UNIVERSITY OF ILLINOIS

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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

VALERIE RATTS

ENTITLED THE CLONING OF THE AMINO TERMINUS OF THE SV40 LARGE T ANTIGEN GENE INTO ESCHERICHIA COLI

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

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THE CLONING OF THE AMINO TERMINUS OF THE SV40 LARGE T ANTIGEN GENE INTO ESCHERICHIA COLI

BY

VALERIE RATTS

THESIS

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in
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INTRODUCTION

The physical simplicity of Simian virus 40 (SV40) provides a model system for studying the regulation of DNA replication and gene expression. SV40 is among the smallest viruses known. The genome of SV40 consists of a covalently closed circular, double-stranded molecule of DNA which is 5243 base pairs long. The SV40 genome has been completely mapped (1) and codes for 5 different proteins as shown on Figure 1. The late region of SV40 DNA codes for 3 structural proteins VP1, VP2 and VP3 (2). The early region of SV40 DNA codes for 2 proteins referred to as small and large T antigen. Small T antigen has a yet undefined role. Large T antigen, a 90 kdal protein, is thought to be necessary for the initiation of viral replication and cell transformation. Permissive cells are able to support complete replication of the SV40 virus which results in lysis. Nonpermissive cells do not support replication, but are stably transformed. The expression of the large T antigen gene has been shown to be necessary to produce colonies of cells transformed by SV40 (3). The process by which large T antigen influences the growth of transformed host cells seems to involve altered DNA transcription and replication. T antigen is necessary for the initiation of rounds of viral DNA replication (4). In addition, large T antigen regulates its own synthesis by

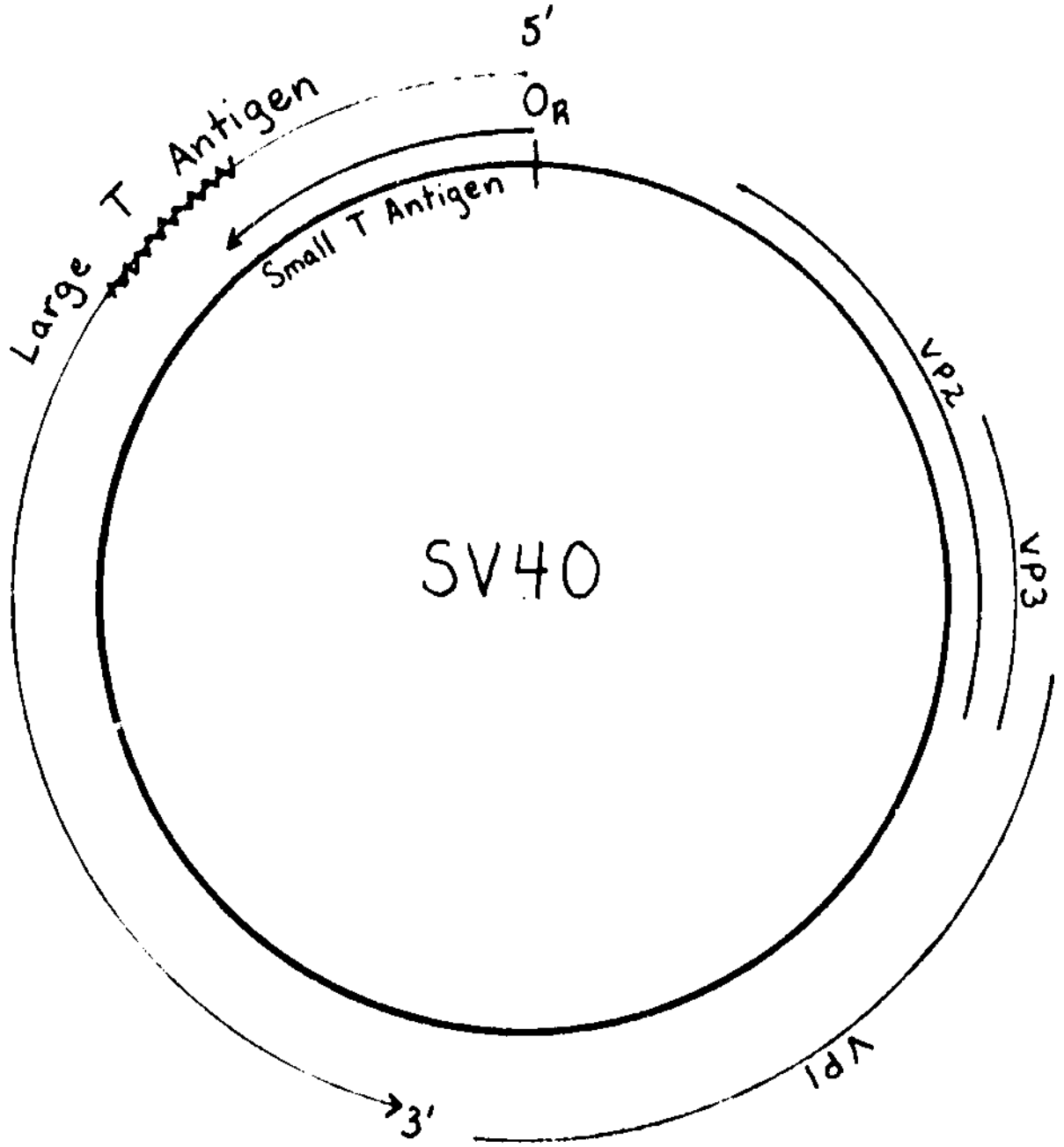


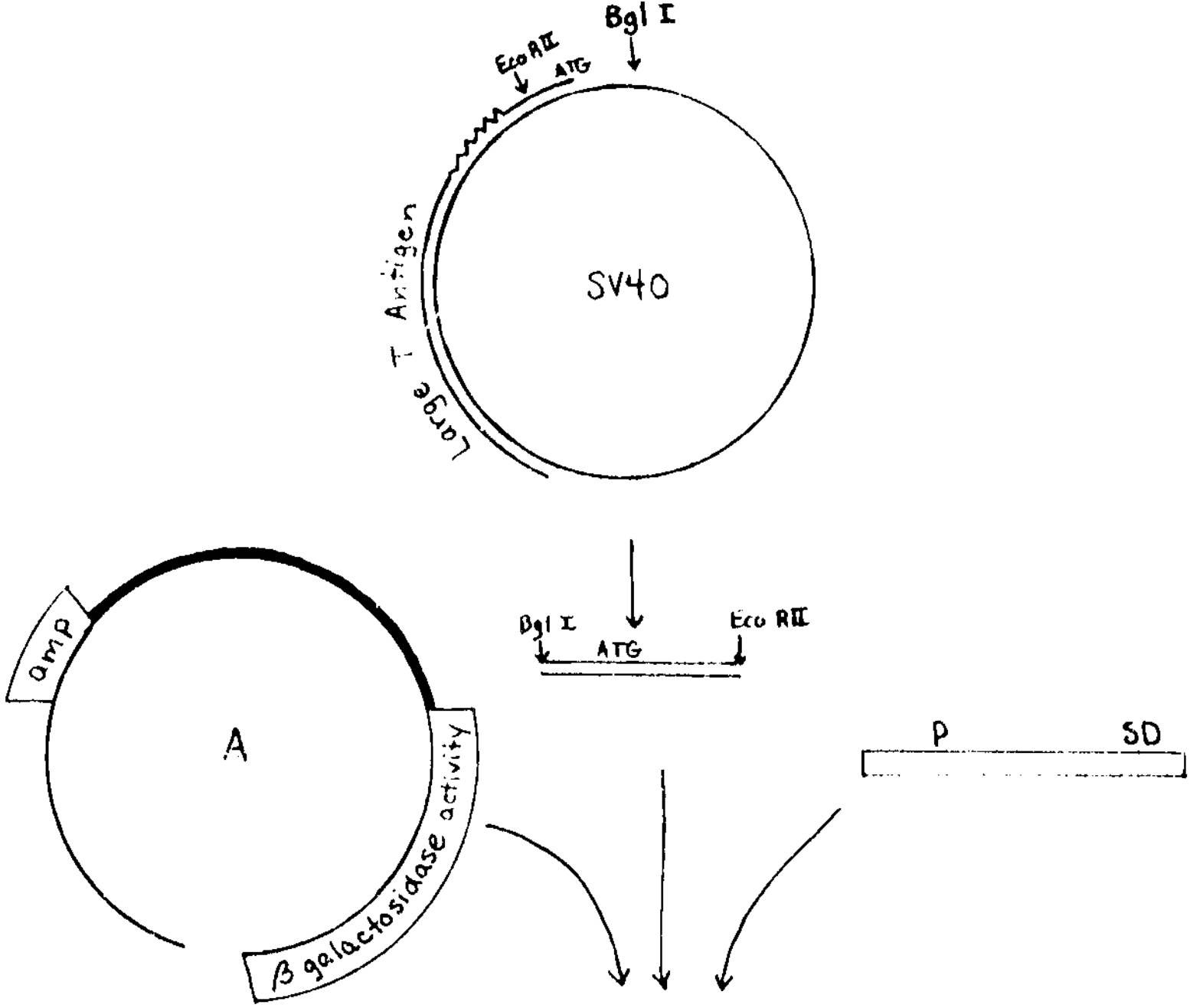
Figure 1. Schematic representation of the Simian virus 40 genome.

