E. coli K12

Capsule

Cell Wall

Plasma Membrane

DNA

Pilus

Cytoplasm

Ribosomes

Bacterial Flagellum

Improvements of E. coli K12 for recombinant DNA methods:

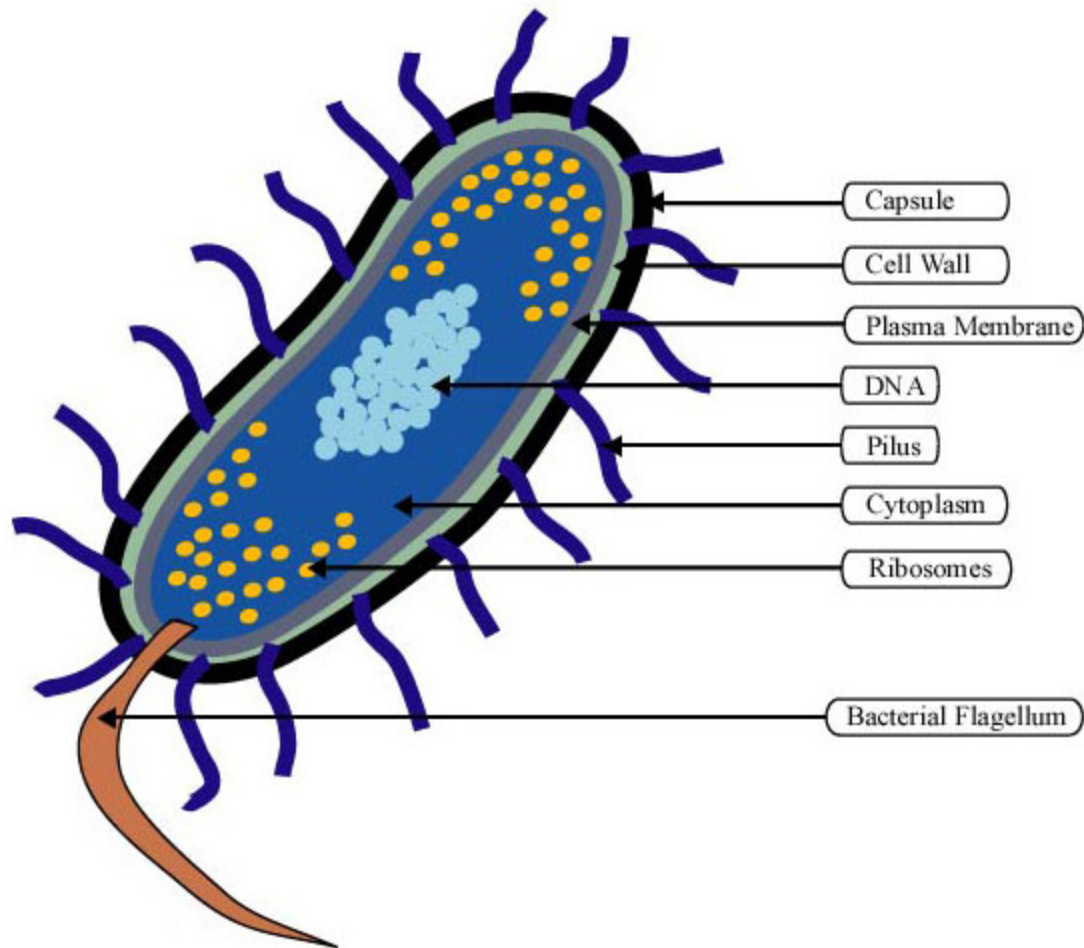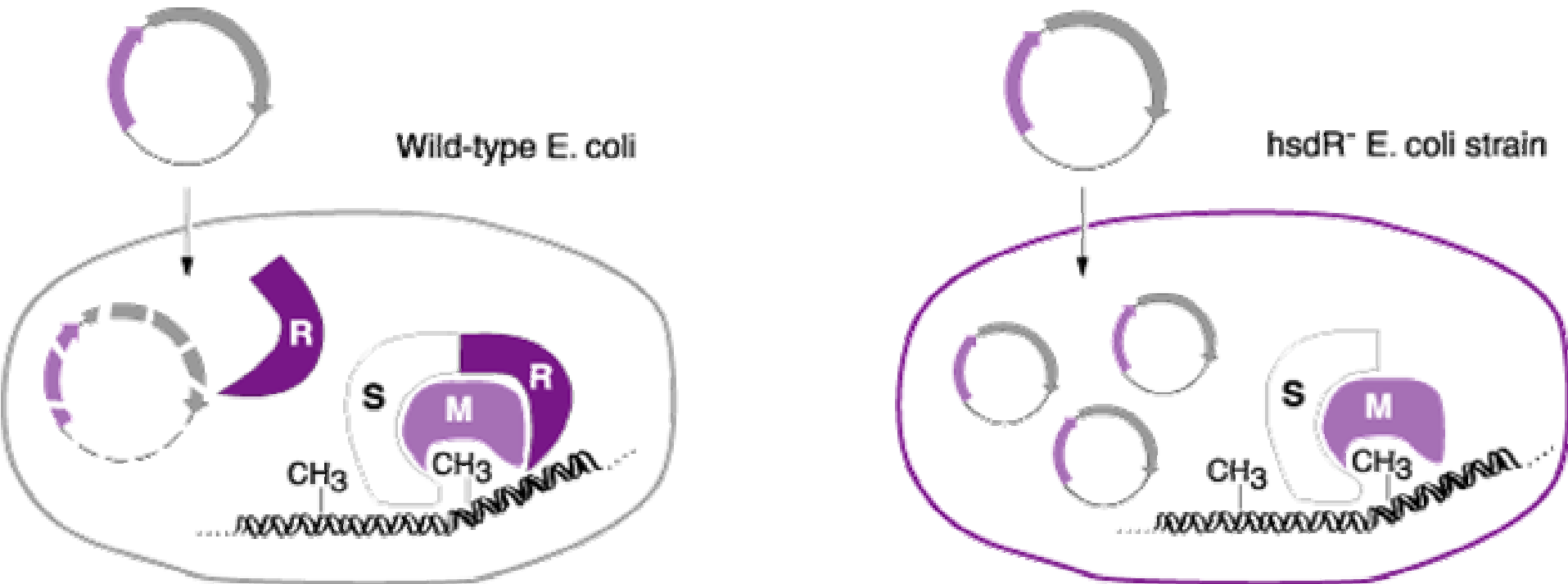- 1) Removal of the EcoK restriction system (hsdR$^-$)

