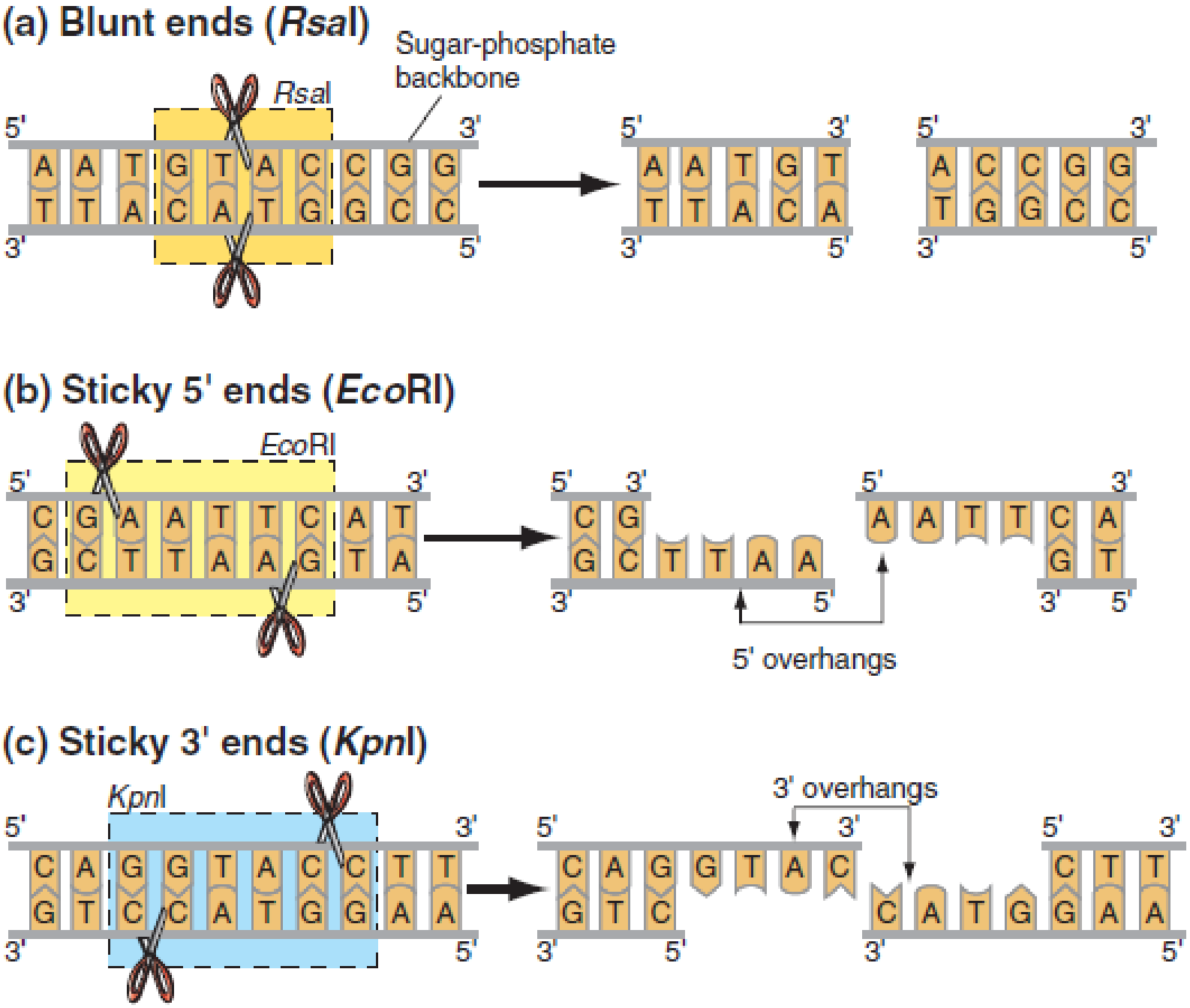
Restriction Enzymes

An endonuclease（核酸内切酶） that recognizes specific nucleotide sequences in DNA and breaks the DNA chain at that sites

Specific nucleotide sequences

Palindrome
回文结构

Enzyme	Sequence of Recognition Site	Microbial Origin
*Taq*I		*Thermus aquaticus* YTI
*Rsa*I		*Rhodopseudomonas sphaeroides*
*Sau*3AI		*Staphylococcus aureus* 3A
*Eco*RI		*Escherichia coli*
*Bam*HI		*Bacillus amyloliquefaciens* H.
*Hind*III		*Haemophilus influenzae*
*Kpn*I		*Klebsiella pneumoniae* OK8
*Cla*I		*Caryophanon latum*
*Bss*HII		*Bacillus stearothermophilus*
*Not*I		*Nocardia otitidiscaviarum*


(a) Blunt ends (RsaI)
Sugar-phosphate backbone
RsaI
5'
3'
A A T G T A C C G G
T T A C A T G G C C
(b) Sticky 5' ends (EcoRI)
EcoRI
C G A A T T C A T
G C T T A A G T A
5' overhangs
(c) Sticky 3' ends (KpnI)
KpnI
3' overhangs
C A G G T A C C T T
G T C C A T G G A A

The Size of the Recognition Site Is the Primary Determinant of Fragment Length

(a) Calculating Average Restriction Fragment Size

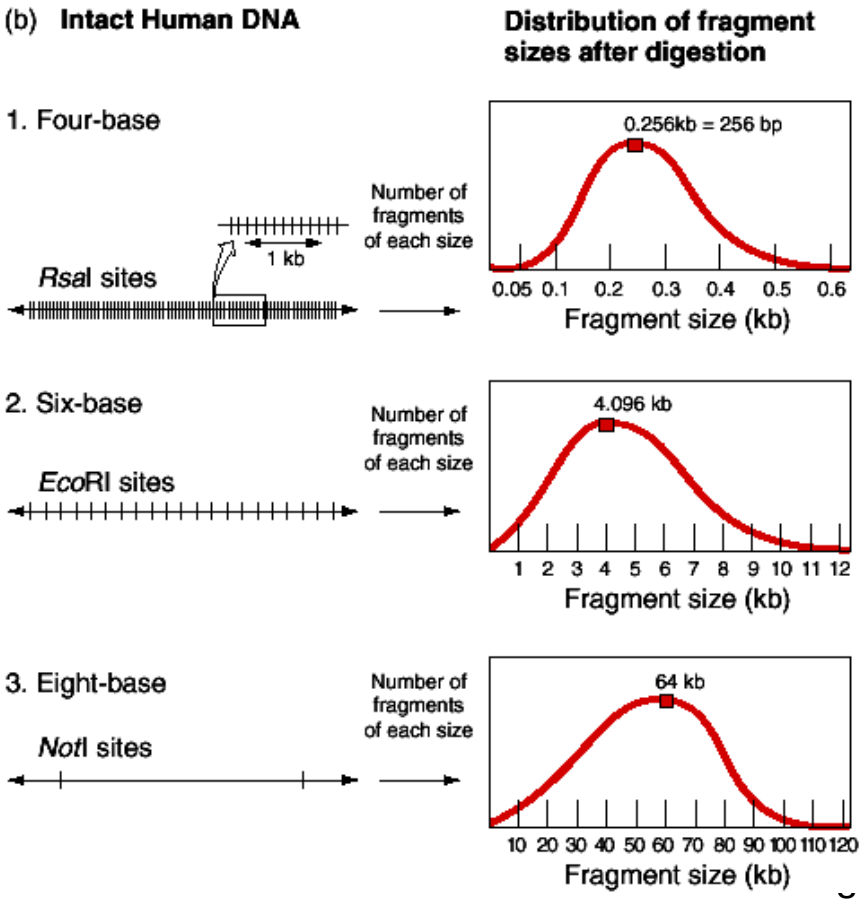
1. Probability that a four-base recognition site will be found in a genome =

$$1/4 \times 1/4 \times 1/4 \times 1/4 = 1/256$$

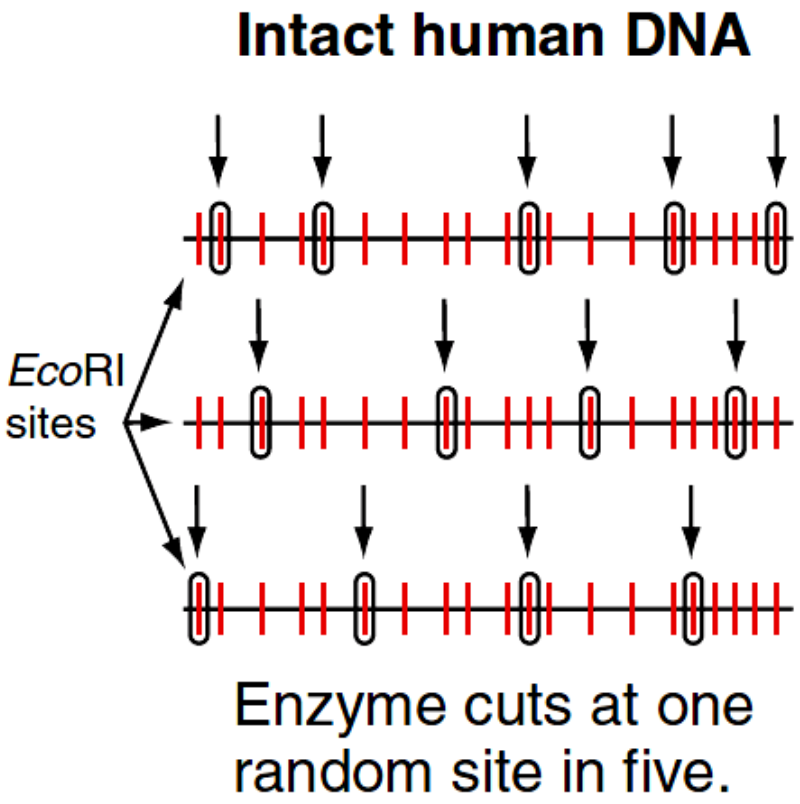
2. Probability that a six-base recognition site will be found =

$$1/4 \times 1/4 \times 1/4 \times 1/4 \times 1/4 \times 1/4 = 1/4096$$

Different Restriction Enzymes Produce Different Lengths of Fragments

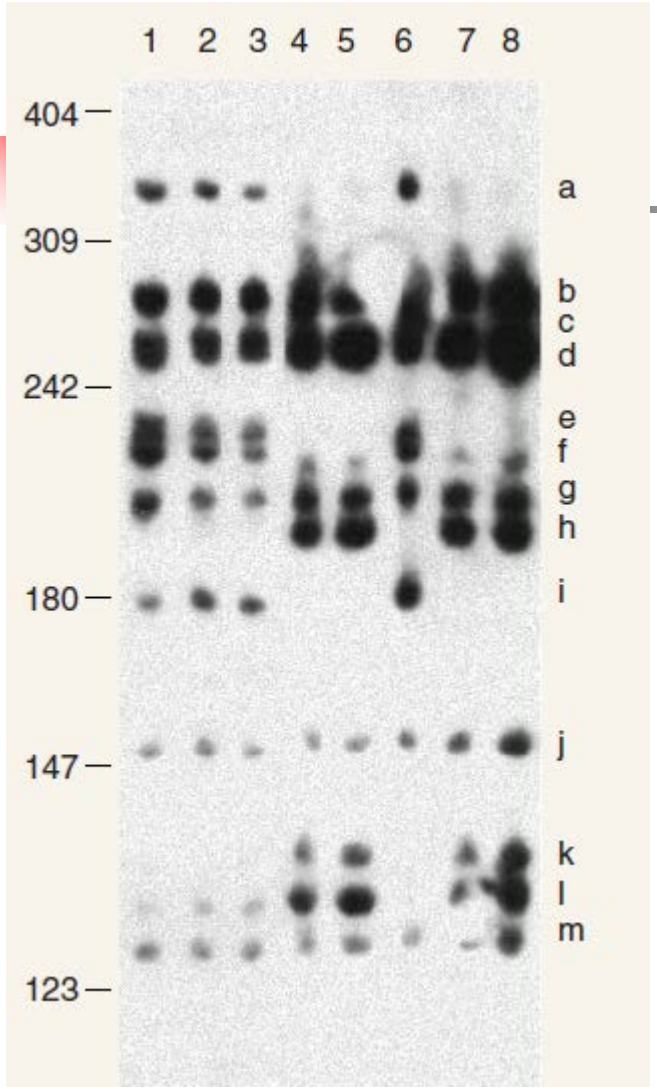


The Timing of Exposure to a Restriction Enzyme Helps Determine Fragment Size

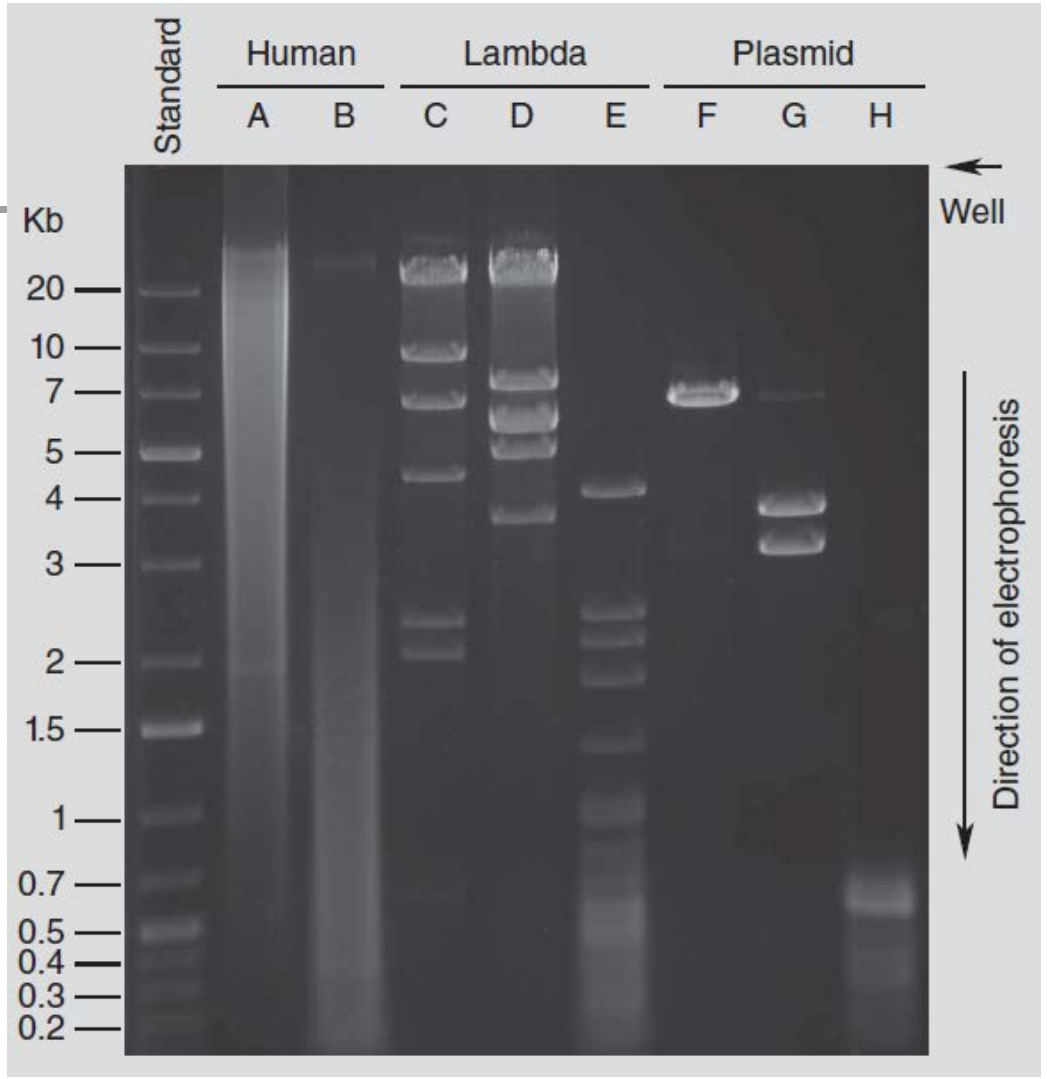


Control the amount of enzyme or the amount of time the DNA is exposed to the restriction enzyme.