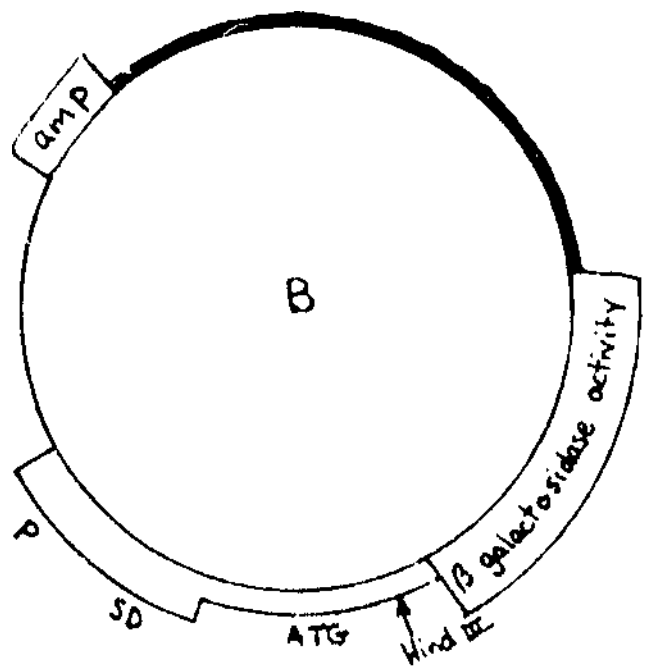
controlling the transcription of the T antigen gene (5). Studying the enzymatic and biochemical properties of T antigen can provide insight into the molecular mechanism through which T antigen controls gene expression and thus, alters cellular metabolism. In order to study the properties of T antigen, it is necessary to have a readily available quantity of the protein. Cloning the large T antigen gene would provide a more efficient, greater yielding method for isolating T antigen than the present procedure of purifying T antigen from tissue culture cells (6).

In order to clone the large T antigen gene into E. coli several factors must be taken into consideration. First, in order to get efficient synthesis of a protein in E. coli frequent transcription of the gene and efficient translation of the mRNA is required. Roberts et al. (7) have developed a method which places a strong promotor and ribosomal binding site in front of a coding sequence in order to maximize the expression of a cloned gene. In this method, a DNA fragment bearing the lac operon and Shine-Delgano sequence is positioned at an appropriate distance in front of the initial codon of the desired gene to be expressed. The lac promotor initiates efficient transcription of the gene, while the Shine-Delgano sequence directs the binding of the mRNA to ribosomes and assists in proper initiation of translation. This method has been used by Roberts et al. (8) to clone the small T antigen gene in E. coli and has resulted

in the production of a protein indistinguishable from small T antigen normally isolated from SV40 infected cells. Guarente et al. (9) developed a modification of the above procedure which allows one to assay for efficient expression of initial region of a gene without assaying for the gene products (see Figure 2). In this method a DNA fragment coding for B-galactosidase activity is inserted 3' to the sequence of DNA that encodes the amino terminal region of the protein to be cloned. The distance between the lac promotor and the initial codon can be varied by carrying out partial digestion with nucleases. The newly constructed plasmid, B, as shown in Figure 2 can be used to transform appropriate strains of E. coli. Bacteria which contain a plasmid with the promotor and Shine-Delgano sequence positioned an optimum distance from the initial codon of the gene direct the expression of the amino terminus of the cloned protein fused to a protein expressing B-galactosidase activity. The B-galactosidase activity of these bacteria can be detected by measuring a color change on indicator plates. Thus, the relative expression of B-galactosidase which is proportional to the expression of the N terminus of the cloned gene can be detected by measuring the intensity of color on the indicator plate. The second factor which must be taken into consideration when cloning large T antigen is that the early region of SV40 DNA appears to contain a non-coding region (10), called an intron, which is normally spliced out

Figure 2. Outline of the major steps in cloning the SV40 large T antigen gene. The first step involves isolating the amino terminus of the T antigen gene. This is inserted into plasmid Λ in front of a region coding for β galactosidase activity. It should be noted that the β galactosidase protein is not expressed unless a sequence containing the promotor region (P) and the Shine-Delgano sequence (SD) is placed 5' to the β galactosidase gene. The amino terminus of the SV40 large T antigen is inserted in between these two sequences (B). Expression of the amino terminus of T antigen is measured by the expression of β galactosidase protein. Finally the carboxy terminus of T antigen (in the form of cDNA) is inserted to reconstitute the entire gene (C). This plasmid (C) can now be used to transform E. coli. Hopefully, intact, functional large T antigen will be produced.



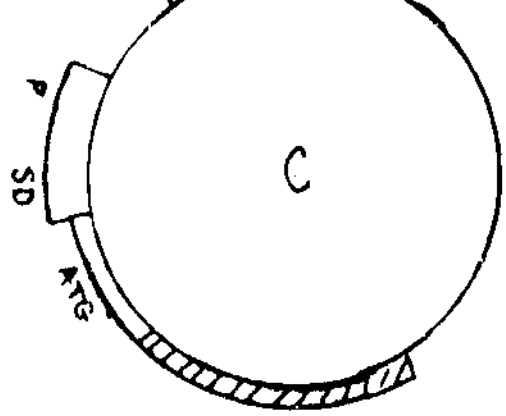


Hind III +

Large T Antigen cDNA

Hind III

(Carboxy Terminus)



Plasmid containing entire large T antigen gene without an intron

of mRNA transcripts by the host cell (11). The splicing of this sequence and the joining of the neighboring segments is necessary for the production of large T antigen because it eliminates several chain termination codons found in this region. A procaryotic system such as E. coli does not possess the capability to eliminate intervening sequences. In order for cloned large T antigen gene to be expressed in E. coli the intron must be eliminated prior to cloning. The method suggested by Guarente et al. (9) would allow the elimination of the intervening sequence when cloning the large T antigen gene. This method utilizes cDNA which is DNA with the intervening sequences eliminated. In this method, after the amino terminus is cloned and optimally expressed, a cDNA of the carboxy terminus of the gene is inserted using appropriate restriction enzymes and ligases. Thus, the entire gene can be reconstituted, cloned and maximally expressed in E. coli. This procedure is illustrated in Figure 2. Guarente et al. (9) utilized this method to clone rabbit B globin in E. coli.

There are several steps in the cloning of the large T antigen gene. The focus of my research is to complete one of these steps which is the isolation and cloning of the amino terminus of SV40 large T antigen gene.

EXPERIMENTAL DESIGN

The first step in cloning the initial region of the large T antigen gene is to select an appropriate vector. The plasmid pBR322 as shown in Figure 3 was chosen because it offered several advantages. First, pBR322 exists in high copy number in bacteria (12) and therefore, it is possible to isolate relatively large amounts of DNA from cell cultures. Secondly, pBR322 has genes for resistance to ampicillin and tetracycline. These markers can be utilized after transformation to identify bacteria that contain plasmids with inserted DNA. Finally, the nucleotide sequence of pBR322 is known (13) and can be used to identify specific plasmids through analysis of restriction digestion maps.

The plasmids were constructed by inserting the SV40 fragments from a EcoRII and a Bgl I restriction enzyme digestion into the Pst I site of pBR322. The mode of DNA insertion required the enzyme, terminal deoxynucleotidyl transferase, for the addition of homopolymer nucleotide tracts or "trails" to the 3' end of the double-stranded DNA fragments (14). In this procedure pBR322 is cut with Pst I and the resulting ends are tailed with poly(dG). SV40 DNA is cut with Bgl I and EcoRII, and the resulting ends are tailed with poly (dC). The complementary tails are annealed together, resulting in the insertion of SV40 DNA fragments into the Pst I site of pBR322.

