CHARACTERIZATION OF THE SELF-REPLICATING KIRSTEN MURINE LEUKEMIA VIRAL DNA: REPLICATION AND TETRACYCLINE RESISTANCE

DISSERTATION

Presented to the Graduate Council of the

University of North Texas IN Partial

Fulfillment of the Requirements

For the Degree of

Doctor of PHILOSOPHY

By

Hossein Najmabadi, B.A., B.S., MT (ASCP)

Denton, Texas

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Kirsten Murine Leukemia Virus (MLV-K) is a retrovirus that only infects and replicates in eukaryotic cells. These viruses have not been reported to be able to grow in a bacterial cell. However, when the MLV-K was molecularly cloned into pBR322, it was observed that the retrovirus sequences were excised from recombinant molecules. This DNA can replicate and give tetracycline resistance to bacteria. It was of interest to study the excised retroviral DNA, which was able to replicate and confer tetracycline resistance in Escherichia coli. It appears that during excision of retroviral sequences from the recombinant molecule, some pBR322 sequences are also excised so that MLV-K:E contains about 4.1 Kbp of the retroviral DNA sequences and 3.6 Kbp of the pBR322, from the Hind III to the Pst I site. This portion of pBR322 contains the origin of replication and tetracycline resistance.

The restriction endonuclease and Southern blot analysis revealed a fragment of pBR322 from the Hind III and Pst I site that is located in the 3' end of the MLV-K:E molecule. Single stranded sequencing of the two terminal ends of this fragment verified that the 3' end of MLV-K:E contains identical sequence homology to pBR322. The presence of this pBR322 fragment explains the unusual properties of the MLV-K:E molecule. However, tetracycline resistance is less in E. coli containing MLV-K:E than E. coli containing pBR322 as determined by zone of inhibition assay. This may be due to alteration in the promoter region of the tetracycline gene.

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TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	iii
LIST OF TABLES	vii
LIST OF ILLUSTRATIONS	viii
LIST OF ABBREVIATIONS	x

Chapter

I. INTRODUCTION 1
- History
- Characteristics of Retroviruses
- Replication of Retroviruses
- Kirstin Murine Leukemia Virus
- Molecular Cloning of MLV-K into pBR322
- General Characteristic of pBR32
- Origin of Replication
- Tetracycline Resistance

II. STATEMENT OF RESEARCH PROBLEM 24

III. MATERIALS AND METHODS 25
- Zone of Inhibition Assay for Tetracycline Sensitivity
- Preparation of Competent Bacterial Cells and Transformation
- Large Scale Purification of Small Supercoiled DNA from Bacteria
- Small Scale Purification of Plasmid DNA (Mini Plasmid Procedure)
- Southern Blot Transfer of DNA
- Hybridization of Southern Filters

Radioactive Labelling of DNA
A. Radioactive Labelling of pBR322 Probes
B. Radioactive Labelling Hind III Cut Lambda DNA
Cloning Strategy of MLV-K:E into pT7/T3∝ -18
Preparation of Single Stranded Template
DNA Sequencing
A. DNA Sequencing Reaction
B. Preparation of Sequencing Gel
Agarose Gel Electrophoresis
Geneclean Kit Procedure

IV. RESULTS 44

V. DISCUSSION 65

APPENDICES

A. Retroviral Genome and Integrated Proviral DNA 71
B. The Mechanism of Reverse Transcription . 73
C. Morphological Responses to Murine Erythroblastosis Virus 75
D. Restriction Endonuclease Map of the MLV-K:E Genome 77
E. Electron Micrograph of the MLV-K:pBR322 DNA . . 79
F. Plasmid pBR322, and Plasmid ColE1 Origin of Replication and Maintenance Region . . 81
G. The pBR322 DNA Sequence 83
H. Major Secondary Structural Features of RNAs Involved in ColE1 Replication Control . . 87
I. Method for Transfer of DNA from Agarose Gels to Nitrocellulose Filter Paper 89
J. Biological Map of pT7/T3∝-18 Plasmid and Multiple Cloning Site 91
K. Brief Protocol for DNA Sequencing With Sequenase 93

L. Biological Map of pBR322 95

BIBLIOGRAPHY 97

LIST OF TABLES

Table		Page
I.	Classification of Tetracycline Resistance Determinants	18
II.	Comparison of Tetracycline Resistance. . . .	45

LIST OF ILLUSTRATIONS

Figure		Page
1.	Eco RI Digested Small Circular DNA Isolated from Bacteria Containing MLV-K:pBR322 and Analyzed on 0.8% Agarose Gel	8
2.	Genetic Map of the Class B from Tn 10	20
3.	Mechanism Involved in Regulation of the Tetracycline Resistance Gram Negative Bacteria	22
4.	Comparison of Tetracycline Resistance of E. coli RR1 Containing no Plasmid, pBR322, and MLV-K:E	46
5.	MLV-K:E and pBR322 DNA Digested with HindIII Restriction Endonuclease and Analyzed by Agarose Gel Electrophoresis	48
6.	Autoradiography of the Southern Blot of the Agarose Gel Shown in Fig 4	49
7.	Restriction Endonuclease Digest of MLV-K:E DNA	51
8.	Autoradiograph of a Southern Blot of the Gel Shown in Fig 6	52
9.	Restriction Digestion of MLV-K:E and pBR322 with Hin FI, and Hha I	54

10. Plasmid Isolated from White Colonies were Digested with Pst I Restrictions Endonuclease and Analyzed by Agarose Gel Electrophoresis . 56

11. Cloning Strategy of MLV-K:E into Pst I Site of pT7/T3α-18 57

12. Orientation of MLV-K:E Cloned into the Pst I Site of pT7/T3α-18 by Restriction Enzyme Digestion 58

13. Comparison of Nucleotide Sequences of 3' Terminal End of MLV-K:E and Nucleotide Sequences of pBR322 that encodes Beta-Lactamase Gene. . 59

14. Autoradiograph of an 8% Polyacrylaxpoide 7 M Urea Gel Showing the Products of Sequencing Single-Stranded by Sequence System 61

15. Comparison of Nucleotide Sequence of MLV-K:E to 5' Portion of Tetracycline Gene of pBR222. . 62

16. pBR322 Digested Site with Hin FI 64

17. Model for the Excized Molecule-(MLV-K:E). . . . 69

LIST OF ABBREVIATIONS

Abbreviation:	
amp	ampicillin
cpm	counts per minute
DTT	dithiothreitol
EDTA	ethylene Diaminetetraacetate
h	hours
IPTG	isopropylthio-B-D-Galactopyranoside
MLV-K	Kirstin Murine Leukemia Virus
MLV-K:E	MLV-K:pBR322
min	minutes
SDS	sodium dodecyl sulfate
sec	seconds
SSC	Standard saline citrate
TEMED	N,N,N',N'-tetramethyl ethylene diamine
tet	tetracycline
Tris	Tris(hydroxymethylaminonomethane)

UP-water	Ultra pure-water
X-gal	5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside

CHAPTER 1

INTRODUCTION

A variant of Kirsten murine leukemia virus (MLV- K) was molecularly cloned into the bacterial plasmid pBR322. This MLV-K:pBR322 recombinant molecule was utilizied to transform Escherichia coli strain RR1. During the replication of the recombinant molecule in E. coli the MLV-K portion of the recombinant molecule was excised and was able to replicate independently in bacteria and gave the bacteria resistance to tetracycline. The objectives of this research were to determine how an animal viral DNA could replicate in bacterial cells and how the presence of the viral DNA confers tetracycline resistance in bacteria.

History

Retroviruses are viruses that induce cancer in a number of animal species. The first tumor inducing virus was isolated in 1911 by

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This dissertation follows the style of Cell.

Peyton Rous. For many years he had been studing a spontaneous chicken sarcoma, a tumor of connective tissue. He demonstrated that an agent was present in tumor extracts and that the agent could pass through a filter through which cells and bacteria could not pass (Rous, 1910). Tumor induction was shown to be caused by a virus, later called Rous sarcoma virus (RSV). During the early 1930's, several inbred mouse strains were found to have high frequency of spontaneous cancer. One of these strains, C3H had a high incidence (90%) of mammary cancer (Strong, 1935). When the females were from the C3H strain, greater than 90% of the female progeny developed mammary cancers, whereas when the C3H parent was the male, less than 10% of the female progeny developed mammary tumors (Staff of the Roscoe B. Jackson Memorial Laboratory, 1933). This result and some other experiments suggested that a nonchromosomal factor was transmitted from parent to offspring through the female parent. Finally Bittner in 1944 discovered the etiological agent in mouse milk to be a virus called Bittner virus (Bittner, 1944) which later became known as mouse mammary tumor virus (MMTV).

Discovery of Gross leukemia virus (Gross, 1951) quickly led to the discovery of the viruses responsible for other forms of cancer in mice.

Soon, other forms of murine leukemia virus (MLV) were isolated namely, Graffi virus (Graffi et al., 1955). Moloney virus (Moloney, 1960), Rauscher virus (Rauscher, 1962).

Characteristics of Retroviruses

All retroviruses have common morphological,biochemical, and physical properties that justify their inclusion into a single virus family. The genome of the retrovirus occurs as a dimer of apparently indentical 8-10 kb single stranded RNA molecules in which the 5' ends are linked together. The genome is positive-sense in that it can be used to direct protein synthesis. All nondefective retrovirses contain three genes: gag, codes for internal structural, pol, codes for the reverse transcriptase, and env, codes for envelope proteins. The order of the genes are 5' gag-pol-env 3'. Some of the retroviruses contain an additional oncogene (Appendix A).

Chemical composition of retroviruses shared by all the members of the family. They contain about 60-70% protein, 30-40% lipid, 2-4% carbohydrates, and about 1% RNA (Beard, 1963).

Replication of retroviruses

Retroviruses are unique in their replication cycle in that they go through a DNA intermediate (RNA-DNA-RNA). Retroviruses attach to the cell's specific receptors and penetrate the host cell. Different activities of reverse transcriptase result in generation of a linear double stranded DNA with two long terminal repeats (LTR) at the ends(Appendix B). The free linear DNA is converted into circular viral DNA (provirus) and is the precursor to integration of the viral DNA. At least one DNA copy becomes integrated into the host DNA. Once integrated, the provirus will be replicated and distributed equally to daughter cells along with the host DNA. Viral transcription occurs from the integrated viral DNA. The primary transcript is a 35 S RNA which leaves the nucleus and goes to the cytoplasm either unspliced (35 S RNA) or spliced (30 S RNA). The full length 35 S RNA is translated into gag and pol protein. The 30 S spliced viral RNA is translated to env proteins. The virion proteins are assembled into particles that have encapsidated the viral 35 S RNA subunits. The envelope of the virion is formed at the plasma membrane as the virus is released from the cell by a process called budding.

Kirsten Murine Leukemia Virus

Throughout the two decades of the 30 and 40's when transplantable leukemia strains were being developed, there were frequent attempts to pass the disease by method of extracts of leukemia cells and injection of the extracts to low incidence adult leukemic mice. Nevertheless all the well-documented experiments, gave negative results (Furth et al., 1933 and Engelbreth, 1948).

As it is now known, there were two major reasons for their negative results. First, the use of adult recipient for newly infected cells would stimulate cellular immune responses. Second, some mice are resist to infection by some strains of virus. In 1959, studies were begun by Kirsten and Mayer, to determine if extracts from human leukemic tissue, inoculated into newborn mice would induce leukemia in recipient mice (Kirsten and Mayer, 1967). A murine erythroblastosis virus (MEV) was recovered during cell-free passage of thymic lymphomas from old C3HF/GS mice. The virus induced proliferation of red cell precursors. Kirsten and Mayer (1967) isolated mouse erythroblastosis virus (MEV), later called MLV-K, from cell free passage of thymic lymphoma from old C3HF/GS mice. Passage of cell free extract from mice with erythroblastosis to the rat induced erythroblastosis, lymphoma, and

sarcoma. Murine sarcoma virus was isolated from the rat after infecting them with MLV-K (Appendix C) (Kirsten and Mayer, 1967).

Molecular cloning of MLV-K into pBR322

Normal rat kidney cells, NRK-II were infected with MLV-K or MSV-K complex which contains two viruses, one a replicative competent MLV-K and replicative-defective MSV-K which carries the ras oncogne. Sixteen hours after infection, the unitegrated viral DNA was extracted from infected cells by the Hirt detergent fractionation method (Hirt, 1967). Nucleic acids present in the Hirt supernatant were concentrated by ethanol precipitation, redissolved, and subjected to rate zonal centrifugation (Maniatis et al., 1978). Following rate zonal centrifugation, the density gradient tube was fractionated into 30 fractions. Aliquots of each fraction underwent electrophoresis on 0.8% agarose gels (Sugden et al., 1975). To identify the viral DNA, the DNA in the gel was transferred to nitrocellulose filter (Southern, 1975) and analyzed for the presence of viral DNA by hybridization, under conditions previously described (Wahl et al., 1979), to a nick-translated (Maniatis et al., 1976) probe consisting of the entire genome of Moloney murine leukemia virus (Van Beveren et al., 1981). Gradient fractions containing

retroviral DNA were pooled, concentrated by ethanol precipitation, and digested with Pst I restriction endonuclease. The Pst I digested DNA was subjected to another rate zonal centrifugation, and the fractions containing viral DNA sequences were identified and pooled as previously described. The DNA in the pooled fractions was concentrated by ethanol precipitation and was cloned into the plasmid pBR322.

The unitegrated proviral DNAs were cut with Pst I restriction enzyme which generates sticky ends, and then ligated to Pst I-cut pBR322 at 15^0C for 16 h in the presence of 7 units of T4 ligase. The ligated DNA was added to competent bacteria and was plated on NZYM medium-containing tetracycline (100 ug/ml) and incubated at 37^0C for 16 h. Colony hybridization was used to screen the colonies on plates. The plasmid DNA was purified by a modification of the Hirt procedure.

The recombinant plasmid when digested with Pst I and analyzed by agarose gel electrophoresis and resulted in a viral band of 7.7 Kbp and a pBR322 of 4.3 Kbp. The Eco RI was chosen to cut the recombinant plasmid to generate one band about 12 Kbp. Five bands were observed (Fig. 1) instead of one, one about 12 Kbp corresponding to MLV-K:pBR322 recombinant and one about 4.3 Kbp indentical to free pBR322 (Knesek et al., Unpublished Observations).

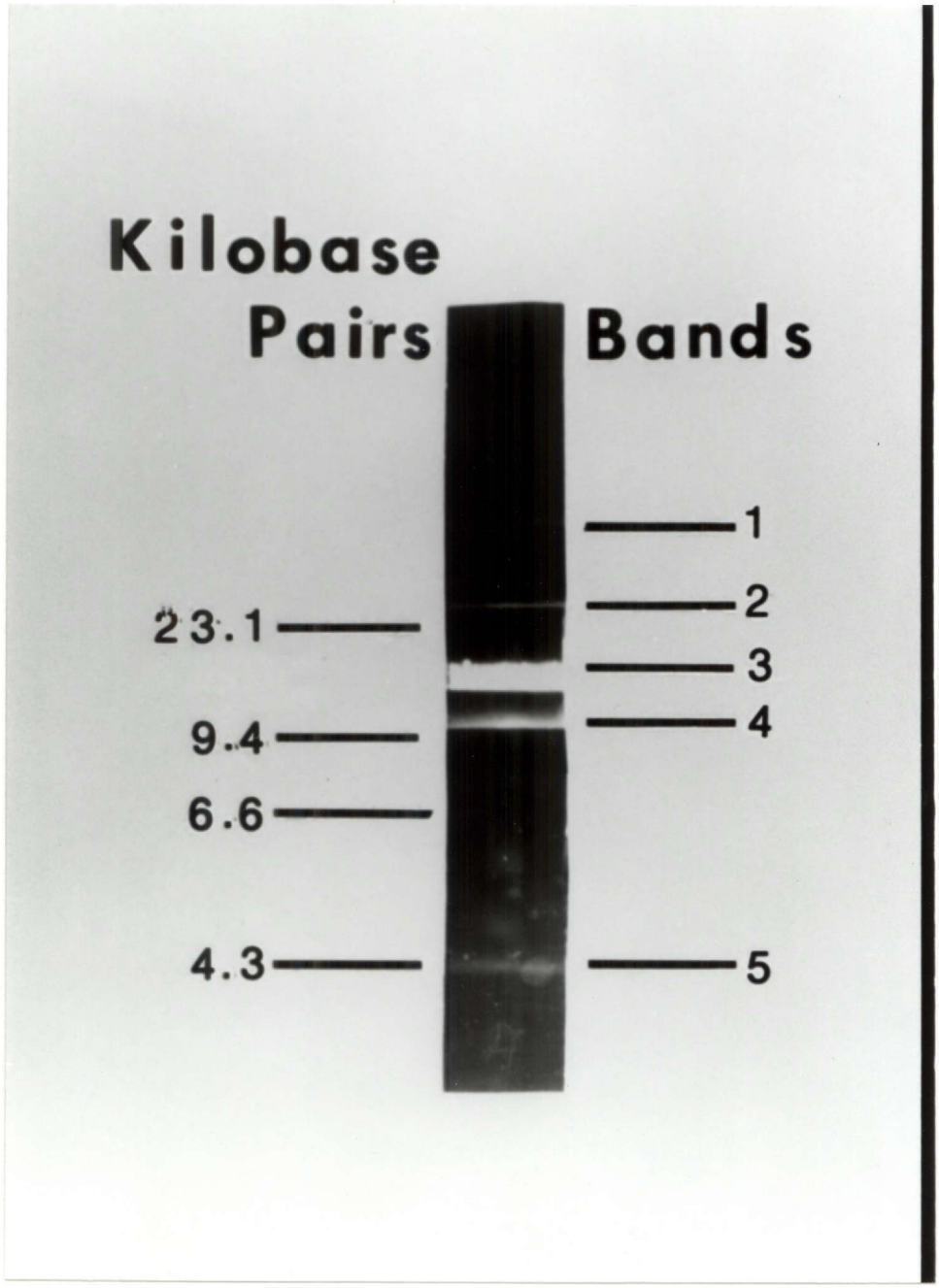


Fig. 1 Eco RI digested small circular DNA isolated from bacteria containing MLV-K:pBR322 and analyzed on 0.8% agarose gel.

Each band was purified from the gel and further characterized. Band 5 was the same size as pBR322 of 4.3 Kbp and had a similar restriction endonuclease map. Band 3, is about 12 Kbp with a restriction map similar to MLV-K:pBR322. Bands 1,2,4, when digested with Xho I a enzyme that does not cut pBR322 but, cuts MLV-K once. All three DNAs came to one band about 7.7 Kbp and the restriction map for this band was similar to MLV-K (Appendix D). An additional experiment was performed, in which DNA from Band 4 was added to competent bacteria. The bacteria grow on tetracycline plates but not ampicillin plates.

The plasmid that was extracted from these bacteria contained 7.7 Kbp viral DNA. Suggesting that the 7.7 Kbp viral DNA was excised from the MLV-K:pBR322 (MLV-K:E) and was able to replicate and give tetracycline resistance to the transformed bacterial cells.

Electron micrograph of the plasmid DNA from bacteria containing MLV-K:E showed some interesting DNA structures, (appendix E). Electron micrographs of DNAs were done in collaboration with Dr. Donald Robberson, the University of Texas System Cancer Center at Houston. The DNA structures show a figure-eight structure with the larger circle measuring 8 Kbp and the small circle measuring 4.3 Kpb in length. These structures are similar to structures proposed by Holliday for DNA

recombination intermediates of circular DNA (Dressler and Potter., 1982)

Two possibilities for replication of retroviral DNA in a prokaryotic organism is as follows. One, the retroviral DNA contains nucleotide sequences that allow it to replicate in E. coli. The second possibility is that during the excision of the 7.7 Kbp retroviral sequence from MLV-K:E recombinant molecules, the origin of replication was removed from pBR322. Another important property of excised 7.7 Kbp retroviral DNA is its ability to allow bacteria that contain it to grow in the presence of tetracycline. Still to be answered is whether the tetracycline resistant genes are part of the retrovirus sequences, or they removed from the pBR322 genome during excision.

