EXPERIMENT 21

Constructing and Characterizing a Recombinant DNA Plasmid

Theory

The discovery of plasmids and restriction endonucleases is largely responsible for the enormous technological advances made in the field of molecular biology over the past 20 years. A desired DNA fragment or gene sequence can be identified, excised from the chromosome, isolated, and cloned into a number of different plasmids or vectors. This gene can then be sequenced, mutated, and/or expressed to gain insight into the function of the protein that it encodes. The plasmid carrying the gene of interest can be introduced into a host cell and faithfully propagated during the cell's normal cycle of DNA replication and cell division. In this experiment, you will subclone the gene for aminoglycoside-3′-phosphotransferase from plasmid pUC4K into pUC19. This gene will confer resistance to the antibiotic, kanamycin, a phenotype that can be exploited in the selection process for cells that obtained the desired plasmid. The resulting pUC19/4K recombinant plasmid will be selected for, and the construction of the plasmid will be verified using restriction endonuclease digestion and agarose-gel electrophoresis.

Plasmid pUC4K is a commercially available *Escherichia coli* vector that contains the kanamycin resistance (kan^R) gene from transposon (Tn) 903 (Fig. 21-1). The gene is flanked on the 5′ and 3′ sides by four different restriction endonuclease recognition sites. Thus, the kan^R gene can be excised from pUC4K and inserted into virtually any other existing plasmid to create a new vector that confers kan^R to its host. The kan^R gene can also be inserted into the open reading frame of a gene of interest, allowing you to study the effect of deletion of the gene (null mutation) on the organism. This type of gene disruption experiment has proven to be very useful in efforts to determine the function of particular gene products in various biological processes.

Plasmid pUC19 (and its derivative, pUC18) is one of the most popular and widely used *E. coli* cloning vectors in molecular biology (see Fig. 21-1). Like pUC4K, pUC19 contains the gene that confers ampicillin resistance (amp^R) to its host (*bla*, β-lactamase). This vector also contains a multiple cloning site (MCS) within the sequence of the LacZα peptide. The LacZα peptide, which encodes the N-terminal 150 amino acids of β-galactosidase, will function in *trans* to complement (restore) β-galactosidase activity in a host strain deleted for the LacZα peptide. The position of the MCS on pUC19 will play an important role in the selection process to identify cells that harbor the desired pUC19/4K plasmid. If cloned into pUC19 in the same reading frame as the LacZα peptide sequence, a gene of interest can be expressed as a fusion protein with the N-terminus of the LacZα peptide. Like the LacZα peptide, the expression of the fusion protein will now be under the control of the *lac* promoter, which is inducible with the addition of isopropylthio-β-galactoside (IPIG) to the growth medium. Recall that the expression of genes under the control of the *lac* promoter is normally low in the presence of the *lac* repressor protein (LacI), the gene for which is also contained in pUC19 (see Fig. 21-1) (See introduction to Experiment 7).

On Day 1 of the experiment, you will digest pUC4K with *Eco*RI and *Xho*I. You will also digest

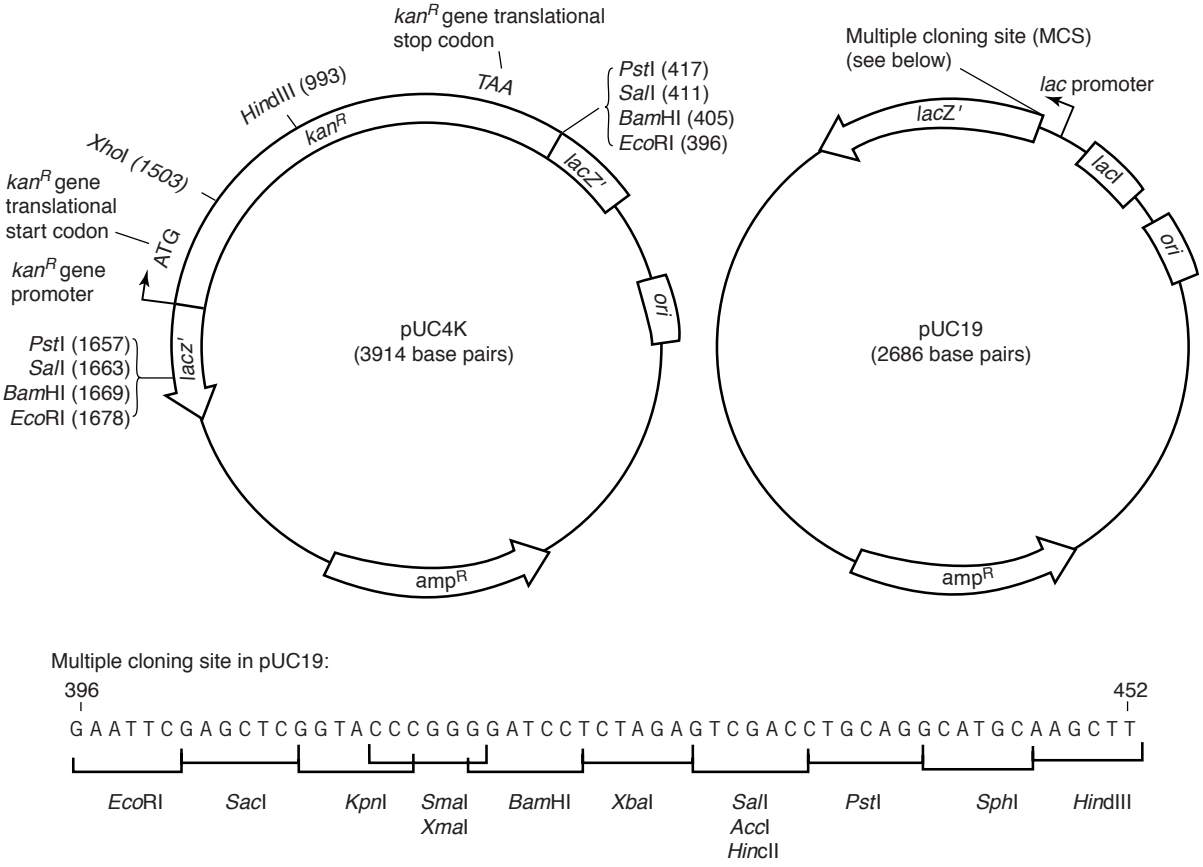

Figure 21-1 The pUC19 and pUC4K plasmids.

pUC19 with *Eco*RI, *Bam*HI, and *Sal*I. Following digestion, the DNA fragments resulting from these reactions (Fig. 21-2) will be mixed together and precipitated with ethanol. Next, T4 DNA ligase and ATP will be added, and the ligation reaction will be allowed to proceed overnight. As shown in Fig. 21-2, the 5′ single-stranded DNA overhang produced in the *Xho*I digest on pUC4K will be compatible with the 5′ single-stranded DNA overhang produced in the *Sal*I digest on pUC19. Although DNA ligase will be able to join these two fragments together, the resulting sequence will no longer be recognized by *Xho*I or *Sal*I. The 5′ single-stranded DNA overhangs produced in the *Eco*RI digests on pUC4K and pUC19 will also be compatible, and will be joined by DNA ligase to regenerate the *Eco*RI site. This type of *directional*, or *forced*, *cloning* ensures that the kan^R gene will ligate into pUC19 in a specific orientation (see Fig. 21-2). If both the kan^R gene and pUC19 were digested with *Eco*RI only, this would not be the case (see Fig. 21-3).

Also note that the *Xho*I digest on pUC4K cleaves off the promoter and start codon (ATG) for the kan^R gene, rendering it incapable of being transcribed or translated by the host cell (see Fig. 21-1). However, the *Xho*I/*Sal*I ligation of the kan^R gene into pUC19 will put it into the same reading frame as the LacZα peptide sequence. What will be produced is a *lacZ/kan^R* gene fusion protein that will confer kan^R to the host cell *and* that is inducible in the presence of IPTG. This differs from the case in pUC4K, where the kan^R gene is *constitutively* expressed under the control of a different promoter.

Since the ligation reaction is performed in the presence of a number of different DNA fragments