Gel Electrophoresis Distinguishes DNA Fragments According to Size



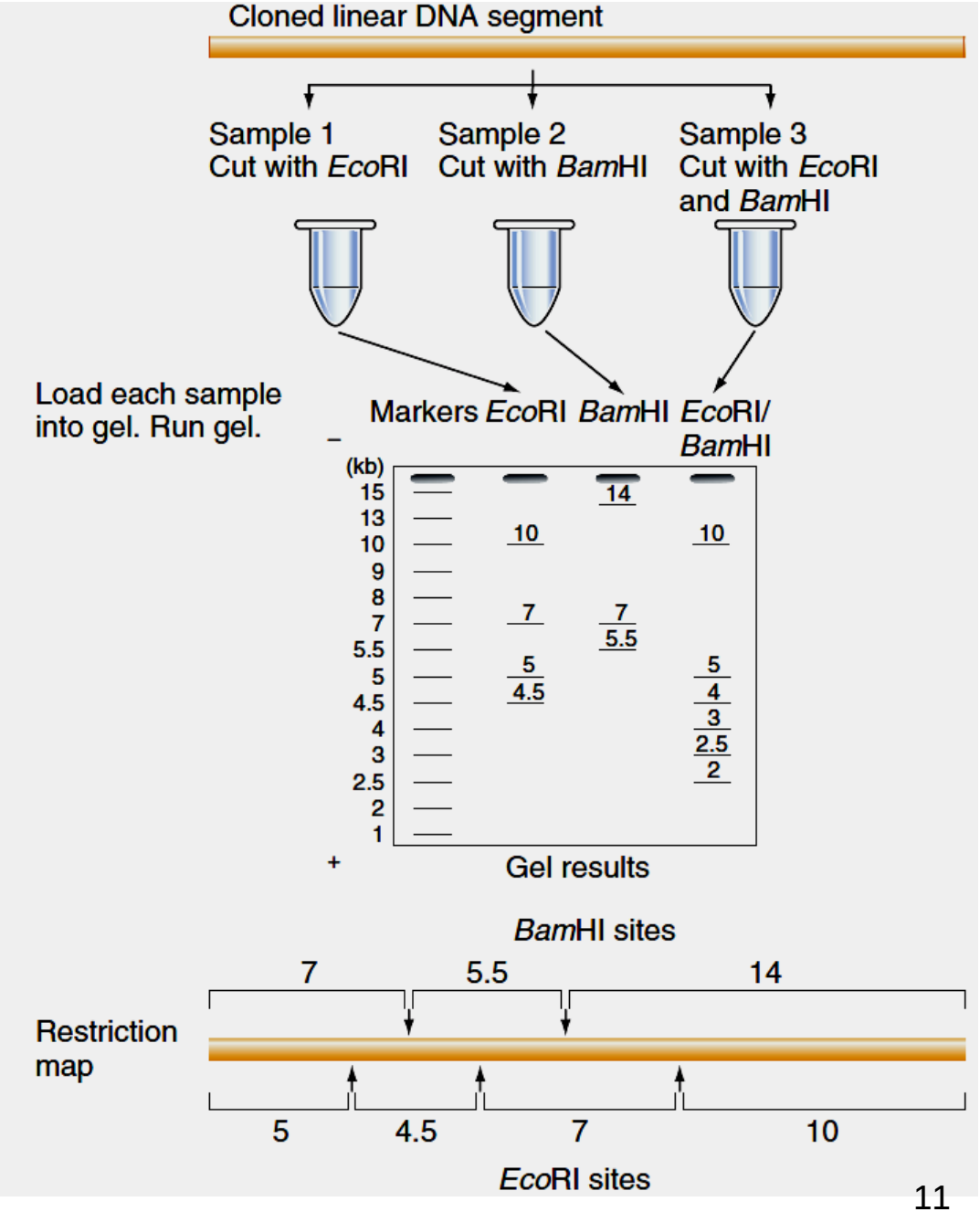
PAGE

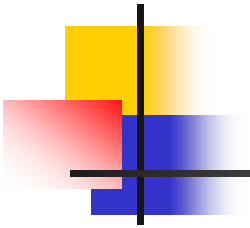


Agarose

Different types of gels separate different-sized DNA molecules

Restriction Maps Provide a Rough Roadmap of DNA Fragment





II. Generating Recombinant DNA Molecules

Cloning Step 1: Ligation of Fragments to Cloning Vectors Creates Recombinant DNA Molecules

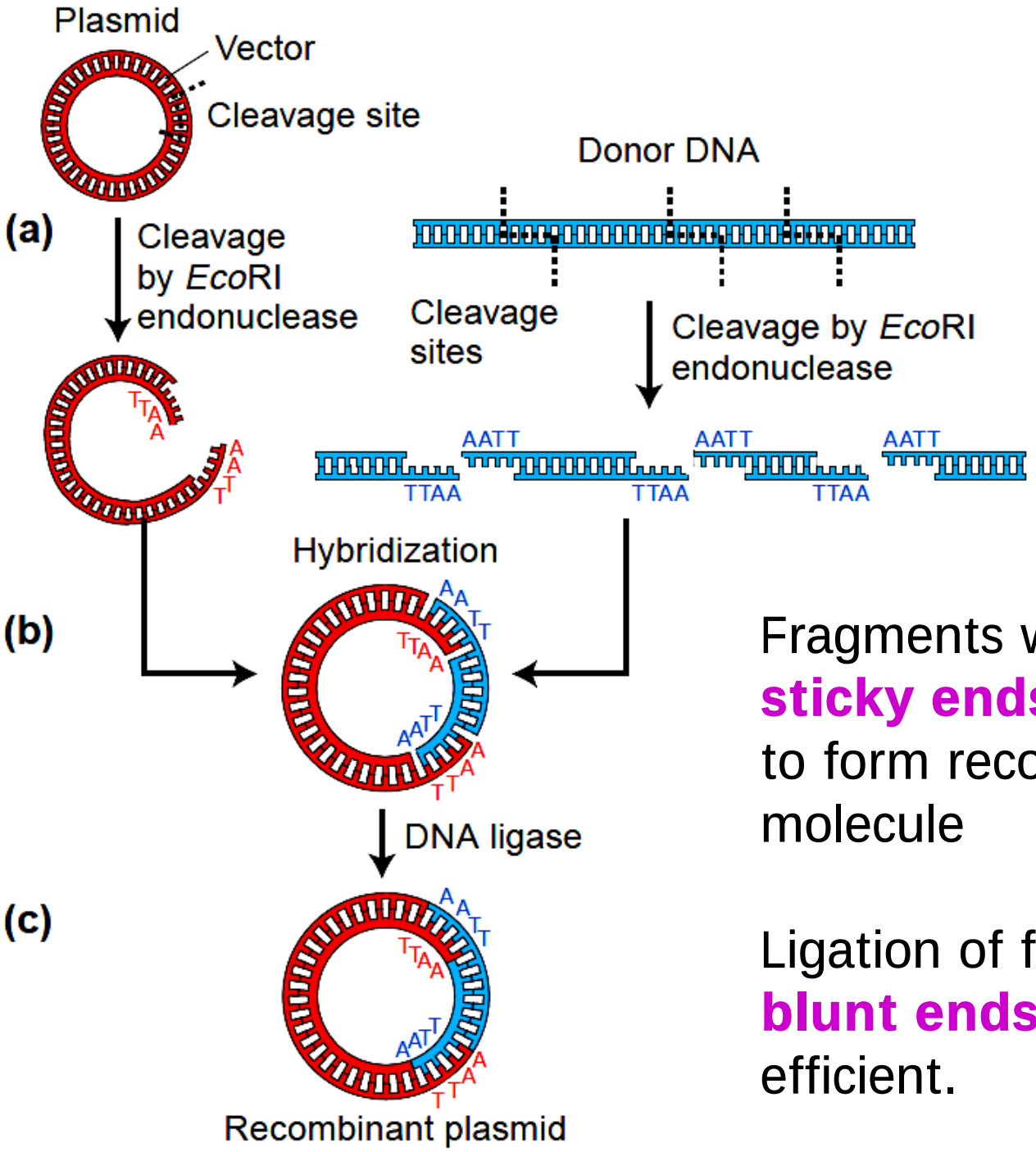
Vectors: To serve as a vector, a DNA molecule must have several properties:

- Autonomously replicate（自主复制）
- Contain convenient restriction sites
- Carry a selector marker gene（选择标记基因）
- Easy to recover from the host cell

There Are Several Types of Vectors

TABLE 9.2 Various Vectors and the Size of the Inserts They Carry

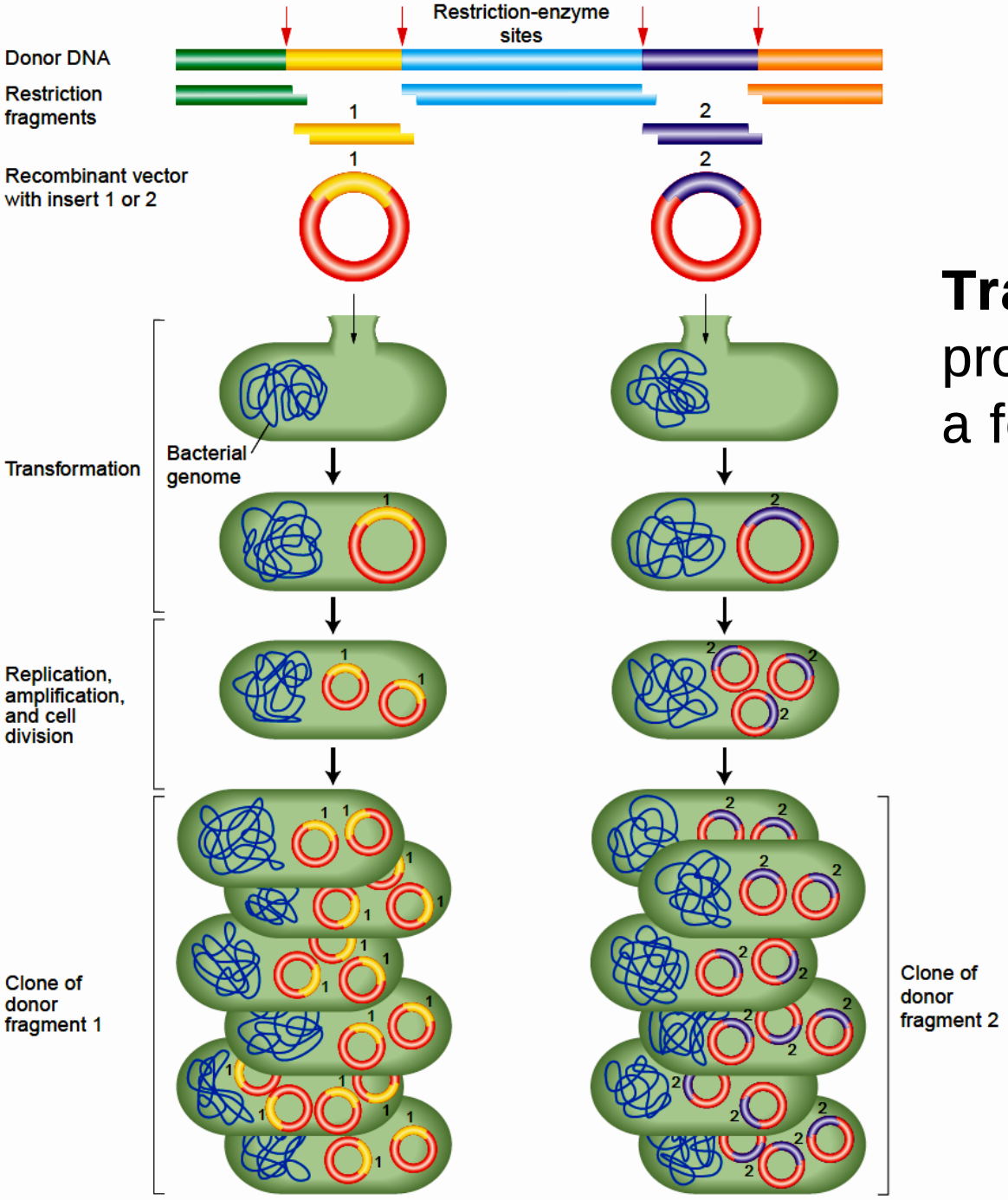
Vector	Form of Vector	Host	Typical Carrying Capacity (Size of Insert Accepted)	Major Uses
Plasmid	Double-stranded circular DNA	*E.coli*	Up to 15 kb	cDNA libraries; subcloning
Bacteriophage lambda	Virus (linear DNA)	*E.coli*	Up to 25 kb	Genomic and cDNA libraries
Cosmid	Double-stranded circular DNA	*E.coli*	30–45 kb	Genomic libraries
Bacteriophage P1	Virus (circular DNA)	*E.coli*	70–90 kb	Genomic libraries
BAC	Bacterial artificial chromosome	*E.coli*	100–500 kb	Genomic libraries
YAC	Yeast artificial chromosome	Yeast	250–2000 kb (2 megabases)	Genomic libraries



Fragments with identical **sticky ends** can be joined to form recombinant DNA molecule

Ligation of fragments with **blunt ends** is much less efficient.