General Characteristic of pBR322

Since the molecular cloning vector was pBR322 (Sutcliffe et al., 1978;Bolivar et al., 1977), it is necessary to point out how pBR322 was originally constructed from three different naturally occurring plasmids. The tetracycline resistance phenotype derives from a fragment of the Salmonella plasmid pSC101 (Cohen and Chang, 1977). The ampicillin resistance gene is from the Tn3 transposon originally carried by the Salmonella. paratyphi B plasmid R 7268 (Datta and Kontomichalou, 1965;

Meynell and Datta, 1967). The origin of replication is derived from pMB1, an E. coli plasmid bearing the genes for ColE1 (colicin immunity) and the Eco RI restriction and modification enzymes (Appendix F and G) (Betlach et al., 1976).

Plasmids are circular duplex DNA molecules that reside stably in the extrachromosomal state in the host bacterium. The duplication of these elements should involve a carefully regulated process that maintains a constant level of plasmid in a bacterium. Replication is initiated at a fixed origin and is unidirectional in the case of CoLE1. The origin of replication pBR322 was derived from CoLE1 plasmid (Betlach et al., 1976). This plasmid require DNA polymerase I for replication. The CoLE1 plasmid differs from most other plasmids in its ability to continue to replicate in the absence of protein synthesis (Bazaral et al., 1970;Clewell et al., 1972).

Origin of replication of pBR322

The plasmid encodes two regulators of replications initiation, one which plays a central role and another which plays an apparently auxiliary role by directly assisting the major regulator. The two regulators work in trans and they have a negative effect on the plasmid and mutation

usually cause increased in copy number.

The major regulator of CoLE1 origin of replication is a small RNA molecule of 108 nucleotides (Lacatena and Cesareni, 1981; Tomizawa and Itoh 1981), because of nucleotides composition of this regulatory RNA, in solution, it forms a tightly folded molecule which contains three stem-loop domains and a free single-stranded 5'- terminal (Tamm and Polisky, 1983); this regulatory RNA is called RNAI. The CoLE1 primer transcript that can serve in vitro as the primer for initiation of leading DNA strand synthesis by DNA polymerase I (Itoh and Tomizawa, 1980), starts 555 base pairs upstream of the replication origin, forms a primer refer as RNAII. This is a large size primer 555 nucleotides in its mature form, RNA II primer functions poorly in the lack of post transcriptional processing by RNAase H. Almost half of the RNAII molecules which extend upstream from the origin of replication make a very stable hybrid with the DNA near the origin of replication. The hybridized RNAII is cleaved at the origin of replication by RNAase H and can then serve as a primer for DNA synthesis by DNA polymerase (Itoh and Tomizawa, 1980; Masukata and Tomizawa 1984). After several hundred nucleotides have been laid down, DNA polymerase I is replaced by a DNA polymerase III. RNAase H cleavage of the primer precursor is the step at which negative regulation

by RNAI is exerted in vitro (Tomizawa et al., 1981).

This RNA (RNAI) is transcribed in the direction opposite that of RNAII. If RNA I is present from the outset of primer transcription, cleavage by RNAase H is inhibited. Binding of RNA I to the complementary region of RNAII stops the cleavage of RNAII by RNAase H at the origin and results in the lack of primer for DNA synthesis (Tomizawa and Itoh, 1981). The structural complex formed between primer (RNAII) and RNA I indicates that binding with RNAI alters primer conformation in structural domains distant from the RNA-RNA duplex domain (Masukata and Tomizawa,1986).

Action of RNA I can be explained by "action at a distance", hypothesis, in which permits interaction of the 5'-terminal end of the RNAII with RNAI affects the fate of the 3'-terminal domains, causing structural alteration, and prevents RNAase H processing. The ability of RNAII to engage in a hybrid with the template strand is depend to certain structural domains of the RNAI. RNAI blocks the formation of a critical primer domain required for hybridization; if this domain does not form, RNA polymerase continues to elongate, and the primer would be released from the template.

The efficiency of interception of the primer (RNAII) precursor by RNAI believed to control the copy number of CoLE1-type plasmid. One of the

factor influence this interaction is protein which is encoded by the CoLE1 plasmid. The product of the gene known as rop (repressor of primer) or rom (RNA one modulater). This gene encodes a small, acidic protein of 63 amino acid that exist as a dimer in solution. This protein accelerates the interaction of primer longer than 135 nucleotides and shorter than about 85 nucleotides, but it serve as inhibitory for interaction of intermediate length primers. This prove the Rop/Rom protein is highly sensitive to RNA conformation (Tomizawa and Som, 1984).

Since it is difficult to detect stable interaction of the protein with RNA, there are not much information available about the mechanism of this acceleration occurs, but, recently, specific but very low affinities for RNAII and RNAI have been shown for purified Rop/Rom protein (Helmer-Citterich et al.,1988). The Rop/Rom protein has been crystallized and its structure solved to 1.7 A^0 by X-ray diffraction (Banner et al., 1987).

Study with CoLE1- plasmid which were capable of replication in RNAase H-deficient cells suggested additional mod of replication may exist in vivo (Naito et al;1984). CoLE1-type replication requires the genetic information contained in a 580 bp region, and any change in this region destroys it ability to replicate. The comparison between

nucleotides sequences of CoLEI and pBR322 demonstrates only few nucleotides differences (Appendix H).

Tetracycline Resistance

The tetracyclines are a family of antibiotics introduced in the late 1940's. The third member of the family, tetracycline was initially prepared by dehalogenation of chlortetracycline, but was later obtained from Streptomyces viridifaciens. Presently about 20 Streptomycetes are known which produce a mixture of different tetracyclines. Studies on the genetics of S. aureofaciens have shown that over 300 genes are involved in the biosynthesis of chlortetracycline. Now various research groups have indicated plasmid involvement in the control of tetracycline biosynthesis (Vanek et al.,1971,Hostaleketal; 1979).

Tetracycline resistance as a therapeutic problem and a widely dispersed determinant, was first recognized in a dysentery epidemic in Japan in the mid 1950's (Akiva et al., 1960). At present, tetracycline resistance is the most common resistance determinant among all species. The exclusive mechanism for resistance involves decreased penetration of the drug into the cell. Tetracycline are broad spectrum antibiotics, which are effective against Gram-Positive and Gram-Negative bacteria,

as well as rikettsias, mycoplasmas, leptospiras, spirochetes and chlamydias. Recent study suggested that the carboxamide group at the C2 position of the tetracycline molecule is required for transport into bacteria (Chopra;1986). Tetracycline is hydrophilic and has a four ring-structure. Researchers have shown that tetracycline inhibits the binding of aminoacycl-tRNAs to the A site on the 30 S ribosome (Sarkar and Thach, 1968; Hierowshi, 1985), showing that primary effect of tetracycline is inhibition of protein synthesis. In other studies, photoincorporation of radiolableled tetracycline into E. coli ribosomes has demonstrated a preferential binding of drug to the 30 S ribosomal protein S7 that is located near the A site (Goldman et al., 1983). Many clinically useful antibacterial drugs have intracellular target sites. Therefore, in order for tetracycline to enter E. coli and the other Gram-negative organisms, it must first pass through the lipopolysaccharide (LPS)-rich outer membrane. Tetracycline passage through the outer membrane requires proteins, designated cmlB because of their cross-resistance to chloramphenicol. These proteins were later found to be deficient in provin IA (Reeve, 1968, Chopra; 1988). Active uptake occurred in EDTA-treated cells and spheroplasts (McMurry, et al. 1981). Inner membrane vesicles (called "right-side-out") from sensitive

E. coli cells showed that tetracycline, as well as minocycline, accumulated in these vesicles in the presence of an energy source. This finding proved that the inner membrane is the site of tetracycline transport.

In Gram negative bacteria, a minimum of four different classes of tetracycline genes (tetA, tetB, tetC, tetD) have been defined (Mendez et al., 1980). Mendez classification is based on strains' sensitivity to tetracycline and tetracycline analogues such as minocycline and chelocardin. These analogues are active against some, but not all, tetracycline strains. The DNA-DNA hybridization was also used to prove the genetic dissimilarity between the different tetracycline resistance determinants (Table. I).

Class A includes the RPI-like determinants. These express lower resistance to tetracyline and little or no resistance to tetracycline analogues. Class B is reserved for determinants with homology to the Tn10 Tc determinant. These express resistance to tetracycline analogues as well as to tetracycline itself. Class C is composed of plasmids with very low resistance to tetracycline (<25 ug/ml) and with sensitivity to the analogues. pSC101 of Salmonella belongs to this category. Class D is represented by a single plasmid determinant, that encoded by RA1

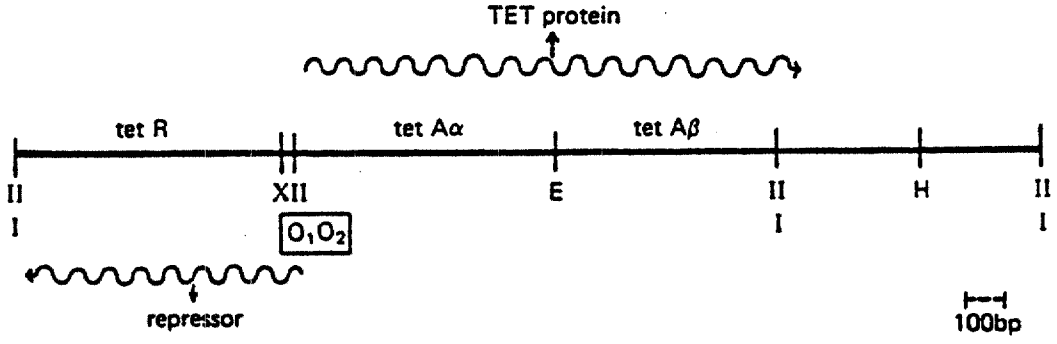
Table 1. Classification of Tetracycline Resistance Determinants

Class	Plasmid	Tet Resistance (ug/ml) Tet	Analog resistance (ug/ml) Mino	Chelo	Prototype Plasmid
A	RP4, RP1 R446b, pIP15 pIP7, pIP113	≥ 75 ≤ 150	≥ 5 < 10	< 5	RP1
A1	R386, N3	75	≥ 5 < 10	≥ 5 < 10	R386
B	R222, pIP69 R136, R455 R688, R7 R621a, R124 R64, R724	≥ 150 ≤ 200	≥ 10	≥ 5	R222
B1	R725, R27	≥ 50 ≤ 100	≥ 5 < 10	≥ 5 < 10	R27
C	pSC101, R144 SP219, pRI20	≤ 25	< 5	< 5	pSC101
D	RA1	100	< 5	< 5	RA1

Table 1. Classification of tetracycline resistance determinants among different plasmid (Mendez et al., 1980).

(Mendez et al., 1980). The DNA sequence of the determinant on pBR322 suggested that there are overlapping proteins which mediate the resistance phenotype (Sutcliffe, 1978). The DNA sequence of the determinant on pBR322 suggested that there are overlapping proteins which mediate the resistance phenotype (Sutcliffe, 1978). Only two protein products are expressed from each of the three tetracyline resistance determinants so far studied, namely an inner membrane TET protein 36,000, and a 23,000-25,000 dalton repressor protein(Fig. 2). Tetracyline-inducible membrane protein (TET) has been found in cells bearing tetracyline resistance determinants of all classes (Altenbuchner et al., 1983). Only a single inducible gene product,corresponding to TET protein, was detectably synthesized from the cloned portion of this region (Wray et al., 1981). Therefore, it was of interest to examine a series of tetracycline-sensitive point mutants in Tn10-mediated resistance were isolated that would compliment to give tetracycline resistance (Curiale and Levy, 1982). Moreover, these mutants could be classified into either of two complementation groups,tetAα or tetAβ. Point mutations in either the tetAα or tetAβ region markedly affected the synthesis and/or size of the TETA protein (Curiale and Levy, 1984).

Since tetracycline-sensitive mutants in one domain can partially



Genetic Map of the Class B Determinant From Tn10.

Fig. 2 Genetic map of the Class B determinant from Tn10. The overlapping DNA segment containing the operator-DNA regions is presented in the box (Xba I-Sau 3A fragment). The location of the two domains of TET protein is approximated. Restriction enzyme sites: I, Hpa I;II, Hin cII; E, Eco RI; H, Hind III;X, Xba I.

complement sensitive mutants in the other domain and some sensitive mutants show dominance over the wild type, a multimeric structure for TET in the membrane has been suggested. To test alone hypothesis, a tetA-phoA gene fusions was constructed, except for last 40 base pairs of the tetA gene all was fused with carboxy terminal of the Alkaline phosphatase (phoA), whose activity requires its dimerezation in the periplasm. Their finding suggest that TET functions as a multimer and that intact beta domains is require for full tetracycline resistance. Expression of tetracycline resistance from Tn10 (Class B) is negatively regulated by a repressor (Fig. 3) (Wray et al., 1981), which also regulated itself (Beck et al., 1982). Genetic organization of the Tn10 determinant consists of separate regulatory and structural regions which are transcribed from overlapping promoters in opposite directions(Wray et al., 1981). Transcriptional analysis of Class C determinants (Stuber et al., 1981), and DNA sequence analysis of Class A determinants (Waters et al., 1983) indicate a similar organization.

Several facts support that the pBR322 tet promoter occurs in the vicinity of the the Hind III site, I) cells containing pBR322 plasmid with DNA inserted at the Hind III site are more sensitive to tetracycline than cells carrying pBR322 (Tait and Boyer, 1978), II) the DNA sequence around

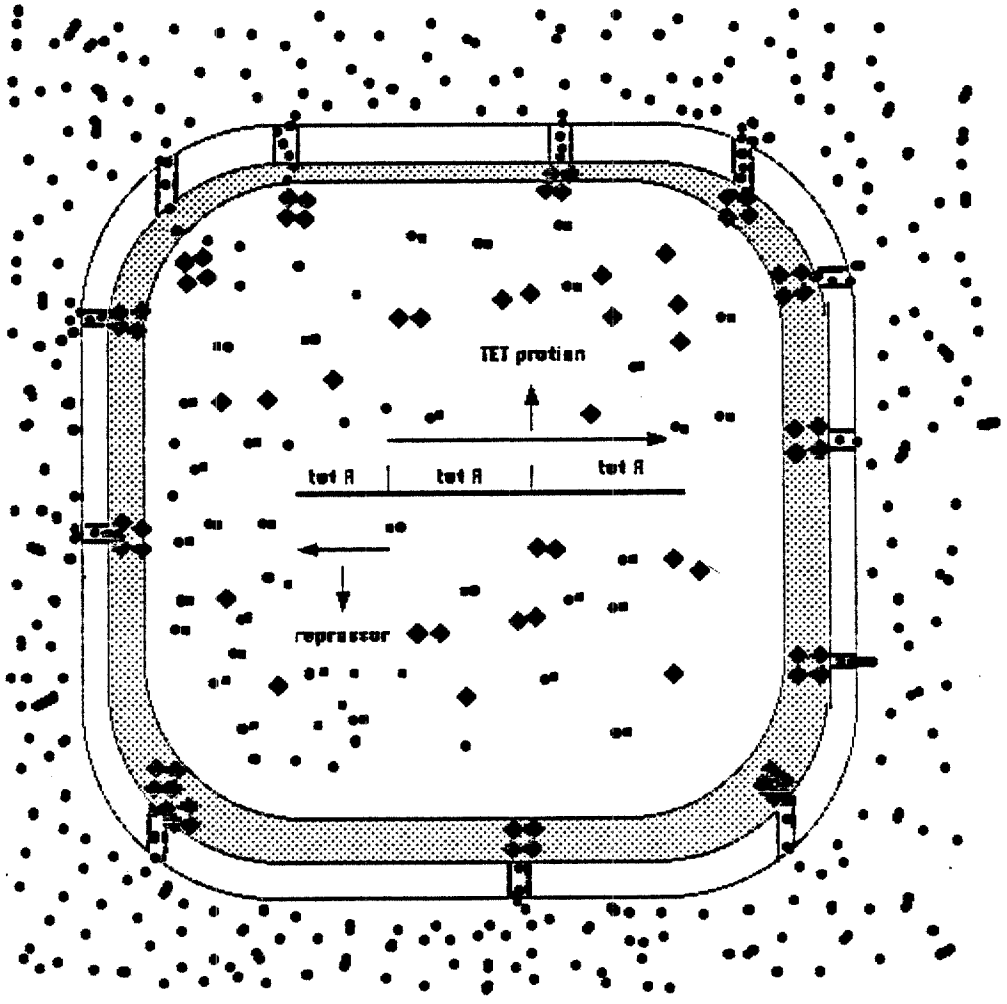


Fig. 3 Mechanism involved in regulation of the tetracycline resistance gram negative bacteria. Tetracycline in the cell binds to the repressor protein and allows the RNA polymerase bind to the operator region and synthesis of TET protein. This protein forms a multimer and localize in the inner membrane blocking the tetracycline entry to the cell.

the Hind III site is homologous with known promoter (Harley and Reynolds, 1987) III) the Hind III site is protected from restriction if E. coli RNA polymerase is allowed to form a stable complex (Rodriguez et al., 1979). These finding propose that the tet mRNA startpoint occurs 6-9 bp downstream of the -10 "pribnow box"region. The position 45 on pBR322 genomic map has been identified as the transcriptional startpoint for tet mRNA (Hawley and McClure, 1983), and the ATG located between position 87 to 89 is the probable start codon for the tet protein (Peden, 1983).

The DNA sequence of the three determinants share a high degree of homology:classes A and C are 70% homologous; classes A,B, and C are about 40% homologous. Comparison of the kinds of amino acids encoded by the three determinants shows remarkable conservation in the amino and carboxyl terminal of TET protein (Curiale et al., 1984). These findings suggest that the determinants share a common origin and function.

CHAPTER II

Statement of Research Problem

This research project deals with the characterization of self-replicating Kirsten murine leukemia viral DNA. The replication of this viral DNA and tetracycline resistance conferred to bacteria by this viral DNA will be studied. The specific aims of this research proposal are as follow:

1. Comparison of nucleotide sequences coding for tetracycline resistance on the viral DNA.

2. Localization of nucleotide sequences coding for tetracycline resistance on the viral DNA.

3. Mapping the origin of replication on the retroviral DNA.

4. Comparison of nucleotide sequences coding for tetracycline resistance in the viral DNA and nucleotide sequence coding for tetracycline resistance in pBR322.

5. Comparison of the origin of replication of the viral DNA with the origin of replication of pBR322.

CHAPTER 3

METHODS AND MATERIALS

Zone of Inhibition Assay for Tetracycline Sensitivity

Zone of inhibition assay was utilized to determine the sensitivity of bacteria to tetracycline. In this assay, Whatman filter paper discs (6 mm) diameter were impregnated with different concentrations of tetracycline (0,5,10,20,40,80,160,320 ug). The discs were placed on NZY plates that had been freshly innoculated with 50 ul of bacteria. The bacteria had been cultured to an O.D. $_{600}$ of 0.6 at 37^{0}C with constant shaking at 210 rpm and were spread with an L-shaped glass rod to obtain an even distribution of the bacterial cells. The culture plates were incubated at 37^{0}C for 16 h and the clear zone of inhibition was measured from the edge of the disc to the point of bacterial growth.

Preparation of Competent Bacteria Cells and Transformation

E. coli will not take up DNA efficiently unless grown and process under selective condition. E. coli that take up DNA efficiently are called

competent. The preparation of competent bacterial cells was a modification to procedure reported by Mandel(Mandel et al., 1970). In brief, E. coli RR1 was grown on NZYM plate overnight at 37^0C. One colony was placed into the 50 ml of liquid NZYM and incubated in a shaker at 37^0C, until the culture reached an $O.D._{600}$ of 0.5 to 0.6. The culture media was centrifuged at 3400 rpm in PRJ INTERNATIONAL centrifuge with 12 place head for 5 min at 4^0C. The supernatant was discarded and the cell pellet was resuspended in 50 ml of ice-cold buffer A (25 mM Tris-HCl, pH 7.5 and 10 mM NaCl). The bacterial suspension was centrifuged under same condition as mentioned above. The bacterial pellet was resuspended in 25 ml of cold buffer B (25 mM Tris- HCl, pH 7.5, 10 mM NaCl, and 50 mM $CaCl_2$). After 30 minutes of chilling on ice, the bacteria were pelleted by centrifugation at 1800 rpm for 5 min at 4^0C. The pellet was gently resuspended in 5 ml of buffer B and these competent bacterial cells were stored on ice until used for transformation experiments.