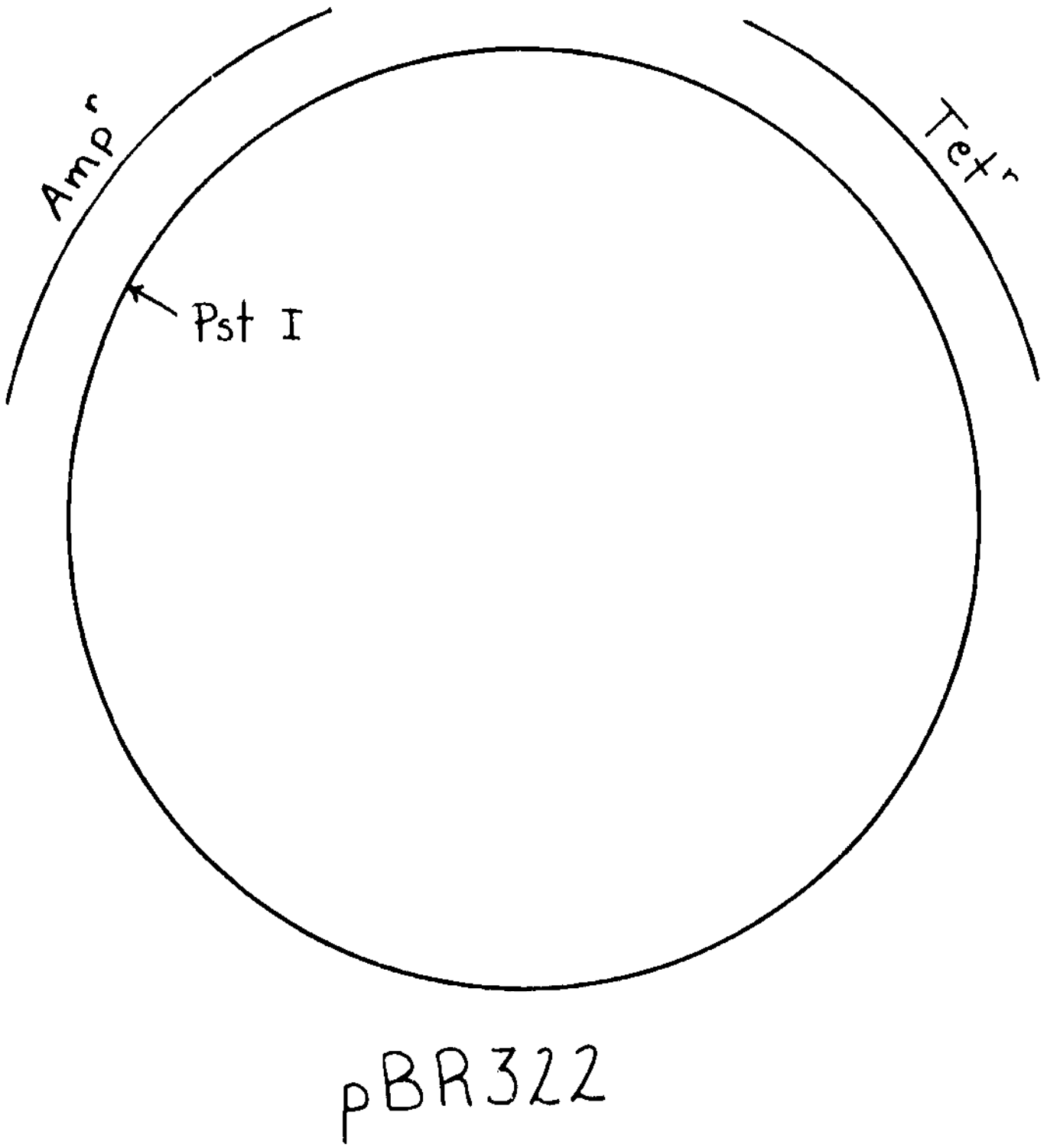
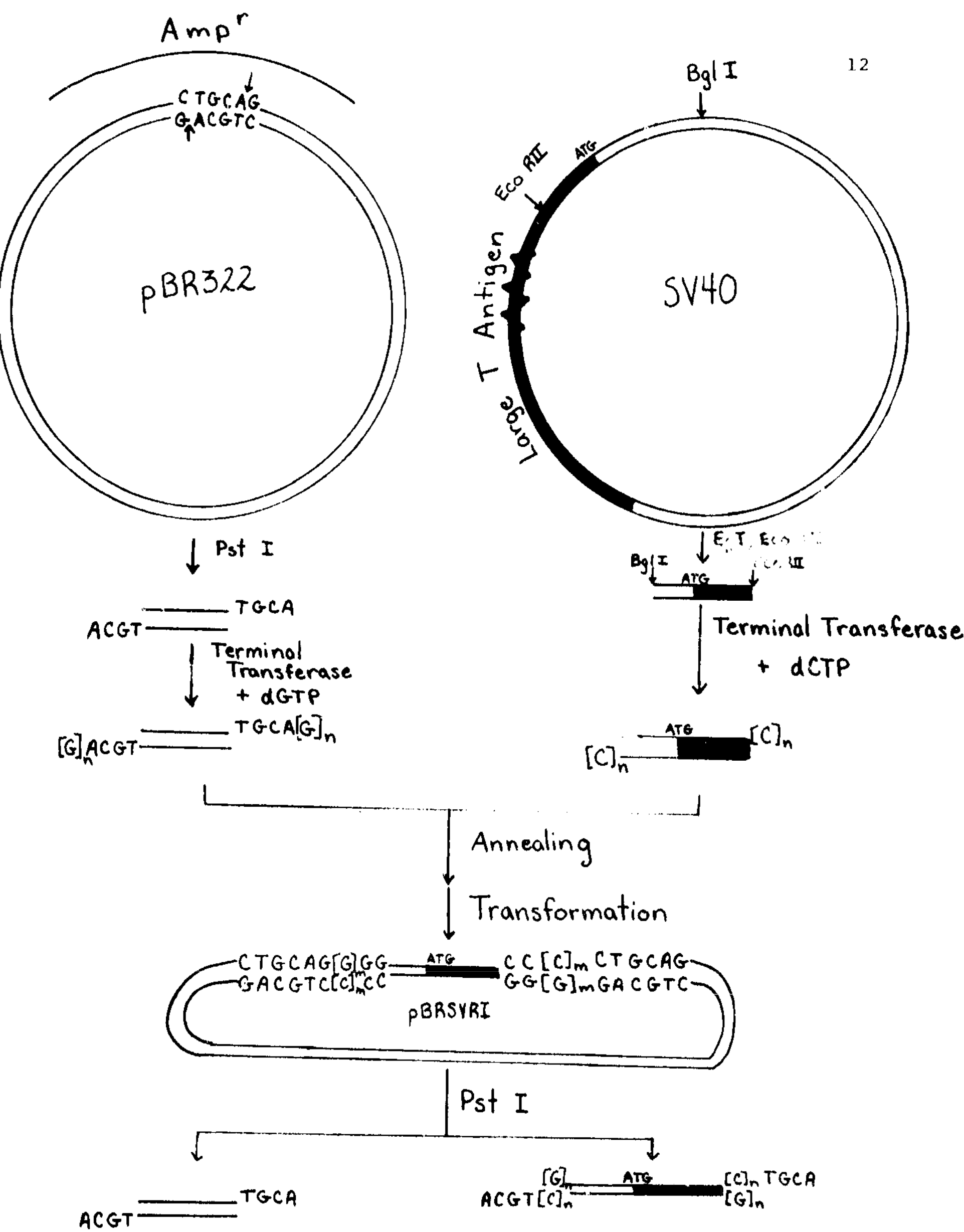


Figure 3. Diagram of the plasmid pBR322.

There are several reasons for constructing the plasmid in this way. First, inserting DNA into the single Pst I site of pBR322 destroys the ampicillin resistance gene while leaving the tetracycline resistance gene intact. Thus, cells transformed with this plasmid can be identified as being viable on tetracycline containing plates and nonviable on ampicillin containing plates. The second reason for using the Pst I is that by tailing Pst I digested pBR322 with poly (dG), the Pst I restriction site is regenerated in the newly formed plasmid (15). The nucleotide sequence which Pst I recognizes is CTGCAG. Pst I cleaves between the A and the G resulting in a CTGCA sequence at the 3' end. Thus, if G is added to the 3' end of this sequence the Pst I site is regenerated as illustrated in Figure 4. Usually restriction sites are destroyed after cleavage and tailing. The advantage of the regeneration of the Pst I restriction site is that it allows for recovery of the inserted DNA fragments. In this experiment, recovery is extremely important because the SV40 fragment is being cloned so that it can be amplified, isolated and recovered for further use. It should also be noted that the orientation of the DNA is not important.

The SV40 DNA was digested with EcoRII and Bgl I for several reasons. The Bgl I site was selected because it cleaves SV40 DNA only once approximately 70 base pairs from the initial ATG codon of the T antigen gene. EcoRII restriction enzyme was selected because it cuts before the intervening

Figure 4. Outline of the construction of pBRSV1. An important reason for constructing the plasmid by using Terminal Transferase is that the Pst I sites are regenerated. This will allow relatively easy isolation and recovery of the fragment containing the amino terminus of the SV40 large T antigen gene.


Amp r
CTGCAG
GACGTC
pBR322
Pst I
TGCA
ACGT
Terminal Transferase
+ dGTP
TGCA[G]n
[G]nACGT
Bgl I
Eco RII
ATG
Large T Antigen
SV40
Bgl I
ATG
Terminal Transferase
+ dCTP
ATG
[C]n
[C]n
Annealing
Transformation
CTGCAG[G]GG
GACGTC[C]mCC
ATG
CC[C]mCTGCAG
GG[G]mGACGTC
pBRSVRI
Pst I
TGCA
ACGT
[G]n
ATG
[C]nTGCA
ACGT[C]n
[G]n

sequence which is necessary for later experiments. One disadvantage of this method is that EcoRII cuts the SV40 genome in 16 places and thus together with Bgl I, 17 fragments are generated by the digestion of SV40. The correct fragment containing the amino (N) terminus of the T antigen gene was not isolated from the mixture. Instead the mixture of fragments was inserted into pBR322.

This mixture of recombinant plasmids containing various fragments of the SV40 genome was used to transform E. coli. The transformants were then analyzed to determine which ones contained plasmids carrying the desired segment of the SV40 genome. By comparing Hind III and Pst I restriction digests of the plasmids and through the use of Southern blot analysis, it is possible to identify a colony containing the correct plasmid. This colony can be grown on a large scale to isolate the SV40 DNA fragment containing the initial sequence of large T antigen gene. A diagram of the desired plasmid containing the correct SV40 sequence is shown in Figure 5.