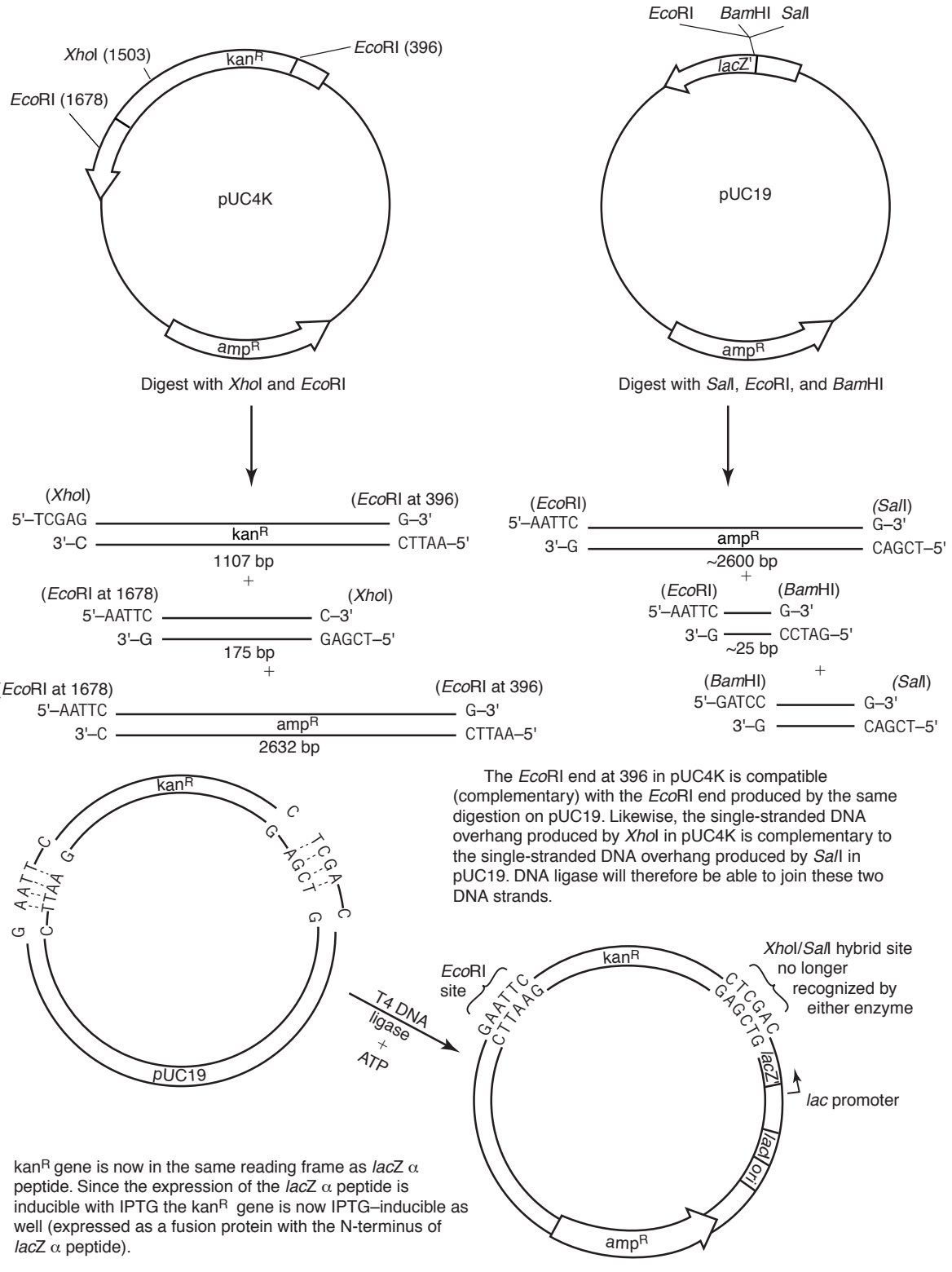

Figure 21-2 DNA fragments produced from pUC4K and pUC19 following restriction enzyme digestion.

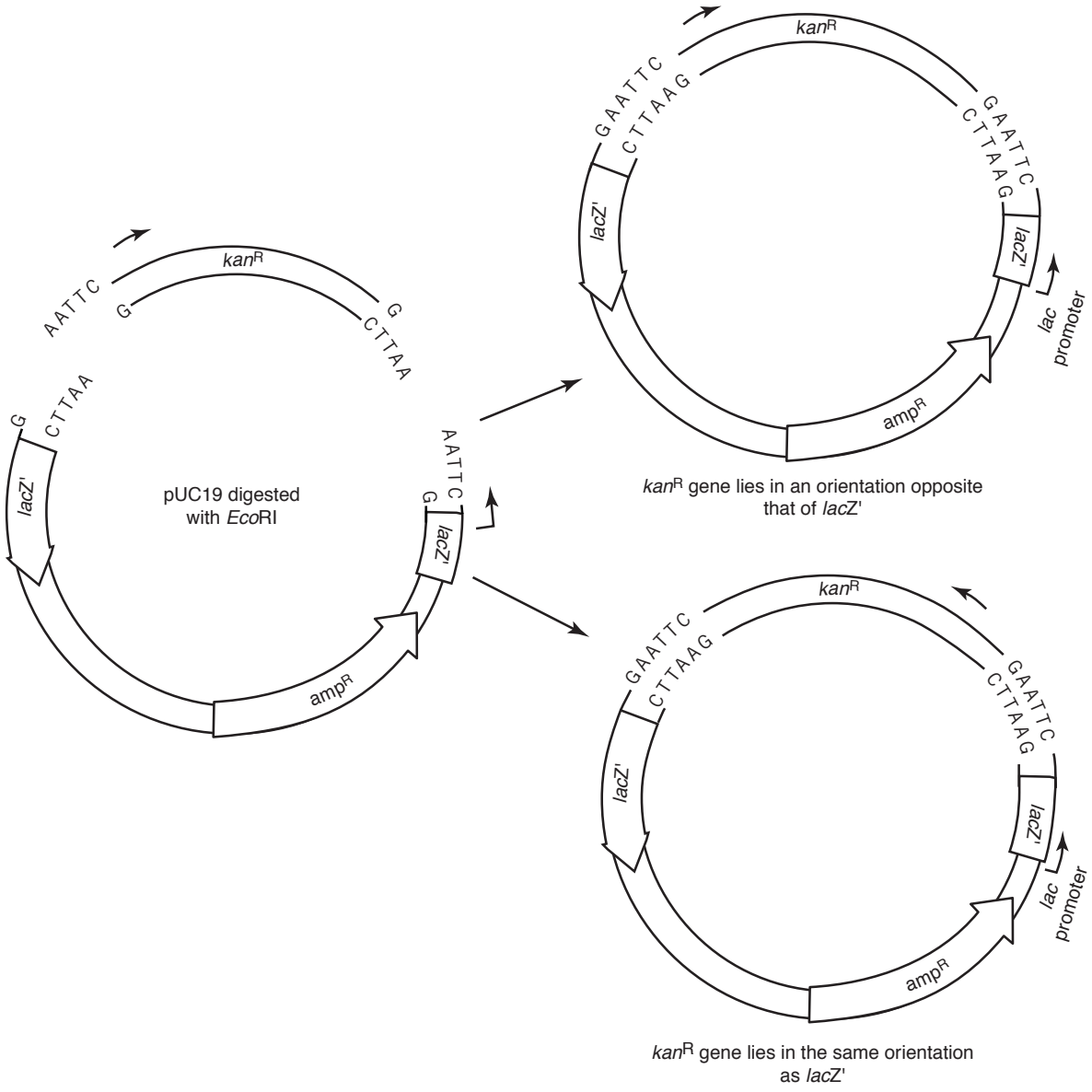

Figure 21-3 Two recombinant pUC19/4K plasmids are possible if both plasmids are digested with a single enzyme. The two resulting plasmids differ in the orientation of the kan^R gene in pUC19. The arrow (→) indicates the direction of transcription of the kan^R gene.

with compatible single-stranded DNA overhangs, the desired pUC19/4K recombinant plasmid is by no means the only plasmid that could be produced during the ligation reaction. For instance, it is possible that the *Eco*RI/*Xho*I fragment carrying the kan^R gene from pUC4K will ligate back into pUC4K. It is also possible that the *Eco*RI/*Sal*I fragment will ligate back into pUC19. The possibility of reforming either of the two parent plasmids is minimized, however, because both events would require a successful three-point ligation (see Fig. 21-4). The two-point ligation that is required to produce the desired pUC19/4K plasmid is statistically much more likely to occur.

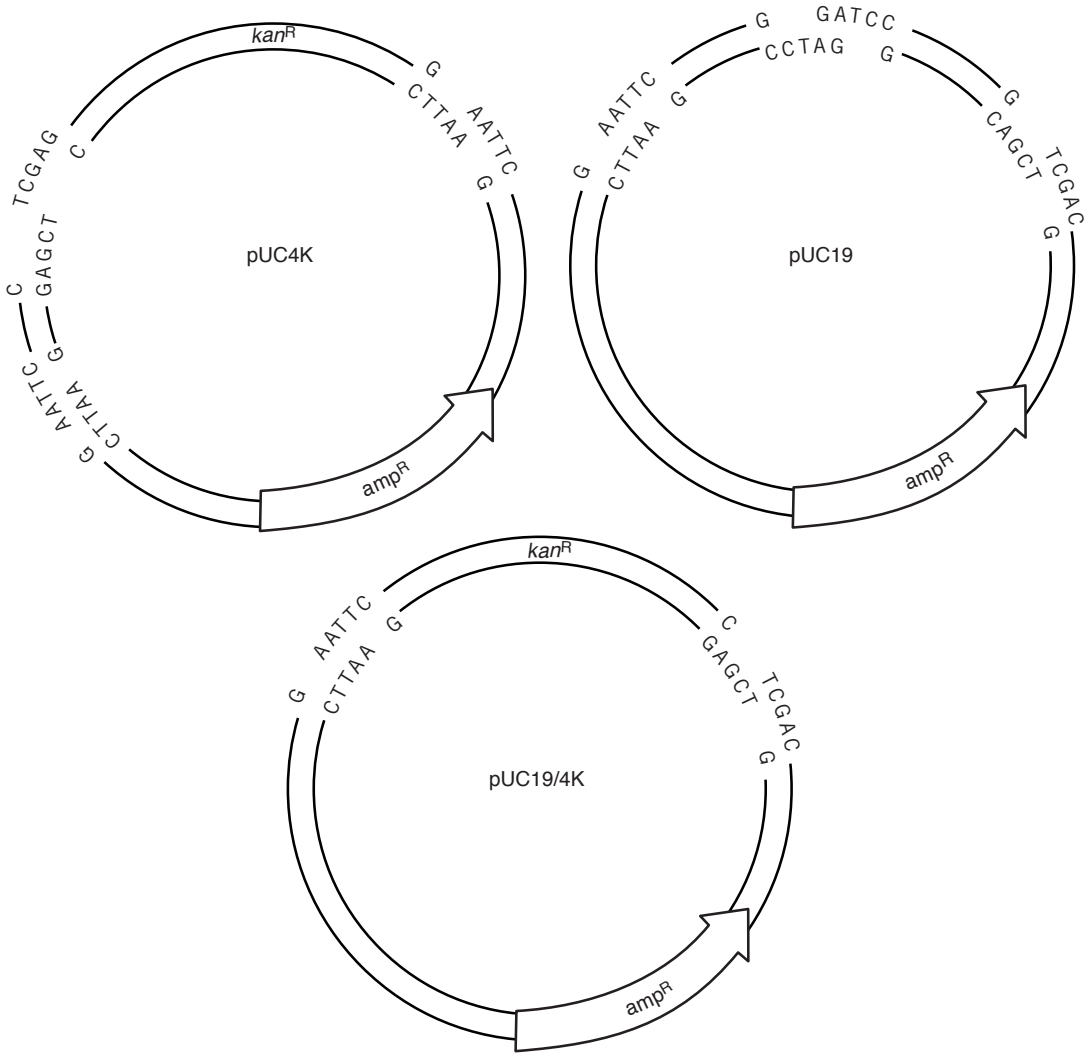

Figure 21-4 On a statistical basis, the two-point ligation required to form the recombinant pUC19/4K plasmid is much more likely than the three-point ligations required to form pUC19 or pUC4K.