The transformation of the competent bacteria was carried out as follows : 200 ul of competent cells was added to 1ug DNA and incubated on ice for 30 min. After incubation the bacterial cells were heat shocked

for 1 min at 42^{0}C. The bacterial cells were then placed on ice for 10 min. Twenty-five microliters of the mixture were placed on NZY plates containing 5 ug/ml of tetracycline and spread with L-shape glass rod and were incubated at 37^{0}C overnight.

Large Scale purification of Small Supercoil DNA from the Bacteria

Small supercoil DNA was purified by a modification of the Hirt procedure (Hirt et al., 1967). NZYM plates containing 5 ug/ml of tetracycline were streaked with bacteria containing small circular DNA plasmid. A single bacterial colony was transferred into one liter flask containing 250 ml of NZYM broth. The flask was incubated at 37^{0}C in shaker at 210 rpm until the bacterial culture reached an O.D.$_{600}$ of 0.8-1.0. Chloramphenicol (50 mg/ml in absolute ETOH) was added to the flask to final concentration of 200 ug/ml. The flask was continuously shaken overnight at 37^{0}C. The bacteria solution was centrifuged in a JA-14 rotor at 5 K rpm for 15 min in a Beckman J21 centrifuge at 5^{0}C. The supernatant was discarded and the bacteria pellet was resuspended in 5 ml of 0.05 M Tris pH 8.0 containing 20% sucrose. Two ml of freshly prepared lysozyme solution (10 mg/ml) was added and mixed gently and

placed on ice for 10 min. Twenty-five ml of a 50 mM Tris and 20 mM EDTA solution, 2.5 mg proteinase K-cellulose, and 1.5 ml of 10% SDS was added to the bacterial suspension and incubated at 56^{0}C for 1 h. After incubation, 38 ml of a 50 mM Tris and 20 mM EDTA solution, 17.5 ml of 5 M NaCl, and 2.0 ml of 10% SDS was added and incubated overnight at 37^{0}C. The mixture was placed at -20^{0}C for 1.5 h and then centrifuged at 12 K rpm at 4^{0}C in JA-14 rotor in a Beckman J21 centrifuge for 20 min. The supernatant was removed and 160 ml of absolute ethanol added and store at -20^{0}C for 4 h. The solution was warm up to room temperature and centrifugation at 6 K rpm for 20 min at 20^{0}C in JA-14 rotor. The supernatant was discard and 200 ml of 70% ETOH was added and allow to set for 1 h at room temperature. The solution was centrifuged at 6 K rpm for 20 min at 20^{0}C in JA-14 rotor. Pellet was resuspended in 9.0 ml of UP- water and 1.0 ml of 0.5 M sodium citrate pH 8.0 was added. The solution was incubated at 68^{0}C for 1 h. After incubation, 9.43 g of CsCl was added to the nucleic acid solution plus 0.4 ml of ethidium bromide (10 mg/ml). The nucleic acid solution was placed into the type 80 Ti quick-seal polyallomer tubes and centrifuged in a Beckman type 80 Ti rotor at 55,000 rpm for 17 h at 20^{0}C. The closed supercoiled DNA (lower band) was removed with a 1 ml syringe with a 18 gauge needle and

extracted four times with and equal volume of isopropanal. The DNA was dialyzed against UP-water with three 50 ml changes. The concentration of the DNA was determined by spectrophotometer in which 1.0 O.D. at 260 nm was equal to 50 ug/ml.

Small Scale purification of Plasmid DNA (Mini Plasmid Procedure)

Bacteria containing plasmid were inoculated into 5 ml of NZYM media in a 125 ml flask with foam stopper. The culture was shaken at 200 rpm at 37^{0}C until the $O.D._{600}$ of 0.8 was reached. Twenty microliter of chloroamphenicol (50 mg/ml dissolved in ETOH) was added to the culture media and was incubated overnight at 37^{0}C. The following day, 1.5 ml of culture media was transferred into screw top 2 ml tubes and centrifuged for 30 sec. Supernatant was discarded and the cell pellet resuspended in 100 ul of ice-cold STET solution (8% succrose, 50 mM Tris pH 8.0, 50 mM EDTA, 5% Trition X100) and 10 ul of freshly made lysozyme (10 mg/ml) was added to the tube and incubated on ice for 10 min.The solution was heated at 90^{0}C for 2 min. After centrifugation the supernatant was removed by needle nose pipets to a new 1.5 ml eppendorf tubes and DNA was purified by Geneclean procedure.

Southern Blot Transfer of DNA

Southern blot analysis of DNA was developed by Southern in 1975 (Southern et al., 1975). The DNA for analysis is first cleaved with restriction endonuclease(s) and sized by agarose gel electrophoresis. After electrophoresis, the DNA in the gel was stained with ethidium bromide (1ug/ml) and the picture of the gel was taken. The DNA(s)in the gel were denatured by soaking gel in 500 ml of solution containing 1.5 M NaCl and 0.5 M NaOH for 1 h at room temperature with constant shaking. The denaturant solution was discard. The DNA was neutralized by soaking in 500 ml of solution containing 1M Tris-Cl pH 8.0 and 1.5 M NaCl for 1 h at room temperature with constant shaking.

A large baking dish was used for the DNA transfer step, in the center of the dish stack of glass plates was loaded, a piece of Whatman 3 mm paper was used to cover surface of the glass plates and about few inches long from each side to fall into the transfer buffer. The dish was filled with 10X SSC almost to the top of the glass plates. The gel was placed on the 3 mm paper, making sure there were no air bubbles between the 3 mm paper and gel. A piece of nitrocellulose filter about 1-2 mm larger than the gel in both dimensions was cut and the nitrocellulose filter was immersed in UP-water and then soaked in 2X SSC. The nitrocellulose

paper was placed on top of the gel and all the trapped air bubbles between the gel and the filter were removed. Three pieces of Whatman 3 mm paper were cut exactly the same size as the gel and were placed on top of the nitrocellulose filter. Paper towels almost same size as the gel were cuts and were placed on top of the Whatman 3 mm papers, and weighted down with a 500 g weight. Transfer of DNA to the nitrocellulose filter was carried out for 16 h (Appendix I). The filter was transferred to 500 ml of 6X SSC at room temperature for 5 min with constant shaking. Filter was allowed to dry at room temperature on piece of Whatman 3 mm paper and baked for two hours at 80^{0}C. The filter was immediately used for hybridization experiments.

Hybridization of Southern Filters:

The nitrocellulose filter containing the DNA was placed in 8"x12" polyester barrier bag (KAPAK/SCOTCHPAK), and a 20 ml of prehybridization solution was added. The prehybridization solution was prepared as follows:1.5 ml of 20X SSC, 3.5 ml of 10X PAF, 2.5 ml UP-H_2O in 17x100 mm plastic tubes. About the same time 2.5 ml of shared calf thymus DNA (5 mg) was added to two different glass tubes, the glass

tubes were transferred to 100^{0}C for two min and then immediately placed into an ice water bath. The cold calf thymus DNA was mixed with the content of the plastic tubes and transferred to the plastic bag. The bag was squeeze to remove as much air as possible and then it was laminated. The bag was placed in 68^{0}C water bath overnight. The prehybridization solution was removed and the hybridization solution contains similar ingredient except 4.8x10^7 cpm of ^{32}P pBR322 nick translate probe (2.4x10^7 cpm/ug) was added and allowed to hybridize overnight at 68^{0}C. Then bag was placed in 68^{0}C water bath overnight. The plastic bag was removed from the water bath and the filters were removed from the bag and washed three times at 68^{0}C in 500 ml of 1X SSC with gentle agitation.

The filter was placed on Whatman 3mm paper and dried in 80^{0}C oven for 30 min. The filters were place next to Kodak X-ray film (AR GBX-2) and exposed for 24 h at room temperature, the film was developed 4 min in developer (KODAK GBX), 30 sec in stop bath, and 2-4 min in fixing solution (KODAK GBX).

Radioactive labelling of DNA

A: Radioactive labelling of pBR322 Probes

The DNA was labeled with ^{32}P by nick translation. In this procdure, E. coli DNA polymerase I was added to a double stranded DNA molecule which nicks by virtue of its enzymatic reaction eliminate nucleotides from 5' side and add simultaneously nucleotides to the 3' side results in movement along the DNA molecule (Kelly et al., 1970). By using ^{32}P-labled nucleotides one can labeled DNA with high specific activity. The pBR322 probe was ^{32}P-labeled by Rigby method of Nick translation (Rigby et al., 1977) by using the reagent provided from Bethesda Research Laboratories. To a eppendorf tube 3 ul of pBR322 DNA (1ug) and 5 ul solution containing: 500 mM Tris-HCl (pH 7.8), 50 mM Mg CL_2, 100 mM 2-mercaptoethanol, 100 ug/ml nuclease-free BSA, 0.2 mM of each dATP, dGTP and, dTTP, eight microliters of ^{32}P dCTP ($2.4x10^7$ cpm/ug) and 29 ul of UP- water to bring the volume to 45 ul, then 5 ul of solution containing, 0.4 U/ul DNA polymerase I, 40 g/ul DNAse I, 50 mM Tris-HCl (pH 7.5), 5 mM Mg-acetate, 1 mM 2-mercaptoethanol, 0.1 mM PMSF,100 ug/ml nuclease-free BSA in 50% (V/v) glycerol. The mixture was incubated at 15^0C for 60 min, 5 ul of stop solution containing, 300 mM Na_2 EDTA, pH 8.0 was added, followed by 3 ul of tRNA (20 ug/ml), 55 ul of 4 M ammonium acetate and 300 ul of ethanol. The sample was

mixed and placed in dry ice acetate bath for 10 min and centrifuged in microfuge for 5 min. The supernatant was discarded and, 50 ul of NT-TE(10 mM Tris, 10 mM EDTA pH 8.0), 50 ul of 4 M ammonium acetate and 300 ul of ethanol were added. This step was repeated twice. Sample was centrifuged and supernatant was discarded and pellet resuspended in 100 ul of NT-TE buffer. Two microliter of this sample was removed diluted to 20 ul and 2 ul of alquotes was placed in 10 ml of Betaphase for scintillation counting. To calculate the total amount ^{32}P dCTP incorporated was obtained by multiplying the cpm by 500 to correct for dilutions.

B: Radioactive labelling Hind III Cut lambda DNA

Radioactive lambda DNA size marker were prepered by the following procedure. Lambda Hind III fragments were labelled by a "fill-in" reaction. The reaction mixture contained the following: 8 ul of Hind III cut Lambda DNA (1ug) heated for 2 min at 68^{0}C, 5 ul of ^{35}S dATP (10 mCi/ml), 5 ul of 10X modified repair buffer (0.5 M Tris-Cl pH 7.5, 90 mM $MgCl_2$ and, 20 mM DTT), 1ul of DNA polymerase Klenow fragment (1 unit) and 31 ul of UP- water was added to a Total volume of 50 ul. The

reaction mixture was incubated at 30^{0}C for 30 min. Five microliter of stop buffer (300 mM Na_2EDTA, pH 8.0), 2.5 ul of t RNA (20 ug/ml), 55 ul of 4 M ammonium acetate and 300 ul of ethanol were added. The mixture was placed in dry ice acetone bath to facilitate DNA precipitation for 10 min and centrifuged for 5 min. After centrifugation 50 ul of NT-TE, 50 ul of 4 M ammonium acetate, 300 ul of ethanol were added and mixed gently, placed in a dry-ice acetone bath for 10 min, and followed by centrifugation in a microfuge for 5 min to pellet the labelled DNA. The supernatant was removed and the pellet was resuspened in 50 ul of NT-TE buffer, 50 ul of 4 M ammonium acetate and 300 ul ethanol. The mixture was shaken and incubated in dry-ice acetone bath for 10 min. Next, sample was centrifuged and supernatant was removed and discarded. The pellet was resuspended in 100 ul of NT-TE buffer. To measure the amount of ^{35}S dATP incorporation into DNA, 2 ul of the sample was removed and 1:10 dilution was made, then 2 ul of the diluted sample was placed in 10 ml of Betaphase for scintillation counting. The Beckman LS 9000 scintillation counter was used. To determine the total amount ^{35}S dATP incorporated the cpm were multiplied by 500 to correct for the dilution. The specific activity of the Hind III lambda marker was 1.39X10^7 cpm/ug.

Cloning Strategy of MLV-K:E into pT7/T3α-18

pT7/T3α-18 is a 2890 Kbp vector, which has been derived from pUC 19 This vector has ampicillin resistance and multiple cloning site (MCS). Furthermore, it contains a 455 bp fragment of the intergenic region of phage F1 and promoters for T7 and T3 phage RNA polymerase which were inserted on opposite sides of the MCS (Appendix J). Due to the unique construction of pT7/T3α-18 plasmid, one can package single stranded DNA of the plasmid into M13 phage particle by infecting bacteria with K13 phage. Single stranded DNA for sequencing procedure (Beck et al., 1981) can be purified from these phages. Therefore, this plasmid was used to clone MLV-K:E. Twelve ug of MLV-K:E and 2 ug of pT7/T3α-18 were digested with 1ul of Pst I (10 U/ul) restriction enzyme for total of 3 h at 37^{0}C. After digestion, the DNA was purified by Geneclean procedure and sample was eluted with 20 ul of UP-water. Eight microliter of digested DNA (0.5 ug/ul), 1ul of 10X Ligase Buffer and 1ul of T4 DNA ligase (1.6 U/ul) were mixed and incubated at 15^{0}C overnight. The content of the reaction mixture was added to 200 ul of competent DH5αF' cells and incubated on ice for 30 min . After incubation the bacterial cells were heat shocked for 1 min at 42^{0}C. The bacterial cells were then placed on ice for additional 10 min.

Twenty-five microliters of bacteria was added to the NZYM plates which contains 50 ug/ml of Ampicillin to which, 50 ul of X-gal (2% stock solution) and 5 ul of IpTG (400 mM) was added 30 min before plating the bacteria. One microliter of uncut pT7/T3α- 18 was added to the 100 ul of competent cells as a positive control. The plates were incubated overnight at 37^{0}C. The plates were checked for white colonies and 13 white colony were observed. Each of these colony were streaked onto NZYM plate containing Amp, X-gal, and IpTG. The cultures were incubated at 37^{0}C overnight. A isolated colony was selected for each of these thirteen isolates. Plasmid DNA from each isolate was obtain by the mini plasmid procedure followed by Geneclean. Five microliter of each plasmid DNA sample was digested with Pst I and analyzed by agarose gel electrophoresis.

Preparation of Single Stranded Template:

This procedure is a modification of M13 single stranded purification by Messing (Messing,J. 1983). E. coli DH5αF' containing MuLV-K:E cloned pT7/T3α-18 was grown in 60 ml of 2X YT medium in 250 ml flask until the $O.D._{600}$ of 0.4 was reach. The culture was infected by adding 100 ul

of K-B phage ($7.4x10^{11}$ phage/ml). Flask was shaken at 37^0C for 1 h, kanomycin (50 ug/ml) and ampicillin (50 ug/ml) added and was incubated additional 1 h. Twenty ml of the culture was removed to a 30 ml corex tube and centrifuged at 9k for 30 min. The supernatant was transferred to different tube. Five ml of a solution containing 20% PEG and 3.5 M ammonium acetate was added and sample was stored at 4^0C overnight. Specimen was centrifuged at 9K for 30 min in IEC centrifuge at 4^0C. Supernatant was discarded and the pellet was resuspended in 400 ul of 10 mM Tris pH 8.0 and 1mM EDTA. The solution was transferred to 1.5 ml microcentrifuge tube and extracted with water saturated phenol, equal volume of phenol/chloroform and equal volume of chloroform. After the chloroform extraction, the aquous solution was measured and an equal volume of 7.5 M ammonium acetate, and three volumes of ETOH was added and stored at -20^0C overnight. The mixture was centrifuged in a IEC centrifuge for 30 min at 4^0C and the pellet was dried under vacuum for 1.5 h. The dried nucleic acid pellet was resuspended in 50 ul of UP-water. Concentration of ssDNA was determined by making 1:30 dilution of sample with to UP- water and reading the O.D. at 260 nm in a spectrophotometer.

--------O.D. at 260 nm x 30 x 40= -------ug/ml

DNA Sequencing

A. DNA Sequencing Reaction

The method of choice for sequencing was chain termination DNA sequencing which has been described by Sanger (Sanger et al., 1977). The chain termination method involves the synthesis of a DNA strand by a DNA polymerase in vitro using a single-stranded DNA template (Messing J, 1983). The sequencing kit "Sequenase" was obtained from Unite States Biochemical Corporation (USB). The sequencing reaction protocals by sequenase procedure were as follow (Appendix K).

For each set of four sequencing lanes, single annealing reaction was used, containing 0.5 pmol of primer, 1-2 ug of single stranded DNA and 2 ul of 5X buffer (200 mM Tris-Cl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl). The tube was warmed to 65^0C for 2 min, then allowed to cool slowly to room temperature over a period of 30 min. This step was followed by addition of 1ul of 0.1 M of Dithiothreitol, 2ul of 1:8 dilution of sequenase enzyme with TE buffer containing 10 mM Tris-Cl, 1mM EDTA, pH 7.5, 2 ul of diluted 5X solution containing 7.5 uM dGTP, 7.5 uM dCTP and, 7.5 uM dTTP. fifteen uCi of ^{35}S dATP (1000 Ci/mmol)was added and incubated for 5 to 10 min at 25^0C. The mixture was distributed in 3.5 ul aliquots into tubes containing either 8 uM ddATP, 8 uM ddCTP, 8 uM ddGTP and,

8 uM ddTTP incubated for 5 min at 37^{0}C. The reaction was stopped by the addition of 4 ul of stop solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and, 0.05% xylene cyanol FF. Samples were stored at -20^{0}C. Sample were heated to 70-80^{0}C for 2 min and loaded immediately onto the sequencing gel.

B. Preparation of Sequencing Gel

The sequencing gel was composed of 8 percent acrylamide and 8.3 M urea and two stock solutions of 250 ml each were prepared as follows: stocks acrylamide solution contained 125 ml of 40% acrylamide (Amresco), 1.66 g of BIS, 100 ml of 5X TBE buffer and UP-water to a final volume of 250 ml. The stock urea solution was prepared by addition of 117 g of urea, 50 ml of 5X TBE and UP-water to a final volume of 250 ml. Twenty-two ml of acrylamide and 33 ml of urea solution were combined in a 50 ml conical tube. At this point, plates were assembled by placing 0.4 mm spacer between 40 cm glass plates and clamped together. The bottom of the plate assembly was taped. The plates were placed in a vertical position. Five ml of this gel solution was transferred to a separate tube and 40 ul freshly prepared 10% ammonium persulfate and 5 ul of TEMED were added. The solution was poured along

the side of the spacers to seal the sides and bottoms. The acrylamid solution was allowed to polymerized for 30 min. Four hundred microliter of 10% ammonium persulfate was added to the remaining 50 ml of gel solution plus 7 ul of TEMED. The solution was transfer slowly between the plates by a LKB pump (P-1) and care was taken to avoid air bubbles. After pouring the gel, the glass was placed in a horizontal position and the comb was inserted and clamped into position. The gel was allow to polymorize for 1 h. Comb, clips, and tape were removed and the plates were transferred to the electrophoresis unit (BRL, Model S2). One liter of 0.5X TBE buffer was added to the chambers (500 each), and the gel was heated by pre-run at 2000 volts for 30-45 min. After the pre-run of the gel, sample wells were rinsed out by washing the electrophoresis buffer up and down in the wells with a pasteure pipette. Three microliter of each sample were added to each well in following order A,C,G, and T. The electrodes were attached and the power supply was adjusted to 2000 volts. The gels were run for 2 to 4 h.

The plates were removed from the BRL unit and the plates were separated by a plastic wedge. The gel will adhere to one of the plates and one single sheet of 3 mm Whatman filter paper was placed on top of the gel. The sandwich was flipped and the gel adhered to filter paper.

The gel filter paper was transfer to a Bio-Rad Model 1125B Slab Gel Dryer and dried at 80^{0}C for 2 h. After drying was completed, the filter paper was wrapped with saran wrap and placed in metal cassette (14" x 17") next to 14" x 17" X-RAY FILM (KODAK, XAR 5) and the cassette was closed. After two days exposure at room temperature, the film was developed 4 min in Kodak GBX developer and 30 seconds in the stop bath (940 ml Glacial acetic acid to 1 liter water) , and 2-4 min in Kodak GBX fixer.

