HIV-1 REGULATORY GENE DEPENDENT EXPRESSION OF A TOXIC GENE

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by
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ABSTRACT

From the first day it was discovered, HIV remains as one the major health threats of 21st century and the methods tried up to now focused on the short-term solutions which were efficient at blocking HIV replication, but also resulted with drug-resistant strains, instead of methods which would completely eliminate HIV-infected cells from potential reservoirs. In this study, the design of a special DNA vector encoding a toxic protein to be expressed only in cells infected with HIV, thereby not damaging to healthy cells and the test of the efficacy of this vector in the cell culture conditions without using HIV infection was aimed. The toxic gene (suicide gene) presumed to create the desired effect was placed under the transcriptional control of HIV promoter LTR and so that the expression of the toxic gene was made dependent upon the tat regulator gene of HIV. In order to prevent leaky gene expression stemming from the basal gene expression from LTR even if it was not induced by Tat, and thereby having potential to damage healthy cells, the prerequisite cis-acting DNA sequences were cloned downstream of the toxic gene. So that, the transcripts produced could retain in the nucleus and would require the function of a second regulator protein 'Rev' which is a molecular chaperone for being transmitted into the cytoplasm. If the efficiency of this model prooved, a full-protective suicide vector will have been designed and this vector may be suggested to be tested in gene therapy trials of HIV infection in the future.

ÖZET

Bulunduğu ilk günden beri HIV 21. yüzyılın en büyük sağlık tehlikelerinden birini oluşturmaktadır ve şu ana kadar kullanılan yöntemler HIV ile infekte hücrelerin tamamen elimine edilmesini sağlayacak yöntemler yerine HIV replikasyonunu bloke ederek etkili olan fakat ilaca dirençli suşların oluşumuna da zemin hazırlayan kısa vadeli çözümler üzerinde yoğunlaşmıştır. Planlanan bu çalışmada, infekte olmayan hücrelere zarar vermeyip sadece HIV ile infekte hücrelerde üretilebilecek toksik bir proteini kodlayan geni içeren özel bir DNA molekülünün oluşturulması ve bu vektörün etkinlik derecesinin HIV infeksiyonu kullanılmadan hücre kültürü ortamında test edilmesi amaçlanmıştır. Arzu edilen etkiyi yaratacağı düşünülen toksik gen (intihar geni) HIV promotoru LTR'ın transkripsiyonel kontrolu altında olacak şekilde klonlanarak toksik proteinin üretimi HIV 'tat' regulator genine bağımlı hale getirilmiştir. LTR promotorunun 'tat' tarafından transaktive edilmediği durumlarda da oluşabilen ve sağlıklı hücrelere zarar verebilecek olan kaçak gen ekspresyonunu önlemek için gerekli cis-acting DNA dizinleri de kullanılmıştır. Böylece oluşan transkriptlerin nukleusta alıkonması ve sitoplazmaya geçerek toksik proteini üretebilmeleri için ikinci bir regulator gene, moleküler şaperon 'rev' e de bağımlılığı sağlanarak tam korumalı bir sistem oluşturulması amaçlanmıştır. Oluşturulan DNA vektörünün etkinliği kanıtlanabilirse, gelecekte HIV infeksiyonunun gen terapisiyle önlemesini sağlamak için yapılan testlerde kullanımı önerilebilecek olan bir DNA molekülü oluşturulmuş olacaktır.

To

Kerem YEĞİN

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
HIV-1	Human Immunodeficiency Virus I
HIV-2	Human Immunodeficiency Virus II
WHO	World Health Organization
HTLV-III	Human T-lymphotropic Virus III
LAV	Lymphadenopathy-associated Virus
T_H	T-helper
APCs	Antigen presenting cells
ZDV	Zidovudine
AZT	Azidothymidine
TAR	Transactivation Responsive Element
Tat	Trans-activator of transcription
Rev	Regulator of viral gene expression
Nef	Negative factor
SIV	Simian Immunodeficiency Virus
LTR	Long terminal repeat
CNS	Central nervous system
SS	Splice sites
mRNA	Messenger ribonucleic acid
RRE	Rev response element
INS	Instability
CRS	Cis-acting repressor
NLS	Nuclear localization signal
NES	Nuclear export signal
NPC	Nuclear pore complex
Ab	Antibody
HSV-1	*Herpes simplex* virus type I
TK	Thymidine kinase
CD	Cytosine deaminase
EF2	Elongation factor 2
TKO	Thymidine kinase obliteration

GCV	Ganciclovir
IL	Interleukin
INF	Interferon
TNF	Tumor necrosis factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
ACV	Acyclovir
VDEPT	Virally directed enzyme prodrug therapy
RAC	Recombinant DNA Advisory Committee
NIH	National Institutes of Health
LB	Luria-Bertani
µg	Microgram
ml	Milliliter
β-gal	Beta-galactosidase
µl	Microliter
Ap	Ampicillin
APS	Ammonium persulphate
Bp	Base pairs
CAT	Chloramphenicol acetyl transferase
CIAP	Calf intestine alkaline phosphatase
CMV	Cytomegalovirus
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
EBV	Epstein Barr virus
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
hyg	Hygromycine
IPTG	Isopropyl-thio-β-D-galactopyranoside
kb	Kilobase pairs
l	Liter
M	Molar
mg	Milligram
min	Minute
mM	Millimolar

neo	Neomycin
ng	Nanogram
nm	Nanometer
OD	Optical density
ori	Origin of replication
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulphonyl fluoride
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethyl-ethylenediamide
Tet	Tetracycline
uv	Ultraviolet
X-gal	5-bromo-4-chloro-3 indolyl-B-D-galactoside
w/v	Weight per volume
E. coli	*Escherichia coli*
V	Voltage
DEPC	Diethylpyrocarbonate
dNTP	Deoxyribonucleotide triphosphate
EtBr	Ethidium bromide
TAE	Tris-Acetate-EDTA
ds	Double stranded
CMF-PBS	Calcium-magnesium free phosphate buffered saline
BCIP/NBT	5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium
EBNA	*Epstein Barr* virus nuclear antigen
IRES	Internal ribosome entry site
CMV	Cytomegalovirus
cDNA	Complementary DNA
orf	Open reading frame
pmol	Picomole
DTT	Dithiothreitol

CHAPTER 1

INTRODUCTION

1.1. AIDS Statistical Data

Following its discovery, HIV-1 remains a major threat to public health and a challenge of the 21st century. According to the latest updates of the reports by UNAIDS, approximately 40 million people are living with HIV around the world (Mikkelsen Henning, 2nd National AIDS Symposium 2004, symposium notes). In 2004, estimated 4.9 million people around the world became infected and 3.1 million people died from AIDS. Though the beginning of the epidemic was seen in Africa, AIDS is, however, not confined to Africa, it is a truly global epidemic. Also in Turkey, the numbers of reported HIV infections have been slowly and steadily increasing and according to WHO estimated 5000 people are now living with HIV in Turkey (Mikkelsen Henning, 2nd National AIDS Symposium 2004, symposium notes).

1.2. Emergence and Overview of HIV-1 Infection

Historically, the disease was recognized before the virus. In 1981, clinicians noted that a very rare cancer, Kaposi's sarcoma, was appearing in young men in New York and that this was associated with immune deficiency (Dimmock&Primrose, 1994). A few of the patients with Kaposi's sarcoma also had *Pneumocystis pneumonia* and other opportunistic infections such as mucosal candidiasis (a fungal infection), disseminated cytomegalovirus infection (a latent herpesvirus), and chronic Herpes simplex virus-induced ulcers. The common feature of the affected patients was that they all had evidence of T-lymphocyte dysfunction. By 1982, it was clear that a new disease had appeared, characterized by severely impaired immune system, its related

opportunistic infections and cancer. It was named acquired immune deficiency syndrome (AIDS) (Levine, 1991).

The human pathogen Human Immunodeficiency Virus (HIV), previously termed Human T-Lymphotropic Virus III (HTLV-III) or Lymphadenopathy-Associated Virus (LAV), is a retrovirus with an unusually complex genetic structure (Levine, 1991). The HIV genome encompasses not only the three structural genes (gag, pol, env) common to other known retroviruses, but also at least four nonstructural gene products. HTLV-III differs from HTLV-I and HTLV-II in many aspects of its structure and biology. While infection of human T lymphocytes with HTLV-I or HTLV-II often results in transformation and immortalization (Poiesz et al., 1980; Miyoshi et al., 1981), infection with HTLV-III generally leads to cell death (Sinoussi et al., 1983; Gallo et al., 1984).

Characteristics properties of lentiviruses including HIV-1 and HIV-2 include high genetic complexity and incubation periods of months to many years before disease development (Coffin et al., 1992).

Upon infection of helper $CD4^+$ T lymphocytes and other $CD4^+$ cells (eg. macrophages) with HIV-1 which causes over 99% of human infections, after a long, symptomless incubation period of approximately 8 years, $CD4^+$ lymphocytes decline to such a low level that the immune system can no longer function efficiently, thus resulting in the immunodeficiency which gives the virus its name (Dimmock&Primrose, 1994).

The most important type of regulatory T-cells are known as helper/inducer cells which are responsible for activating B-cells as well as nearby natural killer cells and macrophages. Once the helper T-cell recognizes a specific foreign antigen, it releases lymphokines which activate B-cells to produce the corresponding antibody (Radunskaya et al., 2002). Thus, the dicrease in T helper cells seen in HIV infected people prevents the synthesis of antibodies and this eventually results with the breakdown of cellular immunity.

Macrophages are a type of very large phagocyte that engulfs and digests foreign agents. Macrophages also secrete cytokines including interferon and tumor necrosis factor, which can stimulate more macrophages and NK cells or kill malignant cells (Radunskaya et al., 2002).

The common feature of the affected patients was that they all had T-lymphocyte dysfunction also lymphadenopathy (the enlargement of the lymph nodes). Decreased

numbers of CD4 cells and a falling ratio of CD4 to CD8 cells (helper to killer T cells) are the common indicators of disease progressing and once the diagnosis of AIDS is made, with all its symptoms, 50% of individuals will not survive for more than 1 year (Levine, 1991).

1.3. HIV-Cell Interactions and Infection

Entry of HIV-1 into host cells requires the expression of the receptor $CD4^+$ and a fusion coreceptor. Several chemokine receptors and closely related 7-transmembrane molecules function as coreceptors for HIV entry. Among these, the chemokine receptors CCR5 and CXCR4, which are the principal receptors for macrophage-tropic and T-cell-tropic viruses, respectively, are the most commonly used (Dorns et al., 2003).

HIV has the ability to infect cells displaying the CD4 cell-surface protein. Although a number of cells, including B cells and certain brain and intestinal cells, have very low levels of CD4 on their surfaces, the two cell types most commonly infected are macrophages and T-helper (T_H cells) (Levy, 1998).

HIV infection normally occurs first in macrophages (Clouse et al., 1995). At the cell surface, the macrophage CD4 molecule binds to the gp120 protein of HIV. The viral gp120 protein then interacts with another macrophage protein, the membrane-spanning chemokine receptor CCR5 which acts as coreceptor for HIV (Bernad et al., 2003). Individuals who express a variant CCR5 protein through homozygosity for a 32-bp deletion in the CCR5 gene do not bind HIV and do not acquire HIV infection (Gallo et al., 1995).Therefore, inhibition of the interaction of HIV with CCR5 in vivo could have a significant effect for preventing disease progression.

After HIV has infected the macrophage APCs (antigen presenting cells), a different form of gp120 is made, which in turn binds to a different coreceptor, the CXCR4 chemokine receptor on T cells (Golding et al., 1996). HIV then enters and destroys the CD4 T-helper lymphocytes, which are responsible for cell-mediated inflammatory responses and B-cell help (Adachi et al., 1996).

Thus, HIV starts as a macrophage infection and progresses to a T-cell infection. The net result of HIV infection is the systematic destruction of macrophages and T cells, leading to breakdown of immunity. This situation has serious health

consequences. In a normal human, CD4 cells constitute about 70% of the total T-cell pool; in AIDS patients, the number of CD4 cells steadily decreases and by the time opportunistic infections become established, CD4 cells may be almost absent (Brock, Biology of Microorganisms, 2003).

Finally, the loss of both humoral and cellular immune function and the appearence of opportunistic infections caused by fungi, mycobacteria, viruses in HIV infected patients indicates the progression of the disease to AIDS. This results in the death of the patient within one year (Levine et al., 1991).

1.4. Possible Treatment Methods for HIV Infection

Today's HIV/AIDS therapy can be divided into three major classes, which are chemotherapy, vaccine, and gene therapy.

1.4.1. The Failure of Chemotherapy

Since the discovery of zidovudine (ZDV, AZT) as an effective antiretroviral agent against human immunodeficiency virus type 1 (HIV-1) (Mitsuya et al., 1985), drug therapy has been widely used in the treatment of AIDS. However, the emergence of resistance mutants during antiviral drug monotherapy interrupted the value of this finding. The inefficient success obtained with drug monotherapy stems from three realities: 1) The large number of HIV virions produced daily (Markowitz et al., 1995; Emini et al., 1995; Ho et al., 1996), 2) The inherent variabiliy yielded by RNA virus replication (Coffin, 1992), and 3) The inability of available drugs to completely suppress replication (Richman, 1994). Combination of antiretroviral drugs targeting the reverse transcriptase (RT) and/or protease of HIV-1 can suppress plasma levels of the virus and delay disease progression in HIV-1-infected people, thus reducing the rate of HIV and AIDS-related mortality (Gulick et al., 1997; Hammer et al., 1997). Despite their ability to suppress viral replication for extended periods of time, current antiretroviral therapies have been unable to eradicate HIV-1 from infected people (Pierson et al., 2000)

Treatment failure related with antiretroviral resistance can be investigated under three categories:

1) Clinical failure: occurrence or recurrence of HIV-related events, 2) Immunologic failure: CD4 count decline to an unacceptable range and failure to adequately increase CD4 count, 3) Virologic failure: Incomplete virologic response or lack of response and virologic rebound (Nesli Basgoz, 2nd National AIDS Symposium 2004, symposium notes). Thus, highly active anti-retroviral therapy (HAART) can suppress the level of detectable HIV in the circulation but does not eliminate integrated provirus from peripheral reservoirs (Fauci et al., 1997; Richman et al., 1997). Therefore, cessation of the treatment can lead to a rapid rebound in circulating virus (Vilde et al., 2003; Gunthard et al., 2003).

Finally, the severe toxic side-effects of the drugs and the frequent emergence of drug-resistant viruses necessitate that the other two approaches should be studied basically and clinically in more detail.

1.4.2. The Failure of Vaccine Development

The handicaps for an effective AIDS vaccine include the genetic and antigenic variability of the virus, the gaps in the biology of virus-host interaction and the complexity of the clinical studies in terms of technical and ethical concepts (Gallo, 2001). The greatest problem with all kind of virus vaccines (subunit formulations, live vector vaccines, all virus formulations-inactivated dead or attenuated live-, and nucleic acid based vaccines) envisaged for HIV infection is that there is no animal model to proove the safety of vaccines. HIV can not infect and cause AIDS in small animals routinely used. The live attenuated vaccines, which have been successful in preventing viral diseases, can not be used for a fatal disease like AIDS. The subunit vaccines, which are safe in general, do not appear to work well for HIV/AIDS as judged by the results in animal experiments (Adachi et al., 1998).

What is mostly important is that most of the vaccines generally stimulate the humoral immunity, ineffective to stimulate both arms of the immune system (Giaretta et al., 1998). Antigenic variability of the virus as well as the necessity of stimulating both arms of the immune system to obtain a good level of protection are important issues that have to be adressed when developing an effective vaccine.

The pitfalls of long-term antiviral treatment together with the difficulties of developing an effective vaccine prompted the scientific community to discover new approaches for the treatment of HIV infection.

1.4.3. A New Treatment Approach for HIV Infection/AIDS: Gene Therapy

Gene therapy of AIDS aims at the long-term reconstitution of the pool of $CD4^+$ T lymphocytes with autologous cells made resistant to HIV infection.

A number of gene therapeutic strategies have been proposed for the treatment of AIDS. One approach intracellular immunization (Baltimore, 1988), aims to render HIV-1 permissive cells resistant to HIV-1 infection by introducing anti-HIV molecules such as antisense RNAs (Chuah et al., 1994; Morgan&Walker, 1996), ribozymes (Paik et al., 1997; Wang et al., 1998; Wong-Staal et al., 1998), RNA decoys (Smith et al., 1996; Fraisier et al., 1998), and trans-dominant negative mutants (Chinen et al., 1997; Davis et al., 1998).

Despite their efficacy in cell culture systems, uncertainties remain about the ultimate therapeutic benefit of these various strategies. For example, delivery of TAR RNA decoys (Sullenger et al., 1990) may lead to interference with the normal function of the cellular TAR-binding proteins. Strategies based upon viral transdominant mutant proteins may unmask toxic interactions with host proteins. Transdominant mutants of Gag, for instance, interfere with release of infectious viral particles (Trono et al., 1989) but may also interfere with normal cellular function of cyclophilin A, to which it may still bind (Luban et al., 1993). The potential also exists for interaction of transdominant Rev mutants with host cell factors in the hematopoietic lineage (Malim et al., 1989). Ribozymes that cleave HIV-1-specific RNAs require an ordered secondary structure for their activity, and double-stranded RNA structures are among the most potent inducers of toxic interferon responses.

A second approach is vaccination using expression vectors for viral proteins, and a large-scale clinical trial based on Moloney murine retroviral vector-mediated transfer of the HIV-1 env and rev genes (Galpin et al., 1994; Su et al., 1997).

Finally, a third approach was the use of suicide gene to induce the death of HIV-1 infected cells, thereby preventing virus spread. To minimize unwanted side

effects, it is essential to develop a technique that will enable tissue-specific gene transfer and expression only in targeted cells. This tecnique which will be told in detail later in this chapter means much in terms of welcoming these requirements.

1.5. HIV-1 Genome

The genome of HIV-1 is complex and contains nine known open reading frames. The different proteins are expressed by ribosomal frameshifting (for Gag-Pol) and by the production of alternatively spliced mRNAs from the full-length precursor RNA (Figure 1. 1) (Pavlakis et al., 1990).

HIV-1 encodes regulatory proteins that promote the expression of the viral genome. Tat and Rev are essential for virus production (Haseltine et al., 1986; Wong-Staal et al., 1986). A third factor, Nef, is not essential for virus propagation in tissue culture (Levy et al., 1987).