Wild-type E. coli

hsdR$^-$ E. coli strain

Improvements of E. coli K12 for recombinant DNA methods:

• 2) Removal of the mcrA/mcrB genes that are responsible for degrading foreign DNA that is methylated, but not at the sites that E. coli K12 would recognize as it's own. (For example human and mouse DNA: CpG-methylated)

Wild-type E. coli

mcrA⁻/mcrB⁻ E. coli strain
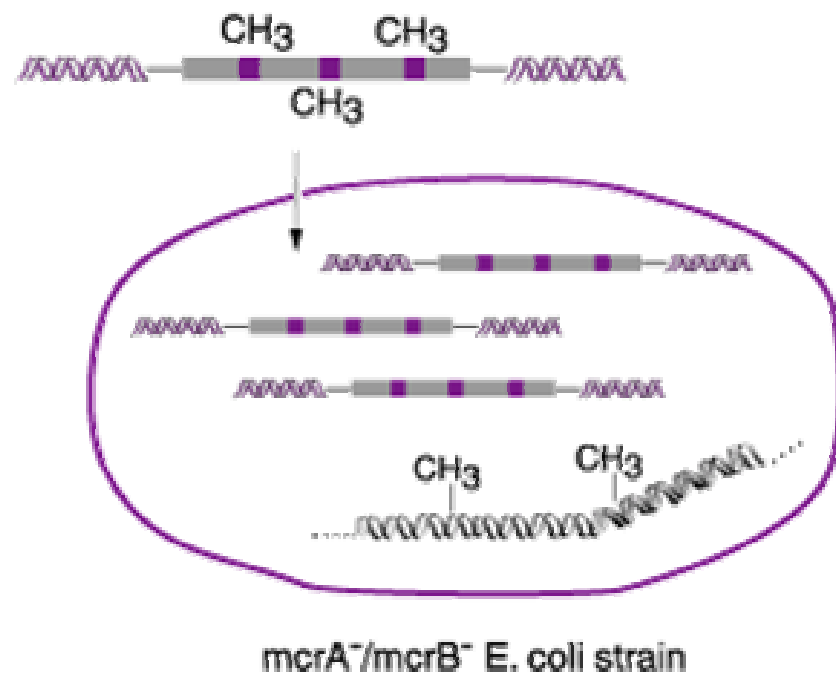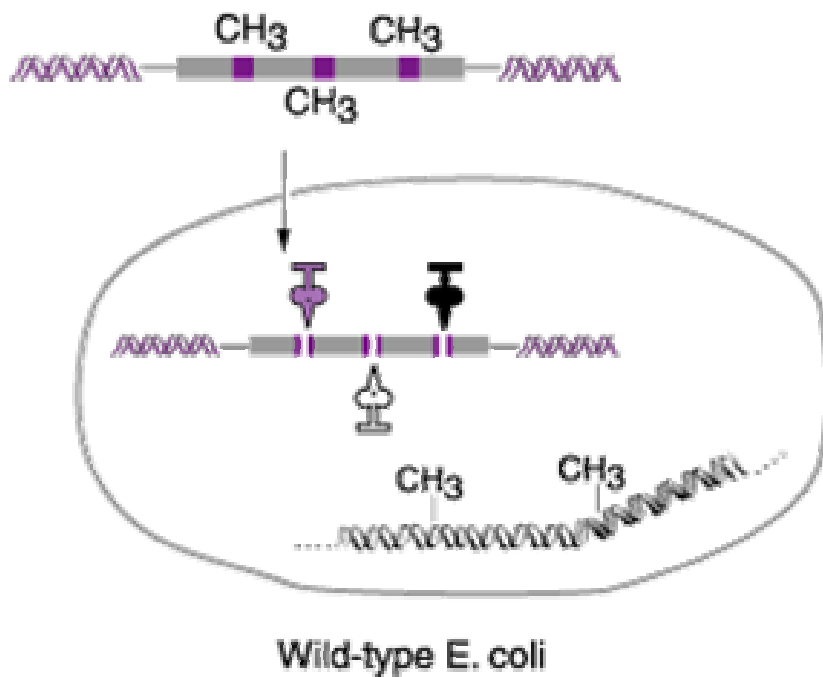
Improvements of E. coli K12 for recombinant DNA methods:

- 3) recA⁻ mutation that suppresses homologous recombination and that makes it more sensitive to UV light.

- 4) Mutation in the endonuclease A gene (endA) that greatly improves the quality of DNA isolated with biochemical techniques.

Genotypes
XL1-Blue strain:
recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'*proAB lacIqZ∆M15 Tn10* (Tetr)]

XL1-Blue MR strain:
∆(mcrA)183 ∆(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac

Major Steps in the Development of Recombinant DNA andTransgenic Technology

1869 Miescher first isolates DNA from white blood cells harvested from pus-soaked bandages obtained from a nearby hospital.

1944 Avery provides evidence that DNA, rather than protein, carries the genetic information during bactetransformation.

1953 Watson and Crick propose the double-helix model for DNA structure based on x-ray results of Franklin and Wilkins.

1955 Kornberg discovers DNA polymerase, the enzyme now used to produce labeled DNA probes.

1961 Marmur and Doty discover DNA renaturation, establishing the specificity and feasibility of nucleic acid hydridization reactions.

1962 Arber provides the first evidence for the existence of DNA restriction nucleases, leading to their purification and use in DNA sequence characterization by Nathans and H. Smith.

1966 Nirenberg, Ochoa, and Khorana elucidate the genetic code.

1967 Gellert discovers DNA ligase, the enzyme used to join DNA fragments together.

1972-1973 DNA cloning techniques are developed by the laboratories of Boyer, Cohen, Berg, and their colleagues at Stanford University and the University of California at San Francisco.

1975 Southern develops gel-transfer hybridization for the detection of specific DNA sequences.

1975-1977 Sanger and Barrell and Maxam and Gilbert develop rapid DNA-sequencing methods.

1981-1982 Palmiter and Brinster produce transgenic mice; Spradling and Rubin produce transgenic fruit flies.

1982 GenBank, NIH's public genetic sequence database, is established at Los Alamos National Laboratory.

1985 Mullis and co-workers invent the polymerase chain reaction (PCR).

1987 Capecchi and Smithies introduce methods for performing targeted gene replacement in mouse embryonic stem cells.

1989 Fields and Song develop the yeast two-hybrid system for identifying and studying protein interactions.

1989 Olson and colleagues describe sequence-tagged sites, unique stretches of DNA that are used to make physical maps of human chromosomes.