Agarose gel electrophoresis

Agarose gel electrophoresis is utilized to separate DNA fragments from 1-20 Kbp in size. The mixture of DNA fragments can be separated based on electrophoretic migration rate of DNA through agarose gel. The DNA bands in the gel are stained with low concentrations of the fluorescent dye such as ethidium bromid (Sharp et al, 1983).

One percent agarose gel was prepared by addition of 1 g agarose (BRL) to 99 ml of 1X TAE buffer (40 mM Tris-acetate and 2 mM EDTA). The mixture was autoclaved for 10 min and cooled to 50^{0}C. The agarose solution was poured into tray and comb placed in position and allowed to solidify. The agarose gel was placed in BRL Model H3 unit and 1X TAE

buffer was added so the buffer covered gel by 1 to 2 mm. Samples were loaded and electrophoresed at 30 volts for 16 h when electrophoresis was completed, the DNA in the gel was stained with ethidium bromid (1 ug/ml). The gel was placed on the UV light box and photographs of gel was made by using transmitted UV light(Spectroline R Model TR-302 nm ulteviolet). Poloroid type 57 was set at f4.5 with 15 orange (39F Tiffen) filters. Exposure was for 1-2 sec.

Geneclean Kit procedure

This is a rapid procedure for purifying and desalting a solution of DNA. Two to three volumes of NaI stock (4 M) was added to the DNA solution plus 1 ul of Glassmilk per ug of DNA suspension. The sample was incubated at room temperature for 5 sec, centrifuged in a microfuge for 5 sec and supernatant was discarded. Pellet was washed three times with 700 ul of New wash. After last centrifugation, 30 ul of UP-water was added and pellet heated in a 45-55^{0}C water bath for 3 min. Sample centrifuged for 5 sec and supernatant was saved.

CHAPTER 4

Results

A variant of Kirsten murine leukemia virus (MLV-K) was molecularly cloned into the plasimd pBR322. During the replication of the recombinant molecule in E. coli the MLV-K portion of the molecule was excized and is able to replicate independently and gives bacteria resistance to tetracycline.

The purpose of this research was to characterized tetracycline resistance conferred to bacteria by MLV-K: E and the replication of the MLV-K:E. To characterized the tetracycline resistance conferred by the viral DNA three different were observed. First, a comparison of tetracycline resistance give to E. coli by MLV-K:E and pBR322, a plasmid that contains a tetracycline resistant gene. Secondly, to localize area of the MLV-K:E which share sequence homology to pBR322 and thirdly, to verify the sequence of the tetracycline resistance which are present in MLV-K:E. To compare the tetracycline resistance between E. coli RR1 containing either pBR322 or MLV-K:E, the disk agar diffusion test was

utilized. In these experiments the relative susceptibility of the E. coli to the tetracycline was determined by size of clear zone around the disk due to inhibition of the bacterial growth. E. coli RR1 with no plasmid showed zone of inhibition at 5 ug of tetracycline,whereas E. coli RR1 containing pBR322 showed a slight zone of inhibition at 320 ug of tetracycline. On the other hand, 10 ug of tetracycline inhibited the growth of E. coli RR1 containing MLV-K:E (Table II). The comparison of the zone of inhibition observed with E. coli RR1 and E. coli RR1 containing either pBR322 or MLV-K:E is illustrated in (Fig. 4).

Table II. Comparison of Tetracycline Resistance

ug of Tetracycline	Zone of inhibition per mm		
	RR1	RR1 with pBR322	RR1 with MLV-K:E
0	0	0	0
5	0.5	0	0
10	2	0	1
20	4	0	1.5
40	8	0	3
80	9	0	4
160	11	0	6
320	14	1.5	9

Table II. Comparison of E. coli RR1 containing pBR322 or MLV-K:E as to the size of the zone of inhibition at different tetracycline concentrations.

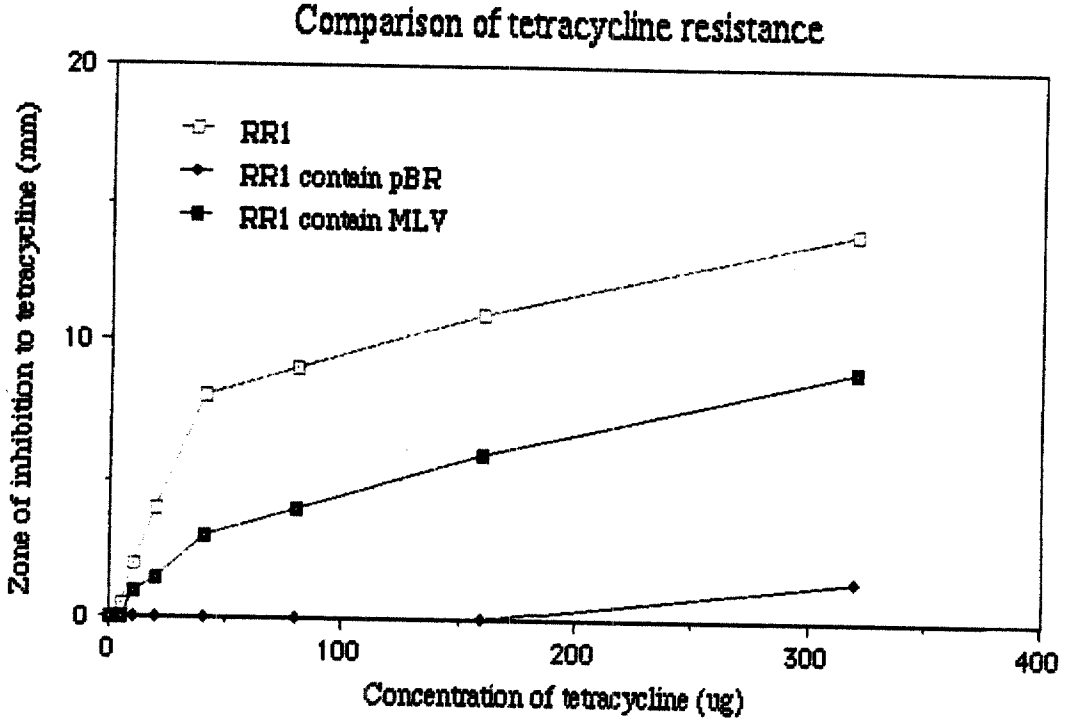
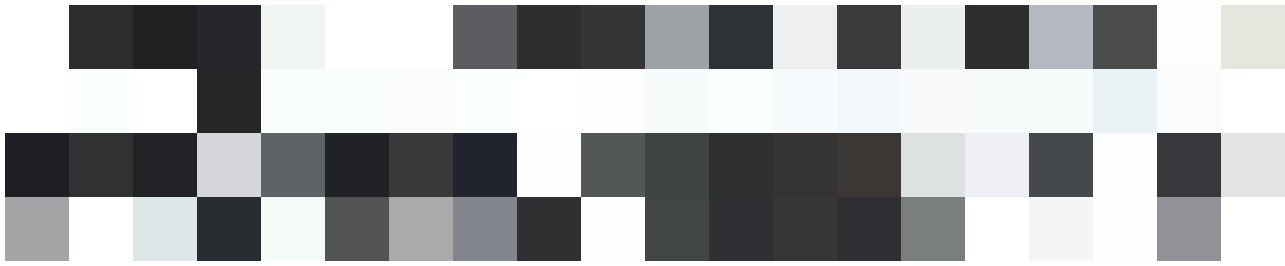
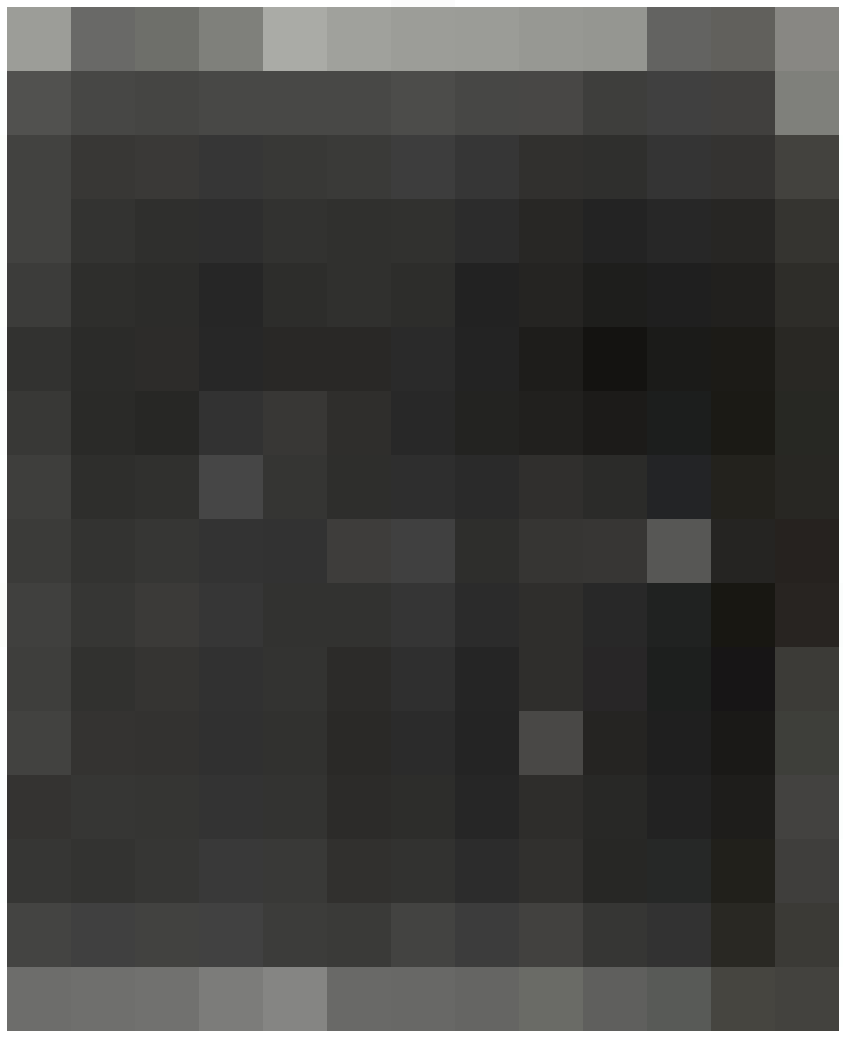


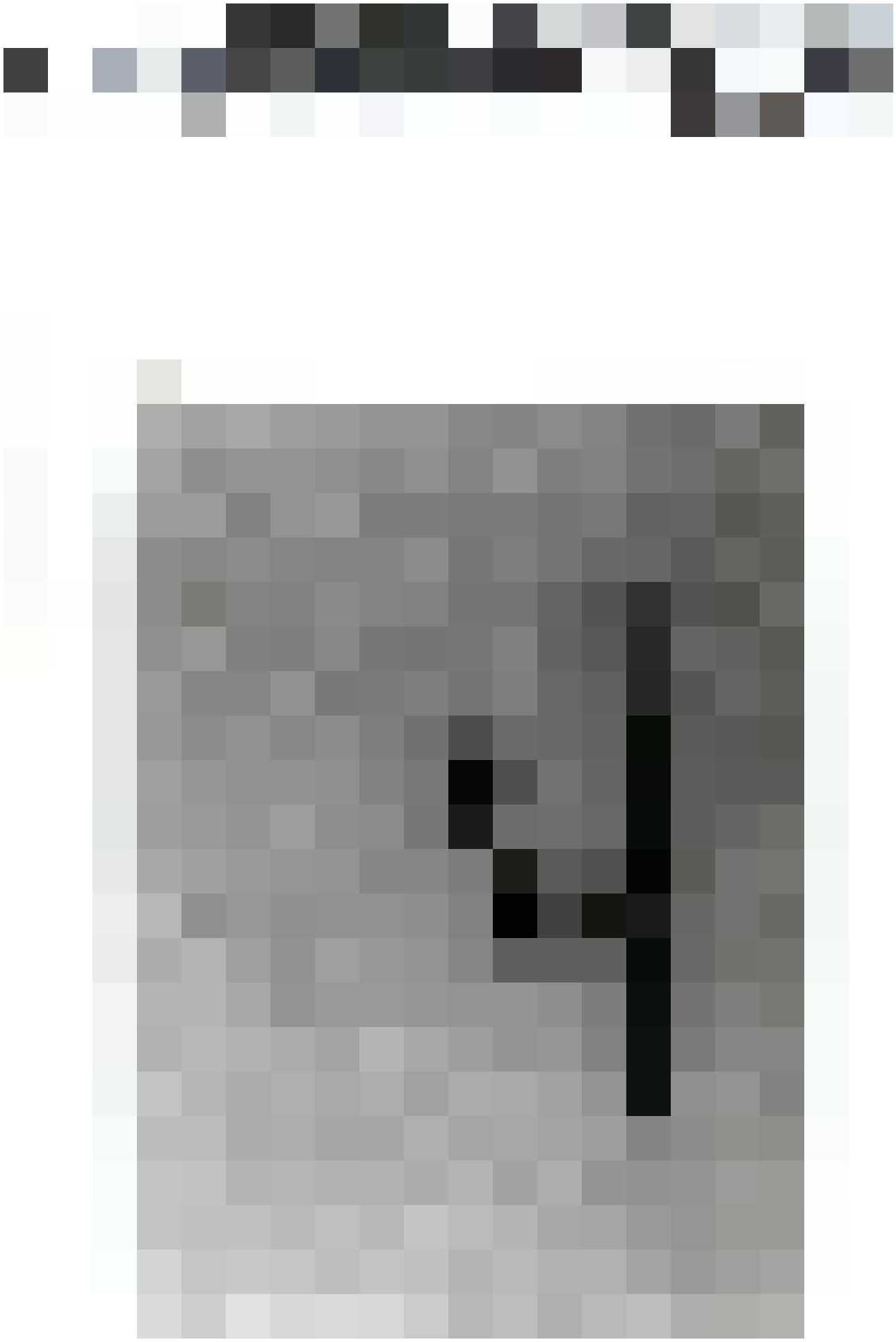
Fig. 4 Comparison of tetracycline resistance of E. coli RR1 containing no plasmid (open square), pBR322 (closed diamond), and MLV-K:E (closed square).

Why were bacteria with MLV-K:E resisted to tetracycline? One possibility is that the bacteria contain two replicating DNAs, one the MLV-K:E plus free pBR322. To rule out the presence of pBR322 in the MLV-K:E DNA preparation, the following experiment was carried out.

The MLV-K:E DNA preparation was digested with the restriction enzyme Hind III, an enzyme that cuts MLV-K:E once and pBR322 once. For a positive control, pBR322 was linearized by the same restriction enzyme. Different concentration of these two DNA were run on an agarose gel and stained with ethidium bromide (Fig. 5). A 7.7 Kbp fragment of viral DNA was seen (Fig. 5, lanes 2,4,6). No band which match the size of the pBR322 was detected in the lanes loaded with viral DNA. Eventhough, no pBR322 DNA band was detected in the MLV-K:E DNA preparation by ethidium bromid staining, trace amounts may not be detected by this procedure. Therefore, molecular hybridization (a more sensitive assay) was employed. The DNA separated on the agarose gel(Fig. 5) was blotted to nitrocellolose using the Southern blot procedure and hybridized to the ^{32}P- labelled pBR322 probe (2.4×10^7 cpm/ug) (Fig. 6).