1.5.1. Mechanism of Action of Tat

The transcriptional transactivator (Tat) is a key regulatory protein of HIV. It is expressed early after the virus integrates into the cell, and stimulates the elongation of RNA polymerase II (RNAPII). This type of transcriptional control had not been previously found. Therefore, work on Tat established a new paradigm in the field of eukaryotic biology.

Tat activation is dependent upon a stable RNA stem-loop structure, that extends from the transcription initiation site to +57, known as TAR (Rosen et al., 1985; Muesing et al., 1987; Feng&Holland, 1988; Garcia et al., 1989; Selby et al., 1989) which serves as the binding site for Tat (Dingwall et al., 1990). Tat, in conjunction with TAR RNA, results in marked stimulation of the elongation properties of RNA polymerase II (Kao et al., 1987; Laspia et al., 1989-1990, Feinberg et al., 1991; Marciniak&Sharp, 1991; Kato et al., 1992) and these effects are probably mediated by association of Tat with RNA polymerase II or other components of the HIV-1 transcriptional elongation complex (Keen et al., 1996; Mavankal et al., 1996). Alterations in TAR RNA structure markedly decrease Tat activation, and viruses containing TAR RNA mutations exhibit several thousand-fold decreases in replication

upon infection of peripheral blood mononuclear cells (PBMCs) or T-cell lines (Harrich et al., 1994-1995-1996; Klaver&Berkout, 1994).

Tat has been shown to activate transcription in trans from the HIV-1 LTR, increasing the steady-state levels of all HIV-1 mRNAs up to 1000-fold (Sodroski et al., 1984-1985; Arya et al., 1985).

The HIV-1 LTR is appoximately 640 bp in length and segmented into the U3, R, and U5 regions (Figure 1.1). The U3 region is further subdivided into the modulatory region, the enhancer element comprising two NF-αB-binding sites and the core region with the TATAA box and three GC-rich binding sites for Sp1 transcription factors (Gaynor, 1992). The R region contains the 59 nucleotide trans-activation-responsive (TAR) element forming a highly stable stem-loop RNA structure (Feng&Holland, 1988) (Figure 1.3).

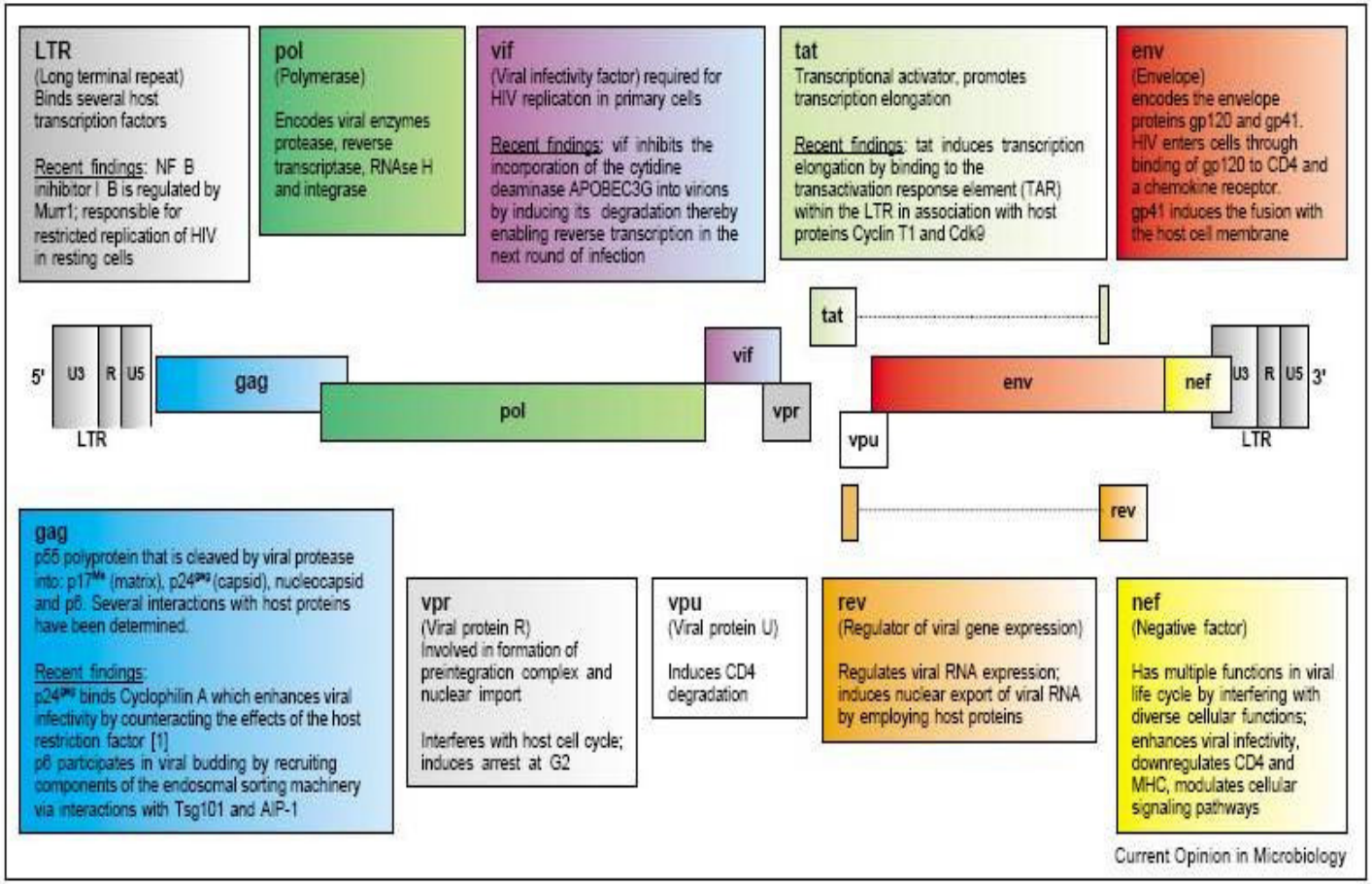

Figure 1.1. HIV-1 genome and most prominent interactions between viral and host proteins

(Source: Alexandra Trkola, 2004)

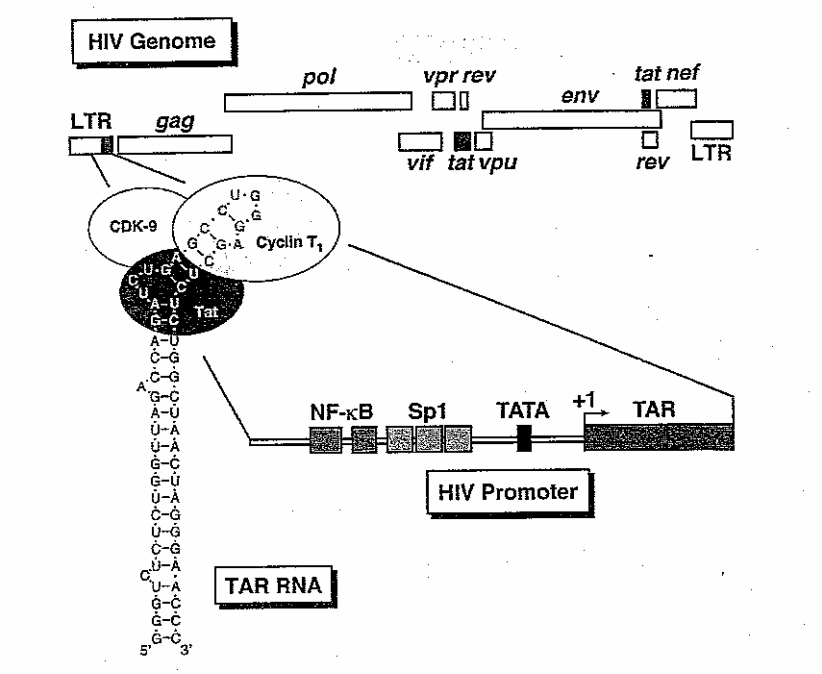

Figure 1.2. Model for Tat-LTR Interaction

(Source: Erik D. A. De Clercq, 2001)

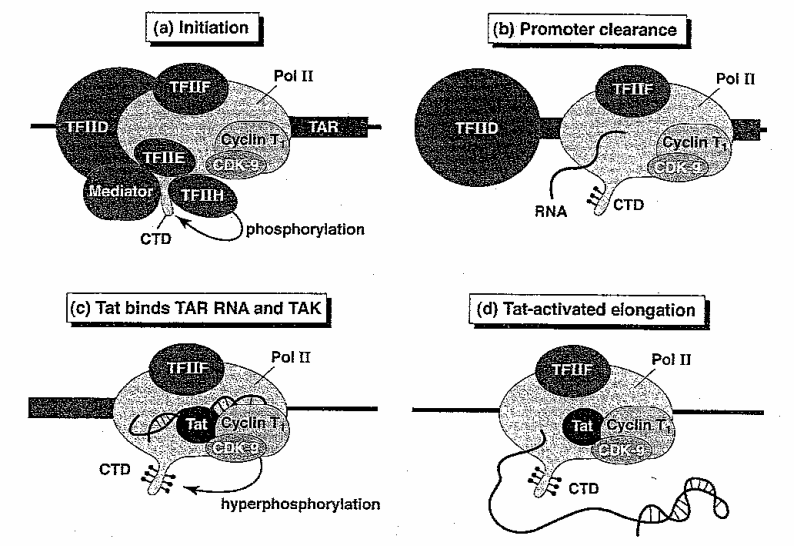

Figure 1.3. Model for the activation of RNA polymerase II by Tat and cellular cofactors (Source: Erik D. A. De Clercq, 2001)

1.5.2. Mechanism of Action of Rev

Retroviral replication requires utilization of incompletely and unspliced RNA and many retroviruses have evolved mechanisms to circumvent the requirement for splicing in nuclear export of RNA. In the case of the complex retrovirus human immunodeficiency virus type 1 (HIV-1), nuclear export of incompletely spliced mRNAs is mediated by the Rev/RRE system, where the viral encoded Rev-protein which is 19-kDa (Feinberg et al., 1986; Sodroski et al., 1986; Felber et al., 1989) binds in trans to a cis-acting sequence termed the Rev-responsive element (RRE) present on intron containing RNAs (Daly et al., 1989; Felber et al., 1989; Perkins et al., 1989; Zapp&Green, 1989; Cochrane et al., 1990; Heaphy et al., 1990; Malim et al., 1990; Olsen et al., 1990). Complete dependence on Rev for the export of RRE-containing RNAs has been demonstrated in several mammalian and amphibian cell lines (Emerman et al., 1989; Hammarskjold et al., 1989; Cochrane et al., 1991; Fischer et al., 1994; Malim et al., 1989).

Over 30 distinct mRNAs falling into three major classes are produced during HIV infection: doubly spliced 1.8 kb RNAs encoding the tat, rev and nef regulatory genes, singly spliced 4 kb RNAs for the vif, vpr, and vpu/env genes, and finally the unspliced 9 kb virion RNA which also acts as the mRNA for the gag/pol gene (Figure 1. 4) (Arrigo et al., 1990; Kim et al., 1989; Purcell&Martin, 1993; Schwartz et al., 1990). Ordered expression of these diverse populations of mRNAs is controlled by the viral regulatory protein Rev, which promotes the export of the 4 kb and 9 kb mRNAs from the nucleus (Malim et al., 1989).

Rev activity requires a 351 nucleotide RNA element called the Rev response element (RRE) which is located in the env gene and is thefore present on each of the HIV mRNAs encoding the viral structural and accessory proteins (Malim et al., 1989; Mann et al., 1994; Rosen et al., 1988). Initially, a monomer of Rev binds to a specific high-affinity site within the RRE (Bartel et al., 1991; Heaphy et al., 1991). Subsequently, up to nine further molecules bind to the RRE in a co-operative manner through protein-protein and protein-RNA interactions (Charpentier et al., 1997; Heaphy et al., 1991; Kjems et al., 1991; Malim&Cullen, 1991; Mann et al., 1994, Zemmel et al., 1996).

While fully spliced RNAs are readily exported from the nucleus, RNAs containing introns are retained in the nucleus by commitment factors, such as the U1 snRNP or SR proteins, until fully spliced or degraded. Rev induces the export of RRE-containing target RNAs and so that either prevents or reverses nuclear retention (Figure 1. 5). Capped, polyadenylated mRNAs are normally efficiently transported from the nucleus to the cytoplasm via channels in the nuclear membrane that are termed nuclear pores. However, if an RNA contains a recognizable intron, this induces an interaction with cellular splicing factors which have been termed commitment factors (Legrain&Rosbash, 1989). These commitment factors include the U1 small nuclear ribonucleoprotein (snRNP) particle as well as members of the serine-arginine-rich (SR) class of splicing factors. If the splice sites are recognized by the nuclear commitment machinery but are then found to be nonfunctional, the defective RNA can be degraded. Most importantly, recognition by commitment factors effectively blocks the nuclear export of the target RNA until the intron(s) present in the RNA are fully removed (Legrain&Rosbash, 1989; Chang&Sharp, 1989). The purpose of the cellular commitment machinery is therefore twofold. First, these factors function in the identification of introns and thus are essential for appropriate splicing. Second, these factors retain immature transcripts in the nucleus and thus prevent pre-mRNAs from encountering the cytoplasmic translational machinery of the cell. The advantage of this retaining process is that the cell is protected from the deleterious effect which could result from the generation of defective proteins which are the products of the translation of pre-mRNAs that include introns within the intended protein coding sequence.

To achieve a balance between the expression of the fully spliced, singly spliced, and unspliced mRNAs, HIV uses a variety of different mechanisms. Splicing rates are reduced by the virus through the use of sub-optimal splice acceptor sequences (Chang&Sharp, 1989; Dyhr-Mikkelsen&Kjems, 1995; McNally&Beemon, 1992; O'Reilly et al., 1995). In addition, at least two types of cis-acting RNA signals in addition to the RRE are required for Rev-dependent export of unspliced mRNA to the cytoplasm. First, splicing rates are reduced by cis-acting inhibitory sequences located adjacent to the splice acceptor sequences of the first and second exons of the tat and rev genes (Amendt et al., 1994; Barksdale&Baker, 1995; Staffa&Cochrane, 1995).

Several studies suggest the presence of additional sequences within the coding regions of the HIV-1 env and gag genes which may participate in regulating HIV gene expression, either independently or in connection with the rev/RRE.

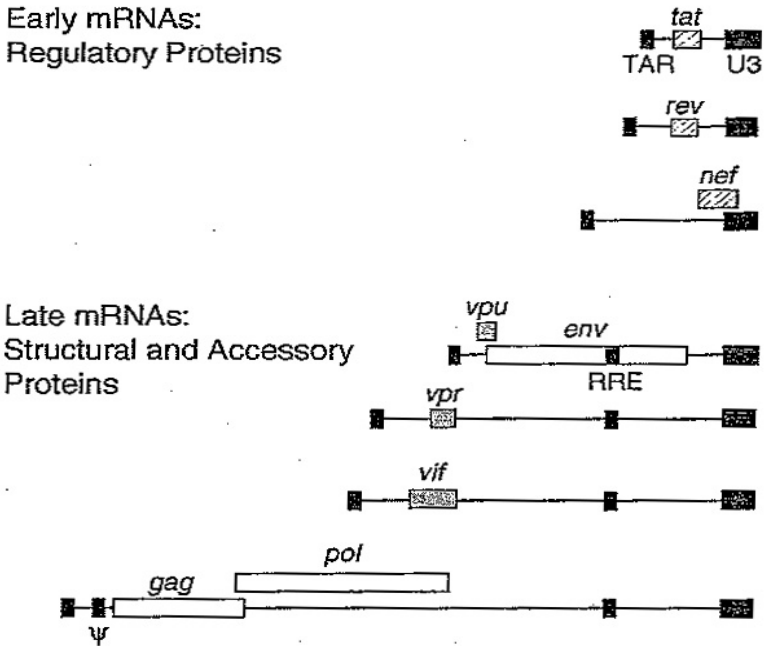

Figure 1.4. HIV-1 viral mRNAs

(Source: Erik D. A. De Clercq, 2001)

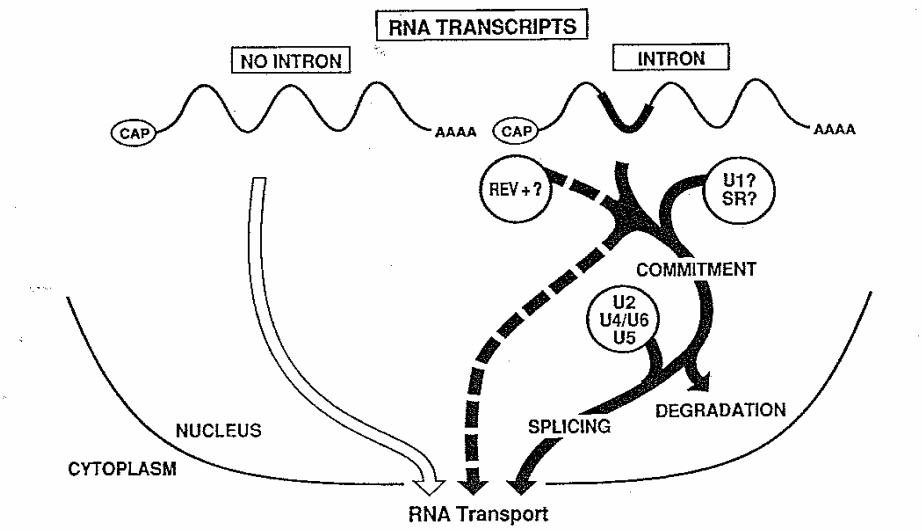

Figure 1.5. Nuclear commitment machinery and splicing

(Source: Bryan R. Cullen, 1998)

The presence of cis-acting repressor sequences (CRS) located within the env gene upstream of the RRE was firstly identified by Rosen et al. using chimeric chloramphenicol acetlytransferase (CAT)-env reporter plasmids (Rosen et al., 1988). The inhibitory effect of CRS sequences was detected in the absence of rev and did not need the splice sites. These inhibitory regions have not been analyzed independently of the RRE. Observations by Hadzopoulou-Cladaras et al. (1989) and Dayton et al. (1988) have shown that expression of the gag gene requires the presence of the RRE in cis and the rev gene product in trans and suggest the presence of cis-acting sequences in the HIV-1 gag gene.