1990 Lipman and colleagues release BLAST, an algorithm used to search for homology between DNA and protein sequences.

1990 Simon and colleagues study how to efficiently use bacterial artificial chromosomes, BACs, to carry large pieces of cloned human DNA for sequencing.

1991 Hood and Hunkapillar introduce new automated DNA sequence technology.

1995 Venter and colleagues sequence the first complete genome, that of the bacterium Haemophilus influenzae.

1996 Goffeau and an international consortium of researchers announce the completion of the first genome sequence of a eucaryote, the yeast Saccharomyces cerevisiae.

1996-1997 Lockhart and colleagues and Brown and DeRisi produce DNA microarrays, which allow the simultaneous monitoring of thousands of genes.

1998 Sulston and Waterston and colleagues produce the first complete sequence of a multicellular organisms, the nematode worm Caenorhabditis elegans.

2001 Announcement of the completion of the draft human genome.

The Number of Human Genes is Certainly Larger than the Initially Published Numbers.

Hogenesch et al. (2001), Cell 106: 413-415
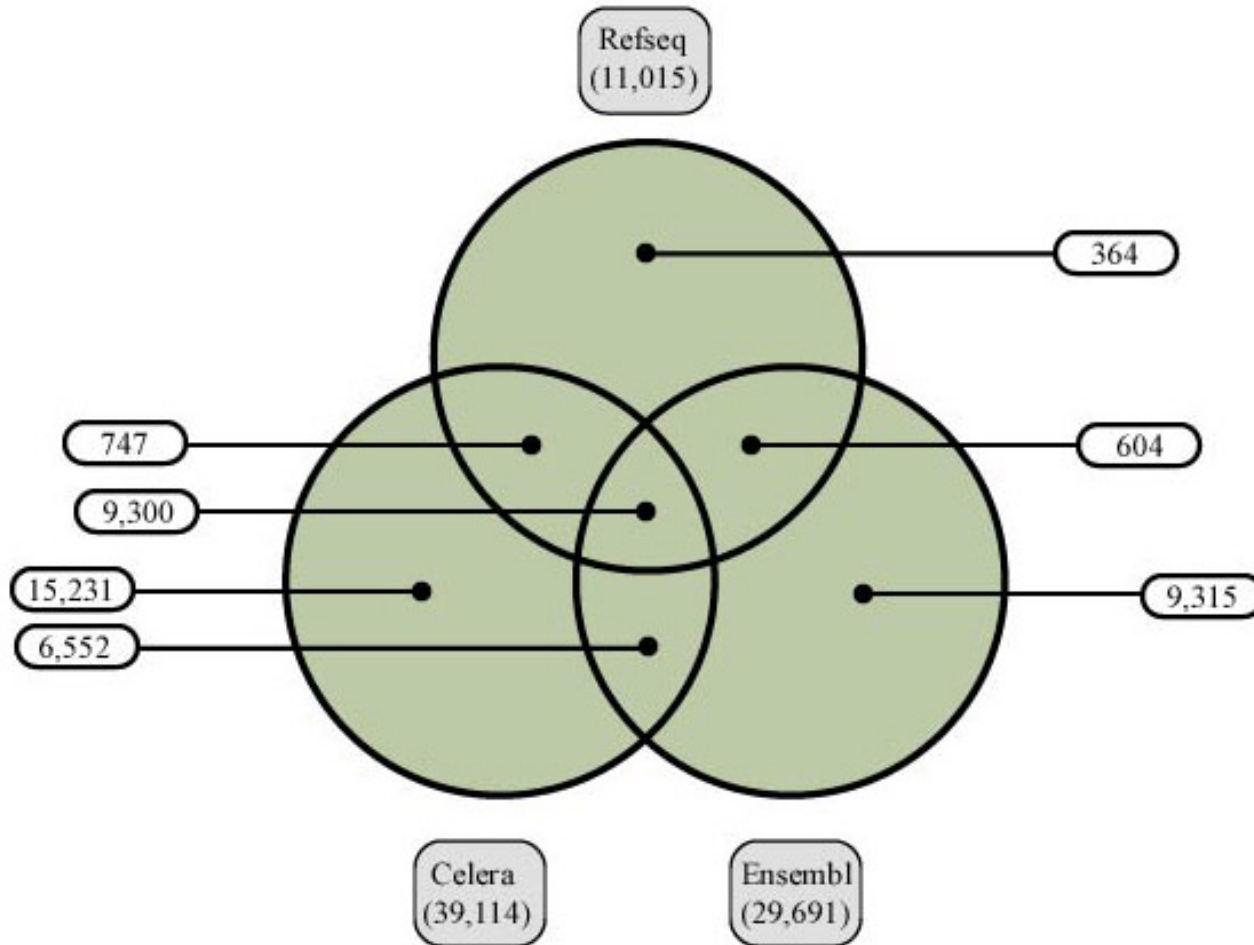
Plasmids
(cloning vectors)