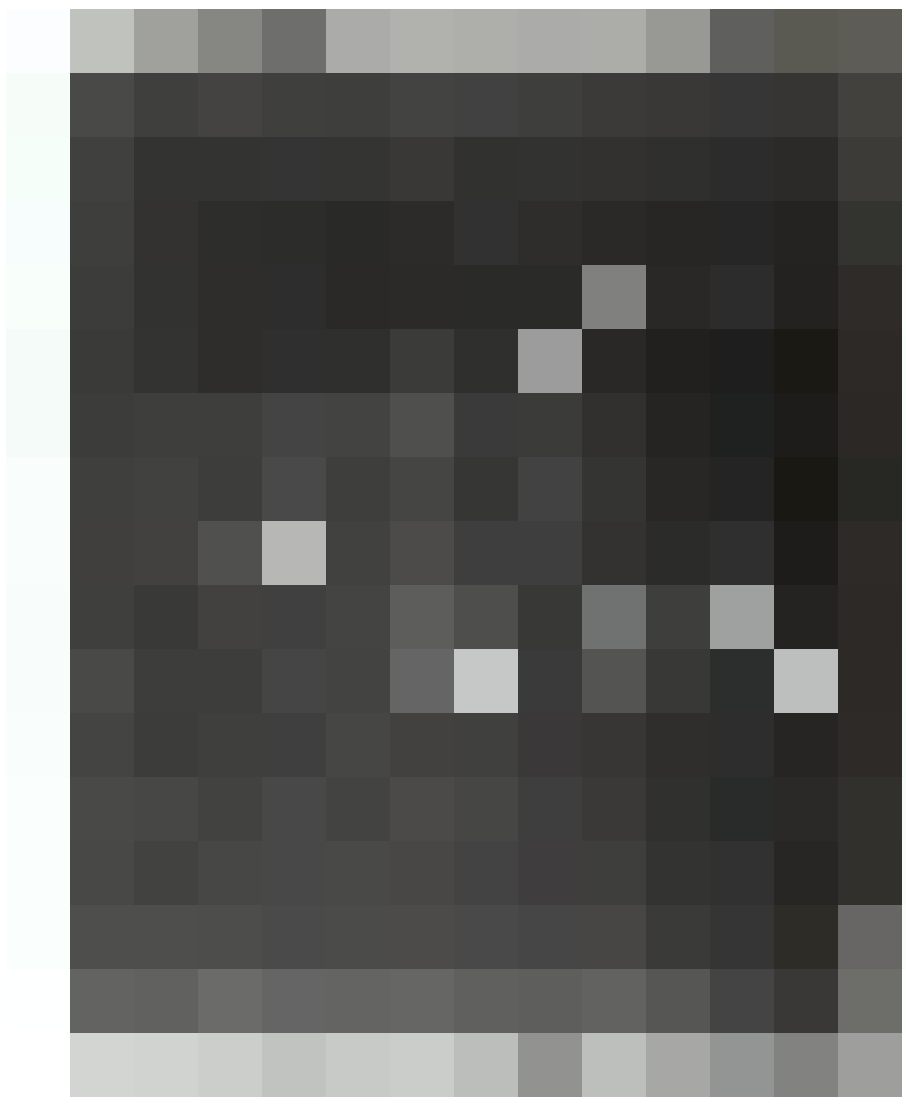


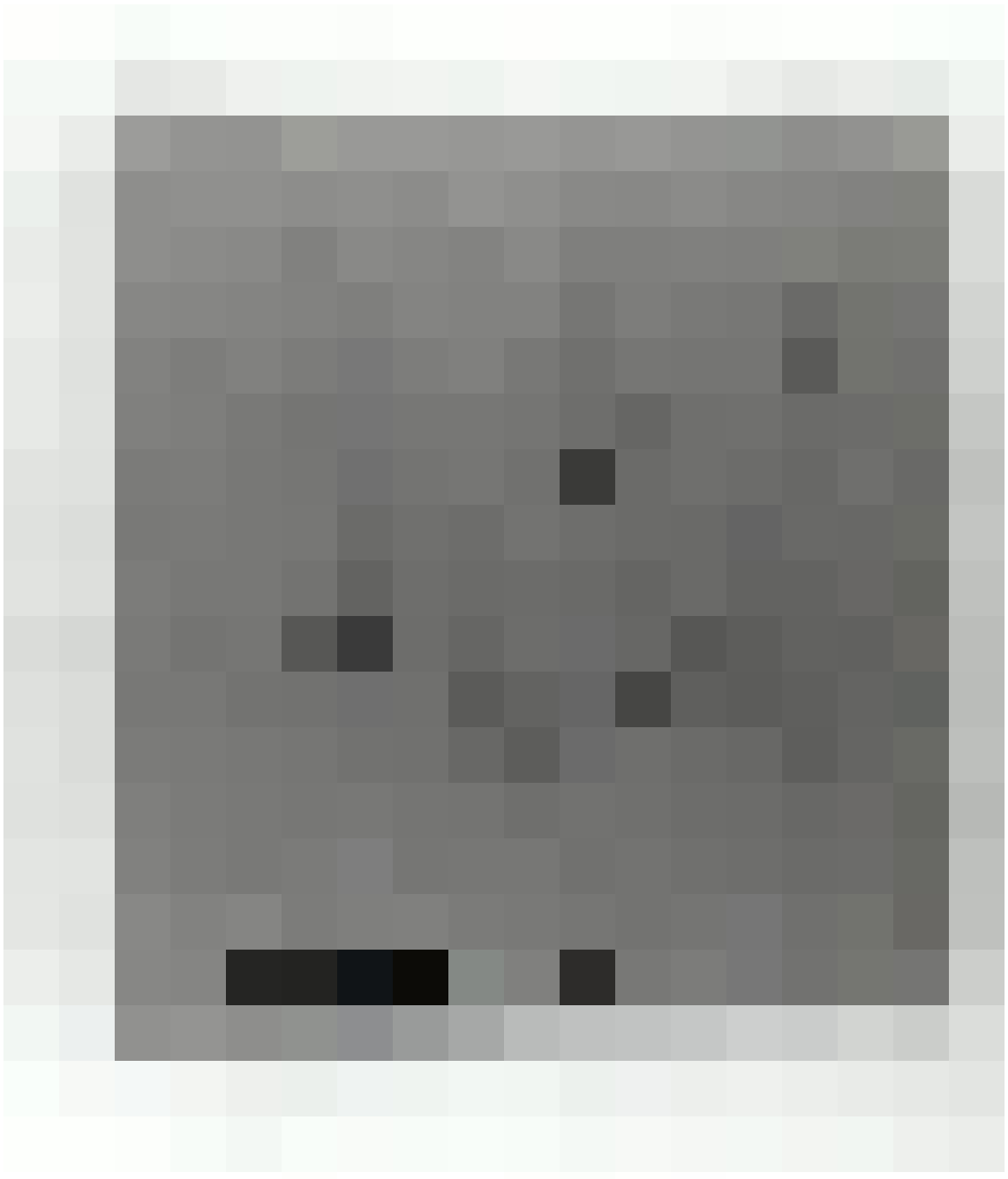




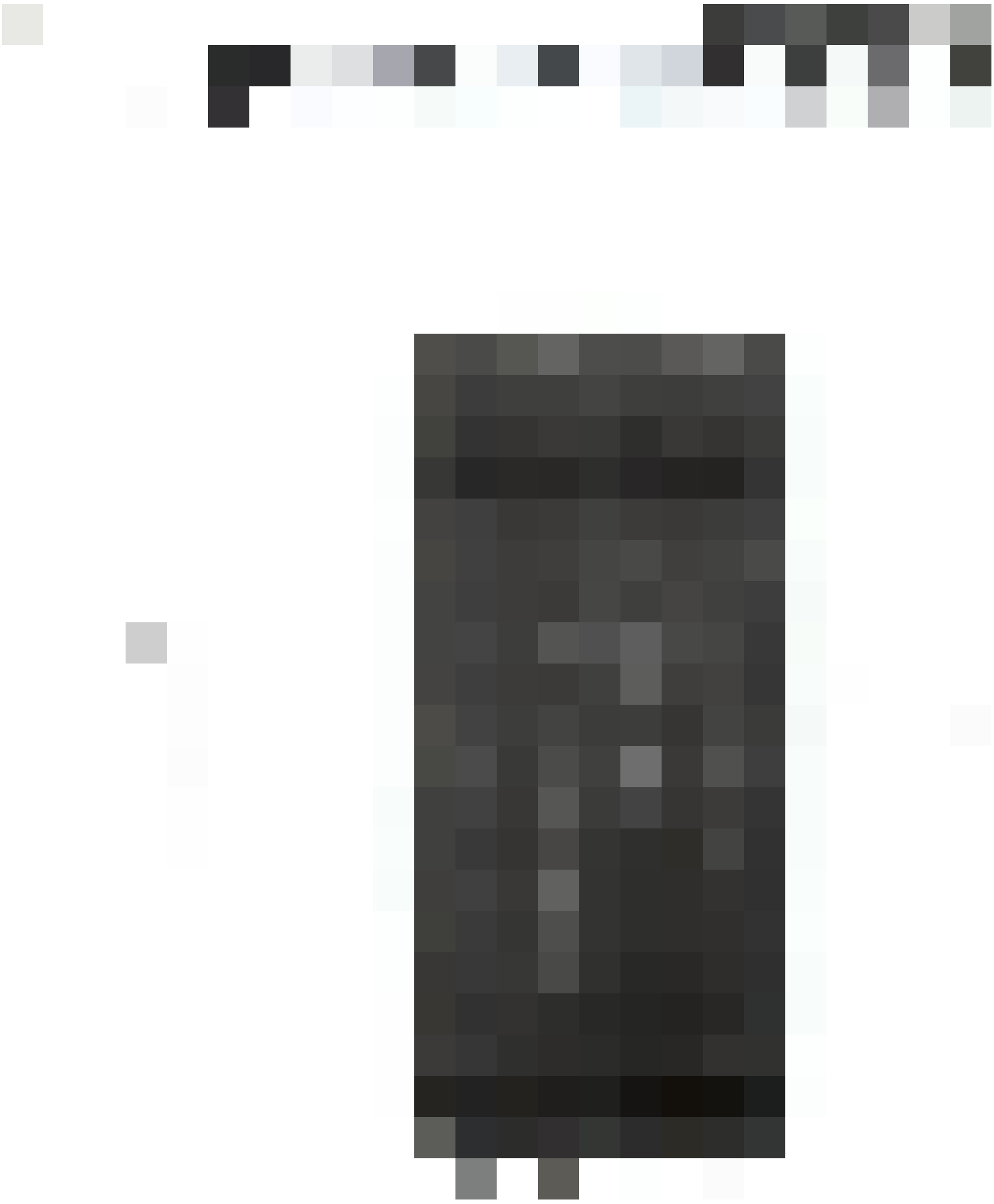
The results of the experiment indicated no free pBR322 was present in MLV-K:E DNA preparation. However; the MLV-K:E DNA hybridized to radioactive pBR322 probe indicating homologous sequences were present. Therefore, an experiment was design to localize the area of the viral DNA which contain sequences from pBR322. MLV-K:E DNA was digested with different restriction enzymes, Pst I, Bam HI, Bgl II, Sal I, Bgl I, Hind III and double digestion with Xho I and Pst I. Digested samples of DNA were electorophoresis on an agarose gel and stained with ethidium bromide (Fig. 7). The DNA in the gel was blotted and hybridized with ^{32}p - labelled pBR322 probe (Fig. 8), as anticipated some of the bands hybridized strongly to the probe, were as others did not hybridized such as, Bgl I 4.1 Kbp, Bgl II 2.5 Kbp and, Pst I- XhoI 0.8 Kbp fragments (Fig. 8).

The results of Southern blot and hybridization of pBR322 to MLV-K:E revealed strong possibility of pBR322 sequences within MLV-K:E. To find out more information about the extend of pBR322 involvement in the MLV-K:E, comparative digestions of MLV-K:E and pBR322 with the restriction enzymes, which cuts many times was undertaken. MLV-K:E DNA (1 ug), pBR322 (1ug) were digested with either Hha I and Hinf I restriction enzyme and the DNA were electrophorsis on a 5% acrylamide gel for 3 h and stained with ethidium bromide. A comparison of DNA bands in the gel revealed that many bands of pBR322 had similar molecular weights as MLV-K:E (Fig 9).





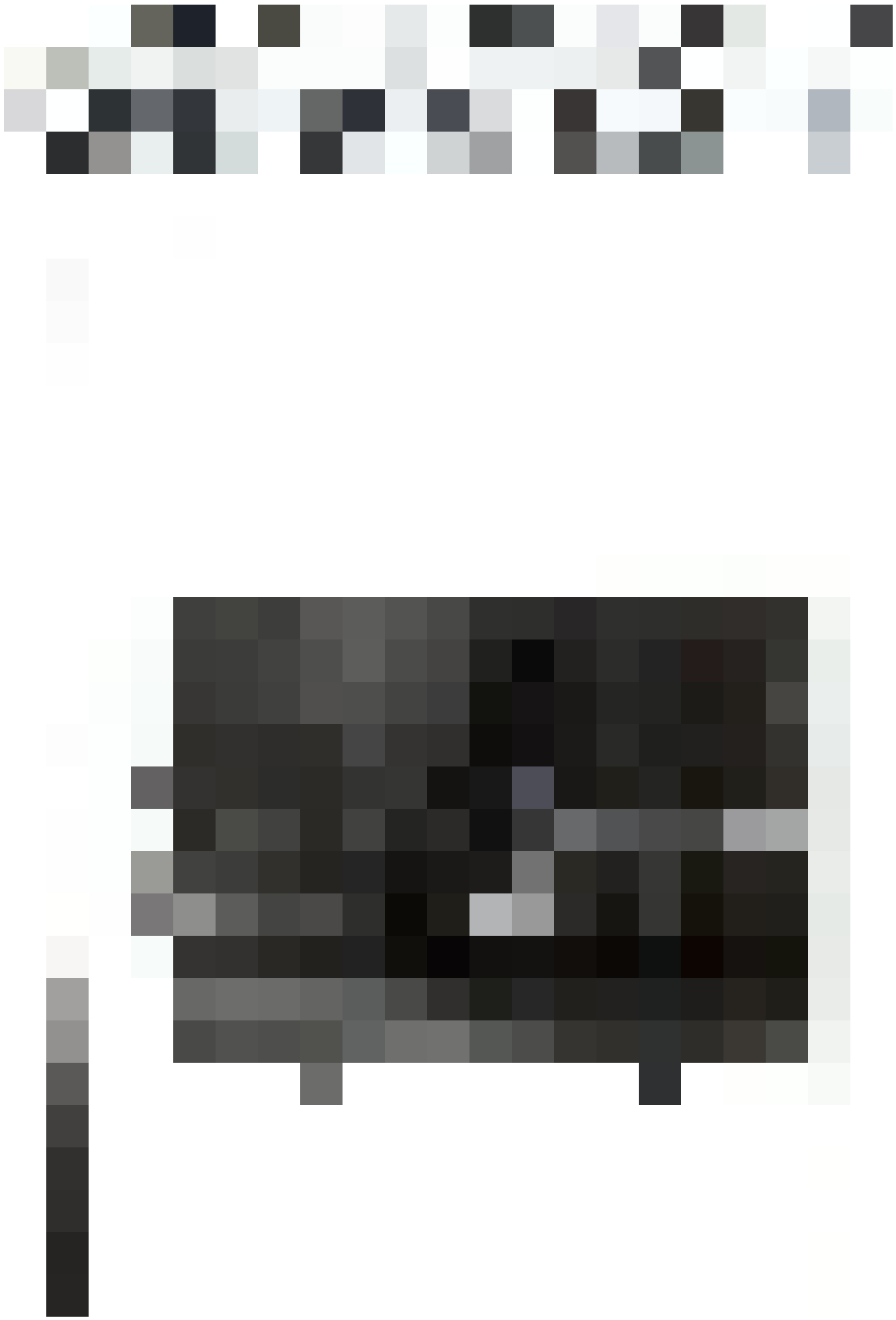
MLV-K:E has similar restriction endonuclease fragments as pBR322 and contain nucleic acids sequence homology with pBR322 as determined by molecular hybridization indicating that the MLV-K:E DNA was composed of retroviral sequences and pBR322 DNA. To further substatement this interpretation sequencing of regions of MLV-K:E DNA was carry out. Since single stranded templete are easier to sequence. The entire viral DNA was cloned into pT7/T3α-18 vector. The cloning strategy has been discussed under method and material section. The experiment was design to clone the viral DNA in to the Pst I site of pT7/T3α-18 which is located in the multiple cloning site of the plasmid. A total of thirteen white colony were isolated and plasmid was purified by mini plasmid purification and Geneclean procedure. These plasmid samples were digested by Pst I to verify the presence of viral DNA in the vector (Fig. 10).



As one could expect after digestion with Pst I the recombinant plasmid showed two bands, one MLV-K:E and other one the same size as the pT7/T3α-18. The result of agarose gel electorophoresis suggested only lanes #4, #9 and #12 have the recombinant molecule.

Since it was necessary to know the orientation of the MLV-K:E cloned to pT7/T3α-18 for sequencing analysis, the three recombinants were digested by Eco RI and Xho I restriction enzyme, and analyzed by agarose gel electrophoresis. If the orientation of MLV-K:E was 5'—— 3', one could expect to see two band on the gel about 0.9 Kbp and 9.7 Kbp, where as if orientation was 3'—— 5', two band will be observed too, but their size will be 3.5 Kbp and 7.1 Kbp bands (Fig. 11). The three recombinant plasmids showed the later case (Fig. 12). Therefore, we concluded the orientation of the fragment is 3' —— 5' for all the MLV-K:E pT7/T3α-18 plasmids.

Single stranded DNA was obtained from the recombinant plasmid as discussed in materials and methods. Sequencing primers were utilized to sequence different regions of the MLV-K:E. This was done by modify procedure for M13 single stranded temperature purification by missing. The sequencing of the 3' end of MLV-K:E DNA use a primer (5-d[CAGGAAACAGCTATGAC]-3) that binds to the position 276 to 293 of the pT7/T3α-18 which is only about 30 bp away from multiple cloning site. The sequencing of 3' terminus of MLV-K:E revealed nucleotides sequence were similar to the region of pBR322 that codes for Beta lactamase (Fig. 13).



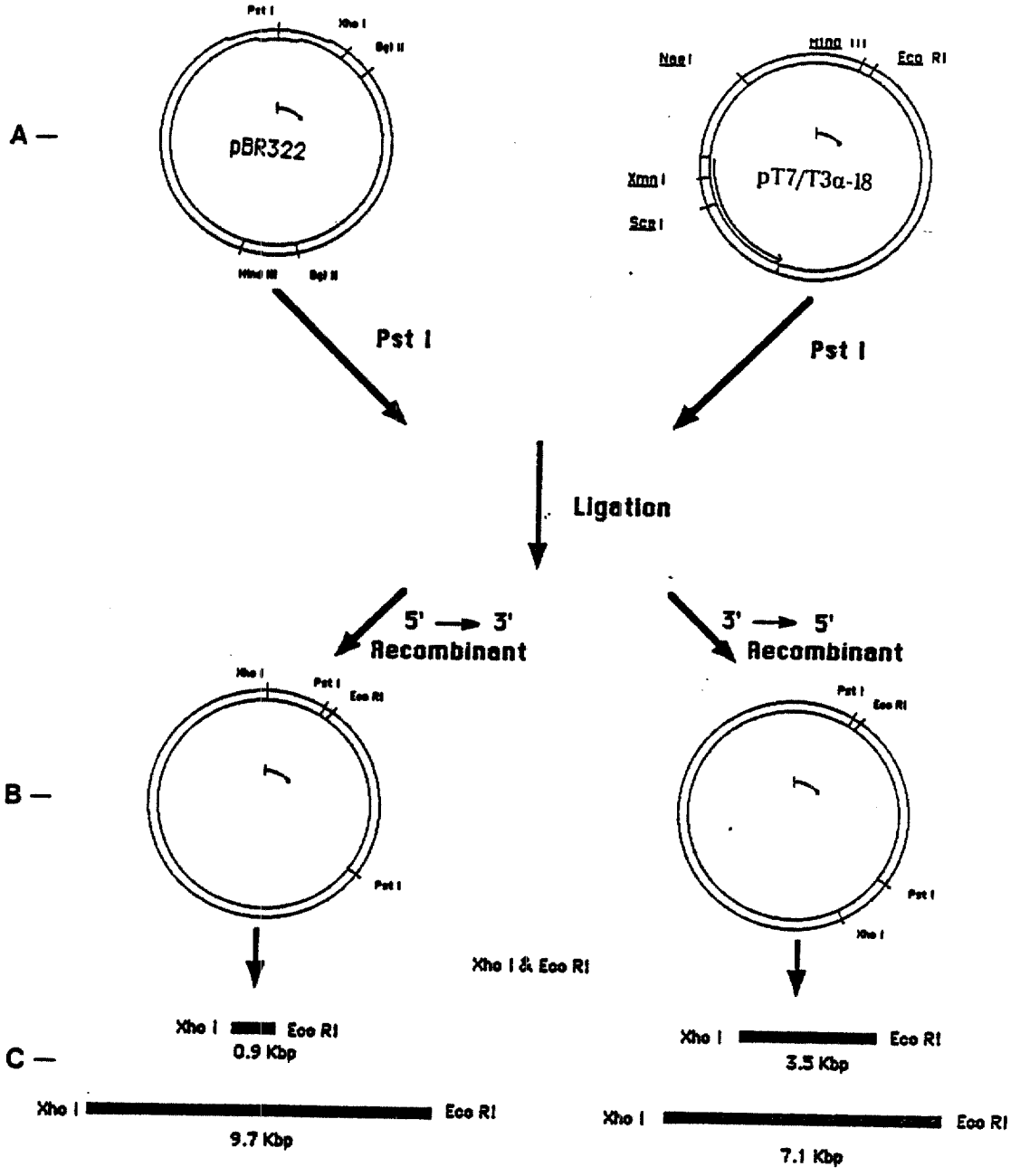
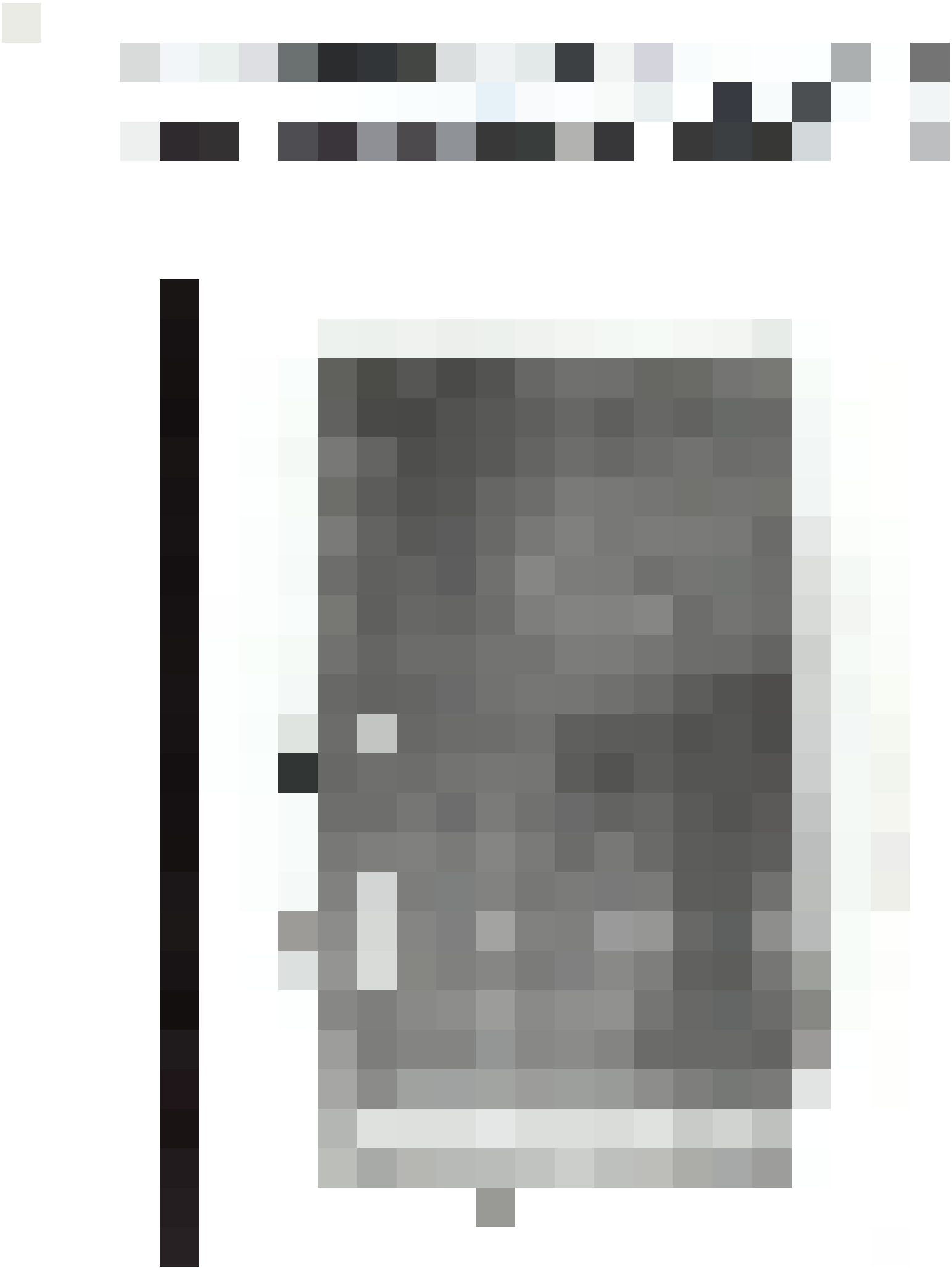


Fig. 11 A. Cloning strategy of MLV-K:E into Pst I site of pT7/T3α-18. B. Two possible orientation of the recombinant molecules. C. Comparison of fragment length resulting from Eco RI and Xho I digestion depending on MLV-K:E orientation in vector.