In the study of Maldarelli et al., two regions, inhibitory region 1 (IR-1) and IR-2, which inhibited expression of reporter genes in two independent test systems were identified in the gag and pol genes, respectively (Maldarelli et al., 1991). The inhibitory effects were orientation dependent and functioned only when they were part of the transcriptional unit. Analysis of the transcripts revealed that IR-containing RNA was synthesized but accumulated in the nucleus of transfected cells. One possible role for these elements is to ensure that unspliced pre-mRNAs are not degraded in the nucleus prior to export to the cytoplasm by sequestering the RNA in nuclear sub-compartments that are inaccessible to the splicing machinery (Berthold&Maldarelli, 1996; Chang&Sharp, 1989; Mikaėlian et al., 1996).

It is interesting that nine copies of the sequence AUUUA exist within the gag-pol region of HIV-1. Sequence analysis has shown that all the lentiviruses studied up to now contain AU-rich regions suggesting that these viruses may have been regulated by the same mechanism (Wagner et al., 1991).

Instability is also important for other types of mRNAs encoding products involved in growth control, such as c-fos, c-myc, lymphokines and cytokines. Histone and transferrin receptor mRNAs are regulated by mRNA stability too.

Though Rev is found to localize predominantly in the nucleolus of transfected cells, it can shuttle between the cytoplasm and the nucleus (Meyer&Malim, 1994). Rev accomplishes this task through the action of various functional domains: 1) a nuclear localization signal (NLS) that directs Rev import into the nucleus (Cochrane et al., 1990; Malim et al., 1989; Perkins et al., 1989; Hope et al., 1990) 2) a RNA binding region that interacts with its target RNA in the nucleus (Cochrane et al., 1990; Hadzopoulou-Cladaras et al., 1989; Heaphy et al., 1990; Malim et al., 1989; Rosen et al., 1988; Zapp&Green, 1989), and 3) a nuclear export signal (NES) that targets the

Rev-RRE complex to the cytoplasm (Fischer et al., 1995; Meyer et al., 1996). The presence of both NLS and NES permits Rev to shuttle between the nucleus and cytoplasm of a cell, but it accumulates mostly in the nucleus and nucleolus (Cochrane et al., 1990; Malim et al., 1989; Perkins et al., 1989; Hope et al., 1990). (Figure 1.6).

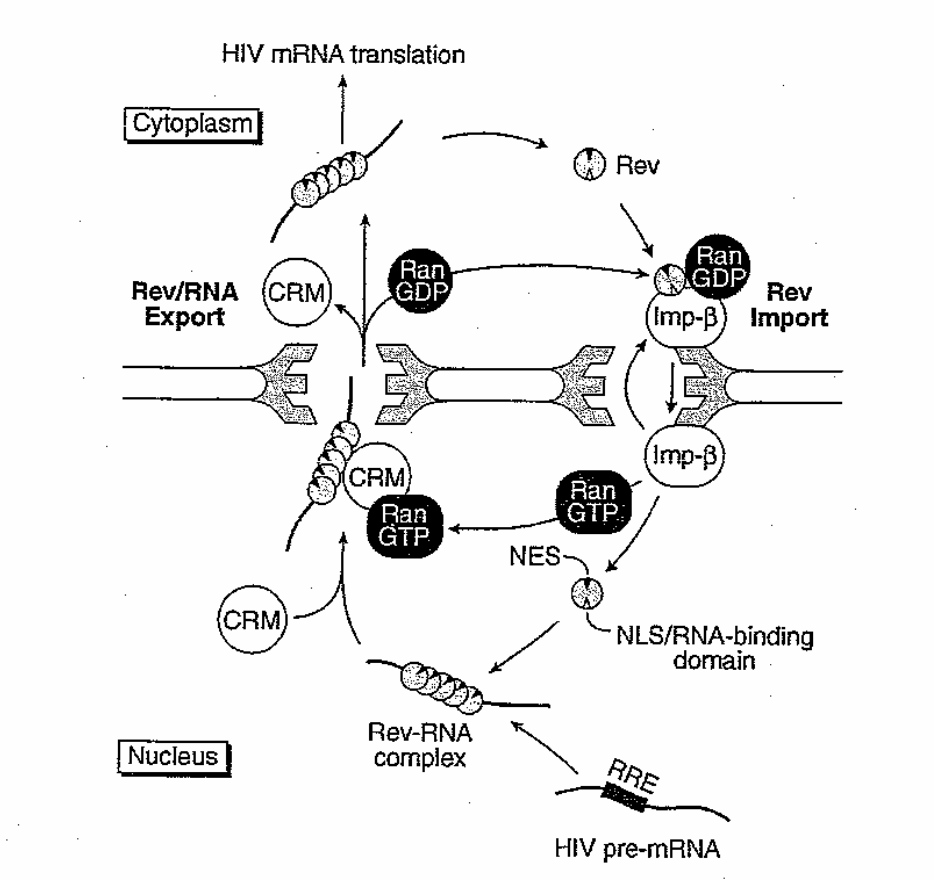

Figure 1.6. The Rev shuttling cycle

(Source: Erik D. A. De Clercq, 2001)

1.6. The Potential Applications of Suicide Gene Therapy

1.6.1. General Features of Suicide Genes

Suicide genes encode for enzymes that can convert a relatively nontoxic prodrug into a highly toxic agent. The ideal suicide gene must be:
1) non-immunogenic 2) have low basal toxicity 3) allow for long-term stable expression in transduced cells 4) its activation should result in the elimination of all transduced cells

Suicide genes may be based on endogenous pro-apoptotic molecules, such as Fas, caspases and human CD20. Inducible Fas is based on a self-protein and is minimally immunogenic (Chen et al., 1996). Its inducer AP1903 does not seem to have any toxicity (Morley et al., 2001). Activation of inducible Fas leads to apoptosis in the majority of transduced cells, but not in complete elimination. Instead of inducible Fas, using inducible caspase mıolecules may be better since caspases are downstream of Fas in the apoptosis-signaling cascade and are far from many anti-apoptotic molecules such as c-FLIP and Bcl-2. Also, human CD20 has been proposed as a non-immunogenic suicide gene. Exposure to a monoclonal chimeric anti-CD20 Ab in the presence of complement results in rapid killing of up to 90% of transduced cells (Bambacioni et al., 2000). Although this result is promising, complete elimination of transduced cells has not been achieved.

Besides with the endogenous suicide genes told above, several metabolic suicide genes have been described including the *Herpes simplex* virus type-1 thymidine kinase (HSV-1 TK) (Moolten, 1986), the *Escherichia coli* cytosine deaminase (Austin&Huber, 1993), and the *Varicella zoster* thymidine kinase genes (Averett et al., 1991). From these metabolic suicide genes, the two, *Escherichia coli* cytosine deaminase and *Herpes simplex* virus type-1 thymidine kinase (HSV-1 TK) have obtained much attention.

Cytosine deaminase (CD) is found primarily in yeasts and bacteria and its expression results with the deamination of cytosine to form uracil (Neuhard et al., 1992). Mammalian cells lack CD and therefore can not convert cytosine to uracil. However, in the transduced cells expressing cytosine deaminase, the relatively non-toxic pyrimidine derivative 5-fluorocytosine (5-FC, 5-Cyt, flucytosine) is converted to

the highly potent chemotherapeutic agent 5-fluorouracil (5-FU, 5-Fura) (Scheiner et al., 1957) which is used in the treatment of cancers (Weckbecker, 1991). Therefore, CD has been used as a suicide gene in animal (Sakai et al., 1985) and human (Cosand et al., 1994) cancer therapy models. 5-FU itself is also a prodrug, which must be converted to cytotoxic fluoronucleotides such as FUTP and FdUMP (Diasio et al., 1990). Gaining an insight about the potential suicide genes is important in terms of developing alternative models and/or replacing the inappropriate ones with the new ones. Debus et al., examined the efficacies of the suicide genes CD and TK alone and in combination in vitro (Dunning prostate adenocarcinoma cell line, R3327 AT-1, which was retrovirally transfected with a suicide gene) and in vivo (via injection to rats) (Debus et al., 2003). According to their experimental system, although the application of each single enzyme/prodrug mechanism resulted in an effective in vitro killing of tumor cells, only the combination of both systems allowed a reliable elimination of tumors in vivo.

While searching for the appropriate suicide gene, it is also important to make a selection according to the purpose. For example, the diphteria toxin, which kills cells at an extremely low concentration (Neville&Hudson, 1986) by enzymatically inhibiting a single cellular target-elongation factor 2 (EF2) to prevent protein synthesis (Bodley et al., 1980) may be a better choice in several situations. However, it does not necessarily mean that the selected suicide gene serves to the desired purpose. For example, Caruso&Klatzmann tested the effect of this toxin under the control of the HIV promoter (Caruso&Klatzmann, 1991). However, viable transformants were not be able to be generated under the control of the HIV promoter.

1.6.2. The HSV-TK/GCV System and Utilization of This System in Several Disease Models

HSV-1 thymidine kinase expression is not deleterious to mammalian cells and it can, unlike mammalian thymidine kinase, selectively phosphorylate certain nucleoside analogs to the monophosphate form(Shaeffer et al., 1977; Grill et al., 1983); subsequently, the nucleoside monophosphate is converted by cellular phosphorylases to its triphosphate form, whose incorporation into DNA by the cellular DNA polymerase α leads to inhibition of cellular DNA synthesis (Elion et al., 1980; Huang et al., 1985) and eventually cell death, a process that has been termed ‘Thymidine Kinase

Obliteration' (TKO) (Evans et al., 1988-1989). The suicide strategy using the HSV-TK gene was originally developed for use in the treatment of cancer (Moolten, 1986). Cancer cells were killed both by the direct cytotoxicity of GCV (ganciclovir)-triphosphate and as a result of the bystander effect in which GCV-triphosphate was transferred from transduced cells to untransduced cells. In the TK/GCV system the bystander effect is caused by the transport of the phosphorylated form of ganciclovir via gap junctions into adjacent cells (Stambrook et al., 1993). In vivo, systemic immune responses may also be effective in tumor regression after HSV-TK and GCV treatment (Barba et al., 1994; Caruso, 1996).

Transducing cells with the *Herpes simplex* virus thymidine kinase (HSV-TK) gene to confer sensitivity to the prodrug (ganciclovir or acylovir) is a well-known strategy and has also been exploited in clinical trials (Moolten, 1986; Bonini et al., 1997). Cell death mediated by the HSV-TK/GCV system can occur via different pathways, depending on the cell type. While apoptosis has been shown to be the cause of death in human colon cancer cells (Freeman, 1993; Vite et al., 1998) and in human and rat glioma cells (Colombo, 1995; Mervelo&Samejima, 1995), nonapoptotic death was in question in TK-expressing hepatocellular carcinoma cells (Tsukamoto&Kaneko, 1995) and in B16 melanoma cells (Vite et al., 1998; Chong et al., 1997). This system prooved its efficiency in various cancer models. For example, in a model of mouse prostate cancer using the RM-1 cell line, injection of an adenovirus (Ad) expressing HSV-TK into a primary tumor followed by GCV therapy not only inhibited local tumor growth but also supressed spontaneous metastatic activity (Thompson et al., 1997). On the other hand, it is important to emphasize that the importance of cell type must never be neglected since while one model works well for a specific cell type, it may not necessarily be resulted with the same effect for a different cell type. According to the investigations carried out up to now, the sensitivity towards GCV varied in different tumor cell lines. In this context, the direct measurement of the enzyme activity in terms of phosphorylating capacity may be a more sensitive indicator of therapeutic efficacy than the determination of the protein content of the enzyme.

Many cytokines (IL-12, IL-4, IL-6, IL-7, INF-δ, TNF-α, and GM-CSF) have been employed for stimulation of immune responses against different tumors (Salvadori et al., 1996; Colombo&Forni, 1994). So, immonogene therapy using cytokines has emerged as a new strategy in the treatment of cancer. Of all the cytokines tested, IL-12 possessed the strongest antitumor activity. Thus, the bystander effect can be further

increased by co-transfer of suicide genes and cytokine genes. Chen et al., demonstrated this synergism in the treatment of colon carcinoma cell lines CC36 and MCA-26. They showed that combined administration of interleukin-2, granulocyte macrophage colony-stimulating factor and HSV-TK was superior to single agent therapy (Chen et al., 1995-1996).

Though the acyclovir can be employed as well, as a substrate for HSV-TK, the ganciclovir seems to be a better choice. In the H9 and CEM T-lymphocyte cell lines tested, it was observed that GCV possessed low cytotoxicity and potent inhibitory properties for an efficient HSV-1 TK gene therapy. For ACV (acyclovir), HSV-1 TK dependence was also observed but ACV was needed at much higher concentrations and longer treatment durations (Drake et al., 1997; Balzarini et al., 1993; Kurtyama et al., 1996).

Besides with its usage as a gene therapy to selectively kill HSV-1 TK-expressing cells among a population of dividing tumor cells, it has also used to study the lineage formation in cultured cells and transgenic animals (Borrelli et al., 1988) and to achieve conditional ablation of targeted cell types in transgenic mice (Heyman et al., 1989). This system has also been utilized to develop novel strategies for controlling parasitic infections. Papadopoulou et al., showed that *Leishmania spp. amastigotes* expressing TK were specifically eliminated by 85% within macrophages when treated with ganciclovir (Papadopoulou et al., 1997).

1.6.3. The HSV-TK/GCV System and Utilization of This System in HIV Infection

Gene therapy as a possible treatment of HIV infection has been investigated in tissue culture experiments (Yu et al., 1994) and in patients (Woffendin et al., 1996). The use of HSV-TK as a therapeutical gene for gene therapy of HIV infection has been proposed by many researches (Venkatesh et al., 1990; Caruso&Klatzmann, 1992; Brady et al., 1994; Caruso et al., 1995).

The characteristics of HIV replication make it suitable for obtaining a rapid HIV-dependent suicide gene of infected cells. First, the HIV promoter is inefficient in the absence of the HIV-encoded regulatory protein Tat (Arya et al., 1985; Cullen, 1990). Second, there is a temporal program of mRNA accumulation during the HIV

replication cycle. In the initial phase, multiply spliced mRNAs encoding HIV regulatory proteins are produced while the mRNAs encoding HIV structural proteins are not significantly expressed until 24 hour after viral infection, at which time virus production becomes apparent (Baltimore et al., 1989). Because of this reason, a toxic gene placed under the control of the HIV regulatory sequences is anticipated to be expressed at a basal low level in noninfected cells and would be switched on in the initial phase of HIV replication, before HIV structural protein synthesis is initiated. This should result in cell death before the release of newly synthesized viral particles and so that prevent viral spread. Such an approach where the conditionally lethal gene was placed under the control sequences of the virus to link the expression of the toxic gene dependent upon the viral replication is generally called virally directed enzyme prodrug therapy (VDEPT) and aims at killing the cells upon infection of the virus, limiting both the virus produced and the generation of latently infected cells (Mullen, 1994; Connors, 1995).

When the problems arose from chemotherapy and the limitations in the development of an effective vaccine for preventing HIV infection are considered, this strategy is quite promising since it makes more sense to simply sacrifice HIV-infected cells instead of trying to interfere with viral replication.

1.7. Aim of the Project

In this study, we aimed to design an efficient DNA plasmid vector to be tested for its selective killing of cells expressing HIV-1 regulatory genes. For this purpose, the first step was the construction of plasmid vectors carrying HIV-1 regulatory genes 'tat' and/or 'rev' since the transfection of these plasmid vectors into HeLa $CD4^+$ cells would generate tat and/or rev expressing HeLa cell lines. Then co-transfection of these cell lines with the suicide plasmid vector whose expression is dependent on the HIV-1 regulatory genes could result in the selective depletion of these cells. For the construction of the suicide plasmid vector, a conditionally lethal toxic gene *Herpes simplex* virus type 1 thymidine kinase (HSV-1 TK) gene was placed under the trancriptional control of the HIV-1 promoter LTR so that the expression of the toxic gene was rendered dependent upon the Tat protein of HIV-1. In order to impose additional constraints on TK expression, other cis-acting sequences INS and RRE

which are important for the action of Rev was cloned downstream of the TK coding sequences so that TK mRNA expressed in the presence of Tat would be retained in the nucleus and would require co-expression of Rev for transport to the cytoplasm since HIV-1 Rev protein was also important as a molecular chaperone to generate the transport of the unspliced transcripts into the cytoplasm where they could be translated. Intron containing RNA (TK and INS-RRE sequences flanked by splice sites in the pre-mRNA) will be synthesized but accumulate in the nucleus of transfected cells. Not only RRE but also INS element of the p17gag gene of HIV-1 are needed for Rev to stimulate the export of unspliced mRNA from the nucleus (Mikaélian et al., 1996). Therefore INS sequences are very important for Rev-regulated expression since the degradation of the unspliced pre-mRNAs prior to the export to the cytoplasm are also achieved by INS sequences (Berthold&Maldarelli, 1996; Chang&Sharp, 1989). Depending on the splice sites flanking the TK and INS-RRE sequences, in Rev-independent expression, these intervening regions will be removed. This model which was created to prevent the expression of the toxic protein in the absence of Rev is anticipated to solve a cumbersome handicap experienced by other researchers in the past. Previous studies have indicated that HIV LTR was not completely silent in the absence of Tat since host transcription factors interacting with specific sequences on the HIV LTR have been shown to provide a basal level of transcription (Westphal et al., 1988; Chinnadurai et al., 1990; Caroso&Klatzmann, 1991-1992; Dinges et al., 1995; Giaretta et al., 1998; Minchin et al., 2004). This resulted with a low but detectable level of TK expression in transduced but not induced cells. Because of this reason, we aimed to construct a more tightly controlled system where the targeted cells could be killed without giving any harm to uninduced cells. If this aim is accomplished, a suicide vector dependent upon the HIV-1 regulatory gene expression will have been constructed without using HIV infection.

It is important to emphasize that in case the desired aim of the project was accomplished, it can not directly be used in clinical trials. Gene therapy trials in the world are still in their infancy and currently, most gene therapy protocols are phase I clinical trials determining toxicity.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

A detailed list of commonly used buffers, solutions and their compositions are presented in Appendix A.

2.2. Methods

2.2.1. Bacteriological Techniques

A list of bacterial strains, plasmids and their sources were presented in Table 2.1. *E. coli* Dh5& strain was used for all the manipulations in the study. Bacteria were grown at 37 °C in LB medium with continuous agitation ranging between 180-200 rpm during 12-16 hours which refers to the definition of 'overnight' term (with the exception of approximately 45-60 min incubation period in order to let antibiotics to be expressed in transformation). When required, the antibiotics ampicillin and tetracyclin were used at final concentrations of 50 and 10 µg/ml, respectively.