Most of the domains or fragments that are used in cloning vectors originated in naturally occurring virulence and resistance plasmids.

TABLE 2.1 Antibiotics and antibiotic-resistance genes are important tools in applied Molecular Genetics

Antibiotic	Mode of action	Resistance gene	Application
Ampicillin	Inhibits cell wall synthesis by disrupting peptidoglycan cross-linking	β-Lactamase (amp^r) gene product is secreted and hydrolyzes ampicillin	amp^r gene is included on plasmid vectors as a positive selection marker
Tetracycline	Inhibits binding of aminoacyl tRNA to the 30S ribosomal subunit	tet^r gene product is membrane bound and prevents tetracycline accumulation by an efflux mechanism	tet^r gene is a positive selection marker on some plasmids (e.g., pBR322, F' derivatives)
Kanamycin	Inactivates translation by interfering with ribosome function	Neomycin or aminoglycoside phosphotransferase (neo^r) gene product inactivates kanamycin by phosphorylation	neo^r gene is a positive selection marker on plasmids commonly used in eukaryotic molecular genetics
Bleomycin	Inhibits DNA and RNA synthesis by binding to DNA	The bla^r gene product binds to bleomycin and prevents it from binding to DNA	bla^r gene is a positive selection marker on plasmids and also used as a marker in eukaryotic cells (*zeo*)
Hygromycin B	Inhibits translation in prokaryotes and eukaryotes by interfering with ribosome translocation	Hygromycin-B-phosphotransferase (*hph* or hyg^r) gene product inactivates hygromycin B by phosphorylation	hyg^r gene is used as a positive selection marker in eukaryotic cells that are sensitive to hygromycin B
Chloramphenicol	Binds to the 50S ribosomal subunit and inhibits translation	Chloramphenicol acetyl transferase (*CAT* or CM^r) gene product metabolizes chloramphenicol in the presence of acetyl CoA	CAT/CM^r gene is used as a selectable marker, and as transcriptional reporter gene of promoter activity in eukaryotic cells

Design of a cloning vector:

1) origin of autonomous replication
   (ori, ColE1 ori)

2) A genetic marker for selection
   (beta-lactamase gene = amp^R)

3) Unique restriction sites
   (MCS)

4) Minimum amount of
   nonessential DNA

Amp^R

BamHI

EcoRI, BamHI, HindIII, XhoI

ori
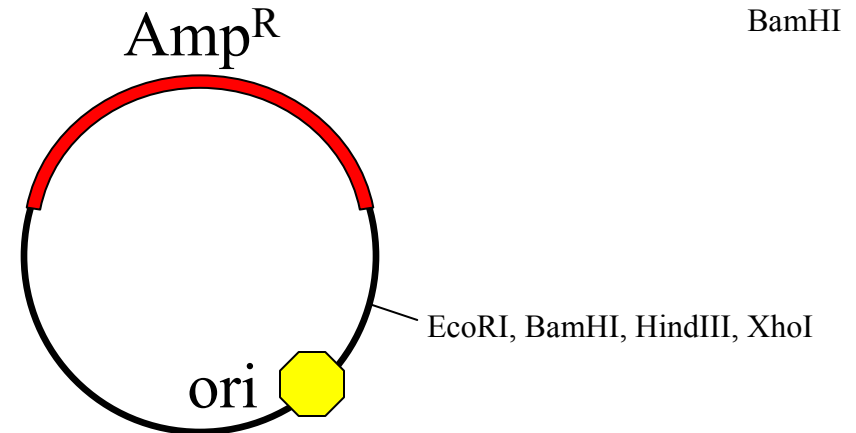
A typical **plasmid cloning strategy** involves **five steps**:

1) **Enzyme restriction** digest of **DNA sample.**

2) **Enzyme restriction** digest of **DNA plasmid vector.**

3) **Ligation of DNA** sample products and plasmid vector.