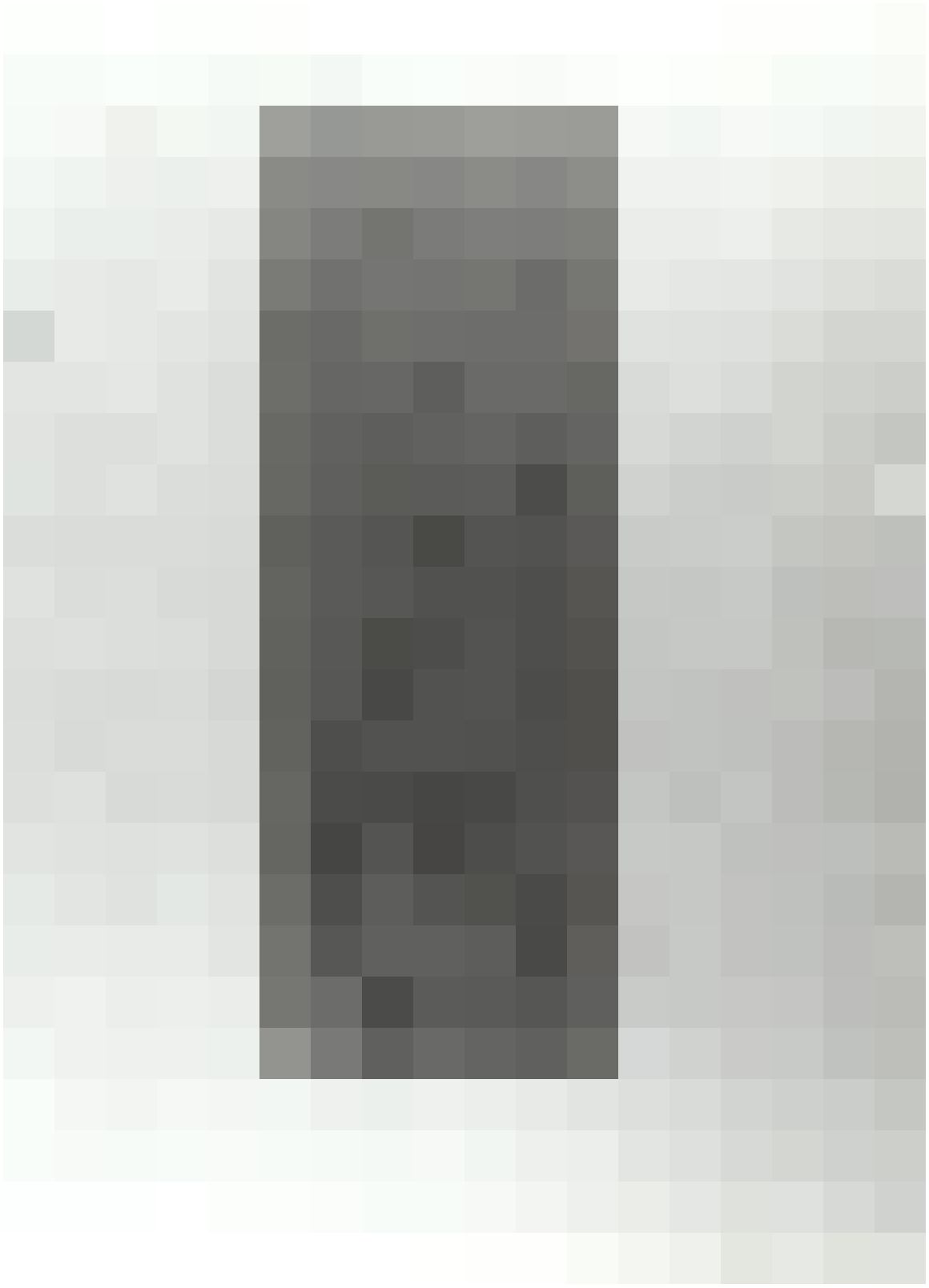


pBR322 5'	AAGTGGTCCT	GCAACTTTAT	CCGCCTCCAT
MLV-K:E 3'	AAGTGGTCCT	GCAACTTTAT	CCGCCTCCAT
pBR322	CCAGTCTATT	AATTGTTGCC	GGGAAGCTAG
MLV-K:E	CCAGTCTATT	AATTGTTGCC	GGGAAGCTAG
pBR322	AGTAAGTAGT	TCGCCAGTTA	ATAGTTTGCG
MLV-K:E	AGTAAGTAGT	TCGCCAGTTA	ATAGTTTGCG
pBR322	CAACGTTGTT	GCCATTGCTG	CAGG
MLV-K:E	-AACGTTGTT	GCCATTGCTG	CAGG

Fig. 13 Comparison of nucleotide sequences of 3' terminal end of MLV-K:E and nucleotide sequences of pBR322 that encodes Beta-lactamase gene starting at nucleotide 3501 to 3614. Deletion are designated as (-).

To prove tetracycline gene sequences are presence in MLV-K:E a primer (5'-d[ATGCCGTCCGGCGTAGA]-3') that bind to position 380 to 397 pBR322 genomic map close to Bam HI site 380 nucleotides from the 5' end of the tetracycline gene. First, the primer was bound to the MLV-K:E template since typical sequence bands were observed (Fig. 14). Therefore, the complementary sequence is present in MLV-K:E DNA and sequence obtained was nearly identical to the 5' end of the tetracycline gene of pBR322 (Fig. 15).

The nucleotides sequence of this region of MLV-K:E with exception of two nucleotides and small area of high GC which was not readable were almost identical to pBR322 nucleotide sequences from position of 101 to 296 which is located in the tetracycline resistance region.



pBR322 5'	GCGCTCATCG	TCATCCTCGG	CACCGTCACC
MLV-K:E 5'	GCGCTCATCG	TCATCCTCGG	CACCGTCACC
pBR322	CTGGATGCTG	TAGGCATAGG	CTTGGTTATG
MLV-K:E	CTGGATGCTG	TAGG-ATAGG	CTTGGTTAT-
pBR322	CCGGTACTGC	CGGGCCTCTT	GCGGGATATC
MLV-K:E	CCGGTACTGC	CGGGCCTCTT	GCGGGATATC
pBR322	GTCCATTCCG	ACAGCATCGC	CAGTCACTAT
MLV-K:E	GTCCATTCCG	ACAGCATCGC	CAGTCACTAT
pBR322	GGCGTGCTGC	TAGCGCTATA	TGCGTTGATG
MLV-K:E	——TGCTGC	TAGCGCTATA	TGCGTTGATG
pBR322	CAATTTCTAT	GCGCACCCGT	TCTCGGAGCA
MLV-K:E	CAATTTCTAT	GCGCACCCGT	TCTCGGAGCA
pBR322	CTGTCCGACC	GCTTTG	
MLV-K:E	CTGTCCGACC	GCTTTG	

Fig. 15 Comparison of nucleotide sequences of MLV-K:E to 5' portion of tetracycline gene of pBR322. Deletion are designated as (-), straight line correspond to high GC area.

The sequencing result of MLV-K:E indicates that tetracycline sequences and 3' end of MLV-K:E contain Beta-lactamase sequences were suggestive that a large portion of pBR322 was presence in MLV-K:E. This sequence on the mLV-K:E genomic map correspond from Hind III site to Pst I, this is about 3.618 kpb fragment which is identical in size to the Hind III-Pst I pBR322 fragment (Appendix L). If this is true, the origin of replication of pBR322 would be present in the 3' end of MLV-K:E DNA. To verify the presence of the pBR322 origin of replication results from restriction digestion of MLV-K:E and pBR322 with Hinf I and Hha I were used.

Enzymes like Hha I and Hinf I cuts pBR322 to many fragments. Comparative digestion of MLV-K:E DNA and pBR322 revealed most of the band which present in pBR322. Digestion by Hinf I or Hha I are seen in MLV-K:E digestion with the same enzymes. Digested pBR322 with Hinf I generate 10 fragments, eight of these 10 fragments were present in MLV-K:E (Fig. 16), these fragments correspond from position 631 to 3362 on pBR322 genomic map. Origin of replication is located between position 2474 to 2914, the presence of the fragments D and B. In the MLV-K:E demonstrate the origin of replication is from pBR322 (Fig. 16).

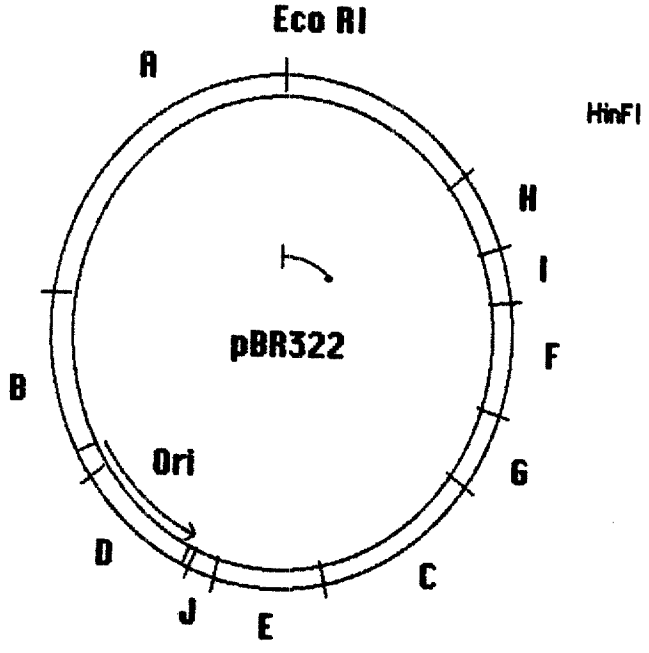


Fig. 16 pBR322 digestion site with Hinf I. Origin of replication is located between the B and D fragments.

CHAPTER 5

DISCUSSION

Retroviruses are eukaryotic viruses, there are not associate with antibiotic resistance . However, the project of our interset was to study MLV-K:E which was able to replicate independently in bacteria cell and give bacteria tetracycline resistance. Bacterial plasmid pBR322 was used as cloning vector for original cloning of Kirsten murine leukemia virus (MLV-K:E). E. coli RR1 were transformed with recombinant molecules. During the replication of the recombinant molecule in E. coli, about 7.7 Kbp portion of the recombinant molecule was excised and was able to replicate independently and conformed tetracycline resistance to bacteria. In preliminary results it was observed that E. coli, which contains either MLV-K:E or pBR322, has a similar generation time when grown in the presence or absence of tetracycline. However, when tetracycline was present, the colony size of E. coli containing MLV-K:E was smaller than the colony of E. coli containing pBR322. This observation was followed by the zone of inhibition assay for tetracycline. The results of this experiment indicated that E. coli

containing MLV-K:E is resistance to tetracycline but only up to a small concentration of tetracycline (10 ug) (Fig. 4) (Table. 2). Since the initial mapping of excized molecule (MLV-K:E) was very similar to the original MLV-K, the possibility of retroviral DNA having bacterial origin of replication and tetracycline resistance gene was proposed.

To rule out presence of free pBR322 associated with MLV-K:E . The MLV-k:E DNA preparation was digested with the restriction enzyme Hind III, which cuts each MLV-K:E and pBR322 once. If there was free pBR322 in MLV-K:E DNA preparation one could expect to see a band about 4.3 Kbp, match the size of the pBR322. However, no pBR322 DNA was detected in the MLV-K:E preparation by ethidium bromides staining (Fig. 5). Ethidium bromides staining can detected 10 ng or more of DNA. Therefore, if there was trace amount of free pBR322, it could have not been detected by this procedure. To role out presence of trace amount of free pBR322, a more sensitive procedure; molecular hybridization was employed. The results of this experiment revealed lack of free pBR322 band in MLV-K:E DNA preparation. Moreover, the MLV-K:E DNA hybridized to radioactive probe suggesting homologous sequences were present. When the MLV-K:E DNA was digested by series of restriction enzymes

which each cuts MLV-K:E to different size fragments, the samples were analyzed on the agarose gel, blotted to nitrocellose paper and hybridized to the ^{32}P labelled pBR322 probe (Fig. 8) with the exception of three bands the remaining bands strongly hybridized with the radioactive ^{32}P labelled pBR322 probe. One of the these three bands Bgl I (4.1kbp) did hybridized but very weakly, suggesting it contain only small nucleotides homology to pBR322, but the other two bands Bgl II 2.5 kbp and Pst I & Xho I 0.8 kbp bands did not hybridized. The Hybridization mapping of the MLV-K:E revealed that about 3.6 Kbp of the MLV-K:E share strong sequence homology to pBR322. This area is from Hind III to 5' Pst I site. Hybridization indicates that when the excision occurred in the recombinant molecules, the excized molecule (MLV-K:E) removed some of the nucleotid sequences from each molecule. It appears now about 3.6 Kbp of MLV-K:E is from pBR322. Furthermore to test the extend of pBR322 involvement in the MLV-K:E, comparative digestions of MLV-K:E and pBR322 with the restriction enzymes that cuts many times was undertaken. Hha I and Hinf I restriction enzymes digestion of MLV-K:E and pBR322, showed that many bands between these two are identical in size (Fig. 9). The analysis of these bands indicates from Hind III to

5'Pst I the restriction sites are almost identical to the pBR322 map.

This portion of pBR322 contains tetracycline resistance gene, origin of replication and portion of Beta-lactamase gene. To test this finding the entire MLV-K:E was cloned into pT7/T3α-18 plasmid by considering 3'———5' orientation, because 3' end of the MLV-K:E was the area of interest. Then the single stranded sequencing was performed. The sequencing result at 3' end of MLV-K:E revealed identical nucleotides sequences to Beta-lactamase gene. To confirmed the nucleotide sequences of tetracycline resistance gene a Bam HI primer was utilized and 196 nucleotide at 5' site of tetracycline gene was sequenced. The results indicated identical homology between MLV-K:E and pBR322 nucleotide sequences. Based on the information which was obtained from sequencing, we concluded that the origin of replication is from pBR322 and it is located between the Beta-lactamase gene and tetracycline gene.

We purpose the following model for the excised molecule (MLV-K:E): the 7.7 Kbp MLV-K:E is composed of 4.1 Kbp of retroviral information and 3.6 Kbp of pBR322 sequences that contain a portion of Beta-lactamase gene, tetracycline gene and origin of replication (Fig. 17).

The results support this model in that the origin of replication and

tetracycline resistance are arrived from pBR322. Why is there a significant difference between MLV-K:E and pBR322 tetracycline resistance? Several possibility could explain the difference, one possibility can be due to the low copy number of the plasmid, results in less TET protein production, therefore the channels which are involved in the transport of tetracycline into the cell are not completely blocked. As a result small amount of tetracycline penetrate the cell membrane and causing slower growth rate of the cell. Defect such as point mutation or dilution or insertion in the TET coding region of tetracycline could lead to the production of TET protein with few amino acid substitution, this could play a significant role in the teritiary structure of TET protein. Change in the tertiary structure of the protein may prevent the complete multimurization of the subunits.

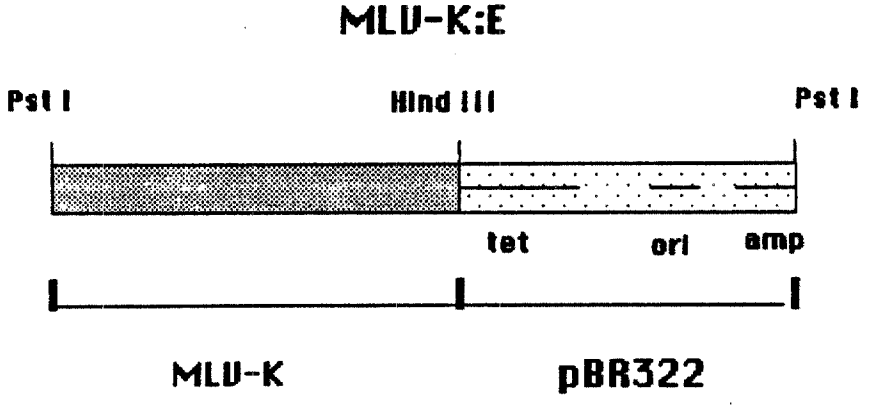
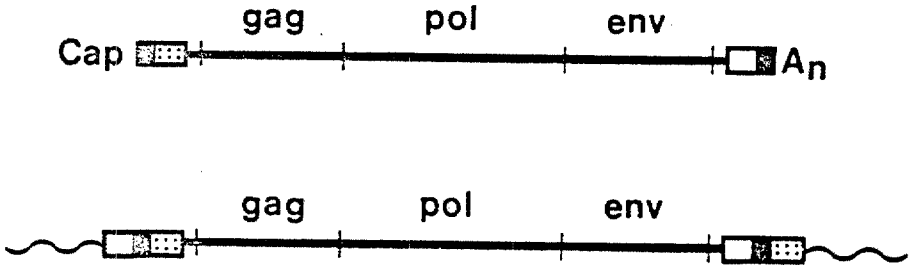


Fig. 17 Model for the excised molecule (MLV-K:E)

Based on the results which was obtained, the latter possibility is most likely. Promoter is consist of to "consensus sequences", 6-7 bp sequence centered about -10 region and 6-7 bp sequence centered about -35 region. Hind III site is located between these two consensus sequences, therefore any deletion or insertion mutations that alter the distance between the 35 and 10 region lead to reduced tetracycline resistance. Insertion at Hind III site make the cell more sensitive to tetracycline than bacterial cells containing pBR322. Insertion of 4, 22, or 33 bp fragments at the Hind III site of pBR322 promoter, reduced the resistance conferred by these plasmid 40% less relative to pBR322, but deletion or insertion of only 2 bp at Cla I site of pBR322 severely affected the resistance and reduced the resistance conferred by these plasmid to 7% (Harley et al, 1988). Cla I site is also located 5 bp upstream from Hind III site. The sequences upstream from Hind III site are necessary for promoter strength, therefore if during excision the sequence upstream from Hind III site have been replaced by retroviral sequences one could expect to see mark decrease in tetracycline mRNA production, due to the weak promoter region.