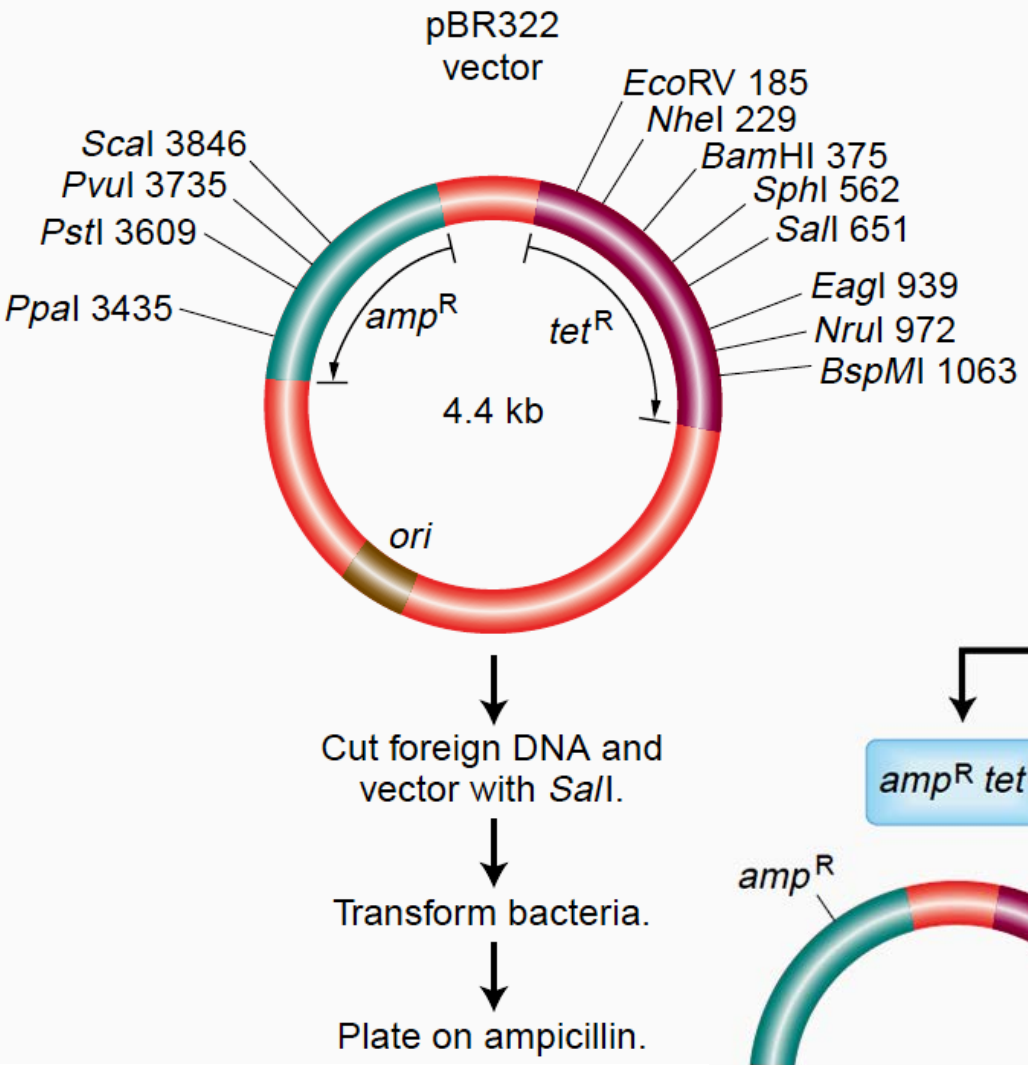
Cloning Step 2: Host Cells Take up and Amplify Vector-Insert Recombinants

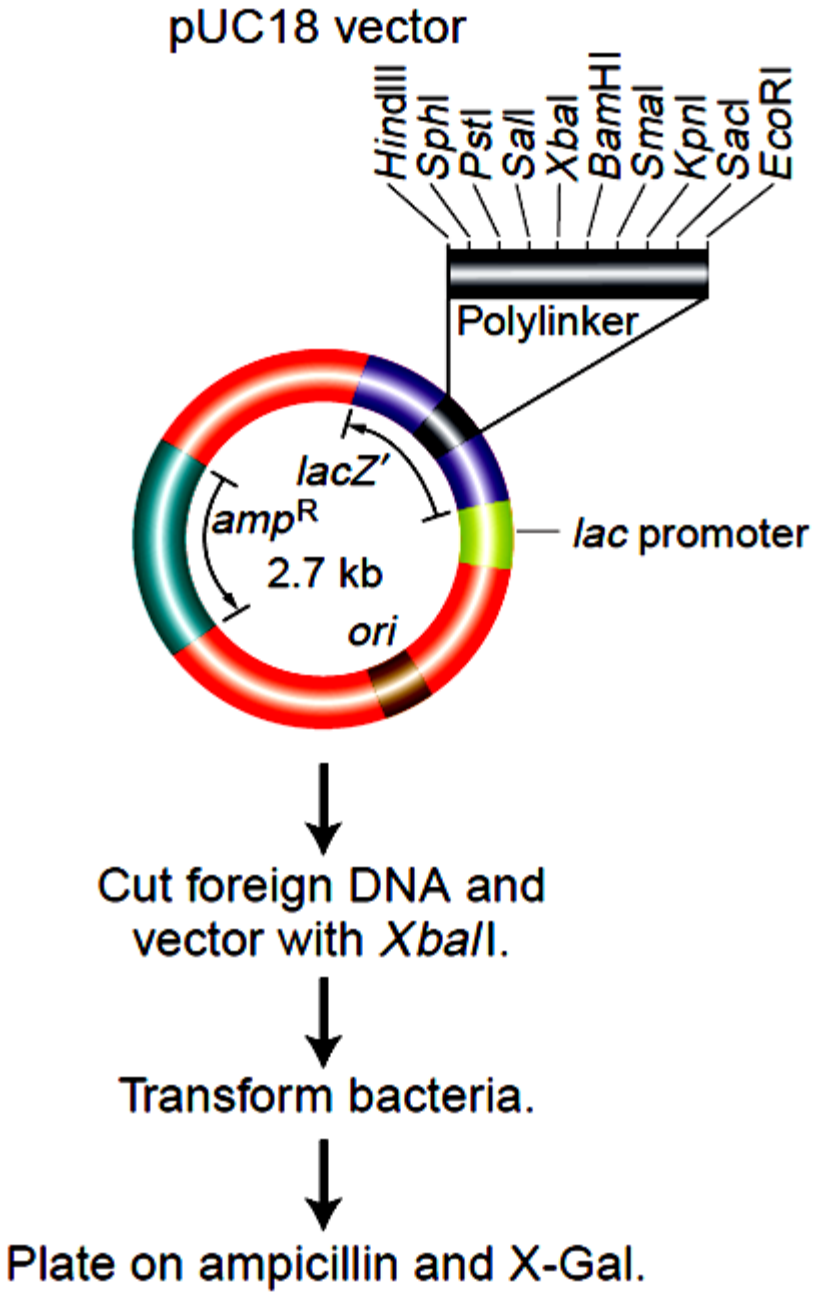


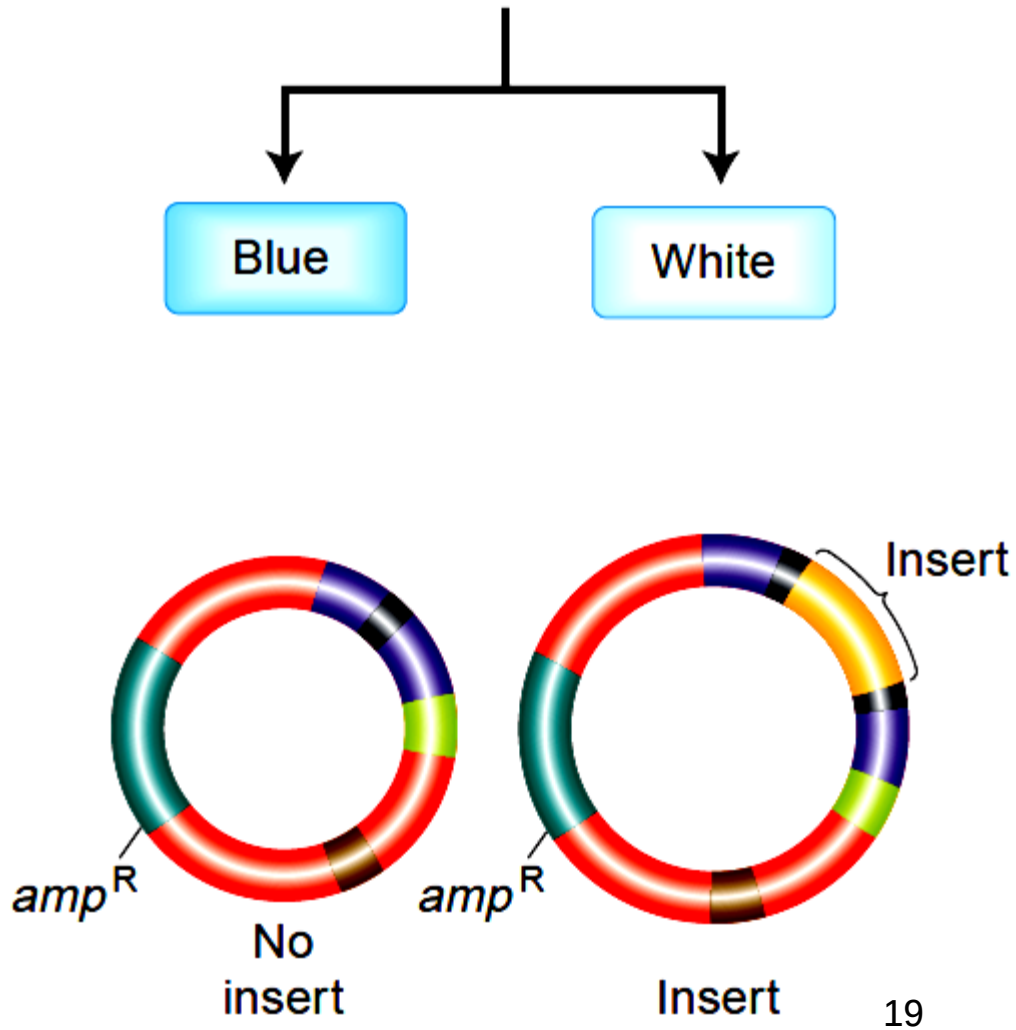
Transformation: a process of cell taking up a foreign DNA molecule

How Do You Know Which Cells Have Been Transformed?

How Do You Know Whether the Plasmids Inside Bacterial Cells Contain an Insert?


pBR322
vector
EcoRV 185
NheI 229
BamHI 375
SphI 562
SalI 651
EagI 939
NruI 972
BspMI 1063
ScaI 3846
PvuI 3735
PstI 3609
PpaI 3435
ampR
tetR
4.4 kb
ori
Cut foreign DNA and
vector with SalI.
Transform bacteria.
Plate on ampicillin.
ampR tetR
ampR tetS
ampR
tetR
ampR
Insert
No insert
Insert


pUC18 vector
HindIII
SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
SacI
EcoRI
Polylinker
lacZ′
ampR
2.7 kb
ori
lac promoter
Cut foreign DNA and vector with XbaI.
Transform bacteria.
Plate on ampicillin and X-Gal.