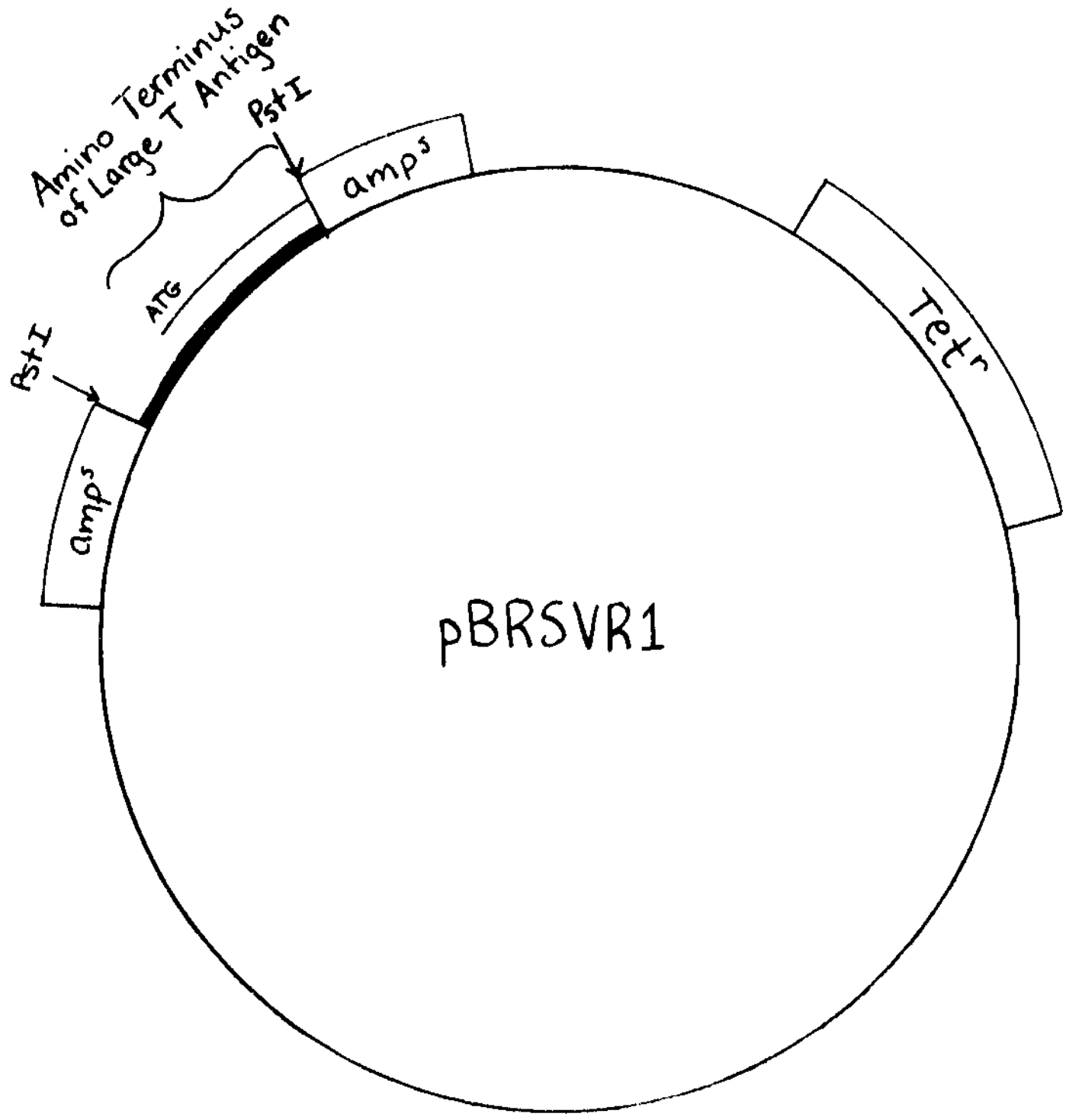


Figure 5. Schematic representation of the plasmid pBRSVR1.

MATERIALS AND METHODS

I. Bacterial Strains and Plasmids

E. coli strain HB101 (rec A^-, amp^-, tet^-) was obtained from Dr. A. Yuki. The plasmid, pBR322, was obtained from A. Mast who worked in our laboratory.

II. Construction of pBRSVR1

A. Digestion of SV40 DNA

Restriction enzyme digestions were carried out according to the procedure of Maniatis et al. (16). Supercoiled SV40 DNA at a concentration of $1.3^{mg}/_{\mu l}$ in 10 mM Tris-HCl (pH 7.4), 5 mM NaCl, and 1 mM Na_2 EDTA was obtained from Bethesda Research Laboratories (BRL). The SV40 DNA was digested with EcoRII and Bgl I which were also obtained from Bethesda Research Laboratories. The following reactants were placed in a 1.5 ml microcentrifuge tube: 15.4 µl SV40 DNA, 10 µl of 10X Core Buffer, 20 µl of Bgl I (6u/µl), 10 µl of EcoRII (4u/µl) and 44.6 µl of distilled water to give a total mixture volume of 100 µl. The 10X core buffer was obtained from BRL. Enzyme units (u), as defined by Bethesda Research Laboratories, is the amount of enzyme required to completely digest 1.0 µg of lambda (or equivalent DNA) DNA in one hour in the recommended buffer and at the recommended temperature. The above reaction mixture was gently vortexed and then incubated at 37°C for 1.5 hours. The reaction was stopped by

extracting the mixture with an equal volume of water-saturated phenol. The microcentrifuge tube was vortexed rapidly and then centrifuged for 2 minutes. The upper aqueous was removed and then extracted as before with another equal volume of water-saturated phenol. The aqueous phase was again removed and placed in a microcentrifuge tube. The digested DNA was ethanol precipitated to remove remaining phenol and other contaminants. Ethanol precipitation was carried out by mixing 10 µl of 3M NaOAc with the aqueous phase followed by the addition of 220 µl of ethanol. The reaction was mixed by vortexing. The DNA was allowed to precipitate for 15 minutes at -70°C. The precipitated DNA was then centrifuged for 5 minutes and the supernatant was discarded. The remaining pellet was dried and resuspended in 18 µl of 10 mM Tris-Cl (pH 8.0), 0.1 mM Na_2EDTA to a final DNA concentration of $1.1^{\mu g}/\mu l$.

B. Digestion of pBR322 DNA

The pBR322 DNA was obtained at a concentration of $0.66^{\mu g}/\mu l$ in 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA. Pst I was obtained from BRL and used according to the restriction digestion procedure described for SV40 DNA. The following reagents were placed in a 1.5 ml polypropylene microfuge tube: 61µl pBR322 (40 µg), 25 µl Pst I (4u/µl), 20 µl 10X core buffer, and 94 µl of distilled water to give a reaction volume of 200 µl. The mixture was gently vortexed and incubated at 37°C for 1.5 hours. As described in the SV40

digestion procedure, the reaction was stopped by phenol extraction, the DNA was ethanol precipitated, and the pellet was re-dissolved in 38 µl of 10 mM Tris-Cl (pH 8.0), 0.1mM Na_2 EDTA to give a final DNA concentration of $1.05^{ng}/_{\mu l}$.

C. Addition of Homopolymer Tails

The method used for 3' DNA terminus tailing was taken from the BRL manual. All of the reactants except for the DNA were obtained from BRL in a 3' DNA Terminus Tailing/Labeling Kit. The tailing of the Pst I-cleaved pBR322 with dGMP was executed in the following manner. Into a 1.5 ml polypropylene microcentrifuge tube were added 2.6 µl of Pst I cleaved pBR322 (2.7 Mg - 2 pmol of 3' ends), 10 µl of 5X reaction buffer (5X = 0.5M potassium cacodylate, pH 7.5, 10mM $CoCl_2$, 1mM DTT), 5 µl of 0.05 mM dGTP in 1mM Tris-Cl (pH 7.6), 29.4 µl water and 3 µl of Terminal Transferase (15 u). The reaction mixture was incubated for 30 minutes at 37°C. The mixture was then extracted with an equal volume of water-saturated phenol. The aqueous phase was removed and transferred to a new tube. One volume of 4.0 M NH_4OAc was added followed by 2 volumes of EtOH (-20°C). The mixture was stored at -70°C for 15 minutes and then centrifuged to pellet the DNA. The DNA pellet was resuspended in 50 µl 10mM Tris-Cl (pH 7.5), 0.1 mM EDTA and the ethanol precipitation step was repeated. The pBR322 DNA was finally dissolved in 27 µl annealing buffer (i.e. 20mM Tris-Cl (pH 8), 0.1mM Na_2EDTA, 0.3 M NaOAc).

The EcoRII, Bgl I-digested SV40 DNA was tailed with dCMP in a similar manner. The following reactants were placed in a 1.5 ml polypropylene microcentrifuge tube: 0.35 1 EcoRII, Bgl I-digested SV40 DNA (0.38 g 4 pmol 3'-ends), 20 ml 5X reaction buffer, 63.7 µl water, 10 µl 0.05 mM dCTP 1 mM Tris-Cl (pH 7.6) and 6 µl Terminal Transferase (15 u). As in the pBR322 reaction, the DNA mixture was phenol extracted, ethanol precipitated twice and then dissolved in 38 µl annealing buffer.

D. Annealing the Fragments

The annealing conditions described were taken from the BRL manual. The annealing reactions were carried out at two different ratios of target EcoRII, BgI-cleaved SV40 DNA to vehicle Pst I-cleaved pBR322 DNA. In reaction mixture A, 0.5 mg pBR322 DNA was annealed to 0.4 mg SV40 DNA and in reaction mixture B, 0.5 mg pBR322 DNA was annealed to 0.08 mg of SV40 DNA. The annealing buffer used was 20mM Tris-HCl (pH 8), 0.1mM Na_2EDTA, 0.3mM NaOAc. The reactions were carried out in a 50 µl reaction volume. The annealing reaction was incubated for two hours at 60°C and then slowly allowed to cool to room temperature. If the DNA mixture is allowed to cool too rapidly, the correct annealing will not take place.