How do you distinguish between cells carrying the pUC19 plasmid and the pUC19/4K recombinant plasmid? Recall that pUC19 carries the gene for amp^R, while pUC19/4K carries the genes for amp^R and kan^R. Any colony that will grow in the presence of ampicillin but not kanamycin most likely harbors pUC19. In addition to the antibiotic selection just described, pUC19 will also confer another unique phenotype to the host cell that will distinguish it from those carrying pUC19/4K. The host cell used in this experiment is *Epicurian coli* XL1-Blue. This strain contains a deletion on the chromosome of the first 450 base pairs of its *lacZ* coding sequence (the LacZα peptide). The pUC19 plasmid will be able to restore β-galactosidase activity in the host strain, since this plasmid contains an uninterrupted LacZα peptide coding sequence (the strain will be *complemented* for β-galactosidase activity). The LacZα peptide expressed from the pUC19 plasmid will be able to

noncovalently interact with the C-terminal fragment of LacZ produced by the host cell, creating a functional β-galactosidase enzyme. This technique of selection is therefore referred to as α-*complementation*. If cells containing pUC19 are placed on an agar plate containing IPTG and 5-bromo-4-chloro-3-indolyl-β,D-galactopyranoside (Xgal), LacZα peptide expression will be induced, and the Xgal substrate will be converted to a blue-colored precipitate. Blue colonies on an IPTG/Xgal plate will therefore be indicative of the presence of an intact LacZα peptide sequence in the plasmid (pUC19). Since pUC19/4K will have the kan^R gene inserted into the LacZα peptide sequence, it will not be able to complement the host strain for β-galactosidase activity, and it will produce white colonies on an IPTG/Xgal plate.

How do you distinguish between cells carrying the pUC4K plasmid and the pUC19/4K recombinant plasmid? This is not as easy as the situation described above, since both plasmids contain an insertion in the LacZα peptide sequence. Both plasmids will confer kan^R and amp^R to the host strain and both will produce white colonies in the presence of IPTG and Xgal. Still, there is one difference between these two plasmids that you will be able to exploit during the selection process. Recall that the kan^R gene is *constitutively* expressed from the pUC4K plasmid, while the kan^R gene is under the control of the *lac* promoter in the pUC19/4K recombinant plasmid. Therefore, any white colony that grows well on a plate containing only kanamycin (no IPTG) most likely carries pUC4K. In contrast, any colony that grows well on a kanamycin plate containing IPTG, but poorly on a plate containing only kanamycin, most likely carries pUC19/4K. The basis for the different types of selection that will be used in this experiment are outlined in Table 21-1.

Table 21-1 Basis of Selection for Clones Carrying the Desired pUC19/4K Recombinant Plasmid

Plasmid	Kan^R?	Amp^R?	Kan^R IPTG-Inducible?	Color on IPTG/Xgal Plate
pUC19	No	Yes	—	Blue
pUC4K	Yes	Yes	No	White
pUC19/4K	Yes	Yes	Yes	White

Supplies and Reagents

pUC19 (0.25 μg/μl)—Pharmacia catalog #27–4951-01
pUC4K (0.5 μg/μl)—Pharmacia catalog #27–4958-01
Distilled water (sterile)
*Pst*I (10 units/μl) with 10X buffer—Gibco BRL catalog #15215-023
*Eco*RI (10 units/μl) with 10X buffer—Gibco BRL catalog #15202-021
*Xho*I (10 units/μl) with 10X buffer—Gibco BRL catalog #15231-020
*Sal*I (10 units/μl) with 10X buffer—Gibco BRL catalog #15217-029
*Bam*HI (10 units/μl) with 10X buffer—Gibco BRL catalog #15201-031
*Hin*dIII (10 units/μl) with 10X buffer—Gibco BRL catalog #15207-020
Water baths at 37°C, 15°C, and 42°C
Phenol (Tris-saturated)
Chloroform
Isoamyl alcohol
3 M sodium acetate (pH 5.2)
Absolute ethanol
Dry ice/ethanol bath (−70°C)
Microcentrifuge
1.5-ml plastic microcentrifuge tubes
70% vol/vol ethanol in distilled water
Vacuum microcentrifuge (Speed-Vac)
P-20, P-200, and P-1000 Pipetmen with disposable tips (sterile)
T4 DNA ligase (1 unit/μl) with 5X buffer—Gibco BRL catalog #15224-025
Epicurian coli XL1-Blue competent cells—Stratagene, catalog #200130
Yeast-tryptone (YT) broth (1% Bactotryptone, 0.5% yeast extract, 0.5% NaCl)
100 mM IPTG solution in sterile distilled water (filter-sterilized)
Ampicillin (100 mg/ml) prepared in sterile distilled water—Sigma catalog #A-9518
Kanamycin (100 mg/ml) prepared in sterile distilled water—Sigma catalog #K-4000
Agar
Culture shaker with test tube racks at 37°C
Petri plates (disposable, plastic)
Sterile glass rod (for spreading bacteria over the plate)
Sterile toothpicks
37°C incubator

Large (16 × 125 mm) glass test tubes (sterile)
1-kb DNA ladder size standard—Gibco BRL catalog #15615-016
Ethidium bromide solution in 0.5X TBE buffer (0.5 μg/ml)—*Toxic!*
Polaroid camera with film and a 256-nm light box
GTE lysis buffer (25 mM Tris, pH 8.0, 50 mM glucose, 10 mM EDTA, 10 μg/ml lysozyme)
Fresh solution of 0.2 N NaOH and 1% SDS in distilled water
Potassium acetate solution (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, 28.5 ml of distilled water)
RNaseA (10 mg/ml in water)—*Boil this solution for 10 min to inactivate any DNase*
6X DNA loading buffer (0.25% wt/vol bromophenol blue, 0.25% wt/vol xylene cyanole, 30% wt/vol glycerol in distilled water)
Agarose (electrophoresis grade)
5X TBE buffer (54 g/liter Trizma base, 27.5 g/liter boric acid, 20 ml/liter of 0.5 M EDTA, pH 8.0)
Agarose-gel electrophoresis apparatus with casting box and 10-well comb
5-bromo-4-chloro-3-indolyl-β,D-galactopyranoside (Xgal), 40 mg/ml solution in dimethylformamide—*Toxic!* (Sigma catalog #B-4252)

Protocol

Day 1: Restriction Endonuclease Digestion of pUC19 and pUC4K, Ethanol Precipitation of DNA Fragments, and DNA Ligation

1. Set up the following reactions in two labeled 1.5-ml sterile microcentrifuge tubes:

Digestion of pUC19	Digestion of pUC4K
14 μl of distilled water (sterile)	14 μl of distilled water (sterile)
2 μl of 10X React Buffer 2	2 μl of 10X React Buffer 2
1 μl of pUC19 (0.25 μg/μl)	2 μl of pUC4K (0.50 μg/μl)
1 μl of *Eco*RI (10 units/μl)	1 μl of *Eco*RI (10 units/μl)
1 μl of *Bam*HI (10 units/μl)	1 μl of *Xho*I (10 units/μl)
1 μl of *Sal*I (10 units/μl)	

2. Incubate both reactions at 37°C for 1.5 hr.
3. Transfer both reactions to a single tube and add 160 μl of sterile distilled water.
4. Add 200 μl of Tris-saturated phenol:chloroform:isoamyl alcohol (25:24:1). Mix vigorously in a Vortex mixer for 1 min and centrifuge for 3 min to separate the aqueous and organic phases. This step is done to denature and remove the restriction endonucleases from the DNA solution.
5. Remove the aqueous (upper) phase and place it in a clean microcentrifuge tube. Repeat step 4 (extraction of aqueous phase) with 200 μl of chloroform:isoamyl alcohol (24:1). This step is done to remove all traces of phenol from the solution, which may denature the T4 DNA ligase during the ligation reaction. Dispose of the organic phase from this extraction in a waste beaker designated by the instructor.
6. Transfer the upper (aqueous) phase to a fresh microcentrifuge tube. *Do not transfer any of the organic phase during this process.* Dispose of the organic phase from this extraction in a waste beaker designated by the instructor.
7. Add 10 μl of 3 M sodium acetate (pH 5.2) to the aqueous phase, as well as 800 μl of absolute ethanol. Cap and invert the tube several times to mix. Submerge the closed tube in a dry ice/ethanol bath (−70°C) for 10 min. This is done to precipitate plasmid DNA.
8. Centrifuge the sample at 4°C in a microcentrifuge for 30 min. Carefully remove the supernatant from the precipitated DNA pellet. *Depending on how pure the DNA is, this pellet may be translucent and difficult to see. It may help you to place the outside hinge of the microcentrifuge tube toward the outside of the rotor during the centrifugation so that you will know where the DNA pellet is at the bottom of the tube. You may also find the use of Pellet Paint to be helpful during this procedure. This product, sold by Novagen (catalog #69049-3), is a pink chromophore that will bind the DNA and give the pellet a color that will be easier to identify at this point.*
9. Add 1 ml of ice cold 70% ethanol to the DNA pellet, centrifuge at 4°C for 5 min, and carefully remove the supernatant from the precipitated DNA pellet. This step is done to remove the salt that was added to the DNA to aid in the precipitation. *This salt, if not removed, could interfere with the DNA ligation reaction.*

10. Allow the DNA pellet to air dry (~10 min) or dry it down briefly (~2 min) in the vacuum microcentrifuge.
11. Resuspend the DNA pellet in 20 μl of sterile distilled water. Store the DNA on ice until ready to proceed with the ligation reaction.
12. Carefully mix the following together in a sterile, 1.5-ml microcentrifuge tube:

    15 μl of DNA sample (from step 11)
    4 μl of T4 DNA ligase buffer (250 mM Tris, pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM dithiothreitol, 25% wt/vol polyethylene glycol-8000)
    1 μl of T4 DNA ligase (1 unit/μl)

13. Incubate the reaction at room temperature for 1 hr and then at 15°C overnight. Store the reaction at 4°C for use on Day 2. Also, store the remainder of your DNA sample from step 11 (5 μl) at 4°C for use on Day 2.