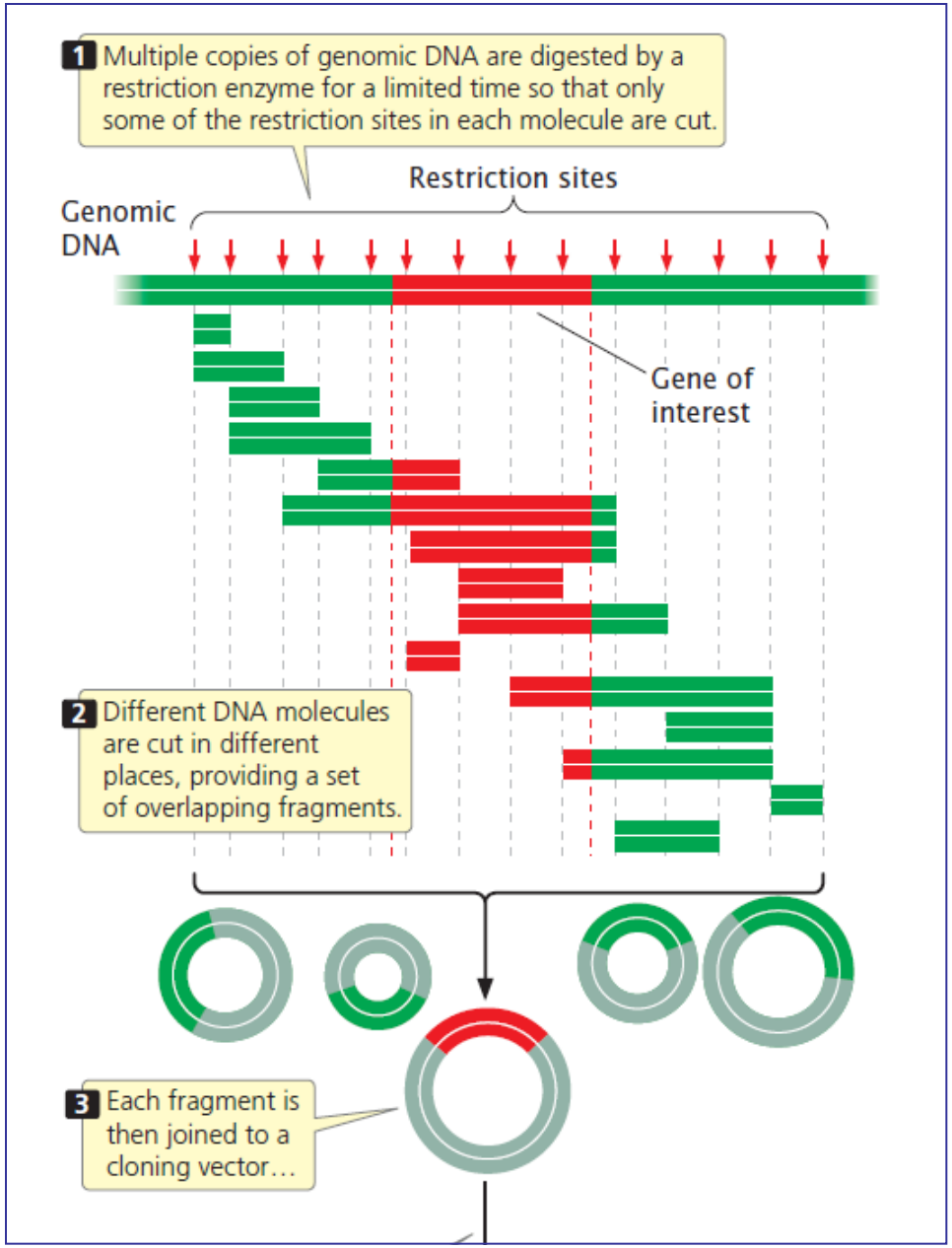

Blue
White
Insert
ampR
No insert
ampR
Insert

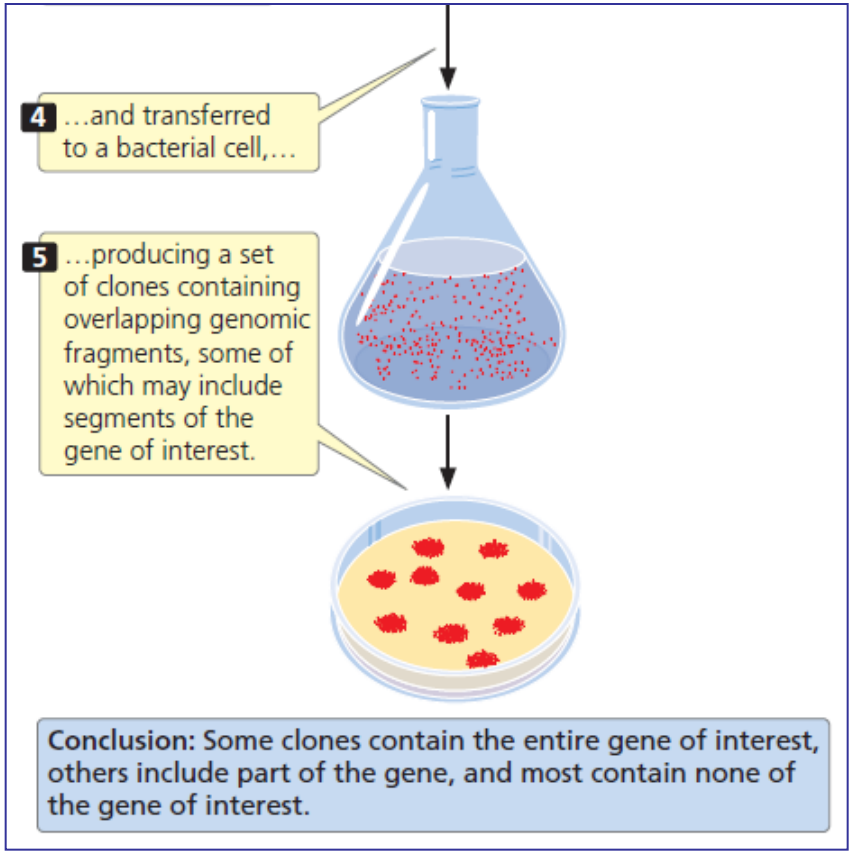
第二节 DNA文库
DNA libraries

- **Library**: a collection of DNA clones that contains multiple copies of nearly every fragment in the whole genome inserted into a suitable vector and placed into storage.

1. Genomic library （基因组文库）

- Making a genomic library





A genomic library contains all of the DNA sequences found in an organism's genome

- Calculate the number of clones in a library

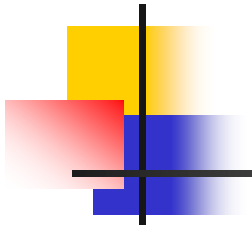
Average size of clone fragment (bp)	Genome size (bp)					
	2×10^6 (bacteria)		2×10^7 (fungi)		3×10^9 (human)	
	Theoretic number		Theoretic number		Theoretic number	
5×10^3	400		4000		600000	
10×10^3	200		2000		300000	
20×10^3	100		1000		150000	
40×10^3	50		500		75000	

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

N: number of required clones

P: the probability of recovering a given sequence

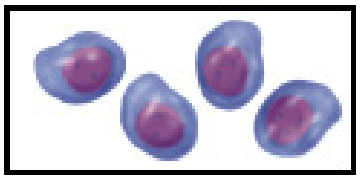
f: the fraction of the genome in each clone



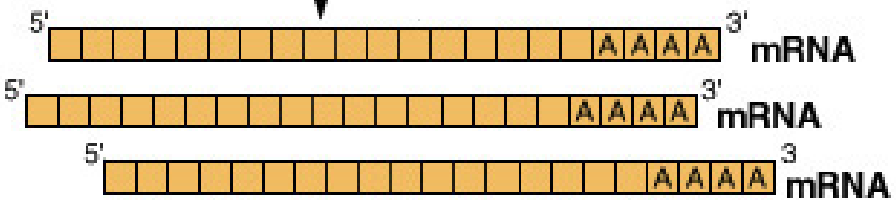
2. cDNA library （cDNA文库）

cDNA cloning

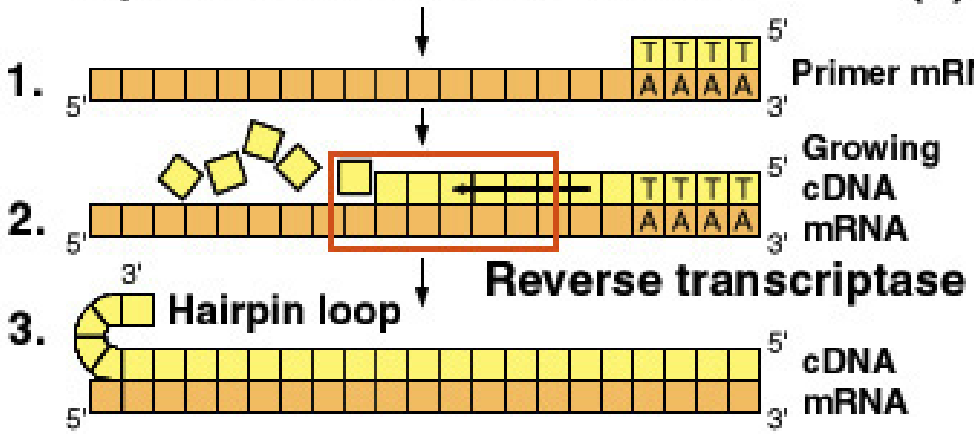
(a) Red blood cell precursors



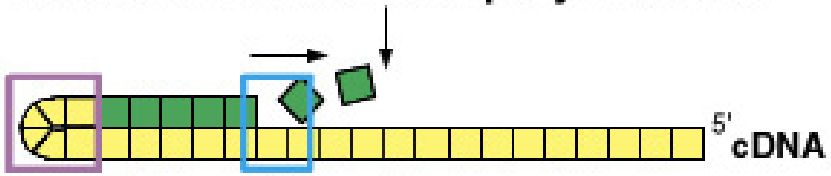
Release mRNA from cytoplasm and purify.



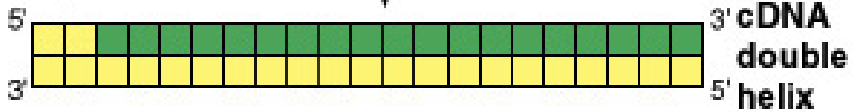
(b) Add oligo(dT) primer. Treat with reverse transcriptase in presence of four nucleotides.



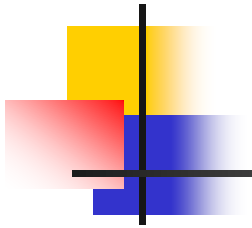
(c) Digest mRNA with RNase. cDNA directs synthesis of second cDNA strand in the presence of four nucleotides and DNA polymerase.



S1 nuclease DNA polymerase cuts hairpin loop

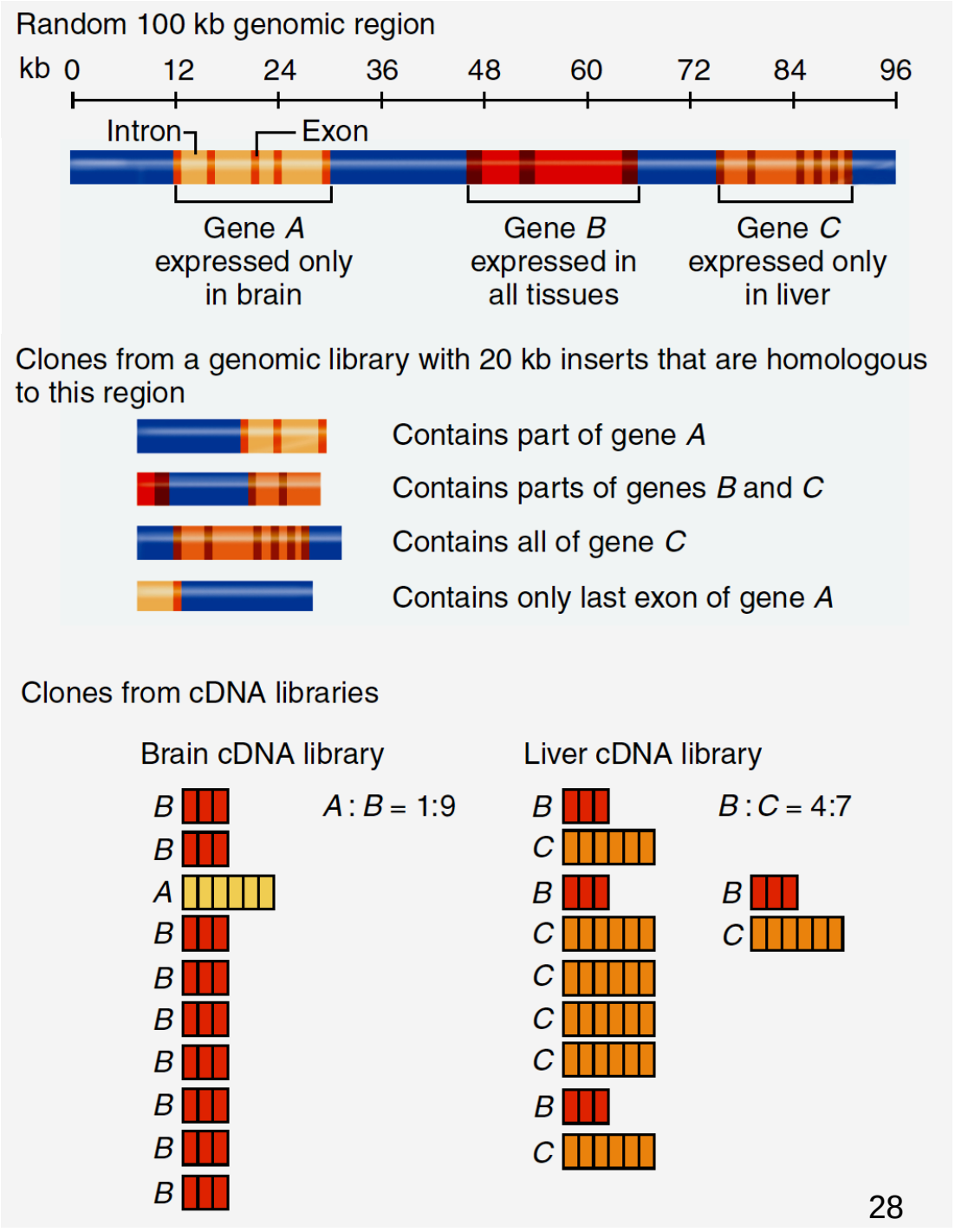


(d) Insert cDNA into vector.



- cDNA libraries represent only the ***expressed*** genes in a given cell type, tissue, or stage of embryonic development.

Genomics *vs.* cDNA libraries



第三节 目的DNA的分离
Screening DNA libraries for genes of interest

1. Screening by functional complementation

利用功能互补进行筛选

Isolate Gal gene of yeast

Making a library of wild type yeast DNA

Recovery of the wild type GAL gene

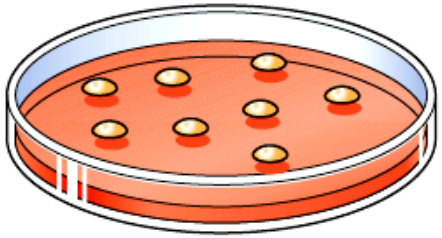
2. Screening with probe （探针）

- **DNA probes**:

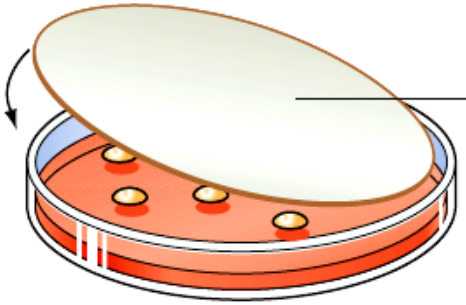
  Short single-stranded DNA, from 10 to several thousand nucleotides in length, are usually labeled by radiation or fluorescent dye（荧光染料）

  Hybridization is used to identify similar DNA sequences

Master plate containing genomic library of mouse clones.

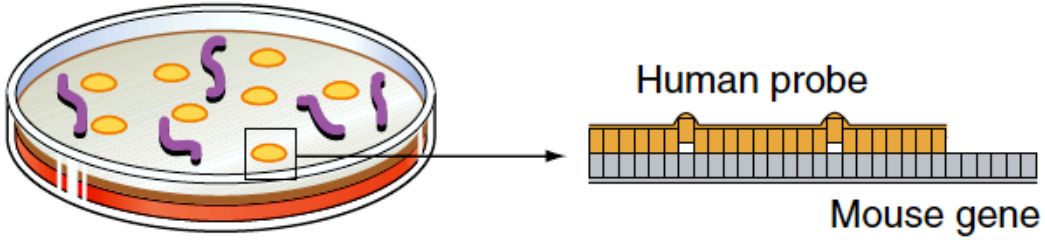


Overlay a nitrocellulose disk to make a replica of the plate.