III. Transformation of E. Coli with pBRSVR1

The protocol used for the transformation of E. coli was taken from Cohen et al. (17). This procedure treats

E. coli with calcium chloride which then makes them capable of taking up closed circular plasmid DNA. A loop of E. coli strain HB101 is used to innoculate 20 ml of L-broth. L-broth is prepared by dissolving 10g bactotryptone, 5g yeast extract and 5g NaCl in one liter of water. The culture is grown overnight at 37°C in a rotary shaker. Then, 0.3 ml of the overnight culture is transferred into 30 ml of fresh L-broth. This is grown at 37°C in a rotary shaker to an optical density of 0.5 at 600 nm. The cells are collected by centrifugation, and resuspended in 15 ml of chilled 10mM Tris-HCl (7.3), 50 mM $CaCl_2$. Once again the cells are sedimented by centrifugation and redissolved in 3 ml of 10mM Tris-HCl (7.3), 50 mM $CaCl_2$. The bacteria were then chilled at 0°C for 5 minutes. To 2 ml of suspended cells, was added 1.0 ml of DNA containing solution (i.e. 0.25 µg recombinant plasmid DNA in 25 µl annealing buffer, 10 µl 1M Tris-Cl_2 (pH 8), and 965 µl H_2O). The mixture was chilled at 0°C for 5 minutes, incubated at 37°C for 2 minutes and then cooled at 25°C for 10 minutes. Warmed L-broth (5 ml) was added to the cell suspension which was then incubated at 27°C for 30 minutes. Aliquots (0.8 ml) were removed and mixed with 2.5 ml of L-broth containing 0.6% agar. The resulting mixture was then plated on nutrient agar plates containing 10 µg/ml tetracycline. The plates were incubated overnight at 37°C to allow transformant colonies to grow.

IV. Characterization of pBRSVR1

A. Rapid Isolation of pBRSV1 Plasmid from E. coli

The procedure used for the rapid isolation of E. coli plasmids was obtained from Maniatis et al. (16) who cited a modified procedure originally developed by Birnboim and Doly (18). A 1.5 ml microcentrifuge tube containing 1.5 ml of L-broth is innoculated with the appropriate transformant. The culture is allowed to grow overnight at 37°C with constant agitation. The cells are then isolated by centrifugation and resuspended in 100 Ml of 50mM glucose, 10mM EDTA, 25mM Tris-Cl (pH 8), and 4 mg/ml lysozyme. The mixture is stored at room temperature for 5 minutes. Then 200 µl of ice cold solution 0.2 NaOH and 1% SDS solution is added. The sample is gently inverted several times and then stored on ice for 5 minutes. Next 150 µl of ice-cold potassium acetate solution is added. The potassium acetate solution is prepared in the following way: 60 ml of 5M KOAc, 11.5 ml glacial acetic acid and 28.5 ml H_2O are mixed. The mixture is chilled on ice for 5 minutes. A white clot of chromosomal DNA forms and precipitates from the mixture while closed-circular plasmid DNA remains in solution. The chromosomal DNA is pelleted by centrifugation and the supernatant solution is transferred to a new microcentrifuge tube. An equal volume of phenol : chloroform (3:1) is added to the supernatant to denature any contaminant proteins. The mixture is vortexed and centrifuged. The aqueous phase containing plasmid DNA

is transferred to a fresh tube. Two volumes of EtOH are added to the mixture. This mixture is allowed to stand at room temperature for two minutes and then centrifuged to pellet the plasmid DNA. The supernatant solution is decanted and 1 ml of 70% ethanol is used to wash the DNA pellet. The DNA is then recentrifuged, the supernatant is again decanted and the pellet is dried in a vacuum desiccator. The dried pellet is dissolved in 30 µl 10mM Tris-Cl (pH 8), 1mM EDTA containing DNase-free pancreatic RNase (20 µg/µl). This procedure led to the isolation of approximately 1 - 2 µg of plasmid DNA per 1.5 ml of culture medium.

B. Hind III Digestion and Analysis by Agarose Gel Electrophoresis

The plasmid DNA isolated from the transformant colonies was digested with Hind III. The restriction enzyme digest was performed as in the SV40 digestion procedure. To 20 µl of plasmid solution (0.05 $^{\mu g}/_{\mu l}$) was added to 1.0 µl Hind III (4u/µl), and 2.3 µl 10X core buffer. The mixture was incubated at 37°C for one hour. To each plasmid sample was added 1/10 volume of tracking dye (0.25% bromophenol blue, 30% glycerol in 6X TBE buffer). The samples were then loaded into agarose gel wells. The agarose gel was prepared by using 1% agarose in 1X TBE buffer (i.e. 0.089 M Tris-borate, 0.089 M Boric acid, 0.002 M EDTA). The gel was run overnight at 25 volts. The next morning the gel was carefully removed and stained in 1X TBE buffer containing 0.5 µg/ml

ethidium bromide for 45 minutes. A photograph of the gel was taken under UV illumination. The bands of DNA stained by the ethidium bromide appear orange and are easily detectable.

C. Pst I Digestion and Analysis by Polyacrylamide Gel Electrophoresis

Plasmid DNA isolated from transformant colonies was digested with Pst I. The restriction enzyme digest was performed as in the Hind III digestion procedure. To 20 µl of plasmid sample (0.05 $^{mg}/_{\mu l}$) was added 1.0 µl Hind III ($5\ ^{u}/_{\mu l}$) and 2.3 ml 10X core buffer. The mixture was incubated at 37°C for one hour. To each plasmid sample was added 1/10 volume of tracking dye (i.e. 0.25% bromophenol blue, 30% glycerol in 6X TBE buffer) The samples were then loaded into a 5% polyacrylamide gel well. The polyacrylamide gel was prepared in the following manner. First a 30% acrylamide stock solution was made by measuring 29 g of acrylamide and 1 g of N, N'-methylene bisacrylamide into a flask and adding water to 100 ml. The solution is deaerated to remove air bubbles. Then into a separate 100 ml flask is mixed 8.3 ml of the 30% polyacrylamide stock solution, 35.7 ml of water, 1.0 ml of 3% ammonium persulfate solution and 5.0 ml of 10X TBE. A cast gel is prepared and checked for leaks. Next 15 ml N,N,N',N'-tetramethylene diamine is added to the polyacrylamide gel mixture. The solution is rapidly, but carefully to avoid air bubbles poured into the cast gel and

allowed to polymerize for one hour. The gel was run overnight in 1X TBE buffer at 15 V. The next morning the gel was carefully removed and stained in 1X TBE buffer containing 0.05 ug/ml ethidium bromide for 45 minutes. A photograph of the gel was taken under UV illumination. The bands of DNA stained by ethidium bromide appear orange and are easily detectable.

D. Southern Blot Analysis

The method described here was taken from a New England Nuclear (NEN) instruction manual for the use of Gene Screen. This is a modification of the initial procedure developed by Southern (19).

i. Transfer of DNA

An agarose gel was prepared containing DNA samples using the exact same procedure as described in "Hind III Digestion and Analysis by Agarose Gel Electrophoresis." The gel was denatured by soaking for 30 minutes in 0.2 N NaOH and 0.6 M NaCl. The gel was then washed for one hour in 0.025 M sodium phosphate buffer (pH 6.5). A piece of Gene Screen that has been soaked in 0.025 M, the sodium phosphate buffer (pH 6.5) is placed on top of the gel. The gel and Gene Screen are then sandwiched on either side by two pieces of Whatman 3MM filter paper and two Scotch-Brite pads. The DNA is then transferred in an electroblot apparatus containing 0.025M sodium phosphate buffer (pH 6.5) at 0.25 A for two hours at 4°C and then for at 1A for two hours at 4°C. The membrane is

then carefully removed from the gel and washed in buffer. The DNA was baked onto the Gene Screen at 80°C for four hours.

ii. Preparation of a Probe

The nick translation procedure for the preparation of a radioactive probe was taken from BRL's Nick Translation Reagent Kit directions. All reagents except the [α^{32}P] dCTP were provided in this kit. Into a 1.5 ml microcentrifuge tube was placed 5 µl Solution A (0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dTTP, 500 mM Tris-HCl (pH 7.8), 50 mM $MgCl_2$, 100 mM 2-mercaptoethanol and 100 µg/ml nuclease-free BSA), 1.0 µl SV40 DNA (1.0 mg), 7.0 ml [α^{32}P] dCTP (156 pmoles dCTP), and 32 µl H_2O. The solution was mixed briefly and then 5 µl of Solution C containing DNA Polymerase I and DNAse I was added. The DNAse I creates gaps in the DNA while the DNA Polymerase I fills up these gaps with nucleotide monophosphates derived from the pool of nucleoside triphosphates. Thus, the reaction incorporates radioactively labeled dCTP into the DNA. The reaction was run at 15°C for 60 minutes. The labeling reaction was stopped by the addition of 5 µl of 300mM Na_2EDTA (pH 8)). The sample was extracted twice with an equal volume of water-saturated phenol. Next, the sample was extracted twice with water-saturated ether. Leftover ether was gently blown off by a light stream of air. The probe was then digested with restriction enzymes as described in the SV40 digestion procedure by adding 2 ml Bgl (4u/.1), 3 ml TaqI (4^u/.1), 6.5 1 10X core buffer and 0.5 µl BSA (100 $^{\mu g}$/ml)

to the sample of labeled DNA. The mixture was incubated at 37°C for one hour and then run on a 1% agarose gel as described in the "Agarose Gel Analysis" section. The low molecular weight band, detected under UV light after ethidium bromide staining, is cut out of the gel with a razor blade. The DNA is recovered from the gel slice (16) by placing the gel slice in a dyalysis bag containing a small amount (1.5 ml) of 0.5 X TBE buffer. The dialysis bag is emersed in a shallow layer of 0.5 X TBE buffer. The DNA is electroeluted out of the gel slice at 50 V for 2 hours. The polarity is reversed for 2 minutes to release the DNA which has been bound to the inner wall of the dialysis bag. Then the liquid inside the dialysis bag is then removed. The DNA recovered is in 0.5 X TBE buffer at a concentration of approximately 0.5 mg/ml.