For the selection of transformants, LB media containing 1.5% (w/v) agar and appropriate antibiotic were used.

Table 2.1**.** List of bacterial strains and plasmids

Strain	**Description**	**Reference**
E.coli Dh5&	F^- , Φ80 Δ lacZ Δ MIS, Δ (lacZYA- argF), U169	Woodcock, et al., 1989
Plasmid	**Decription**	**Reference**
pIRES	$P_{CMV\ IE}$, IVS, SV40 pA, IRES sequence, f1 ori, Neo^r, Amp^r	Clontech
pMEP4	Amp^r, Ori P, ColE1 ori, $P_{hmetIIa}$, SV40 pA, Hyg^r, EBNA-1	Invitrogen
pMEP-IRES	P_{hmetII} in pMEP4 episomal expression vector replaced by IRES, CMV enhancer, CMV promoter, and IVS fragments in pIRES	This study
pMEP-IRES-rev	HIV-1 rev cDNA cloned in the forward orientation in pMEP-IRES expression vector	This study
pMEP-IRES-tat	HIV-1 tat cDNA cloned in the forward orientation in pMEP-IRES expression vector	This study

(cont. on next page)

Table 2.1 (cont.)

pMEP-IRES-rev-tat	HIV-1 tat cDNA cloned in the forward orientation in pMEP-IRES-rev expression vector	This study
pCV1	mammalian expression vector containing hybrid regulatory sequences (1.5 kb of pBR322 sequences adjacent to 1.8 kb cDNA sequences which encode both Tat and Rev)	NIH
pNL4-3	Full-length, replication- and infection-competent chimeric molecular clone of HIV-1	NIH
pNL4-3-MunI	a re-ligated 9147 bp DNA sequence generated from MunI digestion of HIV-1 molecular clone pNL4-3	This study
pREP9	Amp^r, Ori P, ColE1 ori, P_{RSV} , SV40 pA, EBNA-1, Neo^r	Invitrogen
pREP-LTR	P_{RSV} in pREP9 replaced by 748 bp-length LTR and sd1 sequences in pNL4-3	This study
pREP-LTR-INS-RRE	2007 bp-length INS, RRE and sa7 sequences cloned downstream of LTR in pREP-LTR	This study

(cont. on next page)

Table 2.1 (cont.)

pREP-TK	TK (thymidine kinase) fragment derived from HSV (Herpes Simplex Virus) genomic DNA cloned downstream of P_{RSV} in pREP9	This study
pREP-LTR-INS-RRE-TK	1144 bp-length TK suicide gene cloned downstream of LTR in pREP-LTR-INS-RRE	This study
pREP-CAT	CAT (chloramphenicol acetyl transferase) fragment cloned downstream of P_{RSV} in pREP9	This study
pREP-LTR-INS-RRE-CAT	670 bp-length CAT gene cloned downstream of LTR in pREP-LTR-INS-RRE	This study
pCI-Neo	$P_{CMV\ IE}$, SV40 Late poly(A), f1 ori, SV40 Enhancer and Early Promoter, SV40 ori, neo, Amp^r	Promega
pcDNA 3.1 / HisB / lacZ	P_{CMV} , lacZ, Amp^r, ColE1 ori, SV40 ori, Neo^r	Invitrogen
pREP-LTR-INS-RRE-TK-lac	1471-bp length XbaI-XhoI fragment in pREP-LTR-INS-RRE-CAT-lac replaced by 1945-bp length XbaI-XhoI fragment in pREP-LTR-INS-RRE-TK	This study
pREP-LTR-INS-RRE-CAT-lac	3195-bp length lacZ gene cloned downstream of INS-RRE in pREP-LTR-INS-RRE-CAT reporter expression vector	This study

2.2.1.1. Maintenance of Bacterial Strains

E. coli Dh5& strains were streaked on LB agar supplemented with appropriate antibiotic if necessary and kept at 4 °C for short term storage. In case LB broth culture was needed for experimental procedures, a healthy-looking single colony was picked with a loop and inoculated into LB broth again supplemented with appropriate antibiotic if required. Inoculated bacteria were grown until log or mid-log phase at 37 °C with continuous agitation.

Long-term storage of manipulated strains were generated by transferring them into 1.5 ml vials containing 20% (v/v) glycerol in LB broth which then placed into -80 °C.

2.2.1.2. DNA Isolations

2.2.1.2.1. Small-scale Plasmid Isolation with Alkali-lysis Method

1.5 ml of culture obtained from 10 ml broth culture supplemented with appropriate antibiotic was transferred to an eppendorf tube and centrifuged for 1 min at maximum speed. Supernatant was discarded and this step was repeated once again since more cell volume was used at initial stage, much more plasmid DNA was obtained. After removing supernatant, the pellet was resuspended in 200 µl ice-cold Solution I with vigorous vortex. Glucose in solution I increases the osmotic pressure outside the cell, hence cells are destroyed. Released DNA is protected from degrading enzymes with the presence of EDTA which functions by binding divalent cations necessary for DNAse activity. At this lysis stage, pH is kept constant (8.0) because of Tris. Following treatment of cells with Solution I, 400 µl Solution II was added immediately. The tube was inverted gently for 5-6 times until a transparent view was observed. Because of the presence of NaOH in Solution II, constant pH is no longer continued at this stage. The pH of solution becomes approximately 12 at which value genomic DNA and proteins are denatured but supercoiled plasmid DNA is not affected. This is the crucial point of plasmid isolation method in which the name of the procedure refers to this fact. Thereafter, immediately 300 µl ice-cold Solution III was added and the tube was inverted gently for 5-6 times again. The tube containing

Solution I, Solution II, and Solution III was incubated on ice and solution mix became neutralized at this stage. DNA strands denatured at previous stage because of the effect of NaOH are renatured thanks to the presence of acetic acid in Solution III. Potassium acetate in Solution III precipitates both cell wastes and *E. coli* chromosomal DNA. After incubation period on ice, the tube was centrifuged for 5 min at 12000 rpm. Considering the fact mentioned above; following centrifugation, cell wastes and chromosomal DNA resided in the pellet, and the plasmid DNA in the supernatant. The supernatant was transferred to a new eppendorf tube and subjected to the purification steps.

2.2.1.2.2. Alternative Small-scale Plasmid Isolation Methods

During the study, plasmid isolation kits mentioned below were also employed.

– Macharey-Nagel NucleoSpin plasmid

– Roche High Pure Plasmid Isolation Kit

All the steps were performed according to the manufacturer's recommendations.

2.2.1.2.3. DNA Isolation from Agarose Gel with Applichem DNA Isolation Kit

After restriction digestion of plasmid DNA molecules with suitable enzymes and confirmation of the successful digestion with a small volume of digestion mixture (generally one-tenth) loaded into agarose gel, a new agarose gel ranging between 0.8 and 2% (w/v) agarose depending on the weight of molecules for a better seperation was prepared and the remainder of the digestion mixture was loaded into the wells and molecules were let to be seperated at 80 V current. DNA bands that would be used in the further construction steps were excised with a sterile surgical blade and placed into an eppendorf tube. 3 volumes gel weight of 6 M NaI solution was added to the tube and the tube was incubated for 10-15 min at 55 °C by taking the tube from water bath every 2 or 3 min and vortexing vigorously. When it was clearly visible that the gel slice dissolved, 6-8 µl glassmilk solution was added to the sample and vortexed vigorously. The sample was incubated for 5 min at room temperature by vortexing every 1 or 2 min. For fragments less than 1000-bp length, incubation was carried out at 55 °C in

order to improve binding efficiency. Then, the sample was centifuged for 1 min at maximum speed. Supernatant was discarded and the pellet was washed 2 times with wash solution provided with kit. After washing steps, the pellet was dissolved in the desired volume of sterile dH_2O and incubated for 10 min at 55 °C for the elution of DNA. A final centrifugation step for 2 min at maximum speed resulted with DNA in supernatant. By taking care of not to touch the pellet with pipette tip, the supernatant was transferred into a new eppendorf tube.

2.2.1.2.4. Large-scale Plasmid Isolation

For this purpose, two commercial kits were used according to the manufacturer's recommendations.

─ Macharey-Nagel NucleoBond Plasmid DNA Purification Kit:

This kit was used to obtain high volume recombinant DNA molecules after the confirmation of the cloning. After the required cloning analysis was generated with the presumed recombinant DNA molecules tested, the one that could be called the correct clone was transformed to competent *E. coli* cells, a fresh colony was inoculated in 100 ml LB broth supplemented with the ampicillin, and high-volume plasmid isolation which would be the starting material for the following cloning series was obtained.

─ Roche Genopure Plasmid Midi Kit: DNAs to be used in eukaryotic cell transfection experiments were generated with this kit.

2.2.1.3. Bacterial Transformations

2.2.1.3.1. Preperation of Chemically Competent *E. coli*

E. coli Dh5& strain was used to prepare competent cells with $CaCl_2$ treatment. A single colony was picked and inoculated into 100 ml LB broth and grown overnight at 180 rpm at 37 °C. The next day, in order to allow the culture reach the mid-log phase, 1 ml overnight culture was transferred into 100 ml fresh LB broth (1:100 dilution) and incubation was continued for approximately 3 hours at 37 °C with continuous agitation. Following this incubation step, 100 ml culture was transferred into 2 sterile, ice-cold 50 ml falcon tubes each with the volume of 40 ml culture. Falcon

tubes were incubated on ice for 20 min to cool the culture. Cells were pelleted by centrifugation at 5000 rpm for 10 min at 4 °C. After discarding supernatant completely, the cell pellet was resuspended in 8 ml of ice-cold 100 mM $CaCl_2$ and centrifuged at 5000 rpm for 10 min at 4 °C. Supernatant was discarded and the pellet was treated with 8 ml of ice-cold 100 mM $CaCl_2$ again, and centrifuged at the same conditions told above. Supernatant was removed again and finally the cells were resuspended in 1600 µl ice-cold 100 mM $CaCl_2$ (1600 µl for the cells in one falcon tube) very gently. Then, this final suspension volume was divided into aliquots. Sterile eppendorf tubes were already kept on ice. 160 µl of this $CaCl_2$ treated cell suspension was transferred into one eppendorf tube, then 40 µl sterile glycerol was added and mixed gently.

The prepared competent cells were either used immediately for transformation or stored at -80 °C for later use.

2.2.1.3.2. The Uptake of Plasmid DNA by Competent *E. coli* Cells

Frozen competent *E. coli* cells (200 µl) were thawed on ice. The DNA of interest (0.1-1 µg DNA in 1-10 µl DNA suspension) was added to the cells and incubated on ice for 20 min. The cells were then heat shocked for 90 sec at 42 °C followed by incubation on ice for 1-2 min (no more than 2 min). At this stage, the cells have recently taken up a plasmid. 500 µl LB broth was added to the culture followed by incubation at 37 °C for 45-60 min with continuous agitation. During this short incubation period in LB broth in the absence of antibiotic, plasmid replication and expression would be underway. Therefore, at the later step which was the plating of transformed competent cells on LB agar plates containing appropriate antibiotic followed by overnight incubation at 37 °C, when plated cells encountered the antibiotic, they would already have synthesized sufficient amount of antibiotic resistance enzymes to be able to survive.

2.2.1.3.3. Transformation with TransformAid Bacterial Transformation Kit

The kit was used according to the manufacturer's recommendations for a few trials.

2.2.2. Cell Culture and Transfection Techniques

2.2.2.1. Mammalian Cells

HeLa (Human cervical epitheloid carcinoma) (Scherer et al., 1953) cells were used in the study. HeLa cells were preferred because of the advantages such as rapid growth, high plating efficiency and transfection efficiency they offer.

2.2.2.2. Maintenance and Passage of HeLa Cell Lines

HeLa cell lines were maintained in T25 flasks containing 5 ml of medium (35 mm, 60 mm and 94 mm tissue culture dishes were also used in the study with growth volumes of 2, 4 and 10 ml respectively). HeLa cells were grown in DMEM with 10% (v/v) FBS at 37 °C in a 5%CO_2/95% air humidified incubator. The media of transfected HeLa cells were supplemented with 250µg/ml hygromycin.

Since allowing monolayer cultures to become overconfluent would result with the deterioration of cells, examining the confluency state of cells with an inverted microscobe was a daily practice. HeLa cells were passaged at a ratio of 1:3 or 1:4 every 2-3 days at confluence. Firstly, the medium was removed completely and the cells were washed with Versene solution. The cells were then treated with the proteolytic enzyme trypsin (0.25% w/v) which works by digesting the proteins in the cell membrane that anchor the cell to the surface of tissue culture vessel. Trypsin was washed around the cells gently and trypsinization time was tried to be kept at minimum since trypsin would also digest other cell membrane proteins. Suspended cells were transferred to falcon tubes and serum was added at half volume of the trypsin used to inhibit further action of trypsin on the cells. Following 2 min centrifugation step at 1500 rpm, supernatant was removed and cell pellet was gently recovered in fresh DMEM-10% FBS. Resulting cell suspension was then transferred to the fresh tissue culture vessels at the desired dilution rates.

2.2.2.3. Freezing and Recovery of Cell Stocks

For long term storage, HeLa cells which were just confluent were removed from the surface they anchor by trypsinization and centrifuged to form pellet. Then, the cell pellet was resuspended in 1 ml of freezing mixture (90% serum, 10% DMSO). After gentle mixing, the cells were transferred to 2 ml cryotubes. In order to protect the cells from deleterious effect, freezing was done slowly at -80 °C by wrapping the cryotubes with a thick layer of tissue paper.

When required, cryotubes were thawed in a 37 °C water bath and the cells were directly transferred to a tissue culture dish containing appropriate amount of growth medium. Cells were left undisturbed for 24 hours.

2.2.2.4. Mammalian Cell Transfections

In order to transfect our plasmids of interest into HeLa cells, X-tremeGENE Q2 Transfection Reagent was used according to the manufacturer's instructions.

HeLa cells when they were approximately at 50% confluency were transfected with the plasmid vectors and placed under the appropriate antibiotic selection 2 days after transfection until clones appeared. Untransfected HeLa cells and untransfected but exposed to antibiotic selection HeLa cells served as control groups.

2.2.2.5. Preparation of Genomic DNA from Mammalian Cells

Nucleospin Genomic DNA from Tissue Kit was used for this purpose according to the manufacturer's instructions.

2.2.2.6. Preparation of RNA from Mammalian Cells

Promega SV Total RNA Isolation System was used according to the manufacturer's instructions.

Because of the relatively unstable nature of RNA, RNase-free equipment was used while handling RNA. Electrophoresis tanks, water and other solutions used was treated with DEPC which is a strong inhibitor of RNases. Integrity of isolated RNAs

was verified with agarose gel electrophoresis before employed for RT-PCR experiments.

2.2.2.7. RT-PCR

In order to perform PCR using RNA as a template, RNA must be reverse transcribed into cDNA in a reverse transcription reaction. In this study, 2-step RT-PCR (RT and PCR were carried out seperately) was employed. Fermentas RevertAid™ M-MuLV Reverse Transcriptase kit was used for RT reaction.

Template RNA (0.1-5 µg), oligo$(dT)_{18}$ (0.5 µg) was put into a sterile tube and the volume was completed to 11 µl with DEPC-treated water. This mixture was incubated at 70 ºC for 5 min and chilled on ice. Then, 4 µl 5X reaction buffer, 2 µl 10 mM dNTP mix (final concentration: 1 mM), 1 µl Ribonuclease inhibitor (20 U) were added in the order given and finally 1 µl DEPC-treated water was added to adjust the volume to 19 µl. After the mixture was incubated at 37 ºC for 5 min, 200 units of RevertAid™ M-MuLV Reverse Transcriptase (1 µl) was added into the mixture and then incubated at 42 ºC for 60 min. The reaction was stopped by heating at 70 ºC for 10 min.

The generated cDNA was used as template for PCR reaction carried out with sequence-specific primers which consisted of 30 or 40 cycles, beginning with the initial denaturation step at 94 ºC for 3 min, followed by consisting a 1 min denaturation step at 94 ºC, a 1 min annealing at 60 ºC, and a 1 min extension at 72 ºC. A 10 min primer extension at 72 ºC completed the sequence.

2.2.3. DNA Manipulation Techniques

2.2.3.1. Digestion of DNA with Restriction Enzymes

The restriction enzymes used in the study were purchased from Fermentas and the digestions were carried out with the concentrated buffers supplied with the enzymes according to the manufacturer's recommendations.

2.2.3.2. End-fill Reactions

The achievement of the cloning by blunt end ligation necessitates the filling of protruding ends not compatible with each other. For this purpose, T4 DNA Polymerase (Fermentas) was used with all four dNTPs according to the manufacturer's recommendations.

2.2.3.3. Dephosphorylation of DNA

In order to prevent self-ligation of vector termini and thus to facilitate the cloning experiments, phosphate groups at the 5' termini of vector DNAs were removed by CIAP (Calf Intestinal Alkaline Phosphatase) (Fermentas)

5 µl CIAP buffer (supplied with the enzyme), 14 µl dH_2O and 1 U CIAP was added to 30 µl of reaction mixture containing 1.5 µg excised DNA. Dephosphorylation reaction was carried out at 37 °C for 30 min followed by the inactivation of the CIAP at 85 °C for 15 min. DNA was then purified with Applichem DNA purification kit.

2.2.3.4. DNA Ligation Reactions

In order to join double strand DNA molecules that either have blunt ends or compatible cohesive ends, T4 DNA ligase (Fermentas) was used according to the manufacturer's recommendations with some modifications.

Excluding the minority of reactions carried out at room temperature, all reactions were carried out at 4 °C for overnight.

2.2.4. Separation and Purification of DNA Fragments

2.2.4.1. Agarose Gel Electrophoresis

Agarose gels (0.8-1.5% (w/v)) were prepared by adding the required amount of agarose for resolution of linear DNA fragments into 1× TAE electrophoresis buffer and melting the heterogenous mixture in a microwave oven until a transparent view was

observed. After cooling the melted solution, EtBr was added at the concentration of 0.5 µg/ml from a 10 mg/ml stock and it was poured into a horizontal gel apparatus and gel combs were placed and the gel was allowed to harden. Upon removing of the comb from the hardened gel, the gel was placed into an electrophoresis tank containing sufficient electrophoresis buffer overlaying the gel. DNA samples prepared with 6× gel loading buffer were loaded into the wells of the gel and they were exposed to an electric constant at 80V for the movement of the DNA molecules. The movement of the DNA molecules could be observed with bromophenol blue present in the gel loading dye and thus the power supply was turned of when bromophenol blue was exceeding half of the gel. Finally, DNA fragments were visualized in UV transilluminator.