Day 2: Transformation of *E. coli* Host Cells

1. Add 30 μl of sterile distilled water to your 20-μl ligation reaction. Add 2.5 μl of 3 M sodium acetate (pH 5.2) and 200 μl of absolute ethanol. Cap the tube and invert several times to mix. Submerge the capped tube in a dry ice/ethanol bath (−70°C) for 10 min.
2. Centrifuge the sample at 4°C for 30 min. Remove the supernatant from the precipitated DNA pellet and add 1 ml of ice cold 70% ethanol. Centrifuge the sample again at 4°C for 5 min. Remove the supernatant from the precipitated DNA pellet.
3. Air dry the DNA pellet as before or dry briefly in the vacuum microcentrifuge.
4. Resuspend the dried DNA pellet in 5 μl of sterile distilled water.
5. Obtain a microcentrifuge tube from the instructor containing 160 μl of freshly thawed *Epicurian coli* XL1-Blue competent cells (with β-mercaptoethanol added as per the manufacturer's instructions). *Keep these cells on ice at all times!*
6. Add 40 μl of these cells to four *prechilled* (on ice) round-bottomed, 15-ml polypropylene culture tubes. Again, *keep the cells on ice at all times* and label the tubes 1 to 4.
7. To tube 1, add 5 μl of the ligation mixture prepared in step 4. To tube 2, add 5 μl of a 1-ng/ml solution of untreated pUC19. To tube 3, add 5 μl of the 1X ligation buffer (no DNA). To tube 4, add 5 μl of *unligated* DNA sample left over from Day 1 (see Table 21-2 and step 13 of the Day 1 protocol).
8. Incubate the cells with these samples on ice for 15 min, swirling gently every 2 min. Quickly remove the tubes from the ice and place them in a 42°C water bath. Incubate the tubes at 42°C for *exactly* 45 sec. Quickly remove the tubes from the water bath and incubate them again on ice for 2 min. On heat shock, the competent *E. coli* cells will take up the intact plasmid DNA produced during the ligation reaction. *The competent cells are very compromised at this point, and they will die if the incubation is not performed at exactly 42°C for exactly 45 sec. The incubation on ice will prevent the cells from dying after the heat shock.*
9. Add 1 ml of YT broth (use sterile technique) and 30 μl of sterile 100 mM IPTG solution to each tube. Incubate the cells at 37°C with *gentle* shaking for 1 hr. During this time, the cells will recover from the heat shock and begin to express genes (such as kan^R in the pUC19/4K plasmid) under the control of the *lac* promoter.
10. Obtain five agar plates containing IPTG (40 μg/ml), Xgal (40 μg/ml), and ampicillin (100 μg/ml). Obtain two agar plates containing IPTG (40 μg/ml), Xgal (40 μg/ml), and kanamycin (50 μg/ml).
11. Remove 100- and 200-μl aliquots from culture tube 1 and place on two separate IPTG/Xgal/amp plates (see Table 21-3). Plate the same volumes of cells from culture tube 1 on the two separate IPTG/Xgal/kan plates.

Table 21-2 Procedure for *E. coli* Transformation

Tube	DNA Sample	Volume (μl)	Volume of *E. coli* XL1-Blue cells (μl)
1	Ligation mixture	5	40
2	pUC19 (untreated)	5	40
3	Ligation buffer (no DNA)	5	40
4	Unligated DNA	5	40

Table 21-3 Procedure for Plating and Selection of Transformed Bacteria

Transformation Tube	Volume to Be Plated (μl)	Components in Agar Plate
1	100	IPTG/Xgal/amp
1	100	IPTG/Xgal/kan
1	200	IPTG/Xgal/amp
1	200	IPTG/Xgal/kan
2	200	IPTG/Xgal/amp
3	200	IPTG/Xgal/amp
4	200	IPTG/Xgal/amp

12. Remove 200 μl from culture tube 2 and place on an IPTG/Xgal/amp plate. Remove 200 μl from culture tube 3 and place on an IPTG/Xgal/amp plate. Remove 200 μl from culture tube 4 and place on an IPTG/Xgal/amp plate. *Be sure that you label each plate so that you know what antibiotic the plate contains and what transformation mixture was placed on each plate.*
13. Using sterile technique (to be demonstrated by the instructor), use a sterile bent glass rod to spread the bacteria evenly over the surface of each plate. Allow the surface of the plates to dry for 5 min, invert the plates (agar side up), and place them in the 37°C incubator overnight. The colonies that appear on these plates will be grown in culture the next day, and plasmid DNA will be isolated from them on Day 3. The plates must be incubated at 37°C for at least 24 hr. After this incubation, the plates may be stored at 4°C for several days. *We have found that the blue color will develop much better in colonies that contain a plasmid with an intact* LacZα *peptide sequence if this is done. Incubating the plates at 4°C will make it much easier to distinguish between blue and white colonies when the plates are counted (see below).*

Off day: Growth of Transformed Cells

1. Remove your seven agar plates from the 37°C incubator. Obtain four large glass culture tubes from the instructor that each contain 3 ml of sterile YT broth supplemented with 100 μg/ml ampicillin. Label the tubes 1 to 4.
2. Analyze the two IPTG/Xgal/kan plates *containing the transformation mixture from tube 1* to find *four colonies that are white in color*. Obtain two fresh agar plates: one containing kanamycin and one containing kanamycin *and* IPTG. On the bottom surface of each plate, use a marker to divide the plate into four quadrants, labeled 1 to 4.
3. Using a sterile toothpick, stab into the middle of a white colony that is present on this IPTG/Xgal/kan plate. Remove the toothpick and stab the end that came into contact with the bacteria first on quadrant 1 of the kan plate *and then* into quadrant 1 of the IPTG/kan plate. Finally, drop the toothpick (bacteria end down) into tube 1 containing ampicillin supplemented YT broth. Repeat step 3 for each of the other quadrants on the two plates, picking a *new* white colony on the IPTG/Xgal/kan plate each time.
4. Incubate the cultures at 37°C with shaking overnight. These strains will be used on Day 3 to isolate plasmid DNA. Place the two replica plates produced in step 3, agar side up, in the 37°C incubator overnight.
5. Count the number of colonies from transformation culture tube 1 present on the two IPTG/Xgal/kan plates. How many of these colonies are blue and how many are white? Explain these results in terms of what you know about the properties of each of the plasmids that these bacterial colonies may contain.
6. Count the number of colonies from transformation culture tube 1 present on the two IPTG/Xgal/amp plates. How many of these colonies are blue and how many are white? Explain these results in terms of what you know about the properties of each of the plasmids that these bacterial colonies may contain.
7. Count the number of colonies present on the IPTG/Xgal/amp plate containing bacteria from transformation culture tube 2. Why was this control experiment performed? What color are these colonies? Explain. Describe what a low number of colonies on this plate would indicate, as well as possible causes for this result.
8. Count the number of colonies present on the IPTG/Xgal/amp plate containing bacteria from transformation culture tube 3. Why was

this control experiment performed? Describe what a large number of colonies on this plate would indicate, as well as possible causes for this result.

9. Count the number of colonies present on the IPTG/Xgal/amp plate containing bacteria from transformation culture tube 4. Why was this control experiment performed? What color are these colonies? Explain. Describe what a large number of blue or white colonies would indicate, as well as possible causes for this result.

Day 3: Isolation of Plasmid DNA

1. Remove the four culture tubes from the 37°C shaker and add 1.5 ml of each culture to four separate microcentrifuge tubes labeled 1 to 4. Cap each tube and centrifuge for 2 min at room temperature to harvest the cells. Remove the supernatant from the bacterial pellet.
2. Add the remaining 1.5 ml of each culture to the appropriate microcentrifuge tubes and repeat step 1.
3. Add 100 μl of GTE (lysis) buffer to each bacterial cell pellet. Resuspend each cell pellet thoroughly by repeated pipetting or mixing with a Vortex mixer. Incubate the tubes at room temperature for 5 min.
4. Add 200 μl of *freshly prepared* 0.2 N NaOH–1% SDS solution to each tube. Cap and invert several times to mix. The solution will turn from turbid to more translucent as the cells are completely lysed and the DNA and proteins are denatured. Incubate the tubes on ice for 5 min.
5. Add 150 μl of 3 M potassium acetate solution to each tube. Cap and invert several times to mix. The precipitate that forms contains much of the denatured proteins produced in step 4, as well as chromosomal DNA that was not able to renature correctly when the pH was quickly lowered. Incubate the tubes on ice for 10 min.
6. Centrifuge the tubes for 15 min at 4°C. Remove the *supernatant* from each tube and place in four fresh microcentrifuge tubes labeled 1 to 4. The supernatant contains the plasmid DNA, while the pellet contains denatured proteins, chromosomal DNA, membrane components, and other cell debris. The tubes containing this pellet may be discarded.
7. Add 500 μl of Tris-saturated phenol:chloroform:isoamyl alcohol (25:24:1) to each tube containing the supernatant fraction. Mix each tube with a Vortex mixer for 1 min, centrifuge for 5 min at room temperature, and transfer the aqueous (upper) phases to four fresh microcentrifuge tubes labeled 1 to 4. This step is done to remove any remaining proteins and lipid components from the plasmid DNA. Dispose of the organic phase from this extraction in a waste beaker designated by the instructor.
8. Repeat the aqueous phase extraction procedure described in step 7 with 500 μl of chloroform : isoamyl alcohol (24:1). Transfer the aqueous (upper) phases to four fresh microcentrifuge tubes. This step is done to remove all traces of phenol from the solution. Dispose of the organic phase from this extraction in a waste beaker designated by the instructor.
9. Add 1 ml of absolute ethanol to each of the four aqueous fractions. Cap the tubes and invert several times to mix. Incubate the tubes on ice for 10 min.
10. Centrifuge the tubes at 4°C for 15 min. Remove the supernatant from the precipitated DNA pellet (which may also contain significant amounts of RNA, making the pellet appear white in color). Add 1 ml of ice cold 70% ethanol to each DNA pellet, centrifuge for 5 min at 4°C, and remove the supernatant from the DNA pellet.
11. Allow the pellets to air dry for 10 min or dry briefly in the vacuum microcentrifuge. Store the four, dried DNA pellets at −20°C for use on Day 4.