APPENDIX A

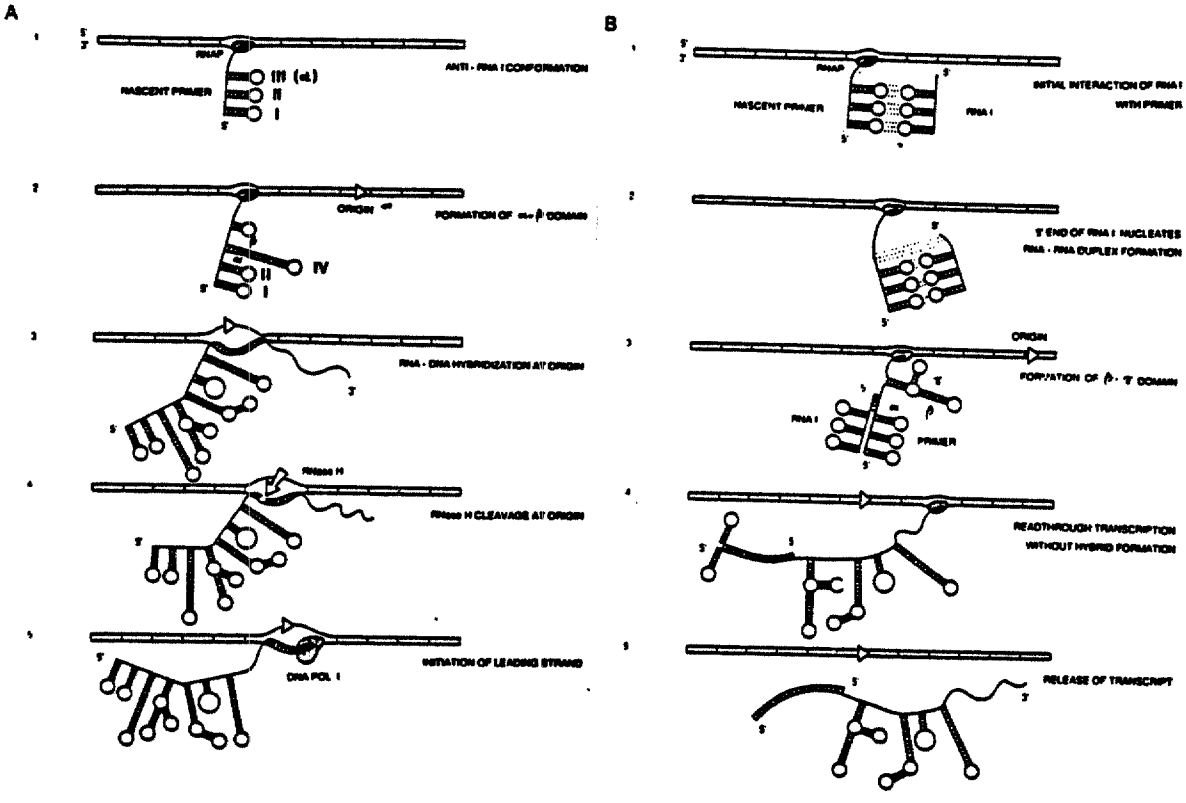
RETROVIRAL GENOME AND INTEGRATED PROVIRAL DNA



Schematic representation of one subunit of a retroviral genome (top) and of an exogenously infected provirus (bottom) (Weiss et al., 1985, Vol. 1)

APPENDIX B

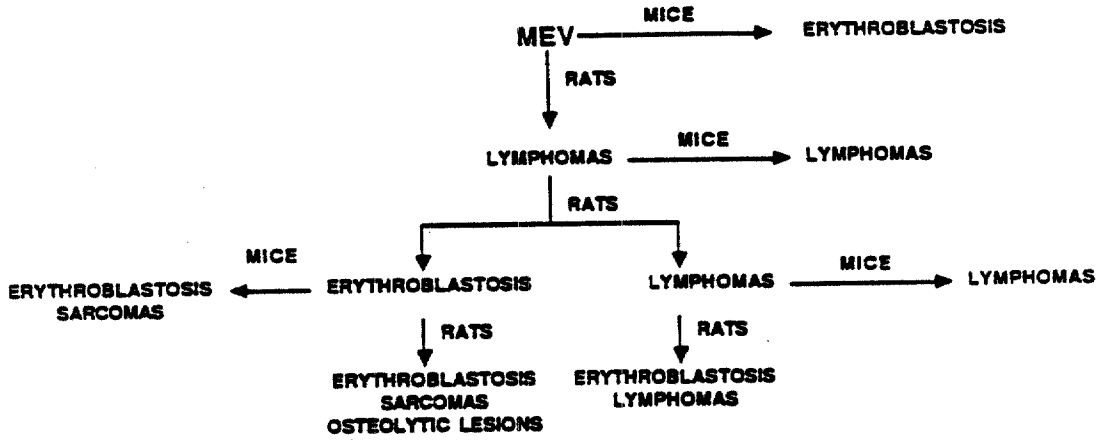
THE MECHANISM OF REVERSE TRANSCRIPTION



Structure I represents one molecule of the dimeric RNA molecule of the virion. In II, minus-strand DNA synthesis is initiated, and in V, plus-strand DNA synthesis is initiated. Various regions of the molecules are denoted by lettters. Upprecase letters refer to sequences in the viral RNA molecule (plus-strand sequences), and lowercase letters refer to minus-strand sequences. The various regions of the molecules are not shown to scale; for instance, region (c/C) is about 18 nucleotides long, while region (d/D) os abpit 7600 nucleotides long. The 3' poly (A) of the viral RNA has been omitted (Gibloa et al., 1979).

APPENDIX C

MORPHOLOGICAL RESPONSES TO MURINE ERYTHROBLASTOSIS VIRUS

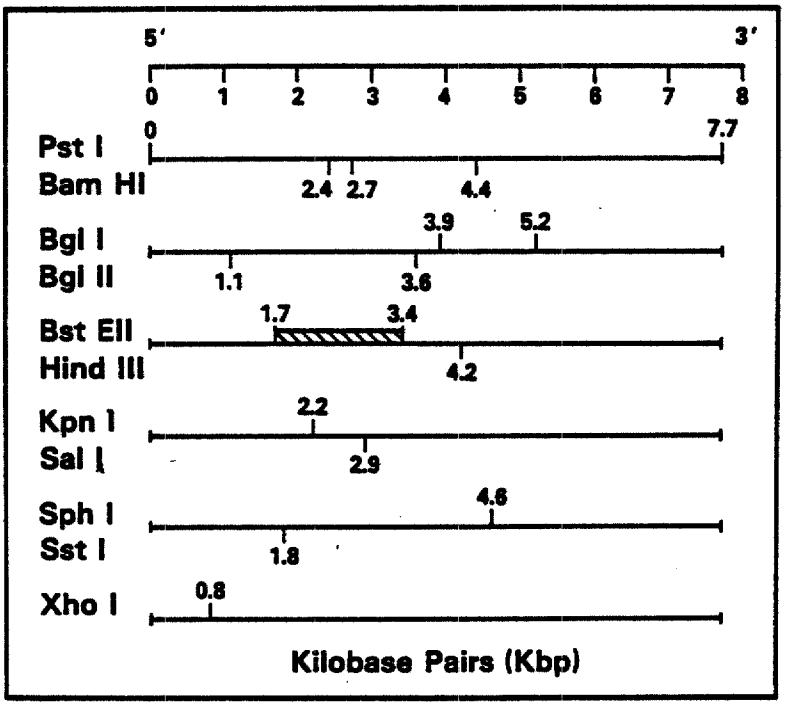


Morophological responses to murine erythroblastosis virus (Kirsten and Mayer, 1967).

APPENDIX D

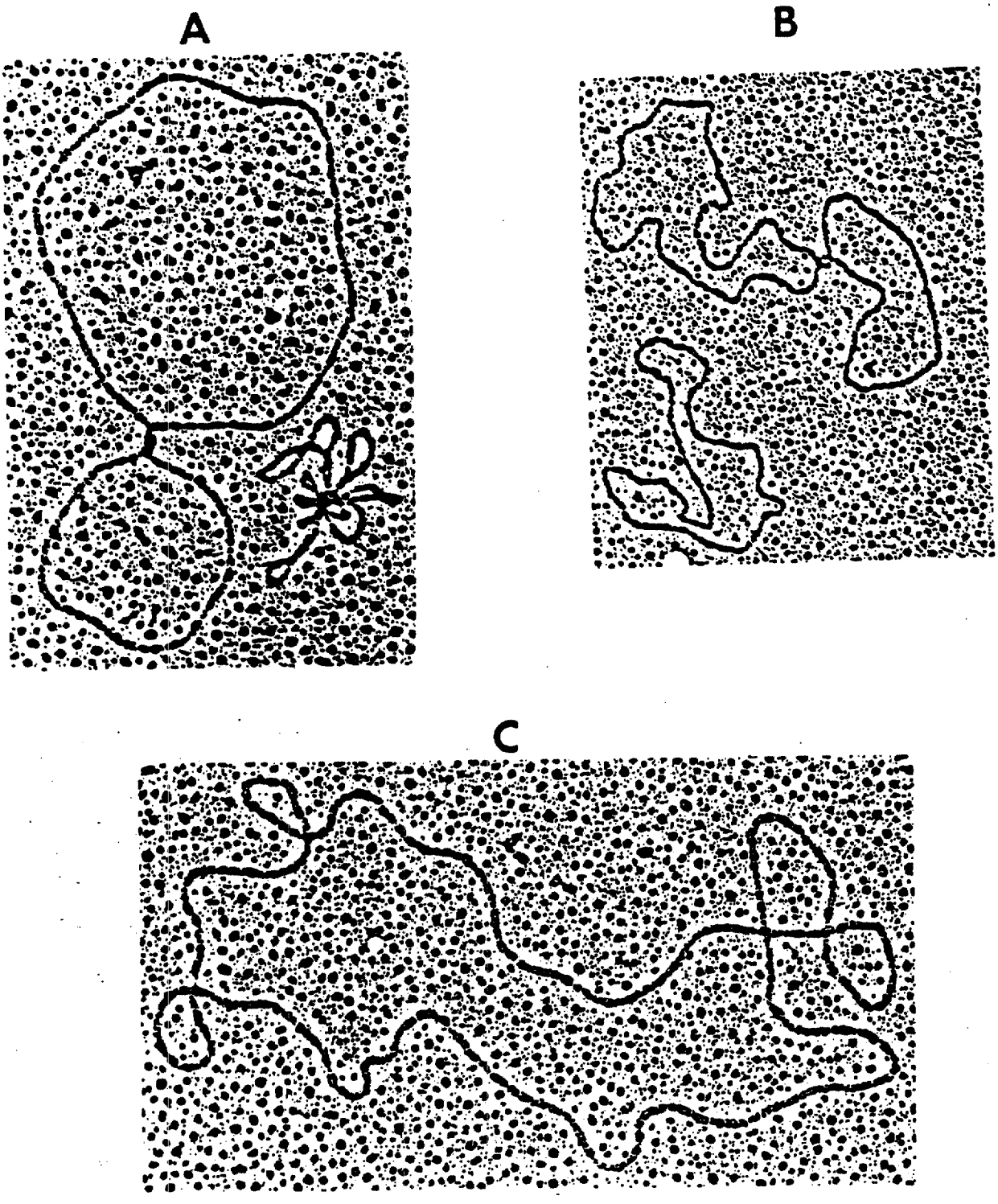
RESTRICTION ENDONUCLEASE MAP OF THE MLV-K;E GENOME

Restriction Endonuclease Map of the MLV-K:E Genome



APPENDIX E

ELECTRON MICROGRAPH OF THE MLV-K:pBR322 DNA



Electron Micrograph of the MLV-K:pBR322 DNA.

APPENDIX F

PLASMID pBR322, AND PLASMID ColE1 ORIGIN OF REPLICATION AND MAINTENANCE REGIO

```
24/27    2475  ACATGTGAGCAAAAGGCCAGCAAAAGG  2501
0.89     760   ACATGTGAGCAAAAGACCAGGAACAGG   734
                           *    *  *

         2493  AGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCC
         753   AGCAAAAGACCAGGAACAGGAAGAAGGCCACGTAGCAGGCGTTTTTCCATAGGCTCCGCC
                     *  .      *  *  *      *    *  *

         2553  CCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGAC
         693   CCCCTGACGAGCATCACAAAAATAGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGAC
                                    *

         2613  TATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCC
         633   TATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCC

         2673  TGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAAT
         573   TGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATA
                                                                      **

         2733  GCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGC
         513   GCTCACGCTGTTGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGC
                        *

         2793  ACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCA
         453   ACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCA

         2853  ACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAG
         393   ACCCGGTAAGGCACGCCTTAACGCCACTGGCAGCAGCCACTGGTAACCGGATTAGCAGAG
                           *    *    *                              *

408      2913  CGA  2915
0.96     333   CGA   331
```

Plasmid pBR322 (upper strand), and plasmid ColE1 (lower strand) origin of replication and maintenance region

APPENDIX G

THE pBR322 DNA SEQUENCES

```
(GAA)TTCTCATGTTTGACAGCTTATCATCGATAAGCTTTAATGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGTCAGGCACCGTGTATGAAATCTAACAAT
                                                                                                        100.
    AAGAGTACAAACTGTCGAATAGTAGCTATTCGAAATTACGCCATCAAATAGTGTCAATTTAACGATTGCGTCAGTCCGTGGCACATACTTTAGATTGTTA

    GCGCTCATCGTCATCCTCGGCACCGTCACCCTGGATGCTGTAGGCATAGGCTTGGTTATGCCGGTACTGCCGGGCCTCTTGCGGGATATCGTCCATTCCG
                                                                                                        200.
    CGCGAGTAGCAGTAGGAGCCGTGGCAGTGGGACCTACGACATCCGTATCCGAACCAATACGGCCATGACGGCCCGGAGAACGCCCTATAGCAGGTAAGGC

    ACAGCATCGCCAGTCACTATGGCGTGCTGCTAGCGCTATATGCGTTGATGCAATTTCTATGCGCACCCGTTCTCGGAGCACTGTCCGACCGCTTTGGCCG
                                                                                                        300.
    TGTCGTAGCGGTCAGTGATACCGCACGACGATCGCGATATACGCAACTACGTTAAAGATACGCGTGGGCAAGAGCCTCGTGACAGGCTGGCGAAACCGGC

    CCGCCCAGTCCTGCTCGCTTCGCTACTTGGAGCCACTATCGACTACGCGATCATGGCGACCACACCCGTCCTGTGGATCCTCTACGCCGGACGCATCGTG
                                                                                                        400.
    GGCGGGTCAGGACGAGCGAAGCGATGAACCTCGGTGATAGCTGATGCGCTAGTACCGCTGGTGTGGGCAGGACACCTAGGAGATGCGGCCTGCGTAGCAC

    GCCGGCATCACCGGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACCGATGGGGAAGATCGGGCTCGCCACTTCGGGCTCATGAGCGCTT
                                                                                                        500.
    CGGCCGTAGTGGCCGCGGTGTCCACGCCAACGACCGCGGATATAGCGGCTGTAGTGGCTACCCCTTCTAGCCCGAGCGGTGAAGCCCGAGTACTCGCGAA

    GTTTCGGCGTGGGTATGGTGGCAGGCCCGTGGCCGGGGGACTGTTGGGCGCCATCTCCTTGCATGCACCATTCCTTGCGGCGGCGGTGCTCAACGGCCTC
                                                                                                        600.
    CAAAGCCGCACCCATACCACCGTCCGGGCACCGGCCCCCTGACAACCCGCGGTAGAGGAACGTACGTGGTAAGGAACGCCGCCGCCACGAGTTGCCGGAG

    AACCTACTACTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGTCGACCGATGCCCTTGAGAGCCTTCAACCCAGTCAGCTCCTTCCGGTGGG
                                                                                                        700.
    TTGGATGATGACCCGACGAAGGATTACGTCCTCAGCGTATTCCCTCTCGCAGCTGGCTACGGGAACTCTCGGAAGTTGGGTCAGTCGAGGAAGGCCACCC

    CGCGGGGCATGACTATCGTCGCCGCACTTATGACTGTCTTCTTTATCATGCAACTCGTAGGACAGGTGCCGGCAGCGCTCTGGGTCATTTTCGGCGAGGA
                                                                                                        800.
    GCGCCCCGTACTGATAGCAGCGGCGTGAATACTGACAGAAGAAATAGTACGTTGAGCATCCTGTCCACGGCCGTCGCGAGACCCAGTAAAAGCCGCTCCT

    CCGCTTTCGCTGGAGCGCGACGATGATCGGCCTGTCGCTTGCGGTATTCGGAATCTTGCACGCCCTCGCTCAAGCCTTCGTCACTGGTCCCGCCACCAAA
                                                                                                        900.
    GGCGAAAGCGACCTCGCGCTGCTACTAGCCGGACAGCGAACGCCATAAGCCTTAGAACGTGCGGGAGCGAGTTCGGAAGCAGTGACCAGGGCGGTGGTTT

    CGTTTCGGCGAGAAGCAGGCCATTATCGCCGGCATGGCGGCCGACGCGCTGGGCTACGTCTTGCTGGCGTTCGCGACGCGAGGCTGGATGGCCTTCCCCA
                                                                                                        1000.
    GCAAAGCCGCTCTTCGTCCGGTAATAGCGGCCGTACCGCCGGCTGCGCGACCCGATGCAGAACGACCGCAAGCGCTGCGCTCCGACCTACCGGAAGGGGT

    TTATGATTCTTCTCGCTTCCGGCGGCATCGGGATGCCCGCGTTGCAGGCCATGCTGTCCAGGCAGGTAGATGACGACCATCAGGGACAGCTTCAAGGATC
                                                                                                        1100.
    AATACTAAGAAGAGCGAAGGCCGCCGTAGCCCTACGGGCGCAACGTCCGGTACGACAGGTCCGTCCATCTACTGCTGGTAGTCCCTGTCGAAGTTCCTAG

    GCTCGCGGCTCTTACCAGCCTAACTTCGATCACTGGACCGCTGATCGTCACGGCGATTTATGCCGCCTCGGCGAGCACATGGAACGGGTTGGCATGGATT
                                                                                                        1200.
    CGAGCGCCGAGAATGGTCGGATTGAAGCTAGTGACCTGGCGACTAGCAGTGCCGCTAAATACGGCGGAGCCGCTCGTGTACCTTGCCCAACCGTACCTAA

    GTAGGCGCCGCCCTATACCTTGTCTGCCTCCCCGCGTTGCGTCGCGGTGCATGGAGCCGGGCCACCTCGACCTGAATGGAAGCCGGCGGCACCTCGCTAA
                                                                                                        1300.
    CATCCGCGGCGGGATATGGAACAGACGGAGGGGCGCAACGCAGCGCCACGTACCTCGGCCCGGTGGAGCTGGACTTACCTTCGGCCGCCGTGGAGCGATT

    CGGATTCACCACTCCAAGAATTGGAGCCAATCAATTCTTGCGGAGAACTGTGAATGCGCAAACCAACCCTTGGCAGAACATATCCATCGCGTCCGCCATC
                                                                                                        1400.
    GCCTAAGTGGTGAGGTTCTTAACCTCGGTTAGTTAAGAACGCCTCTTGACACTTACGCGTTTGGTTGGGAACCGTCTTGTATAGGTAGCGCAGGCGGTAG

    TCCAGCAGCCGCACGCGGCGCATCTCGGGCAGCGTTGGGTCCTGGCCACGGGTGCGCATGATCGTGCTCCTGTCGTTGAGGACCCGGCTAGGCTGGCGGG
                                                                                                        1500.
    AGGTCGTCGGCGTGCGCCGCGTAGAGCCCGTCGCAACCCAGGACCGGTGCCCACGCGTACTAGCACGAGGACAGCAACTCCTGGGCCGATCCGACCGCCC

    GTTGCCTTACTGGTTAGCAGAATGAATCACCGATACGCGAGCGAACGTGAAGCGACTGCTGCTGCAAAACGTCTGCGACCTGAGCAACAACATGAATGGT
                                                                                                        1600.
    CAACGGAATGACCAATCGTCTTACTTAGTGGCTATGCGCTCGCTTGCACTTCGCTGACGACGACGTTTTGCAGACGCTGGACTCGTTGTTGTACTTACCA

    CTTCGGTTTCCGTGTTTCGTAAAGTCTGGAAACGCGGAAGTCAGCGCCCTGCACCATTATGTTCCGGATCTGCATCGCAGGATGCTGCTGGCTACCCTGT
                                                                                                        1700.
    GAAGCCAAAGGCACAAAGCATTTCAGACCTTTGCGCCTTCAGTCGCGGGACGTGGTAATACAAGGCCTAGACGTAGCGTCCTACGACGACCGATGGGACA

    GGAACACCTACATCTGTATTAACGAAGCGCTGGCATTGACCCTGAGTGATTTTTCTCTGGTCCCGCCGCATCCATACCGCCAGTTGTTTACCCTCACAAC
                                                                                                        1800.
    CCTTGTGGATGTAGACATAATTGCTTCGCGACCGTAACTGGGACTCACTAAAAAGAGACCAGGGCGGCGTAGGTATGGCGGTCAACAAATGGGAGTGTTG
```

Nucleotide sequences of pBR322

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GTTCCAGTAACCGGGCATGTTCATCATCAGTAACCCGTATCGTGAGCATCCTCTCTCGTTTCATCGGTATCATTACCCCCATGAACAGAAATTCCCCCTT
                                                                                                    1900.
CAAGGTCATTGGCCCGTACAAGTAGTAGTCATTGGGCATAGCACTCGTAGGAGAGAGCAAAGTAGCCATAGTAATGGGGGTACTTGTCTTTAAGGGGGAA

ACACGGAGGCATCAAGTGACCAAACAGGAAAAAACCGCCCTTAACATGGCCCGCTTTATCAGAAGCCAGACATTAACGCTTCTGGAGAAACTCAACGAGC
                                                                                                    2000.
TGTGCCTCCGTAGTTCACTGGTTTGTCCTTTTTTGGCGGGAATTGTACCGGGCGAAATAGTCTTCGGTCTGTAATTGCGAAGACCTCTTTGAGTTGCTCG