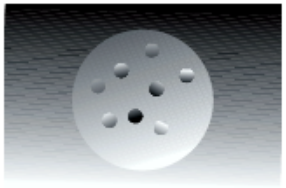


Remove disk from plate and lyse cells on it and denature DNA with NaOH. Bake and treat with UV light to bind DNA strands to disk.

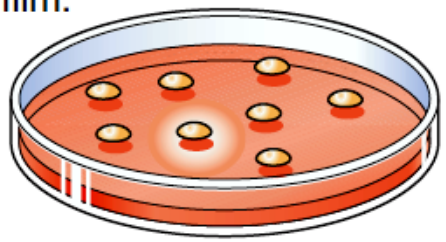
Add labeled probe.
Colonies with complementary DNA sequences hybridize to probe and restrain it.



Wash disk, expose to X-ray film.



Original plate

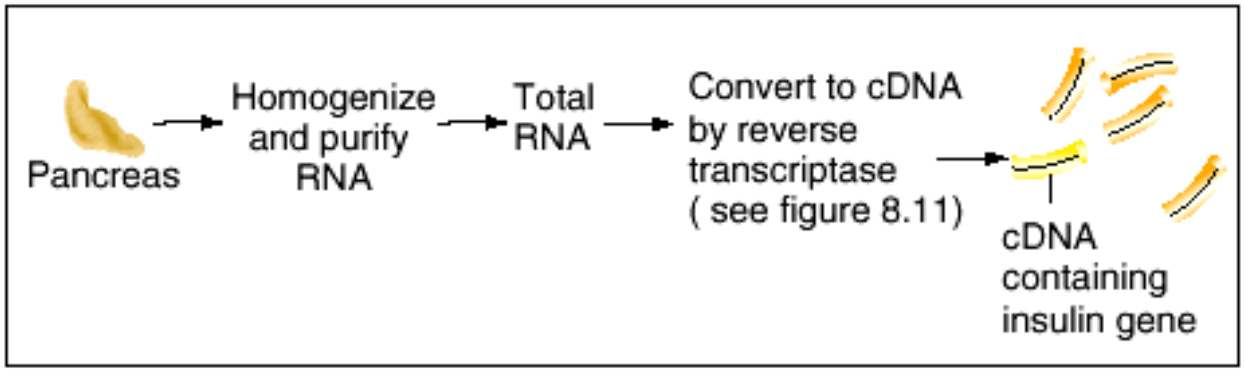


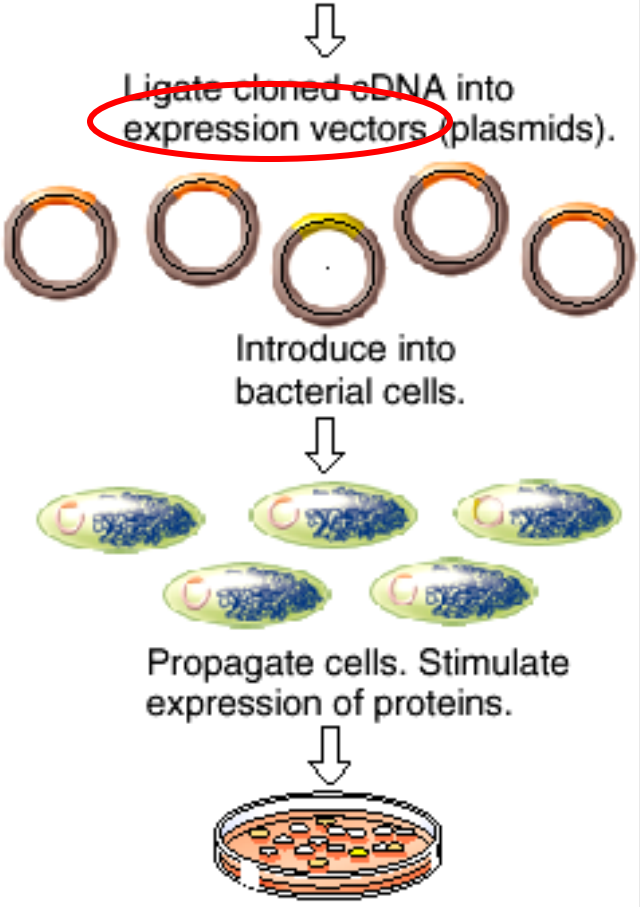
Compare with original plate to locate bacterial clone with desired genomic fragment.

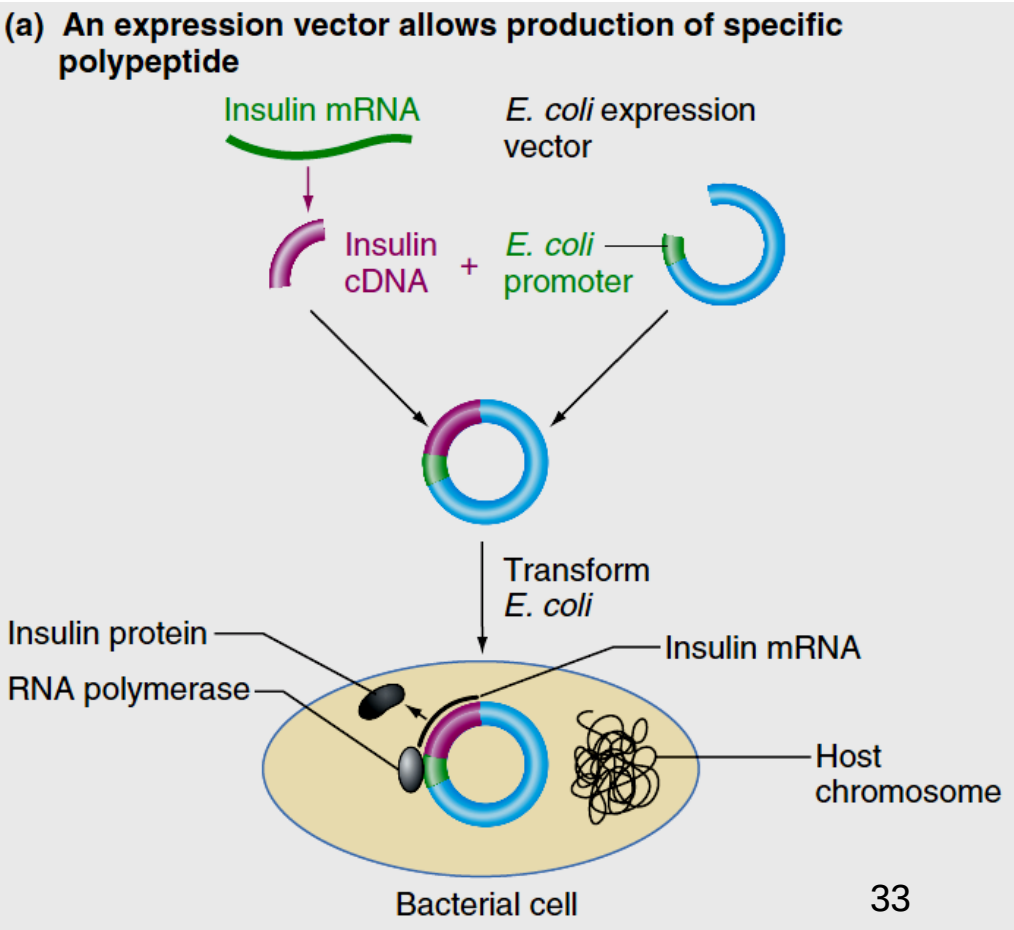
Screening a library of clones by hybridization to a labeled probe.

See movie

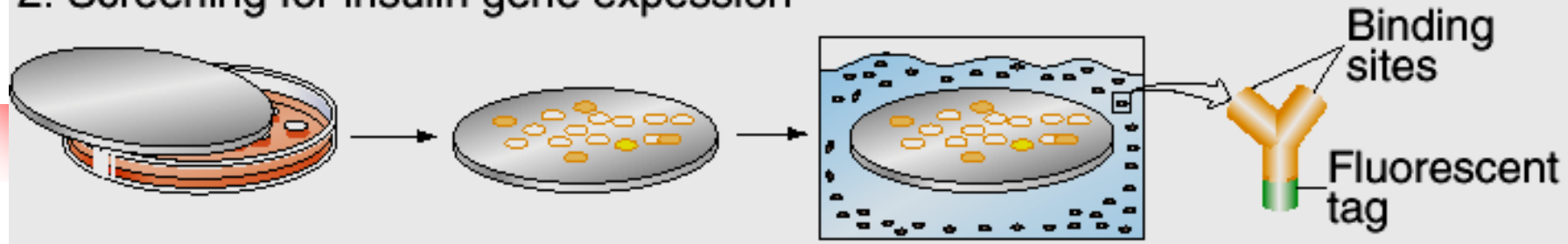
- Antibody can be used as probes







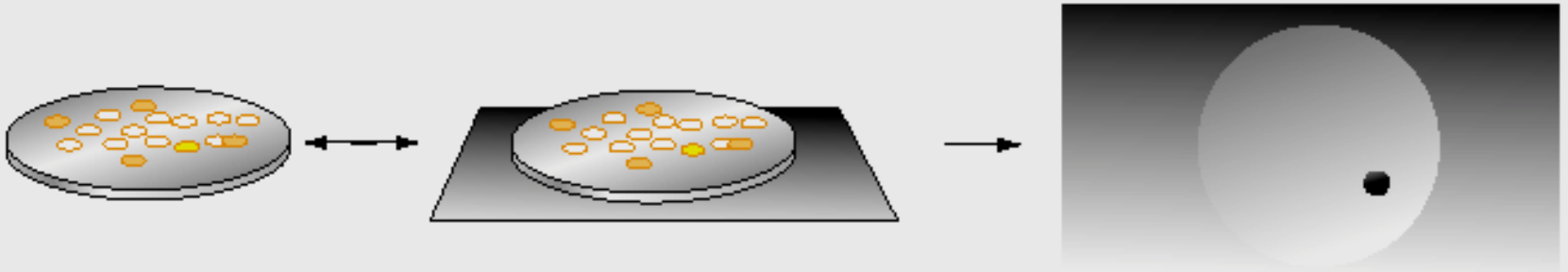
2. Screening for insulin gene expession



Overlay plate with nitrocellulose paper.

Lyse cells. Treat with NaOH. Proteins adhere to paper.

Incubate paper in solution of labeled insulin antibody. Antibodies will bind to insulin protein.

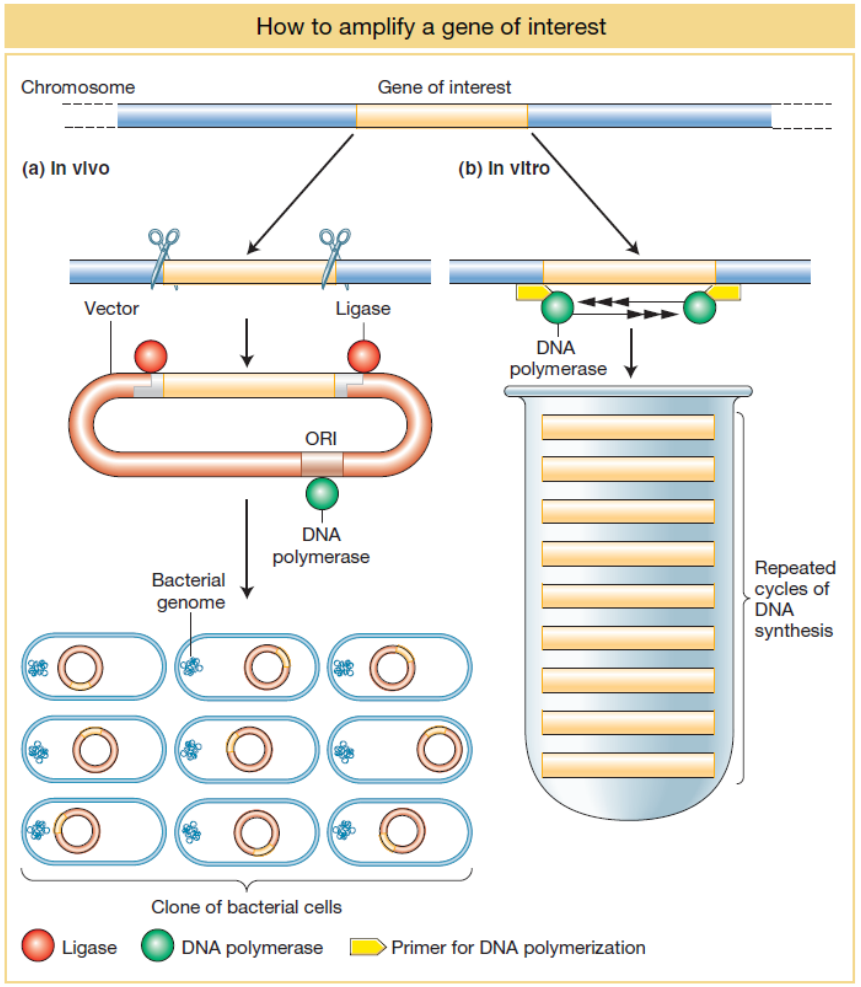


Wash filter. Make autoradiograph. Compare with original plate in order to find bacterial clone containing human insulin gene.