Day 4: Restriction Endonuclease Digestion and Agarose-Gel Electrophoresis of Plasmid DNA

1. In this experiment, you will perform restriction digests on two of the four plasmid DNA samples that you have isolated. If it is possible, select the plasmid DNA isolated from two bacterial colonies that were able to grow well on the IPTG/kan plates, but not as well on the kanamycin plates without IPTG. This is determined by analyzing the growth of the four colonies (clones) on the kan plate and the kan/IPTG plate produced in step 3 from the

previous day. *Remember that our method of selecting between cells carrying the pUC4K and pUC19/4K plasmids is that the kan^R gene on pUC19/4K is IPTG-inducible, while the kan^R gene is expressed constitutively in cells carrying the pUC4K plasmid.* The *lac* promoter is known to be somewhat "leaky," showing some expression of genes under its control (such as the kan^R gene) even in the absence of IPTG. Therefore, you may find that cells carrying pUC19/4K may show some growth on the kan plate without IPTG. Ideally, you will want to analyze colonies (clones) that grew *well* only on the kan plate *with* IPTG. If you only have colonies that grew *equally well* on both the kan plate and the IPTG/kan plate, choose any two of the four plasmids for analysis.

2. Resuspend the two plasmid DNA sample pellets in 32 μl of sterile distilled water.
3. Set up the following reactions *for each of the two plasmid samples* in 1.5 ml microcentrifuge tubes:

***Pst*I Digests**	***Hind*III Digests**	***Sal*I Digests**
8 μl of plasmid DNA	8 μl of plasmid DNA	8 μl of plasmid DNA
1.5 μl of 10X React 2	1.5 μl of 10X React 2	1.5 μl of 10X React 10
1 μl of *Pst*I (10 units/μl)	1 μl of *Hind*III (10 units/μl)	1 μl of *Sal*I (10 units/μl)
1 μl of RNaseA	1 μl of RNAseA	1 μl of RNAseA
3.5 μl of water	3.5 μl of water	3.5 μl of water

The RNAseA is at a concentration of 10 mg/ml, and is added to completely digest all of the RNA remaining in the sample. If this is not done, a large RNA "spot" will be present on the gel following ethidium bromide staining, which will prevent you from seeing low molecular weight DNA fragments on the gel.

4. After a 1-hr incubation at 37°C, add 3 μl of 6X DNA sample buffer to each of the reaction tubes, as well as to the 8 μl of *undigested plasmid* remaining from each of the two samples (see step 2). At this point you should have eight samples for agarose-gel analysis.
5. Prepare a 1% TBE agarose gel by adding 0.5 g of electrophoresis-grade agarose to a 250-ml Erlenmeyer flask containing 50 ml of 0.5X TBE buffer (dilute the 5X TBE buffer stock 1:10 with distilled water to prepare the 0.5X working solution). *Preweigh the flask before heating and record this value.*
6. Microwave the flask for 2 to 3 min, or until the agarose has been fully dissolved (no small pieces of undissolved agarose remain). Place the flask on the balance and add distilled water to the flask until its mass is the same as that before the flask was heated. Water will evaporate from the flask as it is heated. This water must be replaced to ensure that a 1% agarose solution is maintained. Depending on the amount of water that has evaporated, the 1% agarose gel prepared in step 5 may now be greater than 1%.
7. Allow the solution to cool for 5 min, swirling the flask gently every so often to prevent the gel from hardening. When the outside of the flask is cool enough to touch, pour the solution into an agarose-gel cast fitted with a 10-well comb at one end (consult the instructor). Allow the gel to set (~40 min). Remove the comb, and place the gel in an agarose-gel electrophoresis chamber. Add 0.5X TBE buffer to the chamber *until it completely covers the gel and fills the wells.*
8. Load the samples prepared in step 4 as follows:

	Lane 1	**Lane 2**	**Lane 3**	**Lane 4**	**Lane 5**	**Lane 6**	**Lane 7**	**Lane 8**	**Lane 9**
Restriction enzyme	*	*Pst*I	*Hind*III	*Sal*I	None	*	*Pst*I	*Hind*III	*Sal*I
Plasmid	1	1	1	1	†	2	2	2	2

*This lane contains your undigested plasmid samples.

†This lane should be loaded with a solution containing 1 μg of 1-kb DNA ladder size standard, water, and DNA sample buffer (prepared by the instructor).

9. Attach the negative electrode (cathode) to the well side of the chamber and the positive electrode (anode) to the other side of the chamber. Remember that the DNA is negatively charged, and will migrate through the gel toward the positive electrode.
10. Perform the electrophoresis at 50 mA, constant current. You will see two dye fronts develop: one from the bromophenol blue dye (dark blue) and one from the xylene cyanole dye (light blue). The latter of the two dyes will migrate the same as a DNA fragment of approximately 4 kb, while the former dye will migrate the same as a DNA fragment of about 0.5 kb.
11. Continue the electrophoresis until the faster moving of the two dye fronts (bromophenol blue) migrates about three-fourths of the way through the gel.
12. Turn off the power supply, disassemble the electrophoresis apparatus, carefully remove the agarose gel, and submerge it in a small tray containing a 0.5-μg/ml solution of ethidium bromide in 0.5X TBE (enough to completely cover the gel). *Wear gloves at all times when working with ethidium bromide!* Incubate at room temperature for about 20 min. The ethidium bromide will enter the gel and intercalate between the base pairs in the DNA strands. When exposed to ultraviolet light, the ethidium bromide will fluoresce, and the DNA will appear as orange or pink bands on the gel.
13. Destain the gel for 10 min in a solution of 0.5X TBE buffer without ethidium bromide. This is done to remove ethidium bromide from all portions of the gel that do not contain DNA.
14. Place the gel in a dark box fitted with a 254 nm light source. Close the doors of the box and turn on the light source to visualize the DNA bands on the gel. Take a photograph of the gel for later analysis.
16. Prepare a plot of the number of base pairs versus the distance traveled (in centimeters) for each DNA fragment present in the 1-kb DNA ladder lane. If the 1-kb DNA size standards resolved well, you should be able to differentiate between the relative mobilities of the 0.5-, 1.0-, 1.6-, 2.0-, and 3.0-kb DNA fragments (see Fig. 21-5) for use in preparing the standard curve. *Do not attempt to determine the relative mobilities of the larger-molecular-weight DNA size standards on the gel if they did not resolve or separate well. This will only add error to the standard curve over the region where the DNA size standards did display good resolution.*

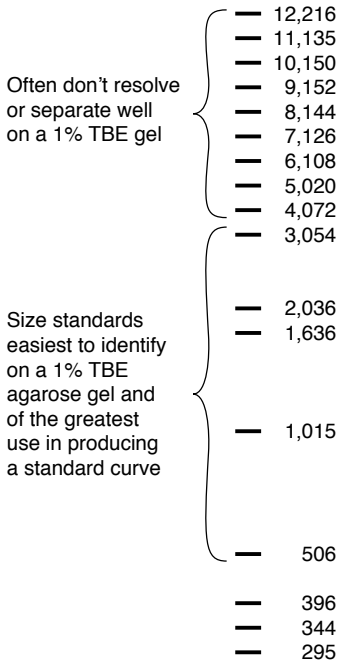

Figure 21-5 Number of base pairs in each band of the 1-kb DNA ladder.

17. Have you successfully constructed the desired pUC19/4K recombinant plasmid? Explain your answer in terms of what size DNA fragments resulted from each digest and your knowledge of the composition of the parent plasmids used in this experiment (pUC19 and pUC4K). Prepare a restriction map of the newly constructed plasmid. The map should include the total number of base pairs in the plasmid, the identity and position of all restriction sites in the plasmid, and the position of all of the different genes present in the plasmid.
18. If you do not think that you have isolated the desired pUC19/4K plasmid, prepare a map of the plasmid that you think you have isolated. Support your drawing with an explanation of how the digestion of this plasmid with the various restriction enzymes would produce the results that you obtained.