2.2.4.2. Phenol-Chloroform Extraction

For the sake of simplicity, DNA volume to be purified was completed to 200 µl with sterile dH_2O and 2 volumes of phenol (400 µl) was added, followed by vortexing and centrifugation at 10000 rpm for 3 min. Leaving yellow phenol phase at the bottom, the upper phase was transferred into a new 1.5 ml eppendorf tube and equal volumes of phenol (200 µl) and chloroform (200 µl) were added followed by vigorous vortexing and centrifugation at 10000 rpm for 3 min. Phenol-chloroform phase remained at the bottom and the upper phase was transferred into a new tube and treated with 400 µl chloroform followed again by vortexing and centifugation at 10000 rpm for 3 min. Upon transfer of the upper phase into a new tube, 1/10 volume of the sample (20 µl) 3 M Na-Acetate (pH 5.2) was added and mixed thoroughly. 2.5 volumes of the sample (500 µl) cold 99% (v/v) EtOH was added and mixed well followed by centrifugation at 10000 rpm for 15 min. Liquid phase was discarded completely and DNA pellet was washed with 2.5 volumes of the sample (500 µl) 70% (v/v) ethanol, followed by centrifugation at 10000 rpm for 3 min. This washing step was repeated once more to make DNA sample of interest completely free of any remaining contaminants. Then, liquid phase was discarded and DNA pellet was dried at room temperature. Finally, pellet was dissolved in the desired volume of dH_2O.

2.2.4.3. Alternative DNA Purification Methods

In the study, some commercial kits mentioned below were taken advantage as well for the purification of DNA samples. All these purification kits were used according to the manufacturer's recommendations though some minor modifications were made whenever they were necessary.

Applichem DNA Isolation Kit

Macherey-Nagel NucleoSpin Extract 2 in 1

Roche High Pure PCR Product Purification Kit

2.2.5. Quantification of DNA Samples

2.2.5.1. Quantification of DNA Spectrophotometrically

Since the heterocyclic cycles of nucleotides have the maximum suction of light at 260 nm wavelength, the suction degree at this wavelength is a measure of the amount of the nucleic acids. According to this, the amount of DNA can be determined by the value at 260 nm where 1 OD (optical density) corresponds to 50 µg/ml of double stranded DNA and 20 µg/ml of single stranded DNA oligonucleotide. So that, the amount of DNA in a nucleic acid solution can be measured with the equation below:

DNA (µg/ml)= OD at 260 nm × dilution ratio × coefficient (50 for dsDNA)

The measurement of absorbance at 280 nm was also important in terms of assessing the purity of DNA since the value of 260/280 was informative about the purity of nucleic acids. This value corresponds to 1.8 for very pure DNA samples, nevertheless the ratio not lower than 1.5 can be tolerated. The presence of phenol or proteins in the solution loweres the value and when lower values were obtained, purifications were found convenient to be repeated.

2.2.5.2. Quantification of DNA with Molecular Size Markers

With the aim of having a general idea about the amounts of DNA samples that were not needed to be determined very sensitively, molecular size markers can also be employed. Though exceptions are present, with most of these DNA size markers the

information given about one or two bands emphasizing how much DNA is present in this size was exploited to compare the size of this band with the sizes of interest to be used in cloning experiments whether as the vector or the insert and the proportional relationship between that size of the marker in which DNA amount was measured and our sizes of interests was informative for having an idea of the amounts of our DNA samples.

2.2.6. Concentration of DNA Samples

2.2.6.1. Concentration of DNA with Ethanol Precipitation

To concentrate DNA samples, DNA was precipitated by adding 1/10 volume of 3 M NaAc (pH 5.2) and 2.5 volume of cold 99% (v/v) ethanol (at -20°C), followed by incubation at -80 °C for 30 min. Then, the mixture was centrifuged for 10 min at 10000 rpm and supernatant was discarded. The pellet was air-dried and dissolved in the desired volume of dH_2O.

2.2.6.2. Concentration of DNA with DNA SpeedVac System

The previous method for the concentration of DNA with ethanol was replaced with DNA SpeedVac System at later stages.

2.2.7. Polymerase Chain Reaction (PCR)

2.2.7.1. Primers

The sequences of all the primers and oligonucleotides used in the study were presented in Appendix B.

2.2.7.2. PCR Amplifications

By using the appropriate primer pairs, target DNA sequences were amplified for 25-40 cycles (25 cycles for plasmid DNA templates and 40 cycles for genomic DNA templates), beginning with a pre-denaturation step at 94 °C for 3 min, followed by consisting a 1 min denaturation step at 94 °C, a 1 min annealing at 60 °C (modified as 2 min annealing at 55 °C for genomic DNA templates), and a 3 min extension at 72 °C. A 10 min primer extension at 72 °C completed the sequence.

PCR reactions were carried out in 50 µl volume of PCR mixture (Appendix A) One tenth of the PCR products was loaded in 1% (w/v) agarose gel with 0.5 µg/ml EtBr and subjected to electrophoresis for the analysis of the PCR reaction. After confirmation, PCR products were purified for later use in the cloning experiments.

2.2.7.3. Colony PCR

Bacterial cells were employed as templates and one bacterial colony was inoculated directly in 50 µl of PCR mixture (Appendix A) with a sterile toothpick. PCR reaction conditions were as explained in chapter 2. 2. 7. 2.

2.2.8. Analysis of Proteins

2.2.8.1. Preparation of Cell Lysates for Western Blotting

Trypsinized cell monolayers were pelleted by centrifugation at 1500 rpm for 2 min and upon removing of the aqueous solution containing trypsin and serum, the cells were washed with ice-cold CMF-PBS and lysed by the addition of 300 µl RIPA buffer (Appendix A). Immediately, the protease inhibitor PMSF was added at the concentration of 2 mM from a 100 mM/ml stock concentration in isopropanol to prevent the degradation of proteins in the lysate. The lysed cells in RIPA-PMSF mix were immediately transferred to a 1.5 ml microcentrifuge tube and incubated for 5 min on ice, followed by vigorous vortexing and centifugation at maximum speed for 10 min. The supernatant was transferred to a fresh tube and stored at -20 °C.

2.2.8.2. Seperation of Proteins by SDS Polyacrylamide Gel Electrophoresis

To enable the discrimination of the searched proteins, the appropriate percents of stacking and seperating gels were prepared in a Thermo vertical gel electrophoresis according to descriptions of Maniatis et al (1989) Cell lysates were heated at 95 °C for 3 min in protein loading buffer and then loaded into the gel. The movement of the molecules were followed by the blue dye (bromophenol blue) present in the protein loading buffer and the current was turned off when the dye was approaching to the bottom of the seperating gel. The current kept constant at the beginning was increased upon passage of the bands to the seperating gel.

2.2.8.3. Transferring Proteins from Acrylamide Gels to Nitro-cellulose Membranes

The run gel was removed and placed in a tray containing transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% (w/v) SDS, 20% (v/v) methanol, pH 8.3) Meanwhile, all other materials to be used for this transfer purpose were soaked in the transfer buffer as well including Scotch-Brite sponge pads, filter papers (cut at the same size of the gel) and transfer membrane (cut at the same size of the gel). For convenience, the gel and one of the filters combined with it were carried together not to harm to the fragile gel and then they were sandwiched on a transfer frame stand that has negative electrode between sponge pad (above the frame with negative electrode)and transfer membrane (in contact with the gel)-filter paper-2 sponge pads and covered with the frame stand of positive electrode. Since proteins were negatively charged because of the SDS in protein loading buffer, their migrations would be from cathode to anode. The construction with 2 frame stands at the top and below were attached with 2 clamps and turned vertical to be placed into the electrophoresis tank full of ice to prevent the harmful effect of heat and sufficient volume of transfer buffer to be in contact with the electrodes in the electrophoresis tank. An electric current of 15 V was applied for 1.5 hours for the transfer of proteins on to the nitro-cellulose filter.

2.2.8.4. Detection of Proteins by Western Blotting

After the transfer of the proteins from to gel to the nitro-cellulose membrane, in order to prevent the nonspecific reactions, the potential protein binding sites on the membrane were covered with irrelevant proteins with a process called blocking. The membrane was incubated in 5% (w/v) non-fat dried milk in PBS for 2 hours at 37 °C with constant shaking. Then, the primary antibodies reactive against the target proteins were added with dilution rates of 1/100 and 1/400 and incubated for 2 hours with gentle agitation. Then, the membrane was washed 3 times with wash solution (1% (v/v) Tween 20 in PBS) Since it was strongly recommended to remove phosphate from the membrane before the secondary antibody treatment, a final wash with the phosphate-free wash solution (150 mM NaCl, 50 mM Tris-Cl, pH 7.5) was done. Then, the membrane was transferred into phosphate-free blocking solution (5% (w/v) non-fat dried milk, 150 mM NaCl, 50 mM Tris-Cl, pH 7.5) containing the secondary antibody (Anti-Mouse IgG Alkaline Phosphatase Conjugate) at 1:30000 concentration and incubated for 1 hour at room temperature with gentle agitation. Then, the membrane was washed with phosphate-free wash solution by four 10 min incubations with gentle agitation. For the detection of target protein bands, chromogenic substrate BCIP/ NBT (5-bromo-4-chloro-3-indolylphosphate/ nitro blue tetrazolium) which was converted in situ into a dense blue compound by immunolocalized alkaline phosphatase) was added on the membrane and waited until the development of the blue colour.

CHAPTER 3

RESULTS

3.1. Episomal DNA Replication in Human Cells and EBV-based Vectors

In order to study the expression profiles of recombinant proteins in eukaryotic cell lines, the starting point is the introduction of the foreign DNA that is anticipated to be expressed into a host cell. Once the DNA has entered the cell, it can follow many routes, but three are most likely (Fig. 3.1). First, it may be destroyed by cellular enzymes called as nucleases. Second, the DNA may be remained in the nucleus or cytoplasm as an extra-chromosomal element (episome). Lastly, it may integrate into the host cell's chromosome and become a stable and permanent part of the genome.

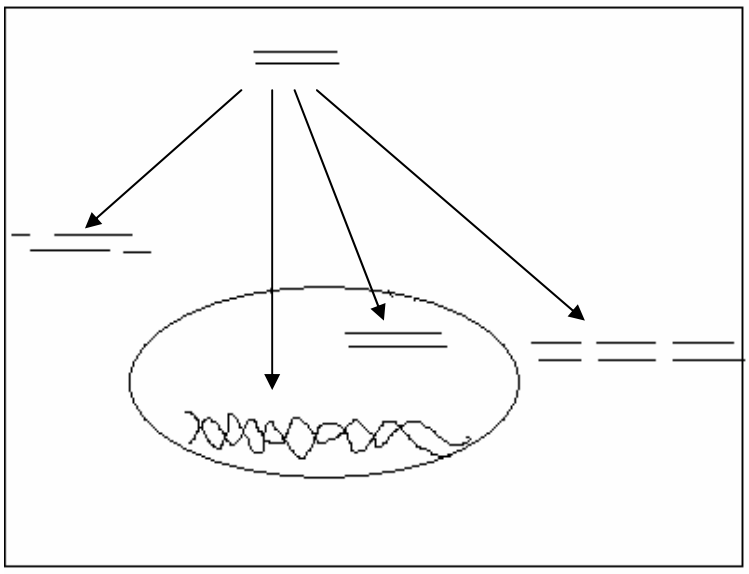

Figure. 3.1. Possible routes of foreign DNA entering a mammalian cell
(Source: Thomas F. Kresina, 2001)

Episomal replication system may be exploited to serve our purpose. If vectors that are maintained as multiple copies/cell by episomal replication in the host cell nucleus are used, recombinant proteins expressed from these constructs may be expected at high levels.

Epstein-Barr virus (EBV), a human herpesvirus, is associated with a number of human malignant diseases. EBV-immortalized cells harbor the viral genome in multiple episomal copies and express a limited number of viral genes, including the genes coding for six nuclear antigens (EBNAs) and three membrane antigens (Strominger et al., 1989).

Since the major risks of toxicity with viral vectors, nonviral vectors have been developed thanks to the recombinant DNA technology. In this study, we took advantage of EBV-based plasmid vectors pMEP4 and pREP9 which possess the EBV origin of replication (ori P) and nuclear antigen gene (EBNA-1) required for ori P function. In the majority of plasmids, the replication functions are clustered within a region of 1-3 kb known as the basic replicon. This was defined originally as the smallest portion of the plasmid to replicate (Kollek et al., 1978). EBNA-1 binds to the viral origin of episomal replication and is required for maintaining the genome as episomes (Sugden et al., 1985). This finding is compatible with the analysis that mutations in the gene for EBNA-1 prevented extrachromosomal persistence of the plasmid (Levine et al., 1985). In this context, it is noteworthy to imply that EBNA-1 and ori P have been conserved during the evolution of the close relatives of EBV that infect Old World primates (Klein et al., 1987; Pagano et al., 1990; Ying et al., 1996).

Cells transfected with EBV-based plasmids (pMEP4 or pREP9) can be selected with the suitable selection marker that plasmids carry (hygromycin in the case of pMEP4 and G418 (neomycin) in the case of pREP9). Besides this marker difference, the two plasmids contain different promoters (metallothionine in the case of pMEP4 and RSV promoter in the case of pREP9). Excluding these two distinct features, two plasmids have the same backbone, as shown in Fig.3.2 and Fig. 3.3.

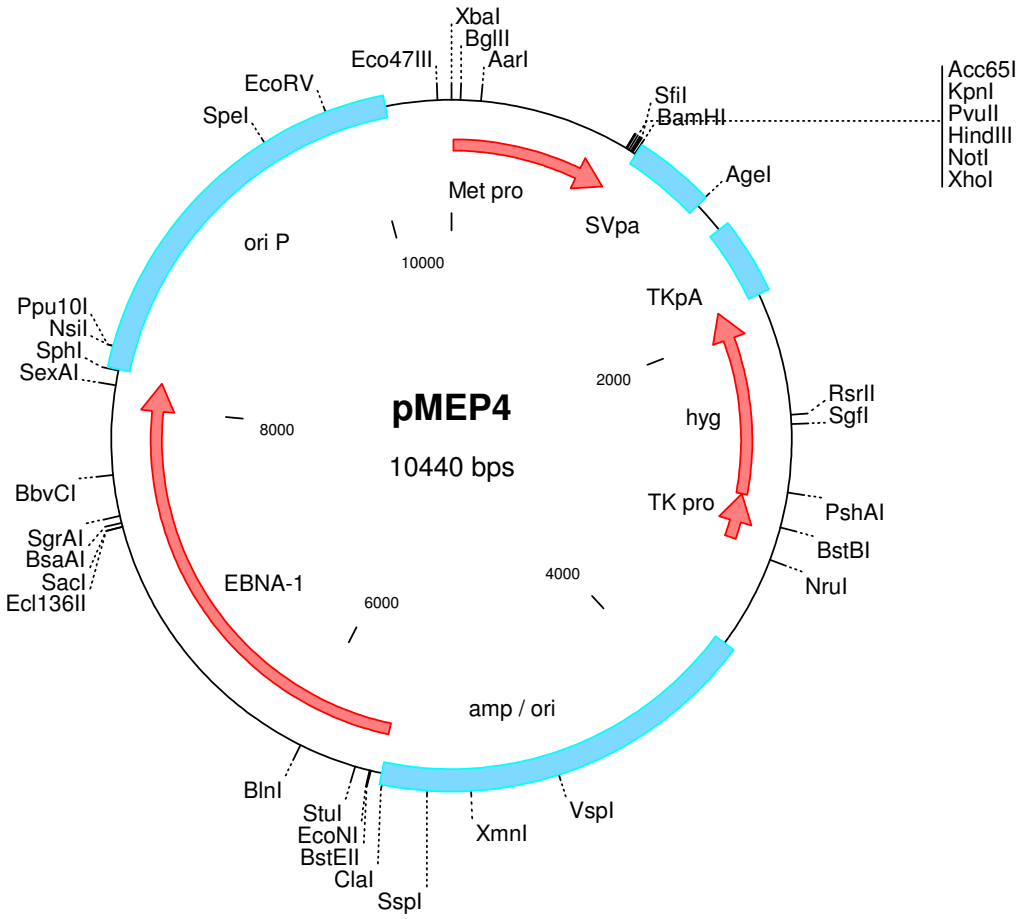

Figure 3.2. pMEP4: An EBV-based episomal mammalian expression vector (Invitrogen) Comments for pMEP4: A polylinker flanked by metallothionine promoter and SV40pA (polyadenylation signal) for the cloning and selection of the recombinant protein; a selectable marker (hygromycin-resistance gene) flanked by TK promoter and TKpA for stable maintanence of the vector; amp/ori: antibiotic resistance gene for the selection in bacteria/ E.coli replication origin; oriP and EBNA-1: EBV replication origin and EBV nuclear antigen necessary for episomal expression of EBV-based plasmids.