iii. Hybridization of DNA

The Gene Screen membrane was prehybridized by treating with 10 ml of a solution containing: 50% formamide, 0.2% polyvinyl-pyrrolidone, 0.2% bovine serum albumin, 0.2% ficoll, 0.05 M Tris-HCl (pH 7.5), 1M NaCl, 0.1% sodium pyrophosphate, 0.1% SDS, 10% dextran sulphate (obtained from Pharmacia Fine Chemicals) and denatured salmon sperm DNA (100 µg/ml). The solution was placed in a small plastic, watertight bag with the membrane and incubated at 42°C for 6 hours. The bag was then opened and 3 ml of the following solution was added: 50% formamide, 0.2% polyvinyl-pyrrolidon, 0.2% bovine serum albumin, 0.2% ficoll, 0.05 M Tris-HCl

(pH 7.5), 0.1% sodium pyrophosphate, 0.1% SDS, denatured salmon sperm (100 mg/ml) plus 400 ml of the radioactive probe solution. The bag was resealed and incubated at 42°C for 24 hours. The membrane was then removed and washed twice for 5 minutes at room temperature with 0.06 M Tris-HCl (pH 8), 0.002 M EDTA. It was then washed twice for 30 minutes at 60°C with 0.3 M NaCl, 0.06 M Tris-HCl (ph 8), 0.002 M EDTA and 0.5% SDS and finally it was twice washed for 30 minutes at room temperature with 0.003 M Tris-HCl (pH 8.0). The membrane was then dried and autoradiographed overnight with an intensifying screen.

RESULTS AND DISCUSSION

I. Transformation of E. coli with pBRSVR1

E. coli was transformed with pBRSVR1. The data from this experiment is reported in Table 1. A viable cell count was taken by plating 0.1 ml of cell culture onto a petri plate containing only nutrient media without antibiotics. This gave a viable cell count of 3200 colonies/plate. A negative control was used to determine contamination by cells which were able to grow on tetracycline plates without incorporation of the pBRSVR1 plasmid. This was found to be 0. This shows colonies are unable to grow without the incorporation of this plasmid, as would be predicted. The transformation efficiency was determined to be 9%. It was also found that 5600 transformants per mg of plasmic DNA were produced.

In Table 2 the data indicate that the molar ratio of SV40 fragments to pBR322 fragments is important to the effectiveness of transformation. A molar ratio of 1:1 gives a transformation frequency of 0.1%, while a molar ratio of 1:2 gives a 100 fold greater transformation frequency of 10%. A possible explanation for this observation would be that in the annealing reaction where SV40 fragments are inserted into pBR322, there is not a high enough concentration of SV40 fragments with poly (dC) tails to find pBR322 with

Table 1. Transformation of E. coli with pBRSVR.

Plate #	Selective Media	# of Colonies (after 18 hours)
1A	Tet +	4
1B	Tet +	0
1C	Tet +	3
1D	Tet +	2
2A	Tet +	196
2B	Tet +	400
2C	Tet +	224
2D	Tet +	304
+*	Tet -	3200
-**	Tet +	0

*This is the + control which gives the # of bacteria plated. This will be used to calculate transformation efficiency.

**This is the - control. In this preparation no plasmid DNA was added. Growth on this plate indicates contamination.

Table 2. Comparison of Different Molar Ratios of Fragments to Transformation Frequency.

Molar Ratio of pBR322:SV40	Average # of Colonies (per plate)	Viable Cell Count (per plate)	Transformation Frequency (%)	# of Colonies per µg plasmid DNA
1:1	2	3200	0.1	40
1:2	281	3200	10	5600

This data shows the importance of molar ratio of foreign DNA to vector DNA. There is a 100 fold difference between the two experiments.

poly (dG) tails. By increasing the ratio of SV40 fragments to pBR322, the chance of SV40 being inserted is increased. When SV40 fragments are inserted, the linear pBR322 becomes circularized. Circular plasmids are required in HB101 for transformation to occur. Thus, the annealing reaction carried out under conditions of a larger SV40 concentration gives a greater transformation frequency because this ratio of SV40 : pBR322 results in a greater number of covalently closed circular plasmids.

The transformants were tested to see if they contained a plasmid with inserted DNA. pBRSVR1 was constructed so that if foreign DNA was inserted into the Pst I site located within the ampicillin gene then the transformants containing pBRSV1 would be tetracycline resistant, but ampicillin sensitive. Bacterial colonies were screened on tetracycline and ampicillin plates. The results of this experiment are shown in Table 3. A small percentage of the colonies were able to grow on ampicillin and tetracycline plates. It is hypothesized that these colonies do not have DNA inserted into the Pst I stie of pBR322. The probable explanation for this observation is in construction of pBRSVR1, small quantities of pBR322 DNA escaped Pst I digestion. Therefore, this small quantity of DNA was not able to insert SV40 fragments. The undigested plasmid was carried along as a contaminant with the recombinant plasmids and used to transform E. coli. Since the pBR322 plasmid is

Table 3. Screening on Ampicillin and Tetracycline Plates.

Colony #	Cell Viability	
	Tet	Amp
E2	+	-
E3	+	-
E4	+	-
E5	+	-
E6	+	-
E7	+	-
E8	+	-
E9	+	-
E10	+	-
E11	+	-
E12	+	-
E13	+	-
E14	+	-
E15	+	-
E16	+	-
E17	+	-
E18	+	-
E19	+	-
E20	+	-
E21	+	-
E22	+	-
E23	+	-
E24	+	+
E25	+	-
E26	+	-
E26	+	-
E28	+	-
E29	+	-
E30	+	-
E31	+	+

Colony #	Cell Viability	
	Tet	Amp
E32	+	+
E33	+	-
E34	+	-
E35	+	-
E36	+	-
E37	+	-
E38	+	-
E39	+	+
E40	+	-
E41	+	+
E42	+	+
E43	+	-
E44	+	-
E45	+	-
E46	+	-
E47	+	+
E48	+	-
E49	+	-
E50	+	-
E51	+	-
E52	+	-
E53	+	-
E54	+	-
E55	+	-
E56	+	-
E57	+	-
E58	+	-
E59	+	-
E60	+	-

intact in these colonies, the bacteria are resistant to ampicillin and tetracycline. Most colonies screened were ampicillin sensitive. Thus, it is most likely these colonies contain a plasmid with foreign DNA inserted the Pst I restriction site.

II. Isolation of pBRSVR1

Plasmid DNA was isolated from 58 transformant colonies. Each of these plasmids could contain 1 of 17 different Bgl I, Eco RII-cleaved SV40 fragments. Therefore, it was necessary to identify the colony containing the plasmid which carried the correct SV40 restriction fragment inserted into the Pst I restriction site.

A. Analysis by Restriction Enzyme Digestion

Since the restriction map of pBR322 and the SV40 genome is known, it is possible to use this information to determine which of the 17 different SV40 fragments has been inserted into the Pst I restriction site of pBR322. Digestion of the SV40 genome by Eco RII and Bgl I gives 17 different fragments which are shown in Figure 6.

Five fragments (including Fragment A which contains the coding region for the amino terminus of the SV40 large T antigen gene) labeled in Figure 6 contain a Hind III recognition site. pBR322 also has a Hind III recognition site. Therefore, plasmids containing one of the five SV40 fragments (A,G,H,I,N) can be recognized by the gel pattern produced