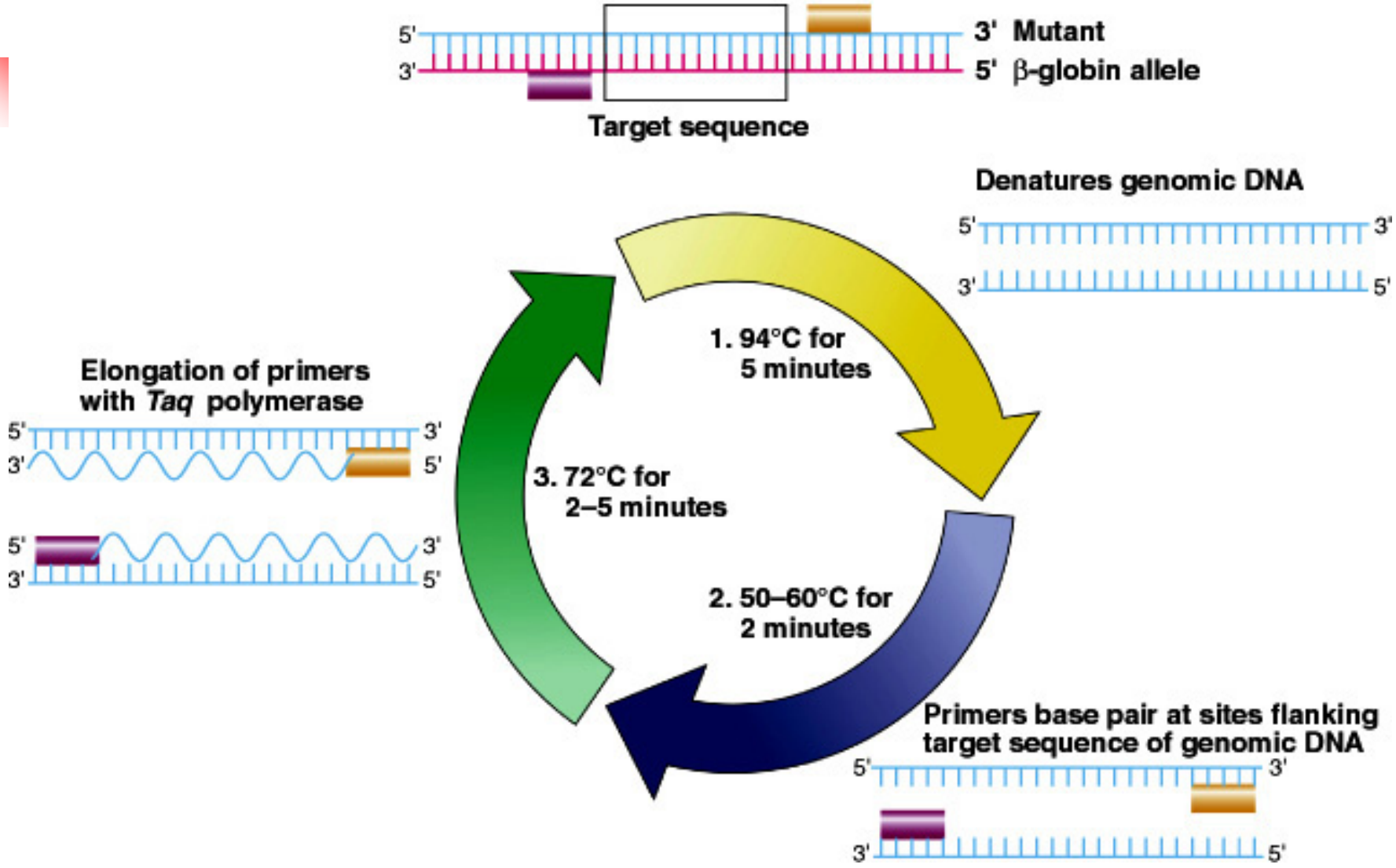
3. PCR Provides a Rapid Method for Isolating DNA Fragments

Polymerase chain reaction, PCR

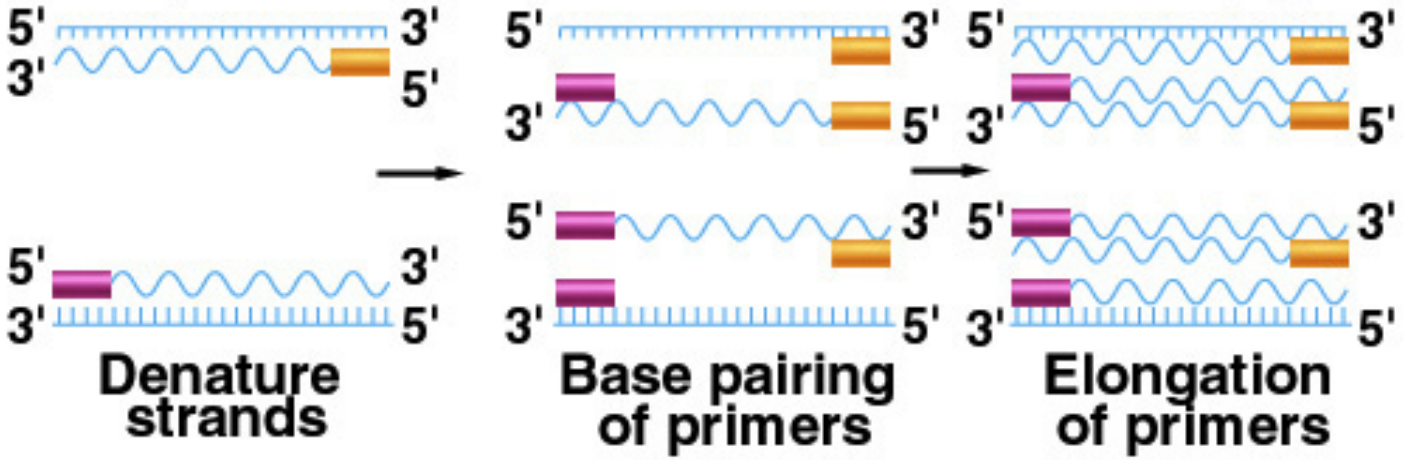
A method for amplifying specific DNA segments that exploits certain features of DNA replication.

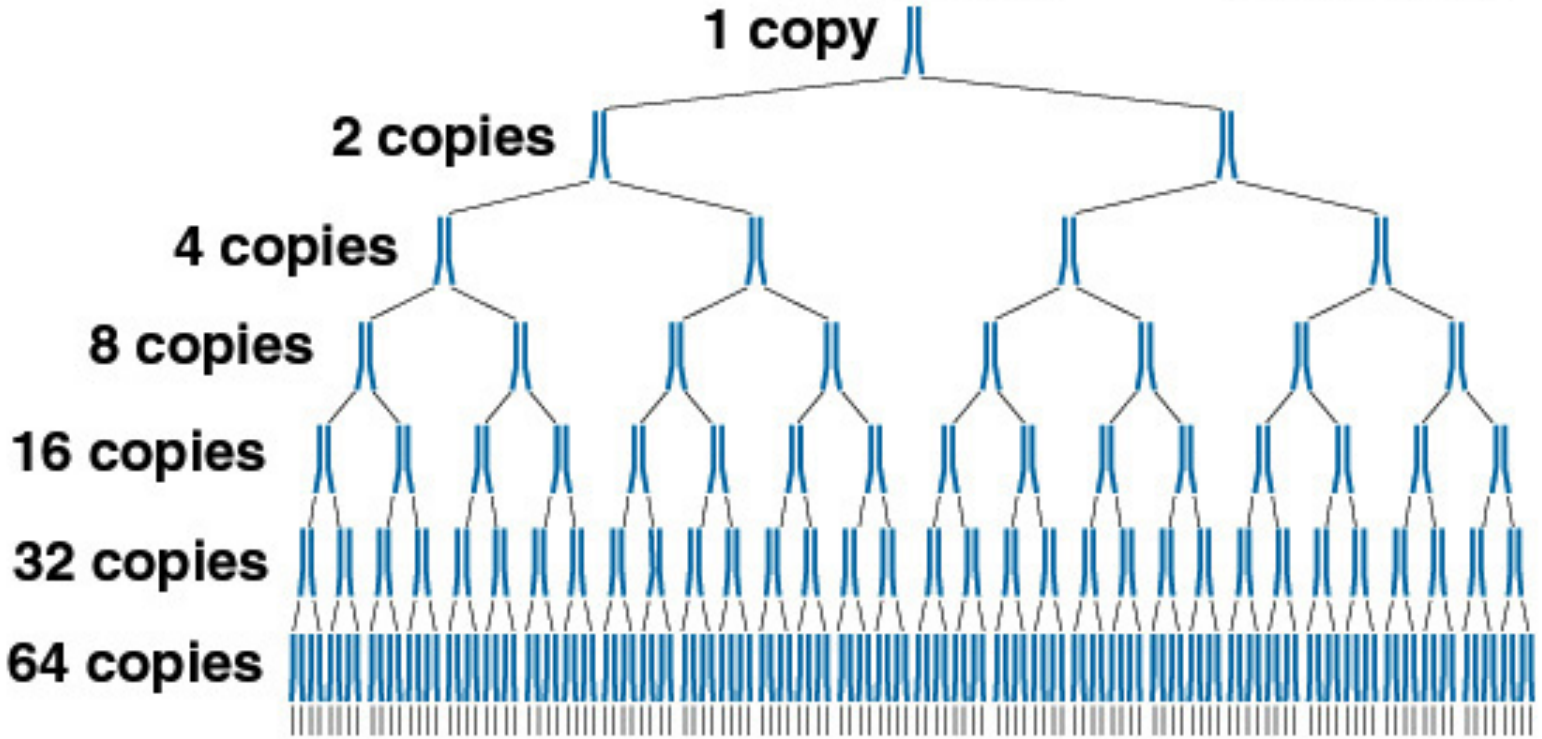


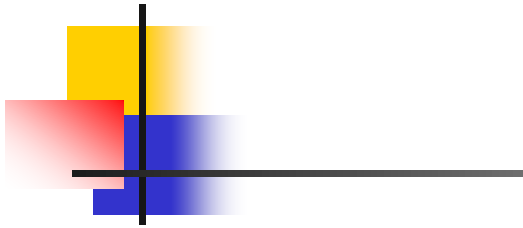
The polymerase chain reaction, part 1



The polymerase chain reaction, part 2







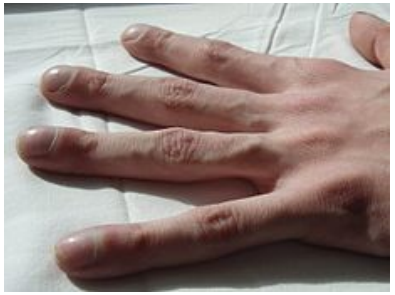
- Amplify a DNA fragment in a genome

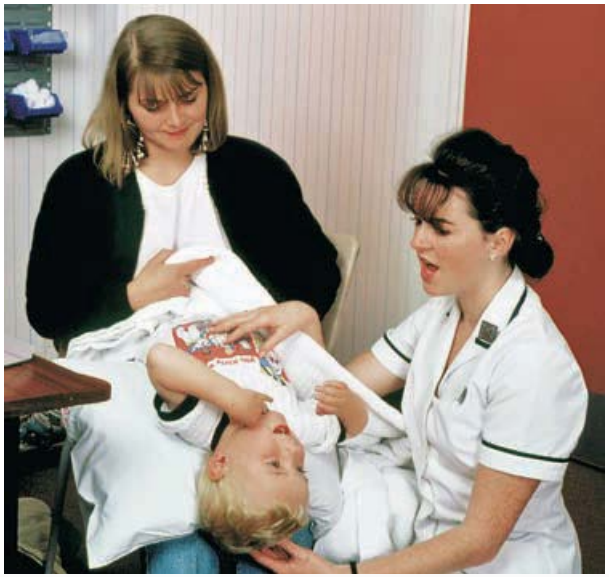
see movie

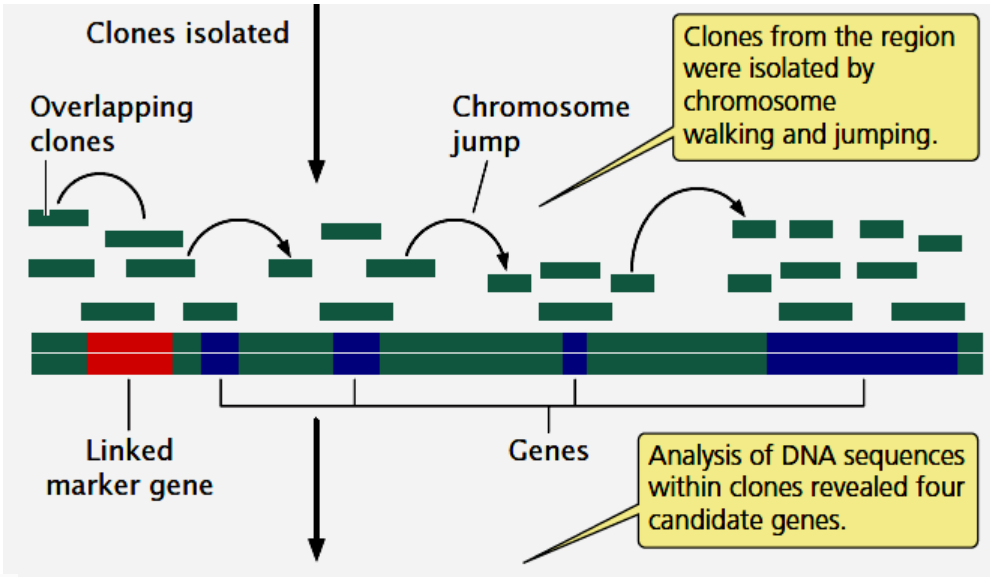
4. Finding Specific Clones On The Basis Of Genetic-map Location (Positional Cloning)

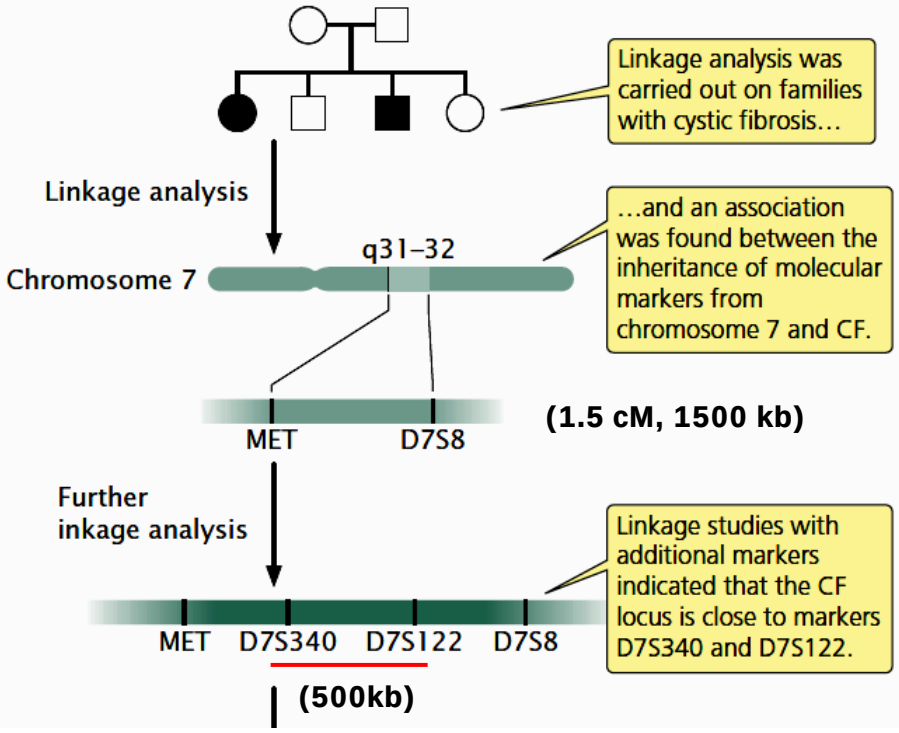
Cystic fibrosis (CF, 囊性纤维化) was the first genetic disease for which the causative gene was isolated entirely by positional cloning