TGGACGCGGATGAACAGGCAGACATCTGTGAATCGCTTCACGACCACGCTGATGAGCTTTACCGCAGCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAA
                                                                                                    2100.
ACCTGCGCCTACTTGTCCGTCTGTAGACACTTAGCGAAGTGCTGGTGCGACTACTCGAAATGGCGTCGACGGAGCGCGCAAAGCCACTACTGCCACTTTT

CCTCTGACACATGCAGCTCCCGGAGACCGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGG
                                                                                                    2200.
GGAGACTGTGTACGTCGAGGGCCTCTGCCAGTGTCGAACAGACATTCGCCTACGGCCCTCGTCTGTTCGGGCAGTCCCGCGCAGTCGCCCACAACCGCCC

TGTCGGGGCGCAGCCATGACCCAGTCACGTAGCGATAGCGGAGTGTATACTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATAT
                                                                                                    2300.
ACAGCCCCGCGTCGGTACTGGGTCAGTGCATCGCTATCGCCTCACATATGACCGAATTGATACGCCGTAGTCTCGTCTAACATGACTCTCACGTGGTATA

GCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTG
                                                                                                    2400.
CGCCACACTTTATGGCGTGTCTACGCATTCCTCTTTTATGGCGTAGTCCGCGAGAAGGCGAAGGAGCGAGTGACTGAGCGACGCGAGCCAGCAAGCCGAC

CGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGG
                                                                                                    2500.
GCCGCTCGCCATAGTCGAGTGAGTTTCCGCCATTATGCCAATAGGTGTCTTAGTCCCCTATTGCGTCCTTTCTTGTACACTCGTTTTCCGGTCGTTTTCC

CCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAA
                                                                                                    2600.
GGTCCTTGGCATTTTTCCGGCGCAACGACCGCAAAAAGGTATCCGAGGCGGGGGGACTGCTCGTAGTGTTTTTAGCTGCGAGTTCAGTCTCCACCGCTTT

CCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTT
                                                                                                    2700.
GGGCTGTCCTGATATTTCTATGGTCCGCAAAGGGGGACCTTCGAGGGAGCACGCGAGAGGACAAGGCTGGGACGGCGAATGGCCTATGGACAGGCGGAAA

CTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCC
                                                                                                    2800.
GAGGGAAGCCCTTCGCACCGCGAAAGAGTTACGAGTGCGACATCCATAGAGTCAAGCCACATCCAGCAAGCGAGGTTCGACCCGACACACGTGCTTGGGG

CCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAG
                                                                                                    2900.
GGCAAGTCGGGCTGGCGACGCGGAATAGGCCATTGATAGCAGAACTCAGGTTGGGCCATTCTGTGCTGAATAGCGGTGACCGTCGTCGGTGACCATTGTC

GATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTG
                                                                                                    3000.
CTAATCGTCTCGCTCCATACATCCGCCACGATGTCTCAAGAACTTCACCACCGGATTGATGCCGATGTGATCTTCCTGTCATAAACCATAGACGCGAGAC

CTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTA
                                                                                                    3100.
GACTTCGGTCAATGGAAGCCTTTTTCTCAACCATCGAGAACTAGGCCGTTTGTTTGGTGGCGACCATCGCCACCAAAAAAACAAACGTTCGTCGTCTAAT

CGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAG
                                                                                                    3200.
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ATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAA
                                                                                                    3300.
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                                                                                                    3400.
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                                                                                                    3500.
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AAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTT
                                                                                                    3600.
TTCACCAGGACGTTGAAATAGGCGGAGGTAGGTCAGATAATTAACAACGGCCCTTCGATCTCATTCATCAAGCGGTCAATTATCAAACGCGTTGCAACAA
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Continue nucleotide sequence of pBR322

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GCCATTGCTGCAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGT
                                                                                                 3700.
CGGTAACGACGTCCGTAGCACCACAGTGCGAGCAGCAAACCATACCGAAGTAAGTCGAGGCCAAGGGTTGCTAGTTCCGCTCAATGTACTAGGGGGTACA

TGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTC
                                                                                                 3800.
ACACGTTTTTTCGCCAATCGAGGAAGCCAGGAGGCTAGCAACAGTCTTCATTCAACCGGCGTCACAATAGTGAGTACCAATACCGTCGTGACGTATTAAG

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                                                                                                 3900.
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CCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTAC
                                                                                                 4000.
GGCCGCAGTTGTGCCCTATTATGGCGCGGTGTATCGTCTTGAAATTTTCACGAGTAGTAACCTTTTGCAAGAAGCCCCGCTTTTGAGAGTTCCTAGAATG

CGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAG
                                                                                                 4100.
GCGACAACTCTAGGTCAAGCTACATTGGGTGAGCACGTGGGTTGACTAGAAGTCGTAGAAAATGAAAGTGGTCGCAAAGACCCACTCGTTTTTGTCCTTC

GCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGT
                                                                                                 4200.
CGTTTTACGGCGTTTTTTCCCTTATTCCCGCTGTGCCTTTACAACTTATGAGTATGAGAAGGAAAAAGTTATAATAACTTCGTAAATAGTCCCAATAACA

CTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCA
                                                                                                 4300.
GAGTACTCGCCTATGTATAAACTTACATAAATCTTTTTATTTGTTTATCCCCAAGGCGCGTGTAAAGGGGCTTTTCACGGTGGACTGCAGATTCTTTGGT

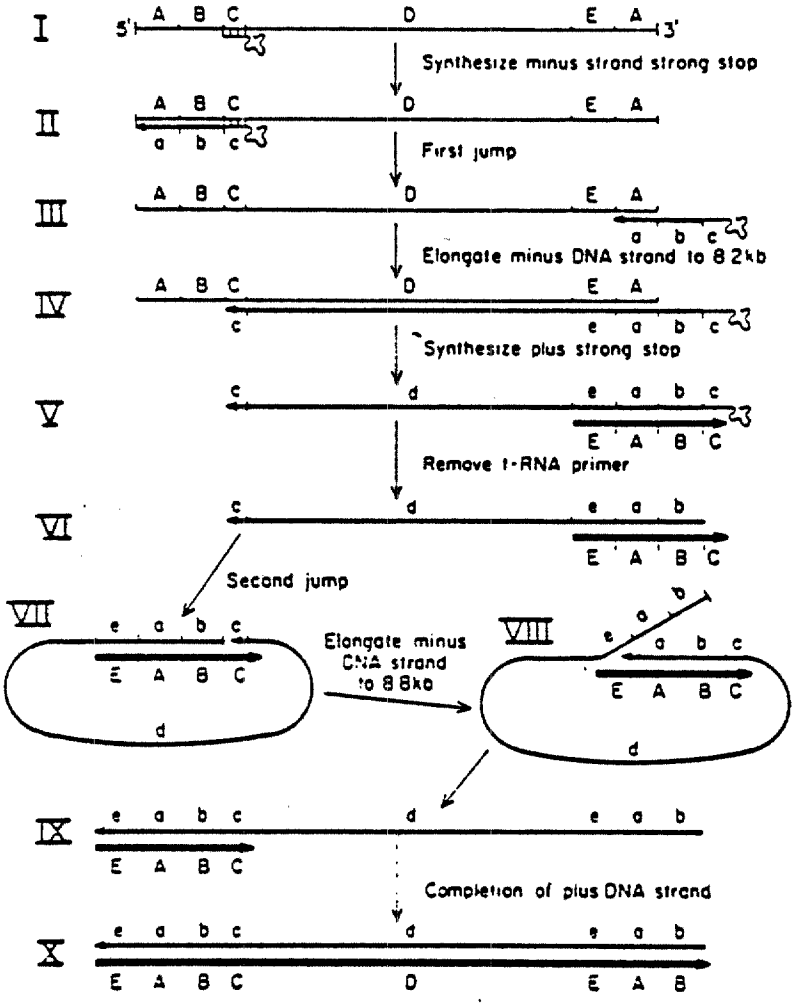
TTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAA(TTC)

AATAATAGTACTGTAATTGGATATTTTTATCCGCATAGTGCTCCGGGAAAGCAGAAGTTCTT
```

The pBR322 DNA sequence. The sequence is presented in double-strand form. The top strand is 5'—3', the lower strand is complementary 3'—5'. The circular nature of the sequence is indicated by the nucleotides in parentheses at the ends. The sequence is numbered such that 0 is the middle of the unique EcoRI site and the count increases first through the tet genes, then pMBI material, and finally through the Tn3 region (Sutcliffe, 1979).

APPENDIX H

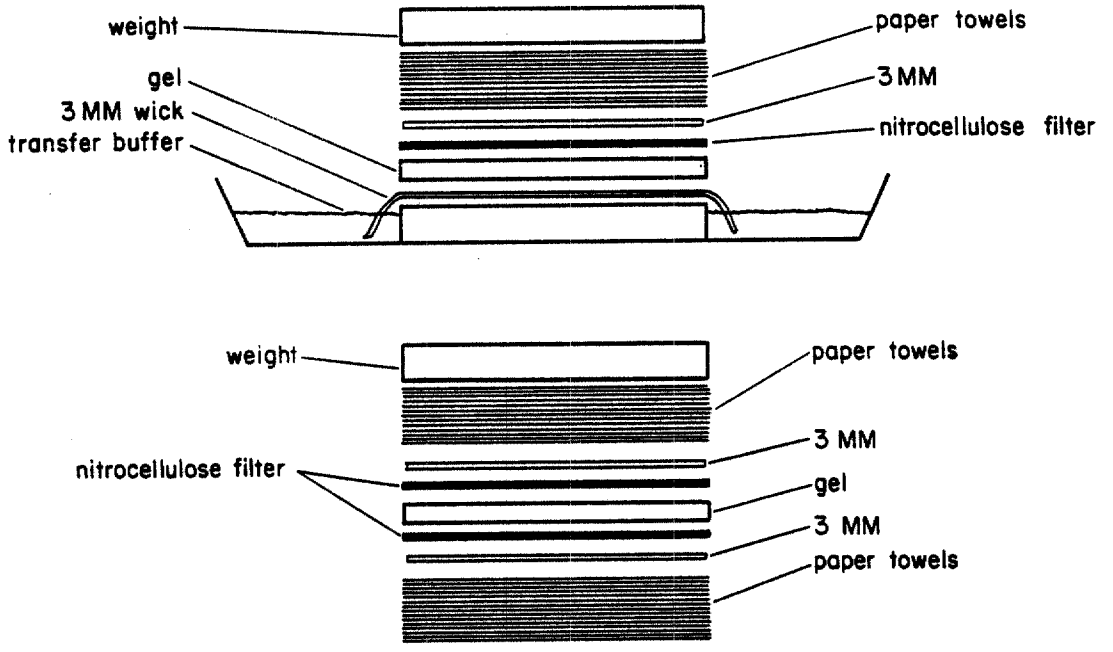
MAJOR SECONDARY STRUCTURAL FEATURES OF RNAs INVOLVED IN REPLICATION CONTROL



A. Primer formation and leading DNA strand initiation. (1) Initial elongation of the primer precursor: the transcript assumes a conformation consisting of three stem-loop domains. The 3'-proximal domain is called alpha. (2) Further elongation results in structural alteration due to interaction of the alpha and beta domains to form stem-loop IV, which eliminates stem-loop III. Stem-loop IV formation precludes interaction of the beta domain with other down-stream primer domains. (3) The primer precursor forms a persistent hybrid in the origin region. (4) RNAase H cleaves the hybridized RNA at specific loci. (5) DNA polymerse I (POL I) adds dNTPs to the processed primer terminus. RNAP, RNA polymerase. B. Inhibition of primer precursor processing by RNA I. (1) RNA I interacts with nascent primer in a reversible reaction between complementary nucleotides located in loop regions. (2) Full-length pairing between RNA I and primer is nucleated from the 5' terminus of RNA I. (3) Pairing precludes interaction of the alpha and beta domains; the domain is free to interact with a downstream domain, y. This interaction alters other, but not all, downstream primer domains. (4) Because of altered RNA conformation, hybrid formation does not occur. (5) Primer transcript is released from the template (Polisky, 1988).

APPENDIX I

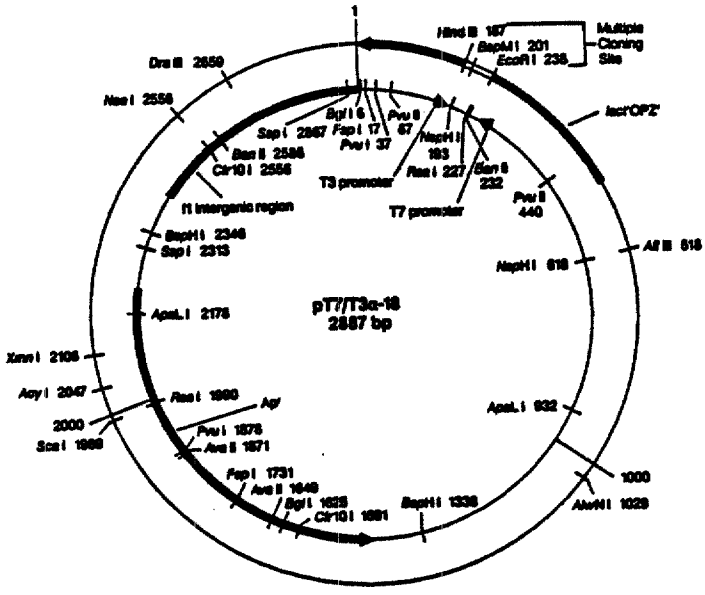
METHODS FOR TRANSFER OF DNA FROM AGAROSE GELS TO NITROCELLULOSE FILTER PAPER



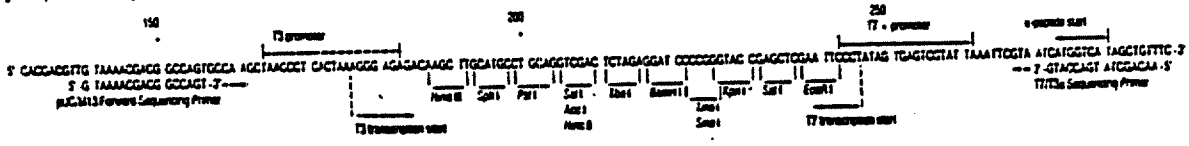
Methods for transfer of DNA from agarose gels to nitrocellulose filter paper. The most common system for transfer of DNA (Top). A system for perparing duplicate nitrocellulose filters from a single gel (Bottom) (Maniatis et al., 1982).

APPENDIX J

BIOLOGICAL MAP OF pT7/T3α-18 PLASMID AND MULTIPLE CLONING SITE



pT7/T3α-18 promoters, multiple cloning site and primer binding region: 131-290



Biological map of pT7/T3α-18 plasmid and multiple cloning site

APPENDIX K

BRIEF PROTOCOL FOR DNA SEQUENCING WITH SEQUENASE

Check off steps as you complete them; typical volumes are given in parentheses.

1. Annealing mixture:

DNA	☐ μl	
H_2O	☐ μl	
Sequencing Buffer	2 μl	☐
Primer	1 μl	☐
Total	10 μl	

Anneal by heating 2 min., 65°C then cool slowly to <35°C.

2. While cooling, label, fill and cap tubes with 2.5 μl of each Termination Mixture. Use Red tubes for dGTP or Orange for dITP. Save for steps 5 and 7.

G ☐ (2.5) A ☐ (2.5)
T ☐ (2.5) C ☐ (2.5)

3. Dilute Labeling Mix 1:5 to working concentration if needed. dGTP (green tube) OR dITP (yellow tube). Retain for use in step 6.

Labeling Mix ☐ μl (2)
H_2O ☐ μl (8)

4. Dilute enough Sequenase™ for all templates in ice cold TE buffer 1:8.

TE buffer ☐ μl (7)
Sequenase ☐ μl (1)

5. Pre-warm 4 termination tubes from step 2 (G,A,T and C) in 37°C bath.

6. LABELING REACTION

To Annealed DNA Mixture (10μl)
Add:

DTT, 0.1 M	1 μl	☐
Diluted Labeling Mix	2 μl	☐
[^{35}S] dATP	0.5 μl	☐
Diluted Sequenase™	2 μl	☐

Mix and incubate at room temp, 5 min.

7. TERMINATION REACTION

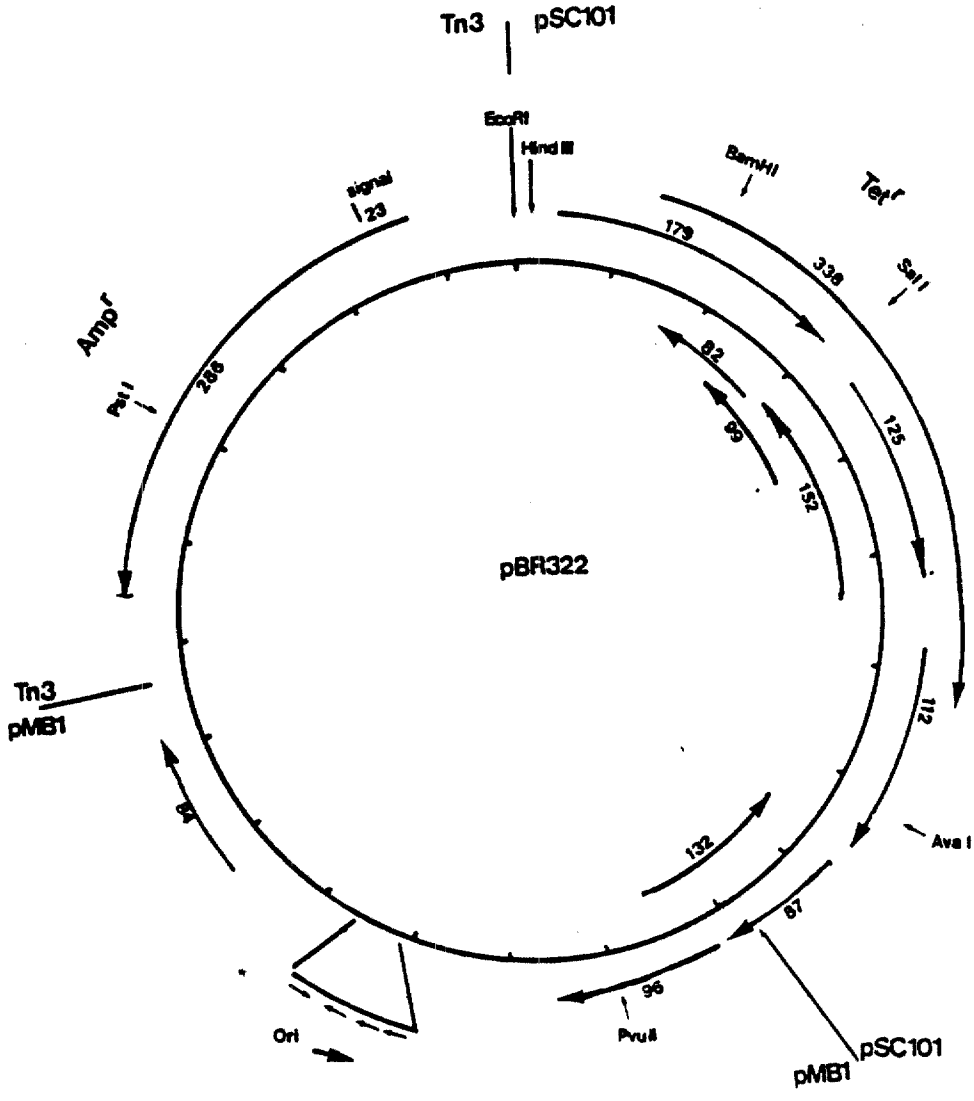
Transfer 3.5 μl of labeling reaction to each termination tube (G,A,T and C), mix and continue incubation of the termination reactions at 37°C for 5 min.

8. Stop the reactions by adding 4 μl of Stop Solution.

9. Heat samples to 75°C, 2 min., immediately before loading.

APPENDIX L

BIOLOGICAL MAP OF pBR322


Tn3
pSC101
EcoRI
Hind III
BamHI
Tet^r
Sal I
signal
23
179
338
Amp^r
Pst I
286
82
99
125
152
pBR322
Tn3
pMB1
112
Ava I
132
87
96
Pvu II
Ori
pSC101
pMB1

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