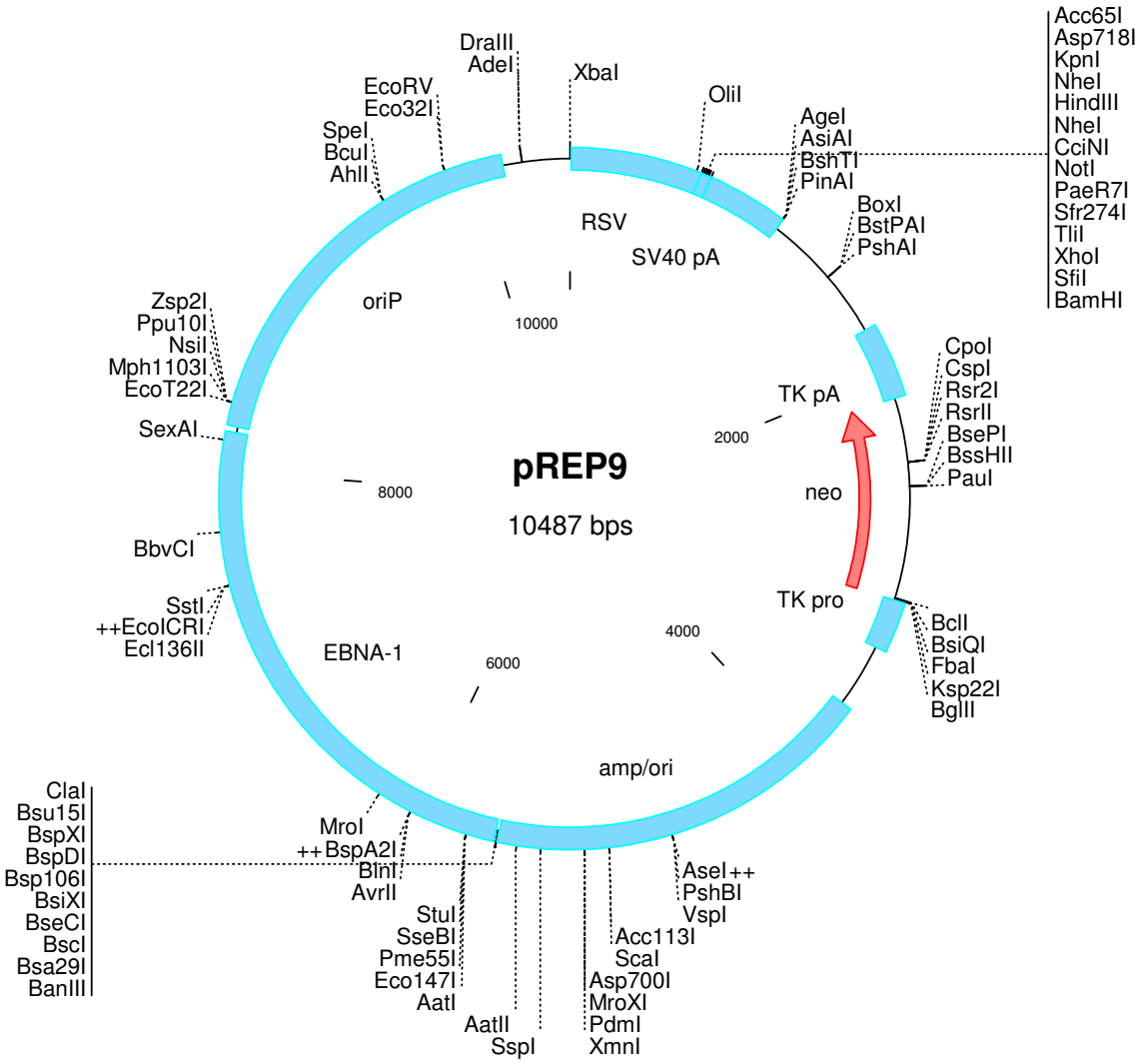

Figure 3.3. **p**REP9: An EBV-based episomal mammalian expression vector (Invitrogen) Comments for pREP9: A polylinker flanked by RSV promoter and SV40pA (polyadenylation signal) for the cloning and expression of the recombinant protein; a selectable marker (neomycin-resistance gene) flanked by TK promoter and TKpA for stable maintanence of the vector; amp/ori: antibiotic resistance gene for the selection in bacteria/ E.coli replication origin; oriP and EBNA-1: EBV replication origin and EBV nuclear antigen necessary for episomal expression of EBV-based plasmids.

3.2. Importance of the IRES in Expression Vectors

In early studies that describe retroviral vectors containing two genes, transcription of the first gene was usually under the control of the retroviral promoter, whereas expression of the second gene was controlled by a heterologous promoter. Although the majority of these studies showed that expression of both genes was detectable, some groups reported that expression of a second gene was absent either due to promoter interference, promoter methylation, or other unknown mechanisms (Emerman and Temin, 1984, 1986; Xu et al., 1989; Hoeben et al., 1991). With the identification of cap-independent translation and the minimal sequence responsible for internal ribosomal entry (IRES) sequence elements that are found in poliovirus (Pelletier and Sonenberg, 1988) and encephalomyocarditis viruses (Jang et al., 1988, 1989) viral RNAs, IRES sequences were used for the construction of bicistronic retroviral vectors for gene therapy purposes (Adam et al., 1991; Aran et al., 1994; Sokolic et al., 1996).

By linking the two genes of interest in a single bicistronic transcriptional unit it should be possible to maintain both functions for a prolonged period of time (Levine et al., 1991; Ghattas et al., 1991; Morgan et al., 1992; Sokolic et al., 1996; Abram et al., 1997; Di Ianni et al., 1997; Gallardo et al., 1997; Murakami et al., 1997; Sugimoto et al., 1997). Considering this fact, the internal ribosome entry site (IRES) from encephalomyocarditis virus was placed between the two genes (rev and tat) for their efficient simultaneous translation as shown in the later figures. In this way, both genes are anticipated to be expressed from a single bicistronic mRNA.

3.2.1. Construction of the Episomal Expression Vector pMEP-IRES

In order to construct pMEP-IRES episomal expression vector, EBV-based episomal expression vector pMEP4 was digested with Bgl II and NotI restriction enzymes and the large fragment was purified by agarose gel electrophoresis. The plasmid pIRES was also digested with the same restriction enzymes and the resulting 1754-bp fragment containing CMV enhancer, CMV promoter, IVS and IRES sequences was subcloned into the Bgl II and NotI sites of pMEP4 after being purified from the gel. Thus, the 879-bp weak metallothionine promoter was replaced by the strong CMV

promoter and IRES sequences to yield pMEP-IRES episomal expression vector (Fig. 3.4)

Following transformation of ligated fragments into competent *E.coli* cells, 5 colonies were obtained. Plasmid DNAs of these ampicillin-resistant transformants were isolated and control digestions were carried out with restriction enzymes XbaI and MunI to prove the generation of the recombinant molecule pMEP-IRES. Firstly, 5 transformants were digested with XbaI and was observed that four digestions yielded the two fragments of expected sizes (Fig. 3.5). Then, one of these samples was taken for further analysis with MunI restriction enzyme to confirm the cloning (Fig. 3.6).

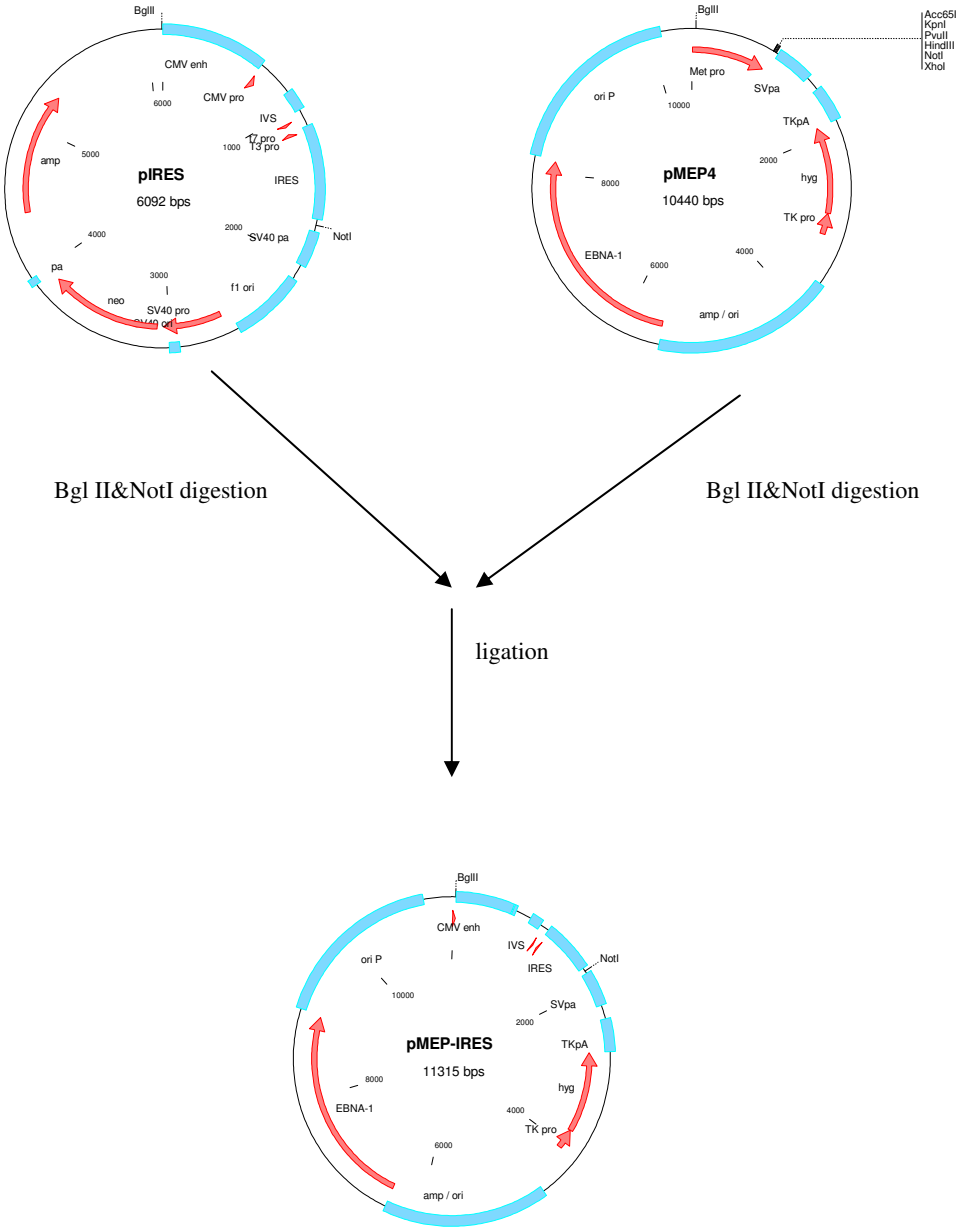

Figure 3.4. Contruction of the episomal expression vector pMEP-IRES

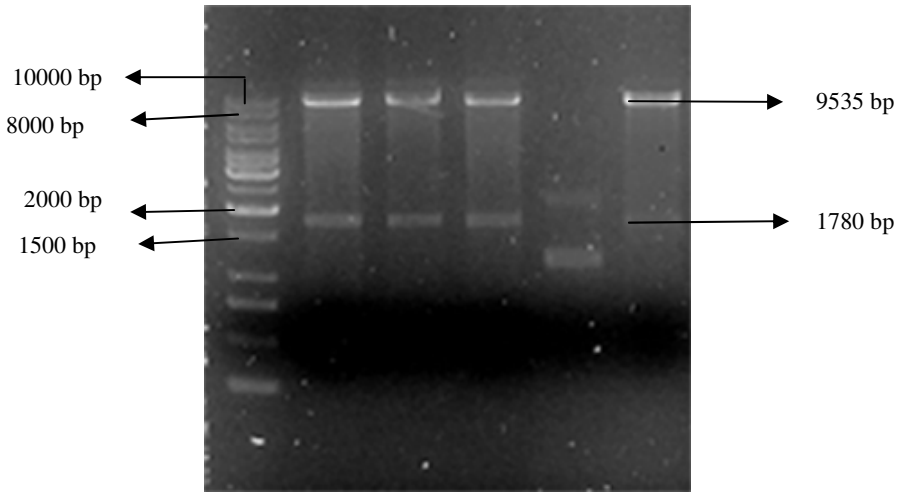

Figure 3.5. Restriction digestion analysis of transformants for pMEP-IRES
Lane 1. 10 kb DNA Ladder; Lanes 2-6: plasmids tested

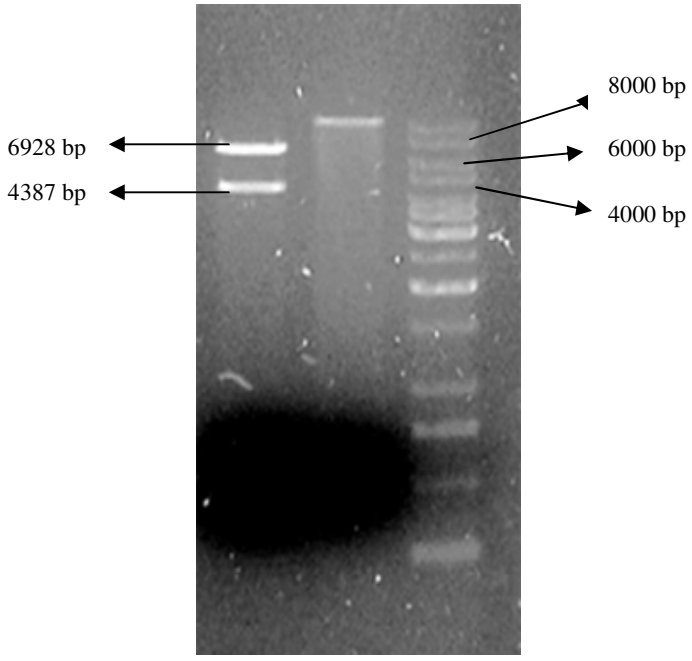

Figure 3.6. Restriction enzyme analysis of plasmid pMEP-IRES
Lane 1. pMEP-IRES digested with MunI; Lane 2. control: pMEP4 digested with MunI; Lane 3. 10 kb DNA Ladder

3.3. Attempted Trials for the Generation of Expression Vectors Carrying HIV-1 'rev' and 'tat' cDNA Sequences

The constructed recombinant molecule pMEP-IRES was used to clone HIV-1 'rev' and 'tat' genes. Since the necessity of these two regulatory genes; tat and rev for the transactivation of HIV-1 promoter LTR and for the regulation of gene expression respectively was discussed in detail in the introduction part, the strategies developed to generate pMEP-IRES-rev, pMEP-IRES-tat, and pMEP-IRES-rev-tat will be explained in this part in terms of the efficiencies of the two methods in the cloning experiments.

Since the effect of rev assumed to be stronger, we focused on generating pMEP-IRES-rev-tat expression vector instead of pMEP-IRES-tat-rev in order to allow rev gene transcribed firstly. Though some resources claim the opposite, generally it is accepted that IRES-dependent expression of the downstream gene in the bicistronic vector is typically between 20% and 50% of the level of the gene located upstream from the IRES (Hayakawa et al., 2000). Certainly, pMEP-IRES-tat-rev expression vector could have been generated as well for a better comparison in terms of the gene expression profiles of these two vectors, however because of time limitations, this step was eliminated.

Two approaches were developed to accomplish the generation of these expression vectors and the first one was cloning by blunt end ligation which then was replaced with cloning by site-directed mutagenesis since the orientation of the cloned fragments was not possible to be detected with the first strategy.

3.3.1. Cloning by Blunt End Ligation

3.3.1.1. Attempts to Construct pMEP-IRES-rev Expression Vector

pCV1 plasmid (NIH AIDS reagent program) was digested with Bsu36I restriction enzyme and the ends of the resulting 722-bp length fragment including rev cDNA together with some small parts of tat and nef genes was made blunt with T4 DNA polymerase enzyme. pMEP-IRES expression vector was digested with NheI restriction enzyme and the ends of the resulting 11315-bp length linear fragment was

also treated with T4 DNA polymerase enzyme. Then, this linearized fragment was used as a vector to clone HIV-1 rev gene (Fig. 3.7).

3.3.1.2. Attempts to Construct pMEP-IRES-tat Expression Vector

The same strategy was used to clone HIV-1 tat cDNA into the expression vector pMEP-IRES. pMEP-IRES was digested with NheI and the linearized fragment was used to clone tat cDNA after treatment with T4 DNA polymerase for end-filling. This time, pCV1 (NIH AIDS reagent program) was digested with SalI and BamHI restriction enzymes to release the 356-bp length fragment containing tat cDNA together with some small parts of vpr and rev genes. Following T4 DNA polymerase treatment, ligation was done followed by transformation of ligated fragments into the competent *E.coli* (Fig. 3.8).

Since the clonings was not achieved by this way, the generated recombinant molecules were found more convenient to be named as 'product 1' 'product 2' in the model figures 3.7 and 3.8.

Though different temperature conditions and variable insert: vector ratios were tried, the ligation reactions were not sucsessful. It was observed that in a wide range of samples tested, the vector molecule was self-ligated and the treatment of the vector with CIAP (Calf Intestinal Alkaline Phosphatase) in order to remove 5' phosphate groups from the vector to prevent self-ligation provided no solution to our problem. Step by step, any other conditions were revised to make a better understanding of where the problem stems from. Then, we concentrated on T4 polymerase enzyme since the incompatible ends of the vector and insert molecules can not be filled with nucleotides, it was impossible to expect these clonings carry out. In order to determine whether there was a problem with T4 polymerase enzyme or not, PCR-amplified kanamycin gene was cloned into the pBluscript KS+ plasmid followed by treatment of both the vector and the insert molecule with T4 DNA polymerase enzyme and lots of colonies were obtained on LB agar plates containing both ampicillin resistance gene for the growth of pBluscript KS+ and kanamycin resistance gene for the selection of the molecule in which kanamycin gene was cloned into the pBluscript KS+ plasmid. Since, pBluscript KS+ plasmid does not contain resistance gene for kanamycin, it can not grow on plates containing the selectable marker kanamycin. Hence, the colonies grew on plates have to

be recombinant molecules. The greatest problem faced was related with supplying adequate amount of DNA. Both the restriction enzymes used for cloning and the restriction enzymes used in the control digestions yielded with very small length of expected fragments which were so difficult to be seperated from other resulting fragments in spite of the various manipulations tried. In some cases, the control tests of colonies obtained showed that the desired genes was cloned into the pMEP-IRES expression vector, however the orientation of the clones could not be determined . Since this is not a positional cloning, we had to control an other important parameter while carrying on our attempts to clone the two regulator genes. Because of all these reasons, this cloning strategy was abandoned and another strategy was tried.