4) **Transformation** of E. coli K-12 with the ligation products.

5) **Growth on agar** plates with selection for **antibiotic resistance**.

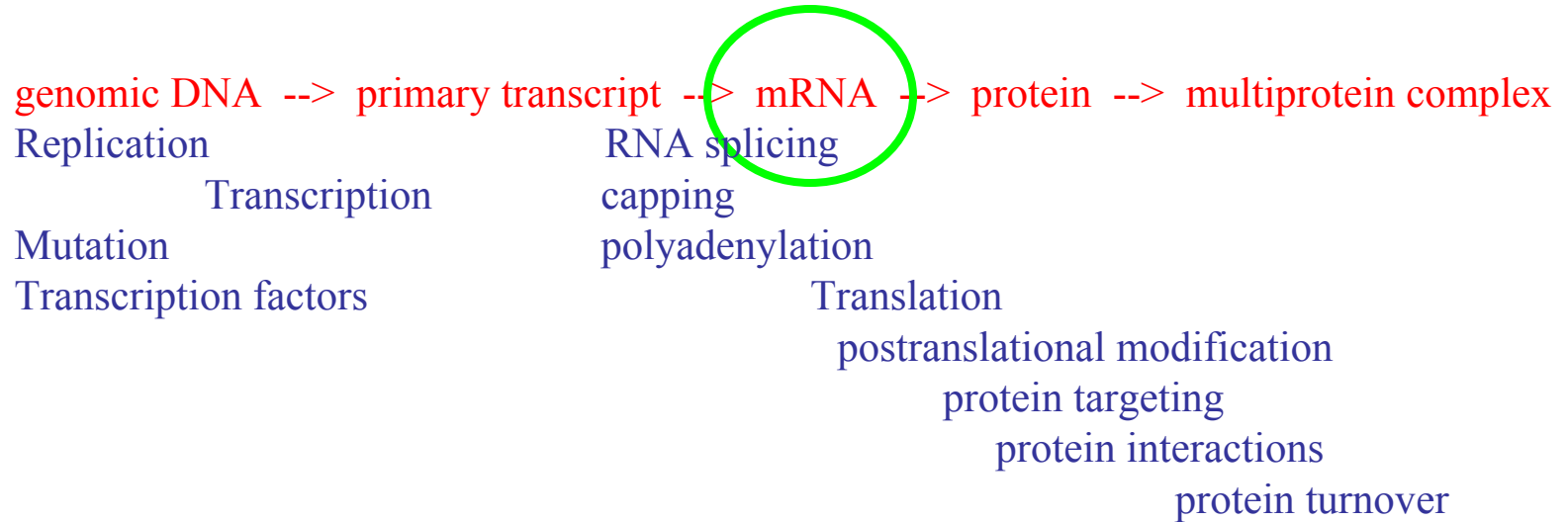See Applied Molecular Genetics, R. Miesfeld, Wiley Publishing, New York, 1999.

Step 3. **Ligation of DNA** sample products and plasmid vector

See Applied Molecular Genetics, R. Miesfeld, Wiley Publishing, New York, 1999.

Step 4. Transformation of E. coli K-12 with the ligation products.

See Applied Molecular Genetics, R. Miesfeld, Wiley Publishing, New York, 1999.

genomic DNA --> primary transcript --> mRNA --> protein --> multiprotein complex

Replication   RNA splicing

           Transcription   capping

Mutation   polyadenylation

Transcription factors   Translation

                                     postranslational modification

                                     protein targeting

                                     protein interactions

                                     protein turnover

One of the milestones of molecular biology:

    cDNA (complementary DNA)   <---   mRNA

    Howard Temin and David Baltimore discovered that the viral RNA-encoded enzyme reverse transcriptase (RT) can synthesize DNA from mRNA templates.

The **Four basic steps** in constructing a cDNA library:

1. **Purification of mRNA** using chemical extraction and oligo-dT purification.

2. **First strand cDNA synthesis** using oligo-dT, random pdN6, or specific primers.

3. **Second strand cDNA synthesis** requires a priming event; done with RNaseH.