Exercises

1. Why is it important to perform a transformation control experiment using undigested plasmid? Describe two possible results that could be obtained in this control experiment, along with an explanation of what each result would indicate.
2. Why is it important to perform a transformation control experiment using no plasmid DNA? Describe two possible results that could be obtained in this control experiment, along with an explanation of what each result would indicate.
3. Why is it important to perform a transformation control experiment using unligated plasmid? Describe all the possible results that could be obtained in this control experiment, along with an explanation of what each result would indicate.
4. The pUC19 and pUC4K plasmids both confer antibiotic resistance to the *E. coli* host strains that they are transformed into. Other than drug resistance, can you think of any other gene(s) that could be carried on a plasmid that would allow you to select for cells that obtained it on transformation? For each of the genetic elements that you propose, describe the relevant features of the genotype of the host strain that you would use in the experiment, as well as how you would select for cells that obtained the plasmid following transformation.
5. Can you think of any advantages of having the *lacI* gene carried on the pUC19 plasmid?
6. You have obtained a plasmid that is able to be propagated in *Escherichia coli* but not in *Bacillus subtilis*. Why is this plasmid able to be propagated in one bacterium but not another? How would you propose to alter the plasmid so that it is able to be propagated both in *E. coli* and *B. subtilis?*
7. Where were plasmids originally isolated from? What function(s) do naturally occurring plasmids serve?

REFERENCES

Berger, S. L., and Kimmel, A. R. (1987). Guide to Molecular Cloning Techniques. *Methods Enzymol* Vol. **152**.

Garrett, R. H., and Grisham, C. M. (1995). Recombinant DNA: Cloning and Creation of Chimeric Genes. In: *Biochemistry.* Orlando, FL: Saunders.

Lehninger, A. L., Nelson, D. L., and Cox, M. M. (1993). Recombinant DNA Technology. In: *Principles of Biochemistry*, 2nd ed. New York: Worth.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning*, 2nd ed. Plainview, NY: Cold Spring Harbor Laboratory Press.

Wilson, K., and Walker, J. M. (1995). Molecular Biology Techniques. In: *Principles and Techniques of Practical Biochemistry*, 4th ed. Hatfield, UK: Cambridge University Press.

EXPERIMENT 22

In Vitro Transcription from a Plasmid Carrying a T7 RNA Polymerase–Specific Promoter

Theory

Transcription is one of two processes that allow a cell to synthesize the proteins encoded in its DNA. In the first process, the genes contained on the chromosome are *transcribed* into molecules of RNA. In the second process, these RNA messages are *translated* by the ribosomes and transfer RNAs (tRNAs) to produce proteins with specific amino acid sequences. RNA polymerase is the enzyme that synthesizes RNA. This enzyme will read a single-stranded DNA template in the 3′ to 5′ direction and construct an RNA molecule in the 5′ to 3′ direction that is complementary to it (Fig. 22-1). In some respects, RNA synthesis is similar to DNA synthesis: both molecules are synthesized in the 5′ to 3′ direction, both of the enzymes involved require a template DNA strand to direct the synthesis, and both of the enzymes involved use nucleoside triphosphates as the basic monomeric units in the synthesis reaction.

Despite these similarities, there are some very important differences between the reactions carried out by DNA polymerase and RNA polymerase. Remember that DNA is synthesized with the use of four *deoxy*ribonucleoside triphosphates (dATP, dGTP, dTTP, and dCTP). In the double-stranded DNA helix, adenine bases hydrogen-bond with thymine bases, and guanine bases hydrogen-bond with cytosine bases. RNA, however, is synthesized with the use of four ribonucleoside triphosphates (ATP, GTP, CTP, and UTP). Adenine and guanine bases in the DNA template will direct the addition of uracil and cytosine bases to the growing RNA molecule, respectively, while thymine and cytosine bases in the DNA template will direct the addition of adenine and guanine bases, respectively, to the growing RNA molecule. The net result of this is that RNA polymerase will produce an RNA molecule identical to that of the nontemplate (coding) DNA strand, with uracil in place of thymine bases.

Another difference between DNA polymerase and RNA polymerase is that the latter enzyme does not require the presence of a single-stranded nucleic acid primer to initiate the polymerization reaction. Given the correct sequence of DNA (a promoter, see below), RNA polymerase will bind the DNA template and begin transcription. This is in contrast to DNA polymerase (see Experiment 24), which requires a short nucleic acid primer to initiate synthesis of DNA. Unlike DNA polymerase, RNA polymerase does not have a 3′ to 5′ exonuclease or "proofreading" activity. As a result, an error (a single base change) will be introduced into an RNA molecule for every 10^4 to 10^6 nucleotides added to the growing macromolecule during synthesis. This error rate is acceptable, since many molecules of RNA are produced from a single DNA template, and because the population of a particular RNA molecule is constantly being regenerated or turned over with time (the half-life of mRNA is relatively short). The 3′ to 5′ proofreading activity of DNA polymerase decreases the error rate in DNA synthesis to one in 10^9 to 10^{10} bases. Because the DNA in a cell provides the *permanent* genetic information, you can understand why the accuracy of DNA synthesis during replication is more critical than that of RNA synthesis in the life cycle of a cell.

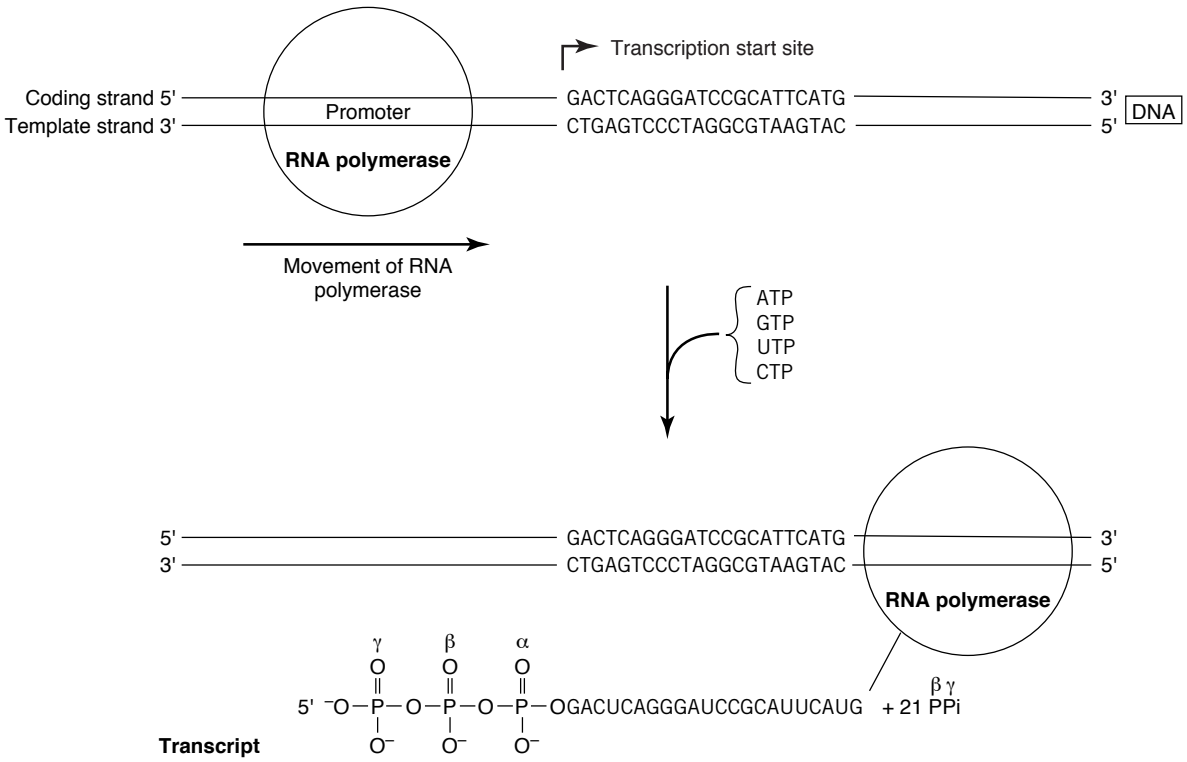

Figure 22-1 Schematic diagram of the process of transcription. RNA polymerase will read the template DNA strand 3′ to 5′ and produce a transcript in the 5′ to 3′ direction that is identical to the sequence of the coding DNA strand, with uracil (U) in place of thymine (T). A radiolabeled transcript can be produced by including an α-[^{32}P] nucleotide, which will become part of the product, or by including a γ-[^{32}P] nucleoside triphosphate that is known to be the first nucleotide in the transcript.

Where does RNA polymerase begin the process of transcription on the DNA template? The DNA element on which RNA polymerase binds and initiates transcription is termed the *promoter*. The simplest promoters are those recognized by viral RNA polymerases. Usually, these are a contiguous sequence of 15 to 30 base pairs that direct the viral RNA polymerase to the transcriptional start site (Fig. 22-2). The viral promoter sequences possess a 5′ to 3′ polarity, allowing you to determine which of the two DNA strands will act as the template for transcription. Bacterial promoters consist of two different but conserved DNA sequences. One of these elements is located about 10 base pairs on the 5′ side of the transcriptional start site, while the other element is located about 35 base pairs to the 5′ side of the transcriptional start site. Although these two elements consist of as little as six base pairs each, the sequence and spacing of these two elements are critical for allowing the bacterial RNA polymerase to bind the DNA template and initiate transcription. Promoters for eukaryotic RNA polymerases are quite variable. Still, there are some recurring elements found about 25, 40, and 100 base pairs to the 5′ side of the transcriptional start site. These sequences, as well as others that are often thousands of base pairs from the transcriptional start site, are believed to be the binding sites for transcription factors (proteins) that regulate the activity of the eukaryotic RNA polymerases. A list of different RNA polymerases, and the promoter sequences that they recognize, are listed in Table 22-1.