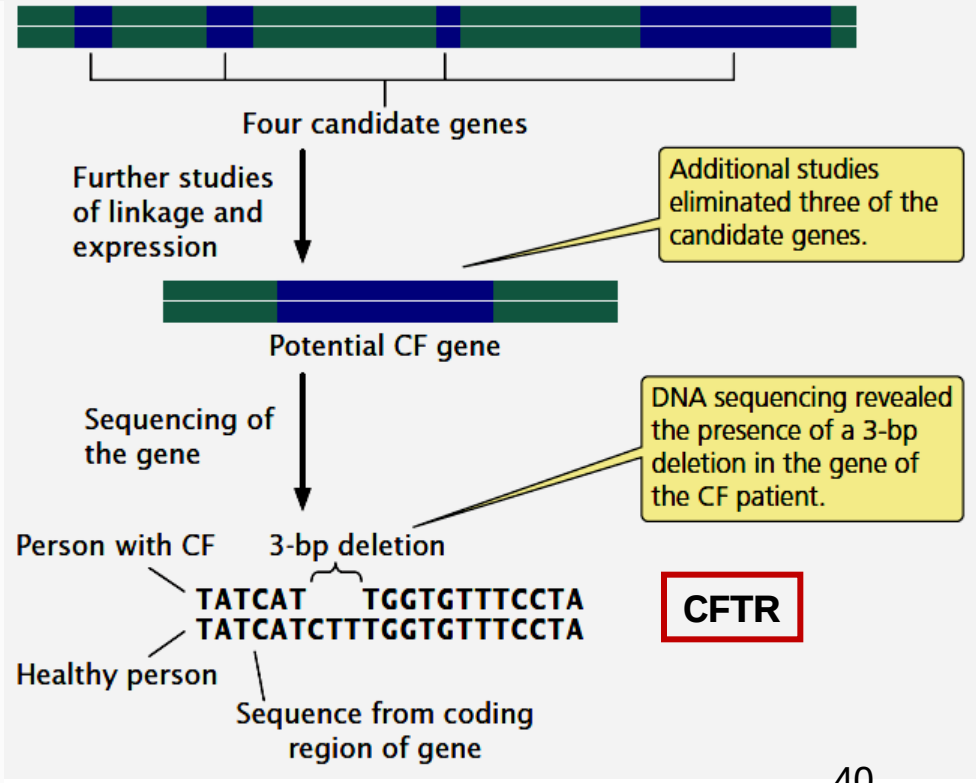
- An autosomal recessive disorder characterized by chronic lung infections, insufficient production of pancreatic enzymes that are necessary for digestion, and increased salt concentration in sweat.
- It is among the most common genetic diseases in Caucasians, occurring with a frequency of about 1 in 2000 live births. About 5% of all Caucasians are carriers of the CF mutation.



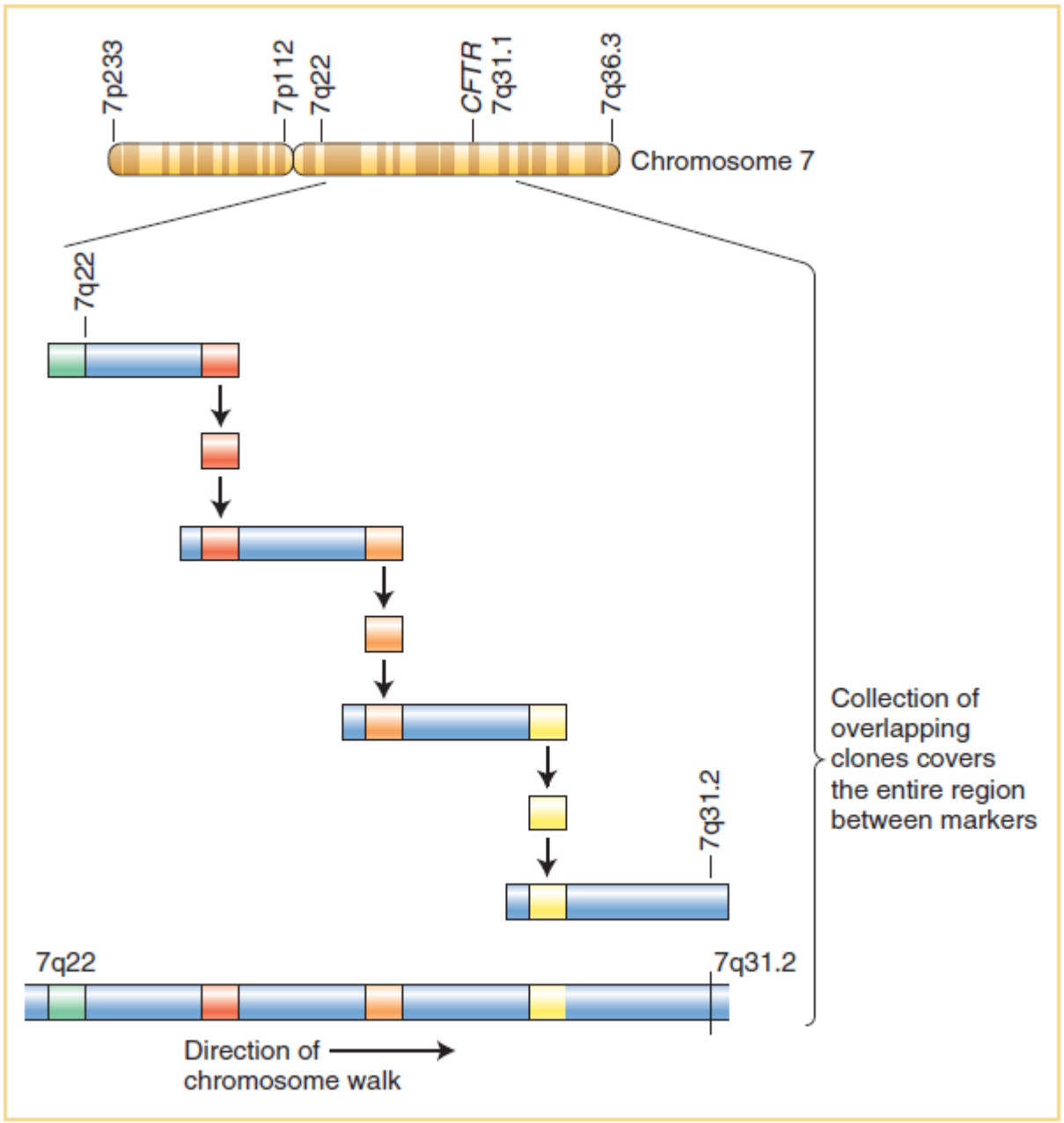



Clones isolated
Overlapping clones
Chromosome jump
Clones from the region were isolated by chromosome walking and jumping.
Linked marker gene
Genes
Analysis of DNA sequences within clones revealed four candidate genes.


Linkage analysis was carried out on families with cystic fibrosis…
Linkage analysis
Chromosome 7
q31–32
…and an association was found between the inheritance of molecular markers from chromosome 7 and CF.
MET
D7S8
(1.5 cM, 1500 kb)
Further inkage analysis
Linkage studies with additional markers indicated that the CF locus is close to markers D7S340 and D7S122.
MET
D7S340
D7S122
D7S8
(500kb)

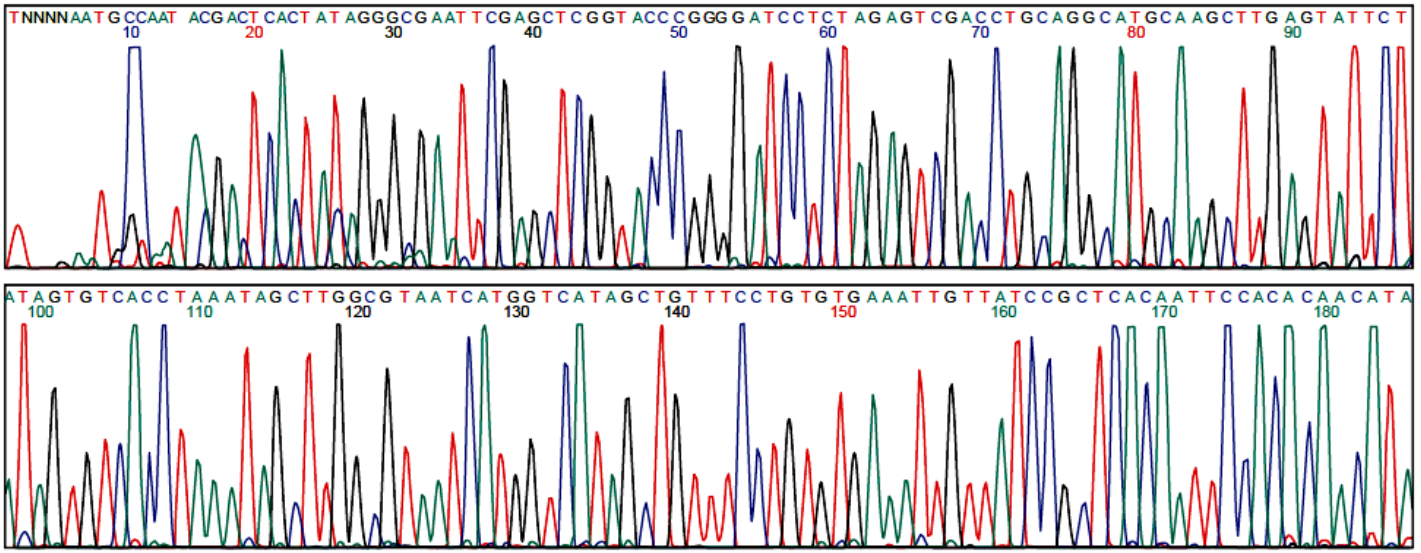

Four candidate genes
Further studies of linkage and expression
Additional studies eliminated three of the candidate genes.
Potential CF gene
Sequencing of the gene
DNA sequencing revealed the presence of a 3-bp deletion in the gene of the CF patient.
Person with CF
3-bp deletion
TATCAT TGGTGTTTCCTA
TATCATCTTTGGTGTTTCCTA
CFTR
Healthy person
Sequence from coding region of gene

Using a chromosomal walk to order a set of clones



5. DNA Sequencing Is the Ultimate Way to Characterize DNA Structure at the Molecular Level

Dideonucleotide chain-termination sequencing (Sanger sequencing)



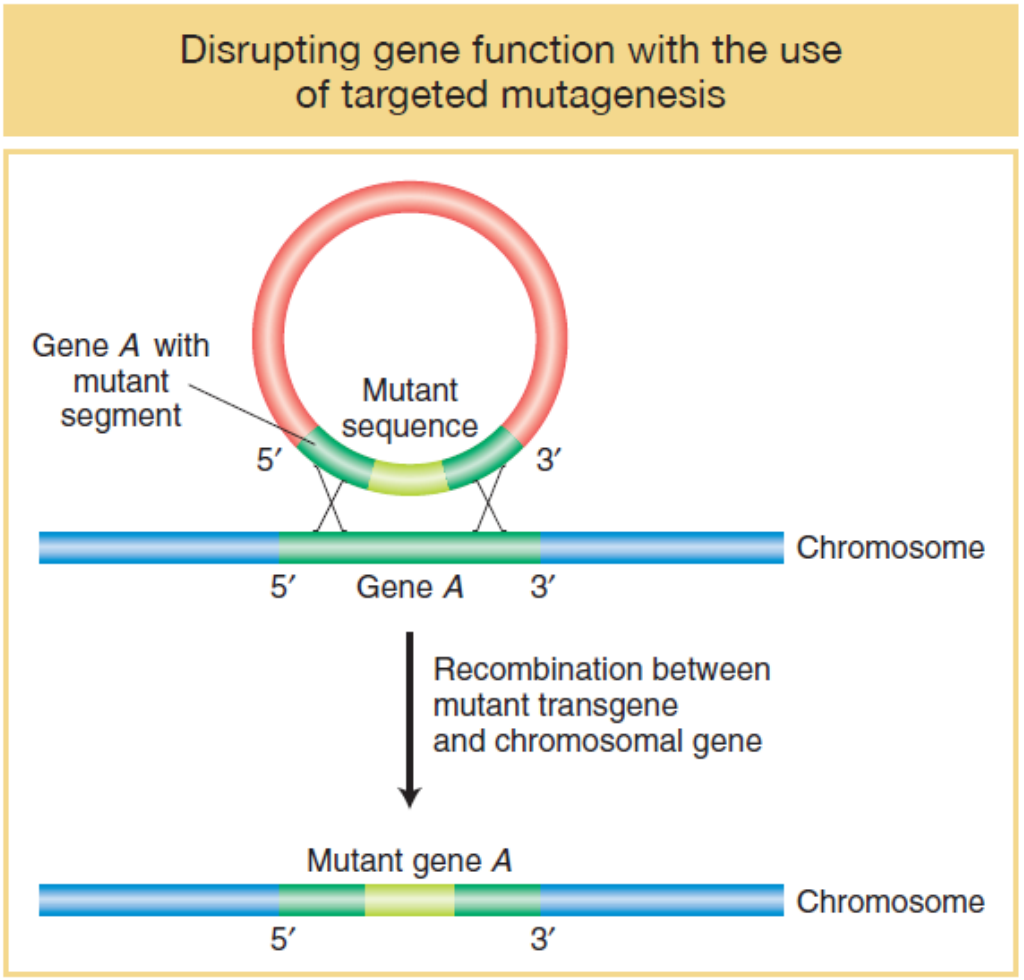
Next generation sequencing technology

第四节　基因功能研究

The gold standard for establishing the function of a gene or genetic element is to disrupt its function and to understand phenotypes in native conditions.