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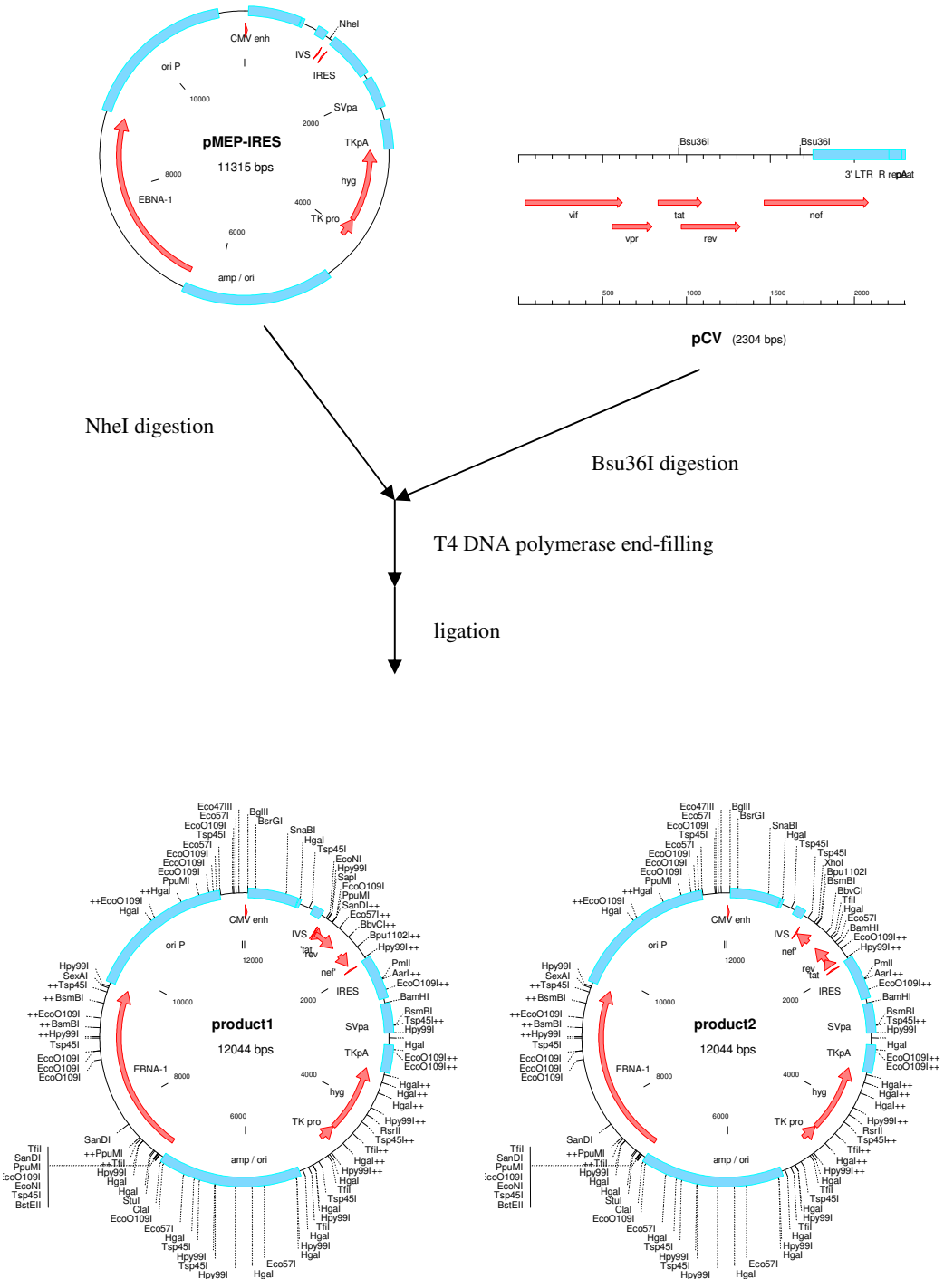


Figure 3.7. The model for the generation of pMEP-IRES-rev vector by blunt end cloning

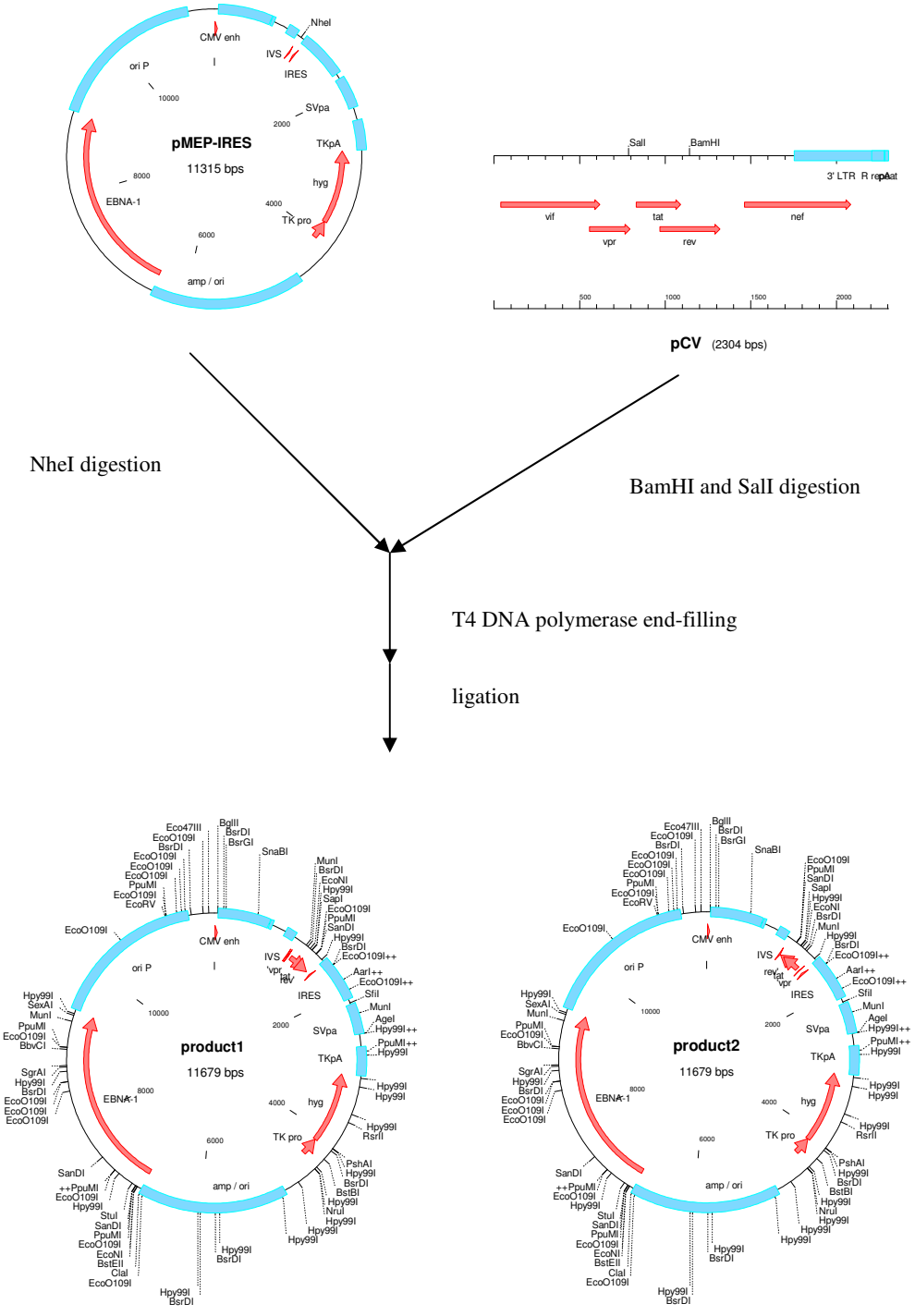


Figure 3.8. The model for the generation of pMEP-IRES-tat vector by blunt end cloning

3.3.2. Cloning by PCR

The biggest problem faced with the previous strategy was related with not obtaining high concentration of DNA molecules to be used in the cloning and the inability to demonstrate the desired orientation of the recombinant molecules. Therefore, primers carrying the appropriate restriction sites (NheI and NotI) were designed in order to amplify rev and tat cDNA sequences then to clone into the pMEP-IRES expression vector digested with the same restriction enzyme of amplified fragment. In order to facilitate the restriction digestion of amplified DNA fragments and thus to raise the ligation efficiency, 8-bp length sequences were placed before the restriction sites as this was the case for all primers used in this thesis work. Also, 20-bp length 2 oligonucleotides IRES and ires-2 were designed. Since their recognition sequences and binding orientations to these sequences were designed in a way to allow an easy determination of the orientations of rev and tat cDNA sequences, by taking advantage of this strategy, pMEP-IRES-rev, pMEP-IRES-tat and pMEP-IRES-rev-tat recombinant molecules were constructed.

3.3.2.1. Construction of pMEP-IRES-rev Expression Vector

pMEP-IRES episomal expression vector was digested with NheI restriction enzyme and the digestion mix containing the 11315-bp length linearized molecule was purified directly after the confirmation of the digestion with a small volume of digestion mix run on the agarose gel. Then, linearized fragment was treated with CIAP (Calf Intestinal Alkaline Phosphatase) and purified once more before it was used as the vector in the cloning. HIV-1 rev cDNA sequence was amplified from pCV1 (NIH AIDS reagent program) plasmid using the forward primer REV-NHE5 and the reverse primer REV-NHE3 both of which have Nhe1 sites. The 379-bp length amplified fragment was gel purified and digested with Nhe1 which resulted with 357-bp length fragment. Following purification, this fragment was cloned into the pMEP-IRES plasmid to yield pMEP-IRES-rev.

Two recombinant molecules of the same 11672-bp length may be generated with this approach. Since the expression profile of rev was desired in the forward orientation, recombinants carrying the undesired orientation of the rev was ignored and cloning

experiments were repeated until the determination of the recombinant molecule carrying the desired orientation of rev. For convenience, in the figure (Fig. 3.9) showing the construction model of pMEP-IRES-rev expression vector, the undesired molecule was not shown since it did not serve to our purpose. It is also noteworthy to declare that the generation of alternative recombinant molecules were also the case not only for this cloning, but also in the generation of pMEP-IRES-tat and pMEP-IRES-rev-tat expression vectors where the desired expression profiles of cloned genes were in the forward orientation again. So, the statement above is valid for these cloning experiments as well. Therefore, in the figures showing the generation model of these expression vectors, the undesired versions of the recombinants were not shown either.

The strategy based on the detection of the orientation of rev by taking advantage of the oligonucleotide IRES is shown in Fig. 3.10. Since the desired transcriptional activity of the rev gene is in the forward direction, as it was shown with arrow, the amplification reaction carried out with rev reverse primer REV-NHE3 and the oligonucleotide IRES which binds at the nucleotide 724, upstream from the rev, have to be resulted with the correct size of 766-bp length and an amplification reaction using rev forward primer REV-NHE5 and IRES is not possible.

As a result of the transformation of ligated fragments into the competent *E.coli* cells, nine of the plasmid DNAs of obtained colonies were subjected to the PCR amplification to directly determine the orientation of the rev gene and three of them gave positive result (Fig. 3.11)

Then, one of these three plasmids were subjected to the other amplification reactions to prove the cloning (Fig. 3.12) The evaluation of the results shows the construction of pMEP-IRES-rev expression vector.

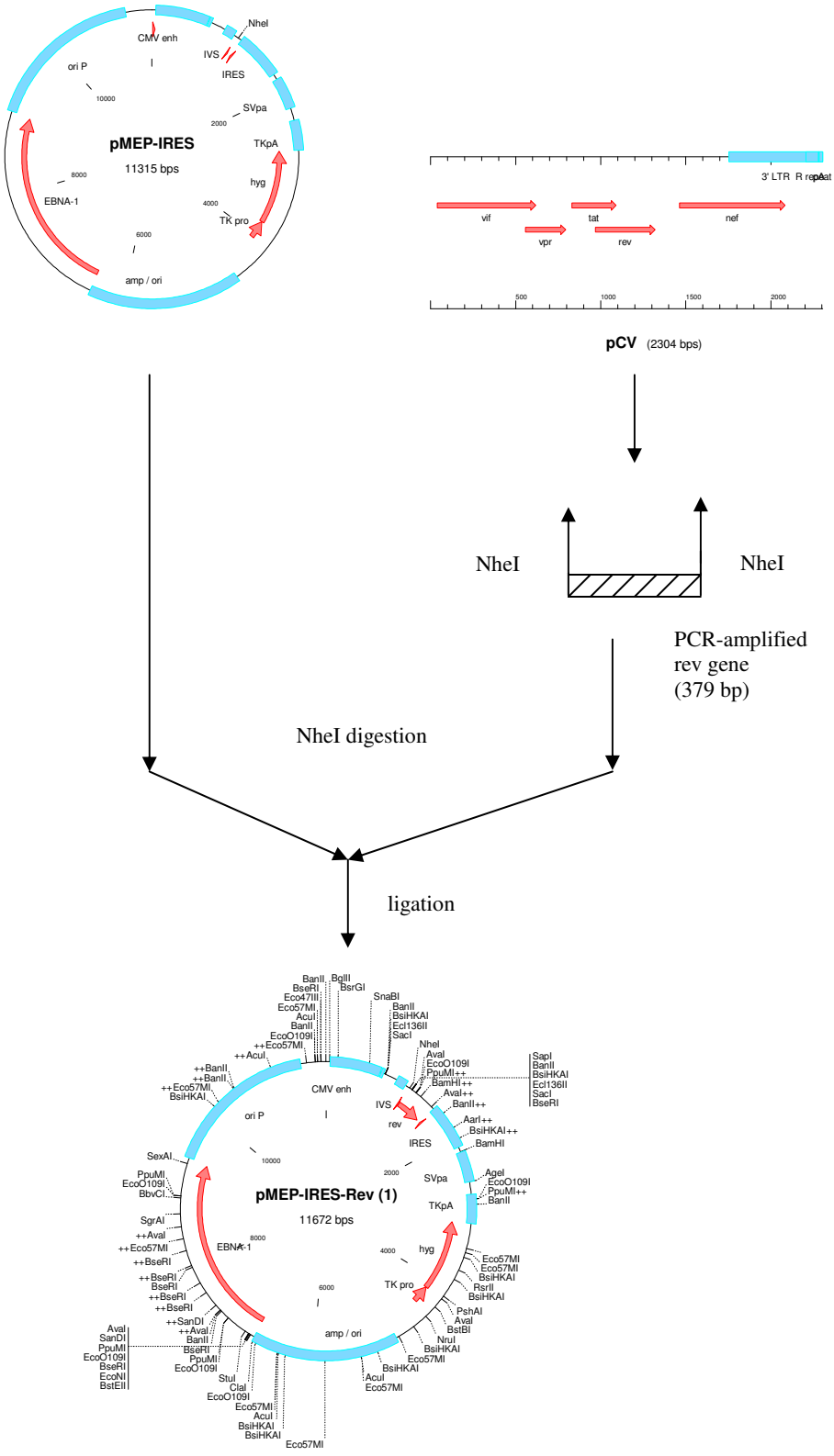


Figure 3.9. Construction of pMEP-IRES-rev expression vector

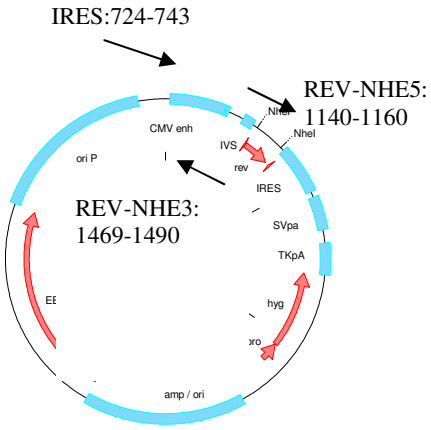


Figure 3.10. The strategy model used to detect the cloning and the orientation of the rev gene in pMEP-IRES-rev expression vector

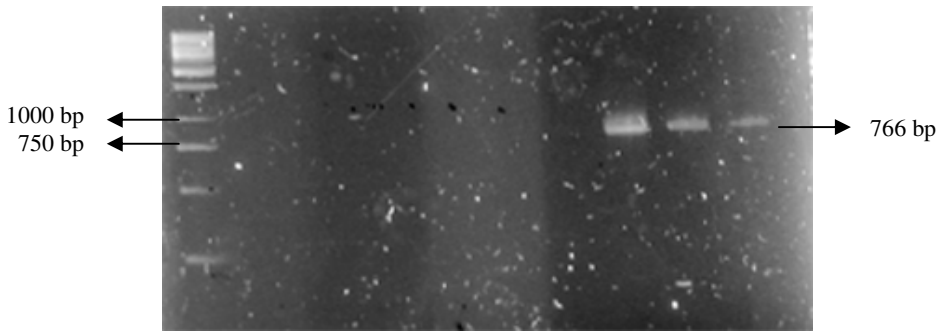


Figure 3.11. PCR analysis of 9 plasmids by primers IRES and REV-NHE3
Lanes: 1. 10 kb DNA Ladder Lanes 2-10: plasmids tested for amplification, last three (no: 7, 8, 9) gave the correct size of 766 bp as it was depicted.

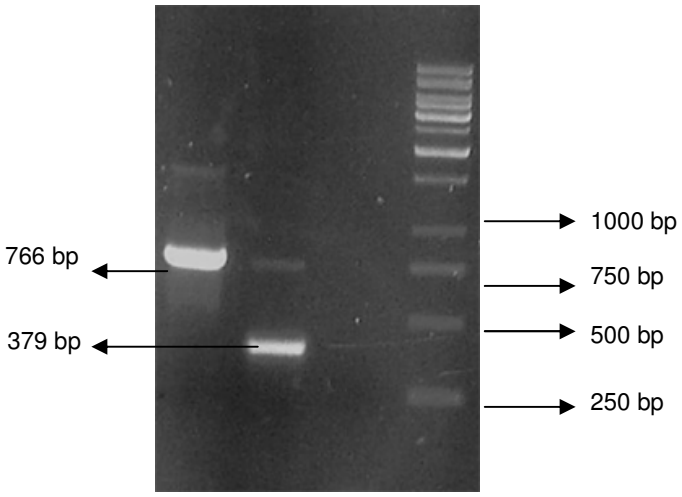


Figure. 3.12. PCR analysis of transformant 7
Lanes 1. amplification by primers IRES and REV-NHE3; 2. amplification by primers REV-NHE5 and REV-NHE3; 3. amplification by primers IRES and REV-NHE5; 4. 10 kb DNA Ladder

3.3.2.2. Construction of pMEP-IRES-tat Expression Vector

pMEP-IRES expression vector was digested with NheI restriction enzyme and the digestion mix was purified, a following CIAP (Calf Intestinal Alkaline Phosphatase) treatment and a last purification step made the linear 11315-bp lenth fragment ready in order to clone the tat cDNA sequences. HIV-1 tat cDNA was amplified from pCV1 (NIH AIDS reagent program) plasmid by primers TAT-NHE5 and TAT-NHE3 both of which were designed to carry NheI site. The 287-bp length amplified fragment was gel purified and digested with NheI which resulted with 265-bp length fragment. After purified, this fragment was cloned into the pMEP-IRES plasmid to generate pMEP-IRES-tat (Fig. 3.13).