Where does transcription of a DNA template end? The process of transcription termination is best understood in bacteria. Rho-*independent* terminators are characterized by a self-complementary

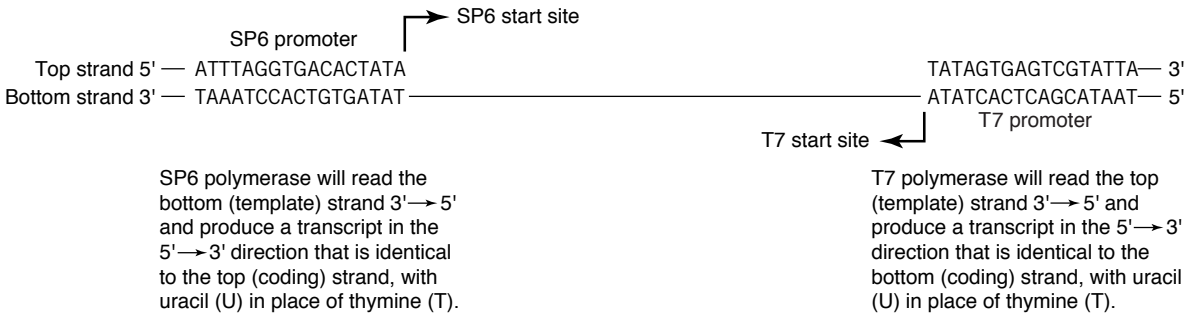

Figure 22-2 Promoters specify where RNA polymerase will bind the DNA and initiate transcription. The polarity of the promoter sequence will specify the coding and template strands of the DNA.

DNA sequence located roughly 20 bases on the 5′ side of the termination site. The RNA produced on transcription of this region is able to form a hairpin structure that causes the RNA polymerase to "stall" and eventually dissociate from the DNA template. A stretch of uridyl nucleotides at the 3′ end of the RNA hairpin is part of the rho-independent terminator sequence that is also believed to help destabilize the enzyme–DNA complex. A rho-*dependent* terminator also contains a self-complementary sequence capable of forming a hairpin structure. In an unknown mechanism, the rho protein hydrolyzes ATP and destabilizes the RNA polymerase–DNA complex as it stalls at the hairpin, eventually leading to the termination of transcription.

How were the promoter sites for the various RNA polymerases determined? If the enzyme binds a particular sequence of bases in the DNA, then that stretch of bases will be protected from chemicals and nucleases that cleave DNA. A single strand of DNA in a double helix is first labeled with a radioactive group at either the 3′ or 5′ end. The DNA duplex is then incubated with the RNA polymerase and treated with a chemical or DNase enzyme that produces a number of different-sized DNA fragments. The DNA duplex is then denatured, and the DNA fragments are resolved by polyacrylamide-gel electrophoresis. Any region of the DNA duplex protected by the polymerase during the DNase treatment will be absent on the autoradiogram of the gel, compared to the same radiolabeled DNA duplex not protected by the polymerase. The "footprint" left on the DNA by the polymerase provides insight into what sequences and what regions of the DNA are bound by the RNA polymerase (Fig. 22-3).

Table 22-1 RNA Polymerases and the Promoters That They Recognize

RNA Polymerase	Molecular Weight	Subunits	Promoter
SP6 (from SP6 phage)	~100 kDa	1	5′-ATTTAGGTGACACTATAGAACTC-3′
T7 (from T7 phage)	~100 kDa	1	5′-TAATACGACTCACTATAGGGAGA-3′
Bacterial (*E. coli*)*	~324 kDa	5	5′-TTGACA-3′ (−35 region)
			5′-TATAAT-3′ (−10 region)
Eukaryotic†	500–700 kDa	~12	5′-GGCCAATCT-3′ (−110 region)
			5′-GGGCGG-3′ (−40 region)
			5′-TATAAAA-3′ (−25 region)

*Promoter sequence recognition by bacterial RNA polymerase is governed by its sigma subunits. The sequence shown is recognized by RNA polymerase with the major subunit found in rapidly growing, well-nourished cells. Other sequences are recognized under other physiological conditions that lead to formation or activation of alternate sigma subunits.

†The promoter sequences for eukaryotic RNA polymerases I, II, and III are numerous and, in some cases, not well defined. The promoter sequence shown is the consensus sequence recognized by RNA polymerase II.

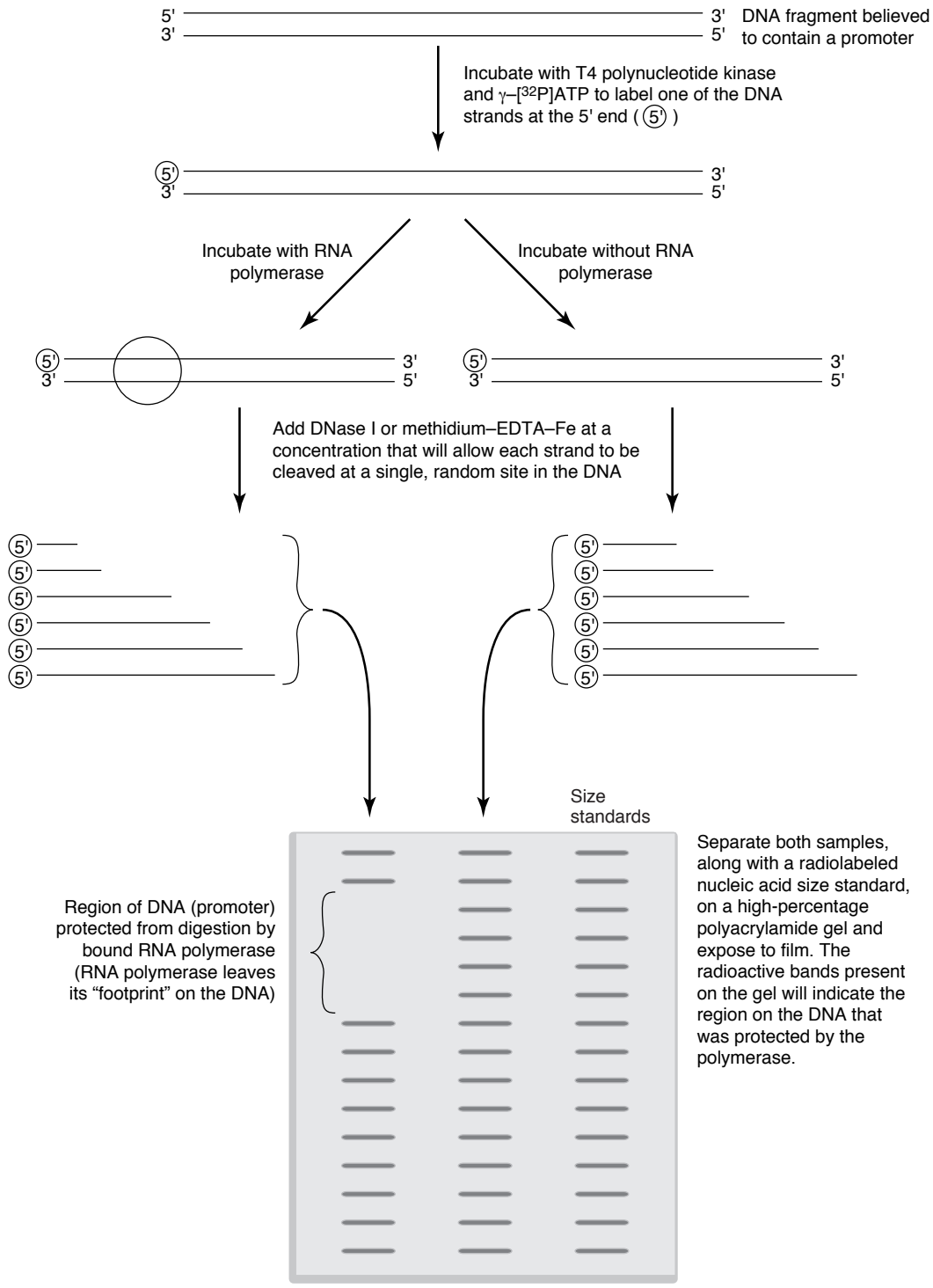

Figure 22-3 DNA "footprinting" to identify the promoter region bound by RNA polymerase.

In this experiment, you will carry out an in vitro transcription reaction from a plasmid (pSP72, Fig. 22-4) containing a T7 RNA polymerase specific promoter. A ^{32}P-labeled nucleoside triphosphate (labeled at the α position) will be included in the reaction to produce a radioactive RNA molecule. The size and sequence of the various transcripts that you will produce can be predicted by the restriction enzymes that the plasmid will be digested with prior to the beginning of the transcription reaction. Since the plasmid template for the transcription reaction will be digested, you will be preparing "run-off" transcripts from the T7 promoter. As a result, the termination of the transcription reaction will not rely on the presence of any transcriptional terminator sequence (rho-dependent or -independent) on the plasmid. The exact molecular weight or size of the various transcripts (the exact number of nucleotides that they contain) will be verified by polyacrylamide-gel electrophoresis at the end of the experiment.