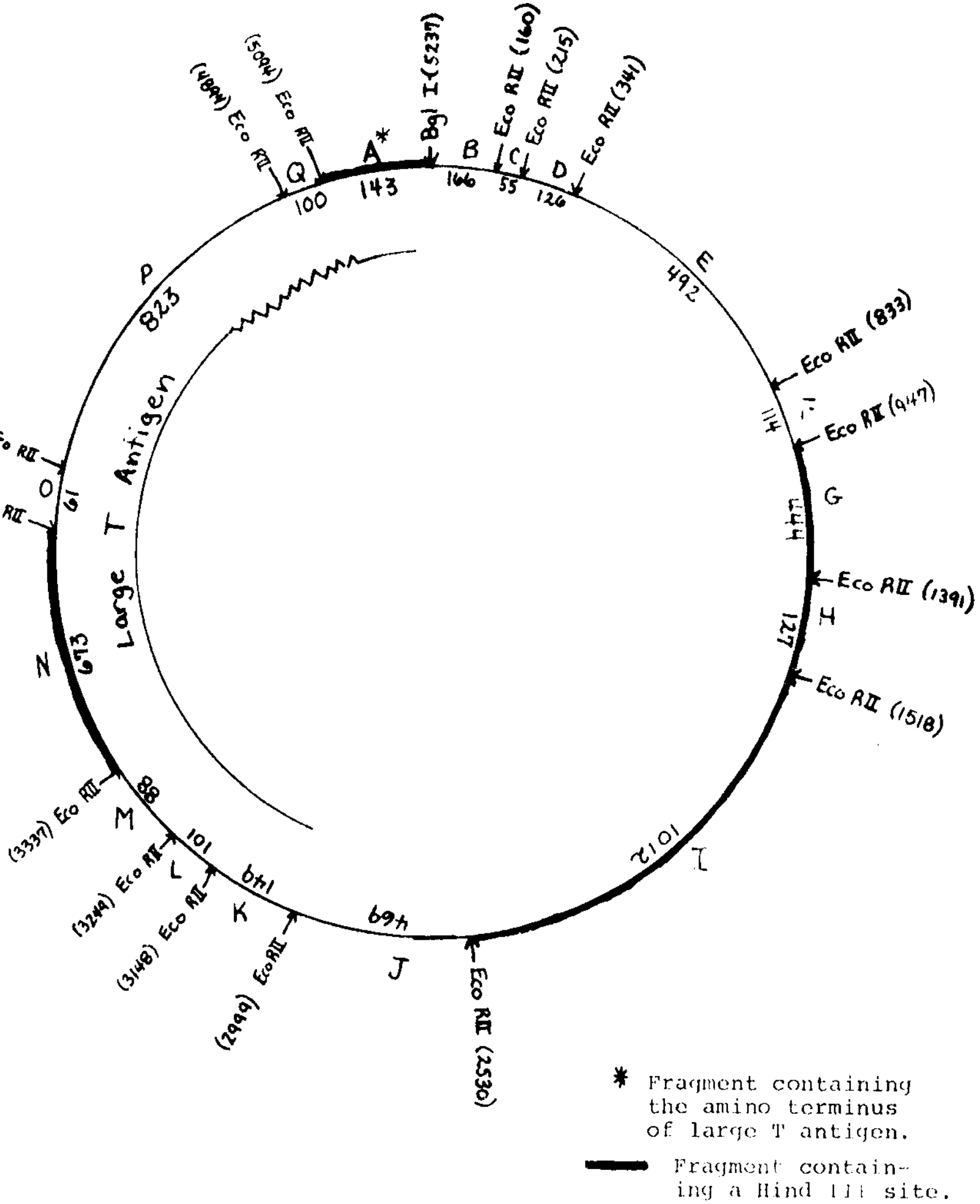
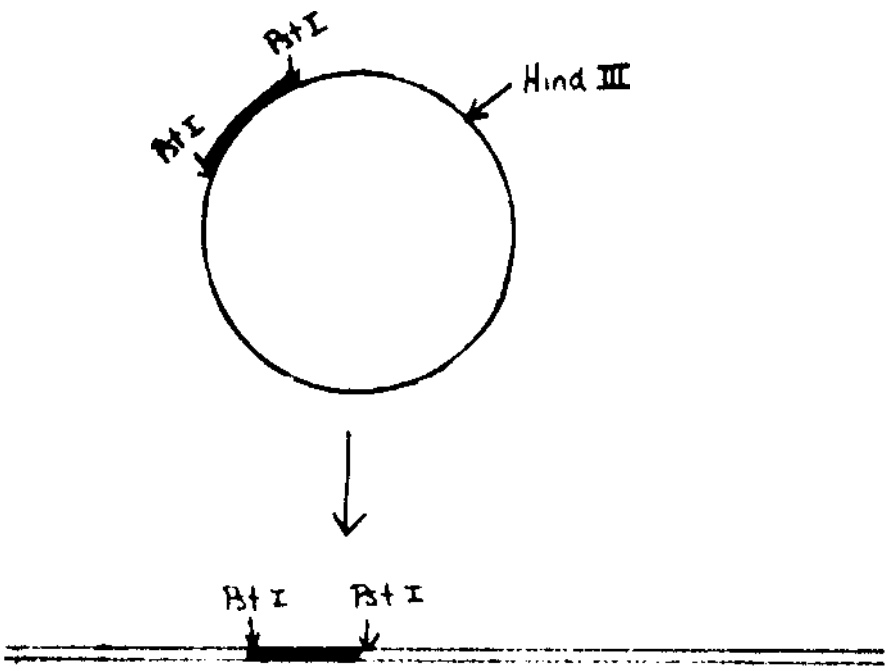


Figure 6. Digestion of SV40 genome by Restriction Enzymes Eco RII and Bgl I.

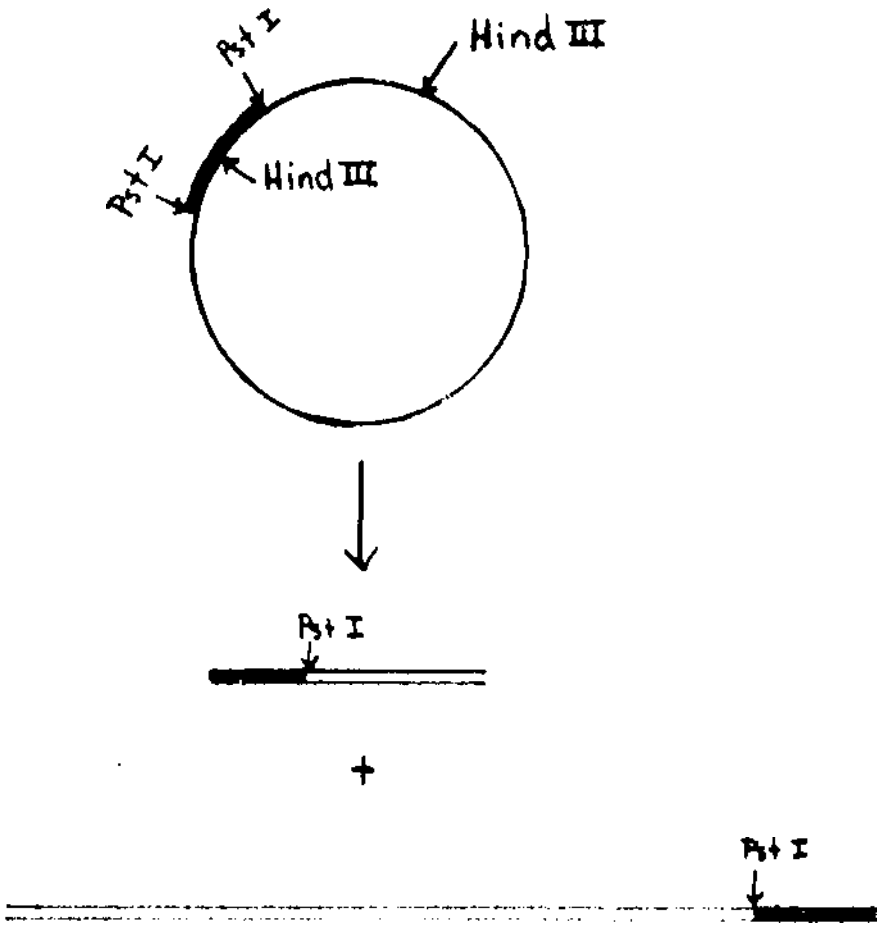
after Hind III digested. In contrast, Fragments A,G,H,I, and N will produce two bands after Hind III digestion. Both bands will move faster on the gel than the band for linearized pBR322 due to their shorter length. Plasmids containing Fragments B,C,D,E,F,J,K,L,M,O,P and Q will be linearized after Hind III digestion and will result in a single band on an agarose gel which moves slower than linearized pBR322. An illustration of the two different types of Hind III digestion patterns are shown in Figure 7. The Hind III digestion patterns of fifty-eight transformant colonies were analyzed. Of this fifty-eight, thirteen transformants contained a plasmid which gave the desired digestion pattern on the gel. A photograph of the gel pattern after Hind III digestion of a plasmid containing a fragment with a Hind III restriction site is shown in Figure 8.

Thus, now there are 12 colonies containing a plasmid inserted with Fragment A,G,H,I, or N. The next step in identifying the correct plasmid was to establish the length of the fragment inserted. As explained in the section on experimental design, the Pst I restriction site has been regenerated. Therefore digestion of the plasmid by Pst I will excise the inserted fragment from pBR322. Then using polyacridimide gel electrophoresis and molecular weight standards it is possible to estimate the size of the insert fragment. A standard curve (Figure 10) was prepared by plotting log (# of base pairs) versus the distance migrated

Figure 7. Comparison of the type of Hind III digestion fragments expected with a plasmid containing an SV40 sequence without a Hind III site (A) versus a plasmid containing an SV40 sequence with a Hind III restriction site (B). In Part A, Hind III digestion is shown to produce the linearized plasmid. The band resulting from this digest will move slower on a gel, due to the extra inserted piece of SV40 DNA, than linearized pBR322. In Part B, Hind III digestion of the plasmid results in two fragments. This plasmid can thus be recognized by the production after Hind III digestion of two bands which both move faster due to their shorter length on a gel than linearized pBR322.