4. Repair of cDNA termini and **ligation of adaptor oligos**; clone into vector.

1. Purification of mRNA

Since RNA is single-stranded, any hydrolysis event that breaks the phosphate backbone will result in cleavage of the molecule into subfragments. Two factors contribute to the biochemical **instability of RNA**.

1. Endoribonucleases (**RNases**) are very stable enzymes that cannot be easily inactivated . In fact, human hands are a rich source of RNase and it is therefore necessary to wear clean gloves during RNA isolation procedures and to use RNase-free labware.

2. RNA is thermodynamically less stable than DNA because of the 2' hydroxyl group on the ribose ring that **promotes hydrophilic attack** on the 5'-3' phosphodiester bond to form a **2'-3' cyclic phosphate**. This cyclic phosphate intermediate is stabilized by Mg^{2+}, a component of many biochemical reactions.
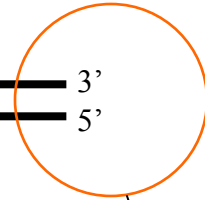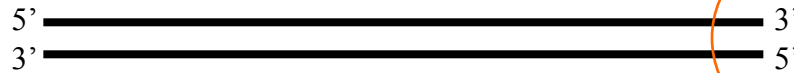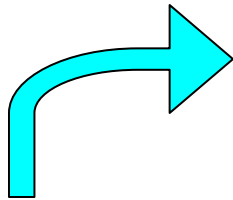
Chicken embryonic cochlea --> Total RNA purification --> mRNA (PolyA$^+$ RNA)
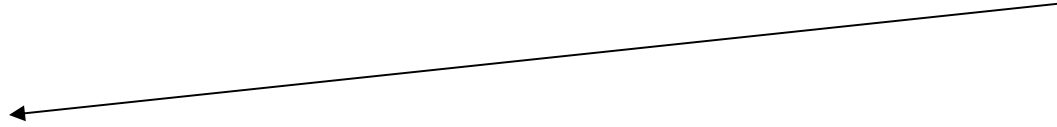
First strand cDNA synthesis --> Second strand cDNA synthesis

Adapter ligation

5' ================================ 3'
3' ================================ 5'

GAATTCGGCACGAG
    GCCGTGCTC

EcoRI adaptor

NNNNAAAAAAAAAAAAAAAAAAAAAAAAAAAAACTCGAG
NNNNTTTTTTTTTTTTTTTTTTTTTTTTTTTGAGCTC

XhoI restriction
site

500 chicken cochlear ducts (enriched sensory epithelium) - embryonic days 14-19

↓

100 µg total RNA

↓

5 µg polyA$^+$ RNA (mRNA)

↓

First strand cDNA synthesis
Second strand cDNA synthesis
Adapter ligation

←————— pAD-Gal4 [EcoRI/XhoI]

↓

cDNA library in pAD-Gal4

5×10^6 independent cDNA clones in pAD-Gal4

→amplification (transformation into E. coli and
   selection with ampicillin -> plasmid preparation

See Applied Molecular Genetics, R. Miesfeld, Wiley Publishing, New York, 1999.

Practical part: Analysis of four cDNA clones randomly selected from a chicken late embryonic cochlea sensory epithelium cDNA library

Day 1 (Lecture #3):   Inoculation of 4 overnight cultures (16hr growth) of plasmid-bearing E. coli XL-1 blue.

Day 2 (Lecture #4):   Harvesting of the bacteria by centrifugation and storage of the cell pellets at -80°C

Day 3 (Lecture #5)   DNA preparation (4 samples)
                      Absorption measurement at 260nm to determine the DNA concentration
                      Sample preparation for automated DNA sequencing
Day 4 (A day before Lecture #6): Sample submission for DNA sequencing

Day 5 (Lecture #7):   DNA restriction digest
                      Agarose gel electrophoresis
                      Hand-out of the sequencing results

Your job: **A)** Find as much information as possible about your clones.
           Remember that the clones stem from an animal model. Why is the chicken an appropriate animal model for inner ear gene discovery? What are disadvantages of the chicken model? Pick one or two clones that may have significance for inner ear function. What is known about these genes?

           B) Document your experiments in a way that someone else could repeat them without having any further information.

Additonal Readings

Applied Molecular Genetics, R. Miesfeld, Wiley Publishing, New York, 1999.