Although this may appear to be a simple experiment, the same techniques may be modified to analyze the effects of different mutations in the RNA polymerase and/or promoter sequence on the process of transcription. Suppose that you wanted

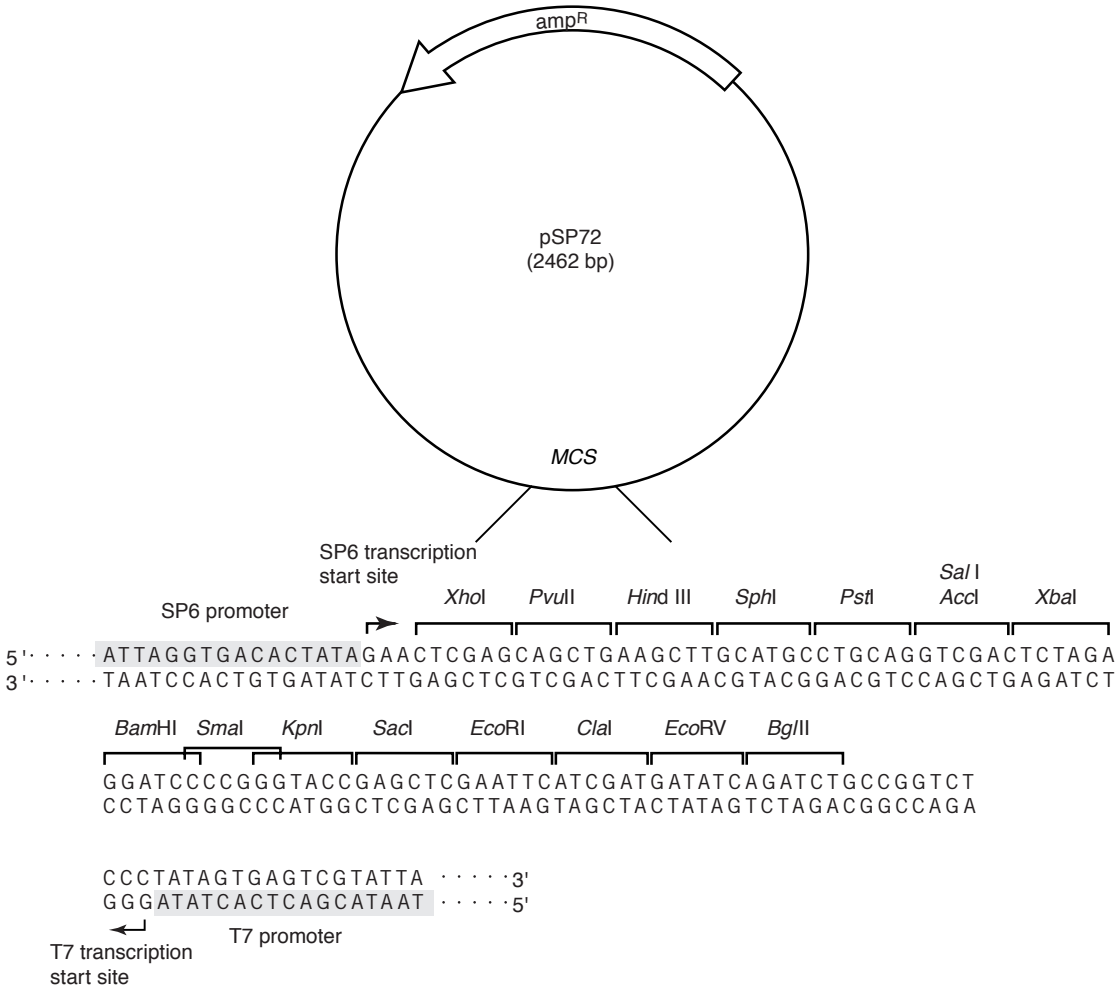

Figure 22-4 The pSP72 plasmid map (simplified).

to determine which of the bases in the T7 promoter were absolutely critical for transcription. You could produce a number of different point mutations within the T7 promoter sequence and use these as templates in the same kind of run-off transcription experiments. By comparing the results of these experiments with those that you will obtain in your experiment (using the consensus T7 promoter in the template DNA), you could make *quantitative* determinations of how each point mutation in the promoter affects transcription. In the same fashion, T7 RNA polymerase mutants could be tested against the wild-type enzyme to determine which amino acid residues are important to carry out transcription from the consensus T7 promoter.

WARNING

Caution: This experiment will require the use of significant amounts of ^{32}P-labeled nucleotide. Wear safety goggles and gloves at all times. Use Plexiglas shields when working directly with the radioisotope. Cover your work surface with absorbent paper to localize any spills that may occur. Consult the laboratory instructor for the proper disposal of the solid and liquid radioactive waste that will be produced in this experiment.

Supplies and Reagents

P-20, P-200, and P-1000 Pipetmen with sterile disposable tips
1.5-ml microcentrifuge tubes
Microcentrifuge
*Hin*dIII (10 units/μl) with the supplied 10X reaction buffer—Gibco BRL catalog #15207-020
*Bam*HI (10 units/μl) with the supplied 10X reaction buffer—Gibco BRL catalog #15201-031
*Eco*RI (10 units/μl) with the supplied 10X reaction buffer—Gibco BRL catalog #15202-021
pSP72 plasmid (~0.1 mg/ml in sterile distilled water)—Promega catalog #P-2191
37°C water bath
Dry ice/ethanol bath (−70°C)
Sterile distilled water
Phenol:chloroform (1:1, water saturated)
Vortex mixer
Chloroform:isoamyl alcohol (24:1)
3 M sodium acetate (pH 5.2)
Absolute ethanol
Vacuum microcentrifuge (Speed-Vac)
TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA)
10X Transcription mix
- 0.4 M Tris, pH 8.0
- 0.15 M $MgCl_2$
- Bovine serum albumin (1 mg/ml)
- 0.5 mM ATP
- 10 mM CTP
- 10 mM GTP
- 10 mM UTP
- 0.1 mM dithiothreitol
- α-[^{32}P]ATP (50,000 cpm/μl)—ICN Biomedicals catalog #32007x

T7 RNA polymerase (20 units/μl)—Gibco BRL catalog #18033-100
Apparatus for polyacrylamide gel electrophoresis
Reagents for 15% polyacrylamide gel containing 7 M urea
- 40% acrylamide solution (380 g/liter acrylamide, 20 g/liter *N*,*N*′-methylenebisacrylamide)
- Urea
- 5X TBE buffer (see below)
- TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine)
- 10% wt/vol ammonium persulfate in water—*prepared fresh*

5X TBE buffer (54 g/liter Tris base, 27.5 g/liter boric acid, 20 ml/liter 0.5 M EDTA, pH 8.0)
Loading buffer (7 M urea in water with 0.1% wt/vol xylene cyanole and bromophenol blue)
RNA size standards (~20, 61, and 97 nucleotides)—prepared by the instructor
Power supply
Film cassette
Plastic wrap
Razor blade
Kodak X-omat film
Film developer or Kodak developing solutions
Dark room

Protocol

Day 1: Preparation of DNA Template and in Vitro Transcription Reaction

1. Set up the following reactions in three labeled 1.5-ml microcentrifuge tubes:

Tube 1	Tube 2	Tube 3
8 μl of pSP72 plasmid	8 μl of pSP72 plasmid	8 μl of pSP72 plasmid
8 μl of 10X React 3	8 μl of 10X React 3	8 μl of 10X React 2
2 μl of *Eco*RI	2 μl of *Bam*HI	2 μl of *Hin*dIII
62 μl of water	62 μl of water	62 μl of water

Mix all the components thoroughly (do not use a Vortex mixer) and incubate in a 37°C water bath for 1.5 hr.

2. To remove the restriction endonucleases from the reaction, add 80 μl of phenol:chloroform (1:1) to each tube and mix with a Vortex mixer for 1 min. Centrifuge the samples at room temperature for 1 min, remove the aqueous phase (top layer) from each tube, and place these in three fresh microcentrifuge tubes. Add 80 μl of chloroform:isoamyl alcohol (24:1) to each of the three aqueous samples, cap, and mix with a Vortex mixer for 1 min. Centrifuge the samples at room temperature for 1 min and transfer the three aqueous phases (top layers) to three fresh microcentrifuge tubes. This last extraction is done to remove all traces of phenol from the solution. *It is critical that all of the phenol and chloroform be removed from the aqueous phases in this final extraction, since they may denature the RNA polymerase during the transcription reaction.* Dispose of the organic phases produced in these extractions in a waste container designated by the instructor.
3. Add 10 μl of 3 M sodium acetate (pH 5.2) to the aqueous phase in each of the three tubes, along with 200 μl of absolute ethanol. Cap the tubes and invert them several times to mix.
4. Incubate the tubes in a dry ice/ethanol bath (−70°C) for 10 min to precipitate the plasmid DNA. Pellet the precipitated DNA by centrifugation for 30 min at 4°C. *Place the microcentrifuge tubes in the rotor with the cap hinge facing the outside during this step. The DNA pellet will be translucent and very difficult to see, but you will know that the pellet will be on the same side of the tube as the cap hinge.* Carefully remove the supernatant from the DNA pellet. You may also find the use of Pellet Paint to be helpful during this procedure. This product, sold by Novagen (catalog #69049-3), is a pink chromophore that will bind the DNA and give the pellet a color that will be easier to identify.
5. Add 1 ml of ice cold 70% ethanol to each tube, centrifuge for 5 min at 4°C, and carefully remove the supernatant from the precipitated DNA pellet. This wash is done to remove the salts that were added to the DNA solution to aid in its precipitation, which may affect the activity of the T7 RNA polymerase.
6. Dry the DNA pellets in each of the three tubes in the vacuum microcentrifuge (~5 min), or allow the pellets to air dry for about 20 min.
7. Resuspend the DNA pellet in each of these three tubes with 8 μl of TE buffer. Label the tubes with your name and either H (*Hin*dIII), E (*Eco*RI), or B (*Bam*HI), depending on which restriction enzyme was used to treat each of the plasmid samples.
8. Add 1 μl of 10X transcription mix and 1 μl of T7 RNA polymerase to each of the three reaction tubes. Mix thoroughly but do not use a Vortex mixer. *Remember that the transcription mix is radioactive. Wear safety goggles and gloves when working with radioisotopes. Anything that touches the solution at this point will be radioactive. Be careful with disposal of radioactive pipette tips, microcentrifuge tubes, etc. Your lab instructor will provide you with radioactive waste storage containers.* Flash spin the samples to the bottom of the tubes for 1 sec and incubate all three reactions at 37°C for 1 hr.
9. After the 1-hr incubation, store the three reaction tubes at −20°C for use on Day 2.

Day 2: Polyacrylamide-Gel Electrophoresis of RNA Transcripts

1. Obtain a 10-well, 15% polyacrylamide gel containing 7 M urea from the instructor. Place the gel in the electrophoresis apparatus.
2. Add 0.5X TBE buffer to the upper and lower buffer chambers. Remove the comb from the gel. Connect the negative electrode (cathode) to the top buffer chamber and the positive