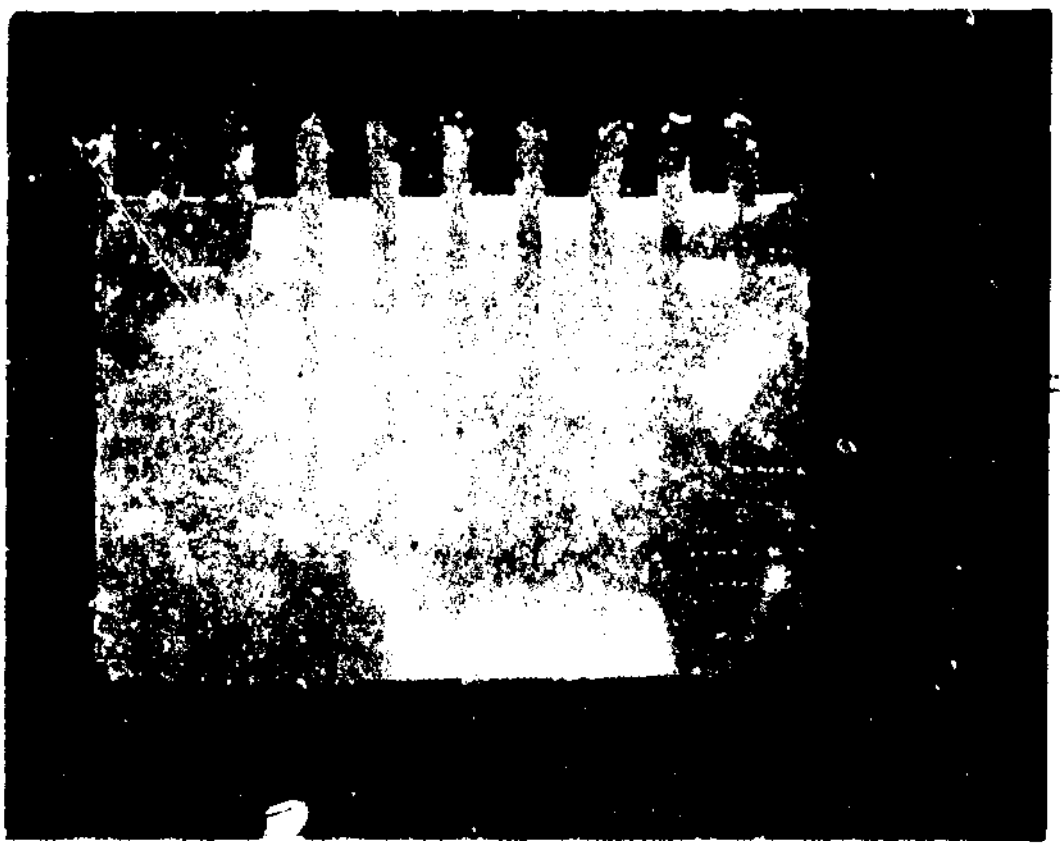

Bst I
Hind III
Bst I
Bst I
Bst I

A


Bst I
Hind III
Bst I
Hind III
Bst I
Bst I

B

Figure 8. Photograph of Hind III produced fragments on a gel. C32 and E16 produce a gel pattern after Hind III digestion which shows they contain an SV40 fragment which has a Hind III site. E4 contains an SV40 fragment without a Hind III site.



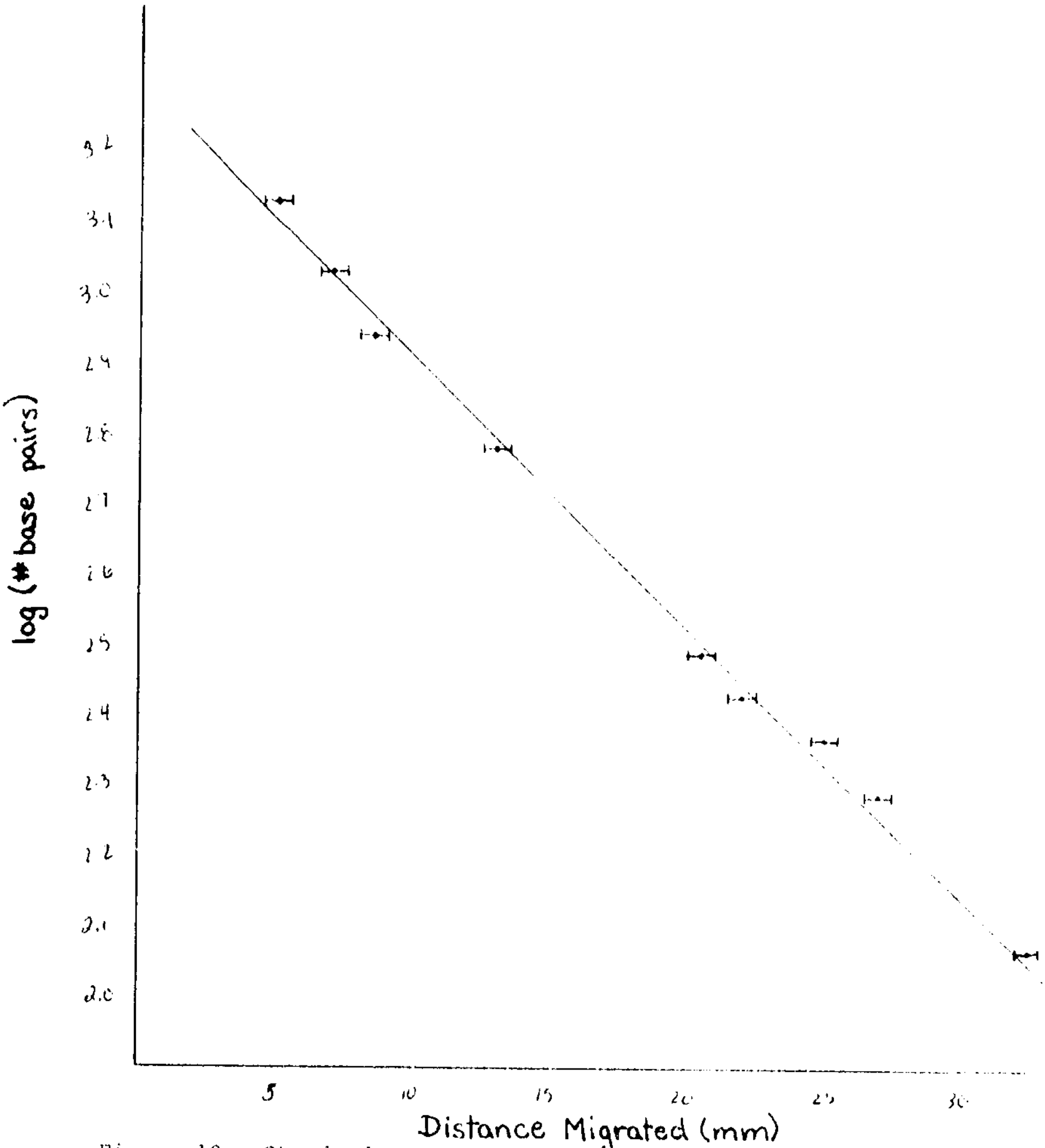


Figure 10. Standards Curve
log (# of base pairs) vs. distance migrated (mm) on 1% agarose gel.

by the band on the gel. This curve is then used to estimate the length of the SV40 fragment. A photograph of a typical Pst I digestion pattern is shown in Figure 9. The length of the SV40 fragment containing the amino terminus for the large T antigen gene is 143 base pairs. The length of fragments A,G,H,I and J are 143, 444, 127, 1102, and 673, respectively. Thus, using this method it was possible to estimate the size of the inserted fragment. A 5% polyacrylamide gel should effectively separate nucleotides of 80-500 base pairs in length. The estimated lengths of the inserted fragments is shown in Table 4. Of the 12 plasmids containing fragments with Hind III restriction sites, 3 plasmids contain fragments approximately 130-140 base pairs in length (2 other plasmids may also contain a 130-140 base fragments). An absolutely positive identification cannot be made at this point, however. Fragment A is 143 base pairs (bp) in length and Fragment is 127 bp in length. Distinguishing between the 143 bp fragment and the 127 bp fragment is not possible, because when these fragments are cut out of pBR322 by Pst I, a poly (dC) tail is present on either end of the fragment. The poly (dC) tail was added originally when constructing the plasmid and should be 20 ± 4 base pairs long. The degree of error, ± 8 bp in the length of the tail makes it impossible to distinguish between 143 bp and 127 bp fragments by gel electrophoresis.

B. Southern Blot Analysis

A summary of the data up until this point is shown in Table 4. In order to make an absolute positive identification of the plasmid containing the 143 bp fragment, it was necessary to prepare a Southern blot and probe with a piece of DNA that is complementary to the 143 bp fragment, but not the 127 bp fragment. The probe used was the 500 bp fragment from Taq I, Bgl I-cleaved SV40 DNA. If the plasmid being probed contains the correct sequence of DNA (i.e. the amino terminus of the large T antigen gene), then it will light up on the film when autoradiographed.

Table 4. Results of Hind III Digestion Analysis, Pst I Estimation of Inserted Fragment Size and Southern Blot Analysis.

Plasmid #	Estimated Length of Inserted Fragment	Hind III Analysis (Contains Hind III site within inserted fragment)
O4	?	+
C18	130-140	+
C32	130-140	+
C34*	993	+
C42	600-700	+
C52	550-600	+
		+
C60	?	+
E4	130-140	-
E12	450-500	+
E15	450-500	+
E16	130-140	+
E18	650-750	+

*If the 993 base pair fragment is inserted into pBR322 it produces a 3 bands instead of 2 bands after Pst I digested. Thus, the identity of this fragment can be confirmed.

CONCLUSION

The data provided indicates that the plasmid pBRSVR1 has been used to transform E. coli. The Hind III digestion analysis, the Pst I idgestion analysis were used to identify plasmids which contain a 143 bp or 127 bp fragment. Blot analysis can now be used to prove that the plasmid contains the 143 bp fragment which codes for the amino terminus of the SV40 large T antigen gene. Thus, the purpose of this project which was to clone the amino terminus of the SV40 large T antigen gene would be completed. The next step is to prepare a large scale plasmid isolation.

This fragment containing the amino terminus of the SV40 large T antigen gene will be used to optimize the distance between the promotor and initial codon of the large antigen gene in order to get maximum expression of the initial region of the T antigen gene. By varying the distance between the promotor and the initial codon, new insight into the mechanics of transcription in prokaryote systems will be gained. In addition, since T antigen is a eukaryotic protein it will be interesting to see ifa after the removal of the intervening sequence whether E. coli is able to produce a functionable form of the protein when the large T antigen gene is cloned. If this is possible, it is feasible to predict that any eukaryotic gene without intervening sequences can be used in cloning.

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