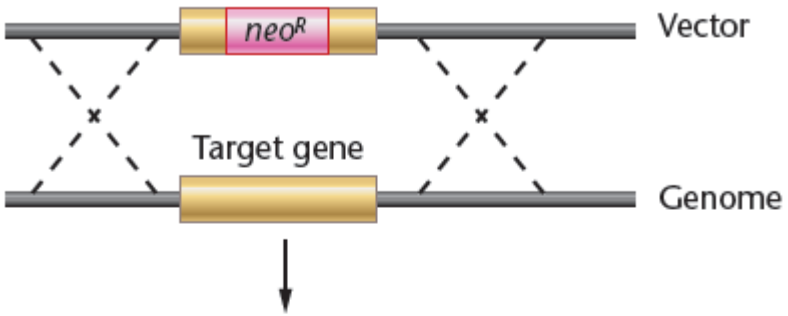
Reverse-genetic analysis starts with a known molecule—a DNA sequence, an mRNA, or a protein—and then attempts to disrupt this molecule to assess the role of the normal gene product in the biology of the organism.

Targeted gene knockout

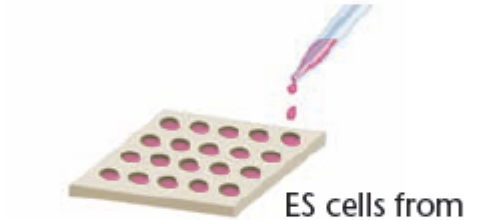


A basic strategy for producing a knockout mouse

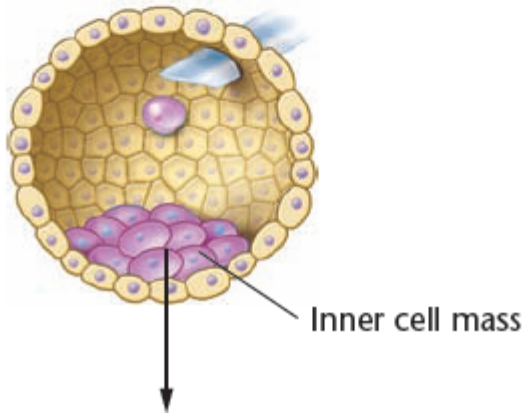
1. Designing the targeting vector



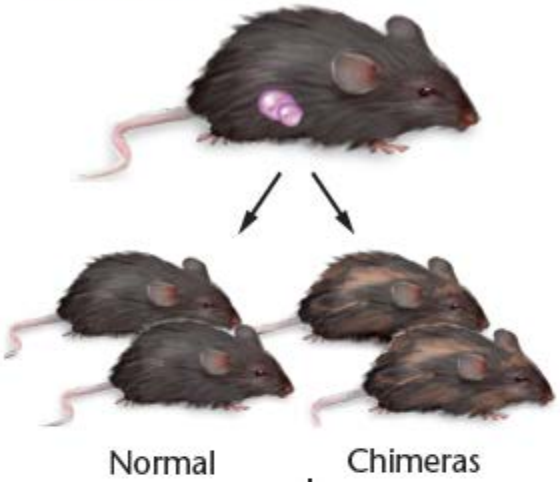
2. Transform ES cells with targeting vector and select cells for recombination



3. Microinject ES cells into blastocyst from black-colore mouse

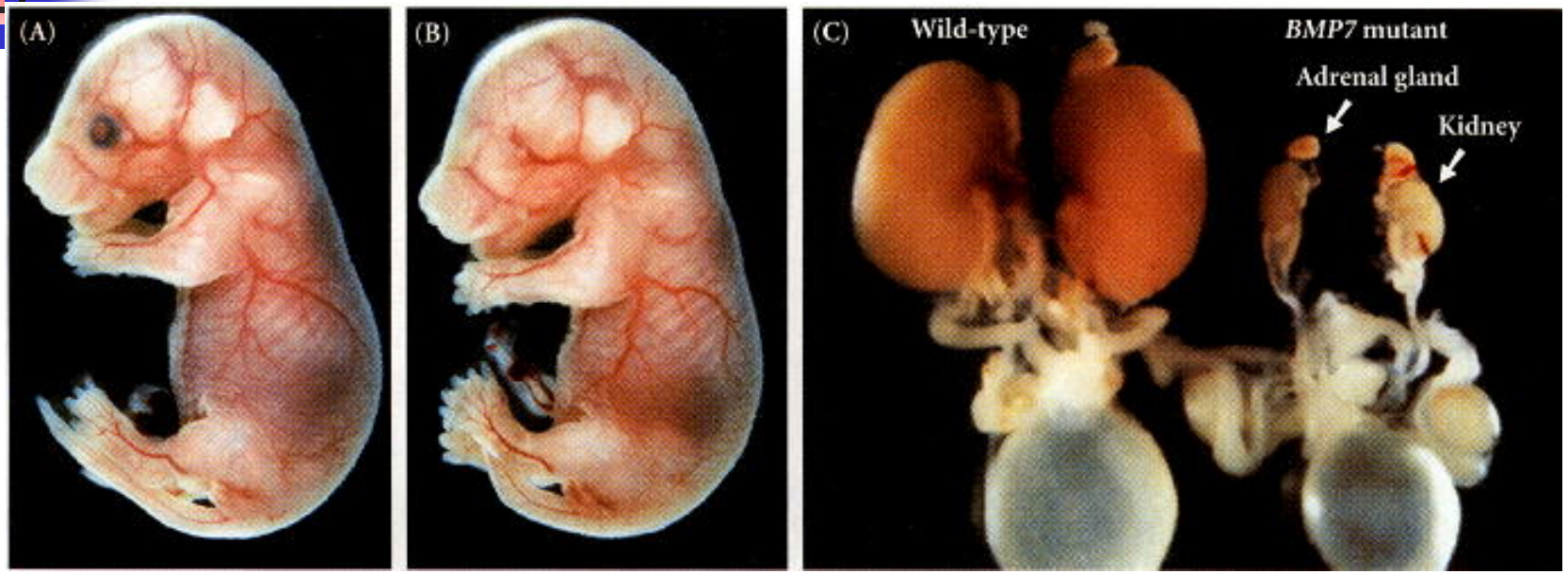


4. Transfer into pseudo-pregnant foster mother, birth of chimeras



5. Chimeric mouse bred to black mouse to create mice heterozygous (+/−) for gene knockout

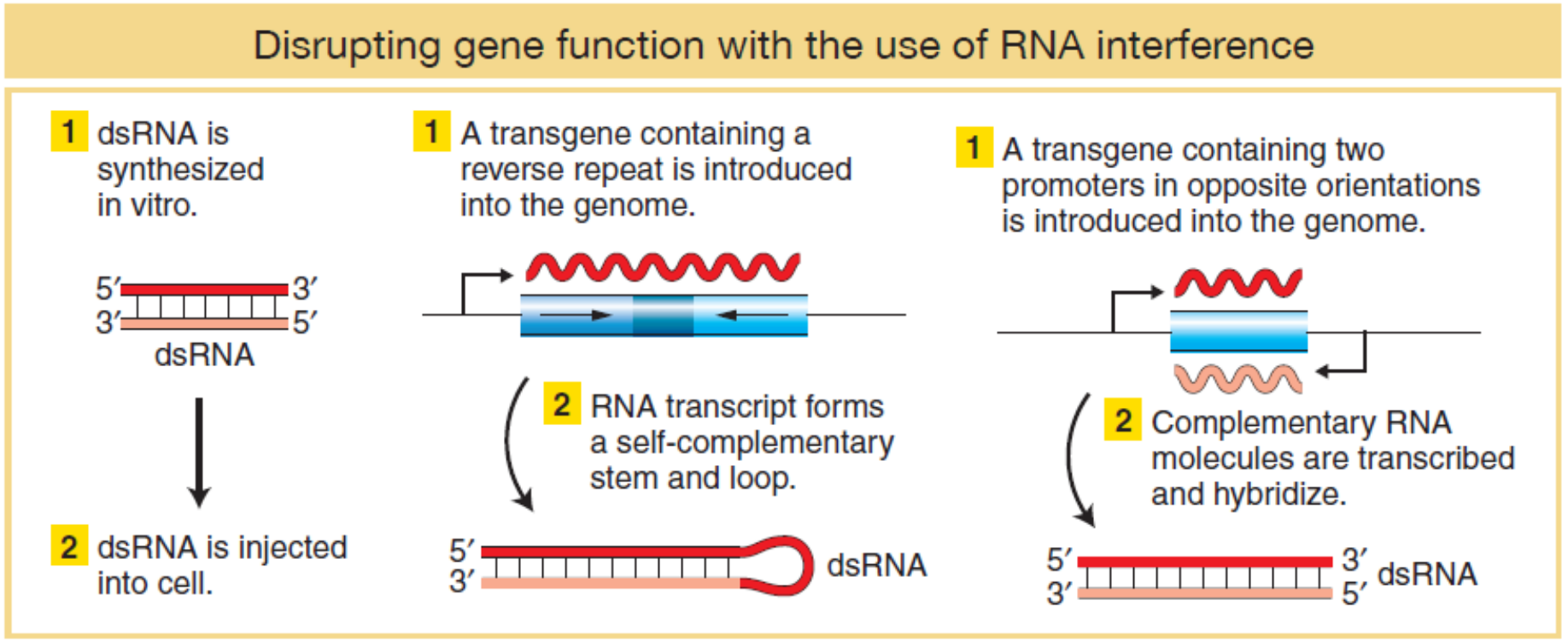
6. Breed heterozygous mice to produce mice homozygous (−/−) for gene knockout



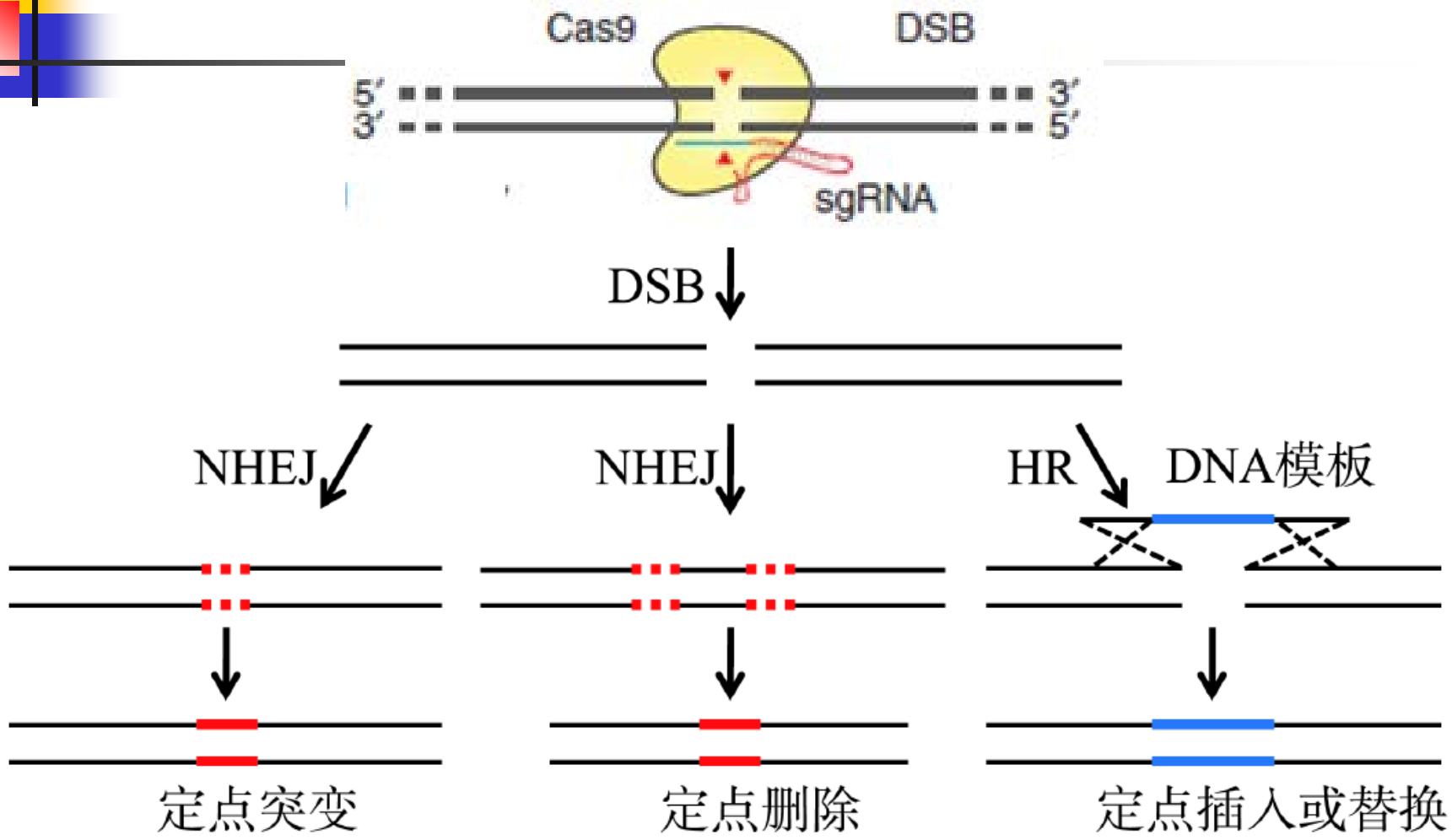
+ / + **— / —**

Morphological analysis of *BMP7* knockout mice

RNA interference (RNAi)



- **CRISPR-Cas9**



Knock-in: making a transgenic organism

In a transgenic, the transgene is often overexpressed in order to study its effects on the appearance and functions

The figures and tables are cited from:

- **Genetics (From genes to genomes)**, Leland Hartwell, Mcgraw-Hill Companies, Inc
- **Concept of Genetics**, William S.Klug, Prentice Hall, Inc
- **Introduction to Genetics Analysis**, Anthony J.F. Griffiths, W.H.Freeman, Inc
- **Principle of Genetics**, D.Peter Snustad, John Wiley & Sons, Inc
- **Genetics-A Conceptual Approach**, Benjamin A. Pierce, W. H. Freeman