32 ampicillin-resistant colonies were obtained following the transformation of ligated fragments and 6 of them gave positive result for tat amplification. The plasmid DNAs of these colonies were isolated and they were subjected to the further analysis to determine the orientation of tat. Restriction digestion of the fragments with MunI yields with 3 fragments, what is important is a 1024-bp length fragment is released when tat is cloned in the desired orientation, and a 905-bp length fragment is released implying the cloning of tat in reversed orientation. 4 of the 6 colonies were positive for this 1024-bp fragment. One of them was chosen to determine the orientation of tat with PCR analysis

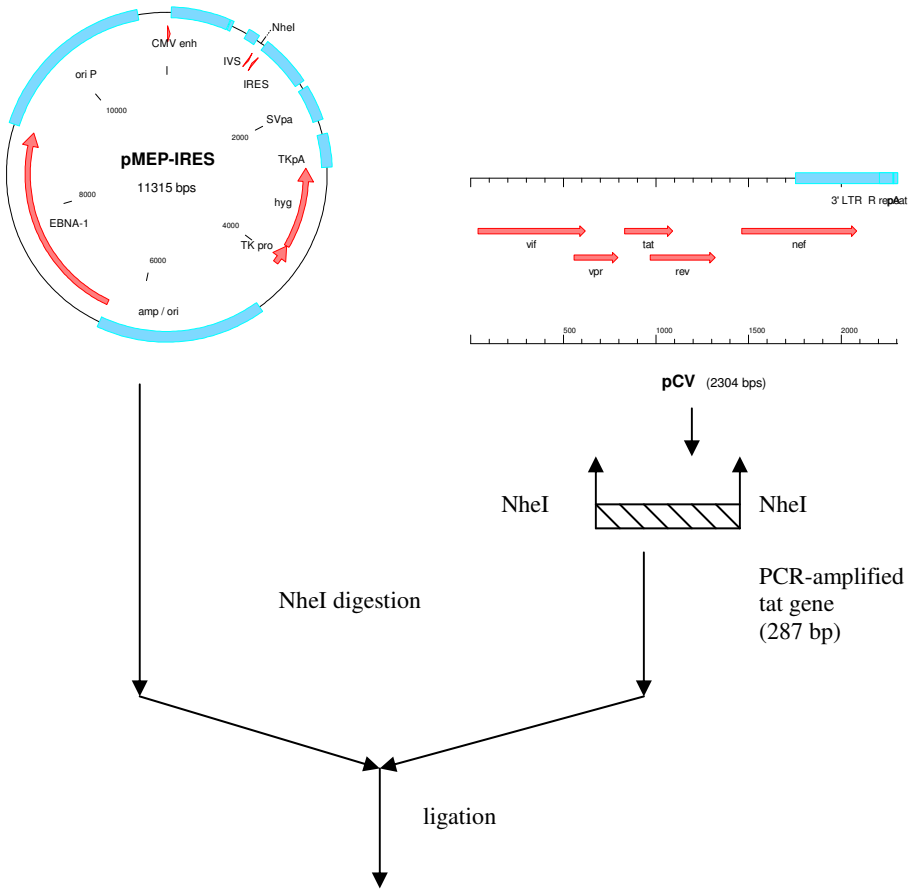
as well not to allow any room for doubt. The previous similar strategy was used to determine the orientation of tat, with the difference this time the primers in question were TAT-NHE5 and TAT-NHE3, together with the oligonucleotide IRES (Fig. 3.14). The amplification reaction by primers TAT-NHE3 and the oligonucleotide IRES which binds at the nucleotide 724, upstream from the tat, has to be resulted with the correct size of 674-bp length and the amplification reaction by primers TAT-NHE5 and IRES is not possible. The generation of pMEP-IRES-tat expression vector was demonstrated with these two tests as shown in Fig. 3.15 and Fig. 3.16.

3.3.2.3. Construction of Bicistronic pMEP-IRES-rev-tat Expression Vector

PCR-amplified HIV-1 tat cDNA (with primers TAT-NHE5-1 and TAT-NHE3-1 which of both were designed to carry NotI site) were cloned into the NotI site of the constructed pMEP-IRES-rev recombinant molecule to generate pMEP-IRES-rev-tat bicistronic expression vector (Fig. 3.17). After the necessary manipulations were carried out as in the case of pMEP-IRES-rev and pMEP-IRES-tat generations, 12 colonies were obtained following the transformation of ligated fragments into competent cells. These ampicillin-resistant colonies were first tested for the amplification and the orientation of tat gene(Fig. 3.19). The orientation of tat in pMEP-IRES-rev-tat can be determined by the tat forward primer TAT-NHE5-1 and the oligonucleotide ires2 which results with a 768-bp length amplicon. From the 12 colonies tested, transformant 12 confirmed the cloning of tat gene in the forward orientation and it was subjected to the further two tests consisting of restriction analysis with MunI (Fig. 3.20) and PCR-based orientation detection with the primers for tat and rev cDNA sequences and the two oligonucleotides designed for this approach (Fig. 3.21).

MunI digestion of the pMEP-IRES-rev-tat molecule is determinative in terms of the resulting 361-bp length fragment released if tat gene was cloned in the desired orientation and 242-bp length fragment released when tat was in reversed orientation in pMEP-IRES-rev-tat vector. The PCR-based orientation determination was also used to prove the generation of the correct recombinant molecule. While the primers REV-NHE3 and IRES determined the orientation of the rev (766-bp length amplicon), the primers TAT-NHE5-1 and ires2 were used to demonstrate the orientation of tat with an

expected 768-bp length amplicon. The amplification reaction carried out by the oligonucleotides IRES and ires2 were diagnostic in terms of the estimating the copy numbers of the cloned tat and rev genes in pMEP-IRES-rev-tat vector, in which case the 2206-bp length fragment was a proof of that pMEP-IRES-rev-tat vector was not interrupted by dimer formations. A nonspesific band was also observed making us think about the potential binding sites of these two oligonucleotides. The last analysis was related with the confirmation of overlapping tat and rev genes. If pCV1 plasmid map is browsed, a better understanding of the situation can be made. The amplification reaction generated by primers REV-NHE5 and TAT-NHE3-1 is not an extraordinary case for pMEP-IRES-rev-tat. However, the control amplification by the same primers in pMEP-IRES-rev is not normally expected to be carried out since there is no tat gene for the binding of the TAT-NHE3-1. If tat and rev genes were located seperately, it was not possible for this reaction to carry out. Whereas, the same 121-bp length amplicon was obtained confirming the fact that tat and rev genes overlap.



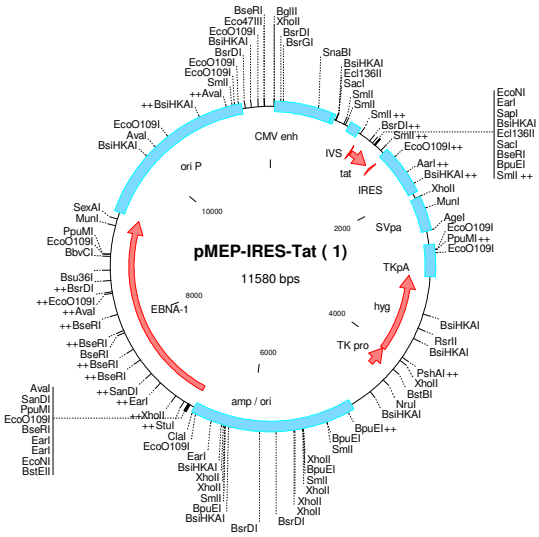


Figure 3.13. Construction of pMEP-IRES-tat expression vector

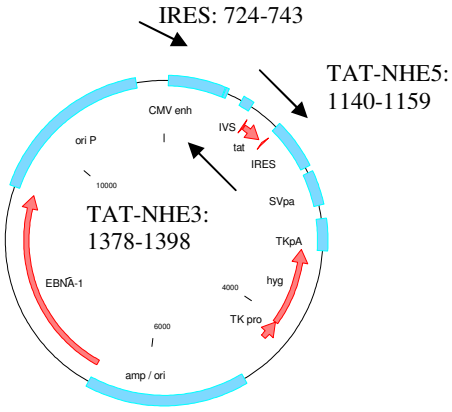


Figure 3.14. The strategy model used to detect the cloning and the orientation of the tat gene in pMEP-IRES-tat expression vector

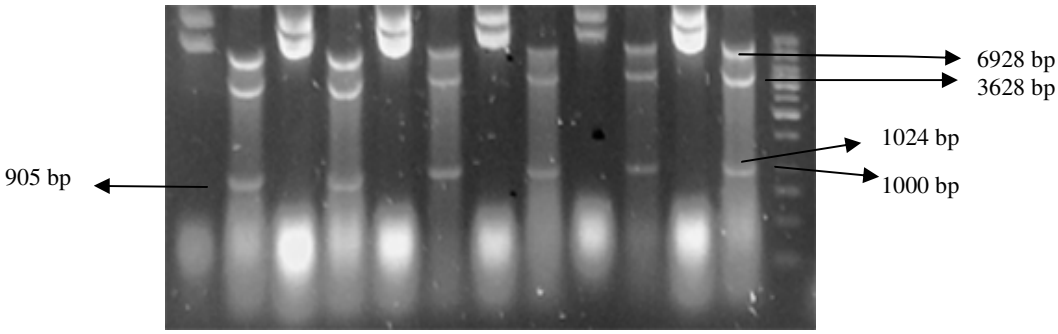


Figure 3.15. Restriction analysis of 6 transformants by enzyme MunI
Lanes 1, 3, 5, 7, 9, and 11 refer to uncut transformants of 1, 2, 3, 4, 5, and 6 respectively. At the right of each of these lanes, MunI digestion profiles of 6 transformants result with the 10 kb DNA Ladder profile.

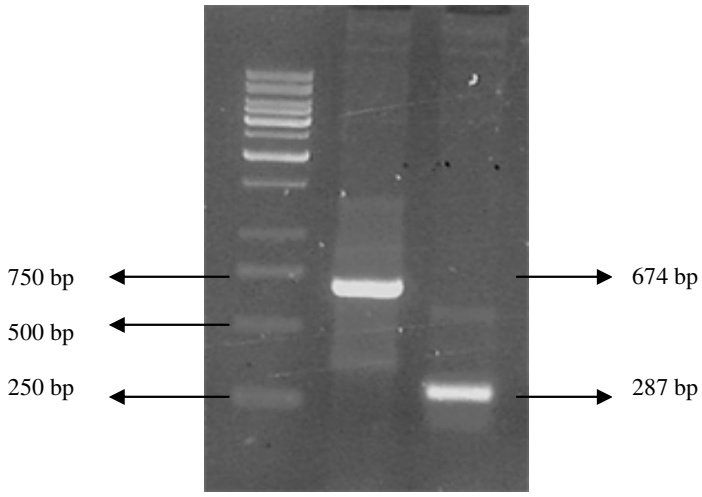


Figure 3.16. PCR analysis of number 3 transformant for the confirmation of the tat orientation
Lanes: 1. 10 kb DNA Ladder; 2. amplification by primers IRES and TAT-NHE3; 3. amplification by primers TAT-NHE5 and TAT-NHE3.

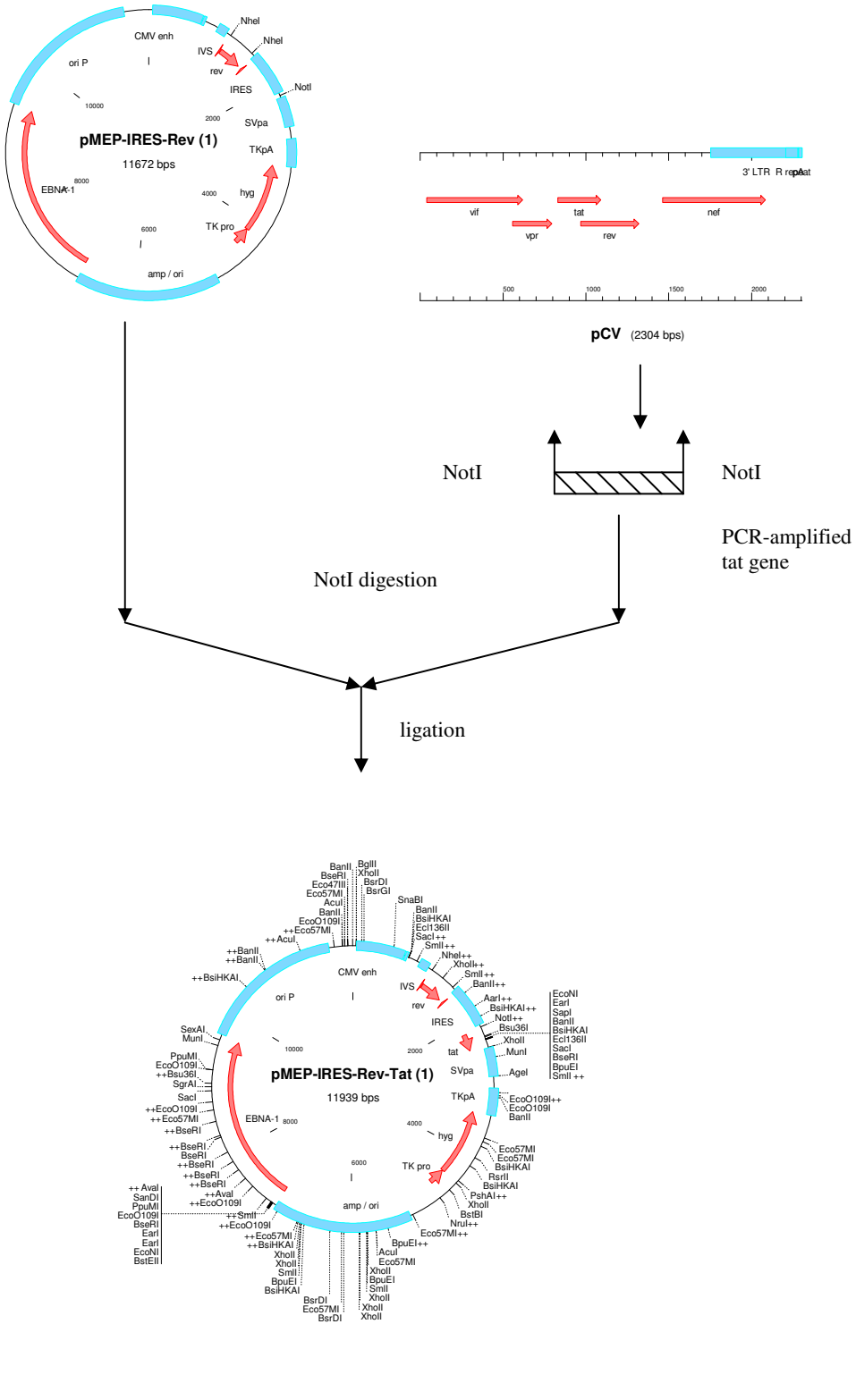


Figure 3.17. Construction of bicistronic pMEP-IRES-rev-tat expression vector

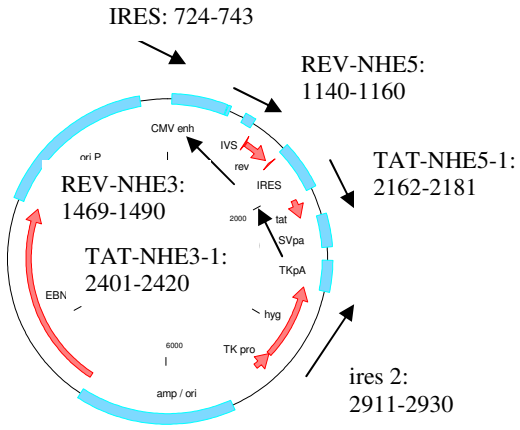


Figure 3.18. The strategy model used to detect the cloning and the orientation of the tat gene in pMEP-IRES-rev-tat vector

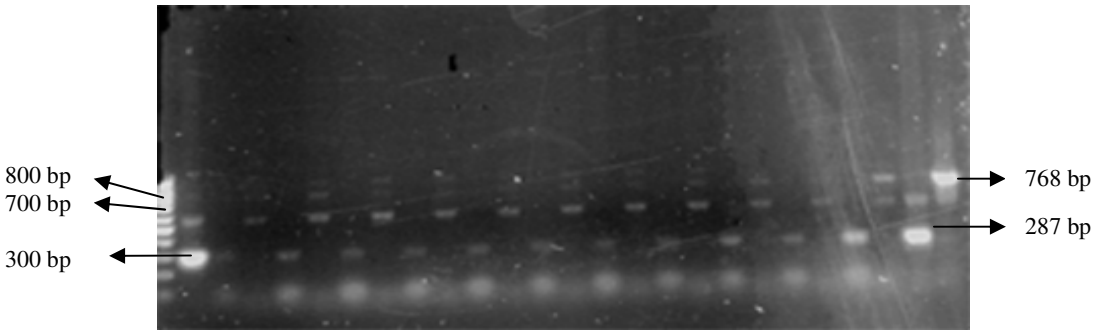


Figure 3.19. Analysis of 12 transformants with PCR
Lane 1: 100 bp DNA Ladder; Lane 2: positive control for tat amplification (pCV1); Lanes 3-26: transformants tested for the cloning and the orientation of the tat gene, where the lane 25 and lane 26 refer to the amplified tat and the orientation of the cloned fragment respectively.

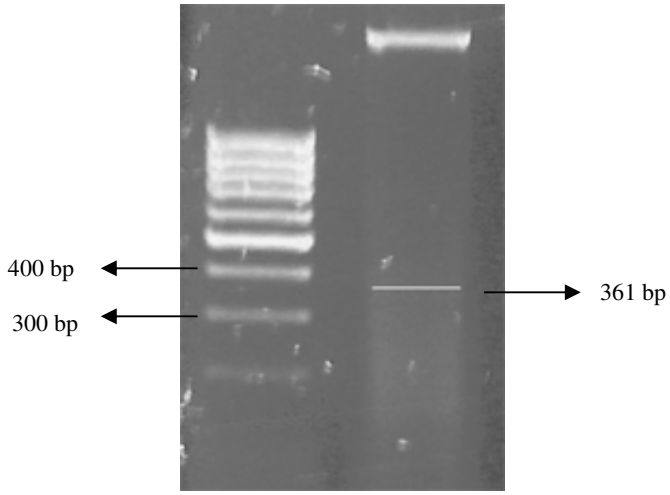


Figure 3.20. Restriction analysis of transformant 12 with MunI digestion
Lane 1: 100 bp DNA Ladder
Lane 2: transformant 12 digested with MunI

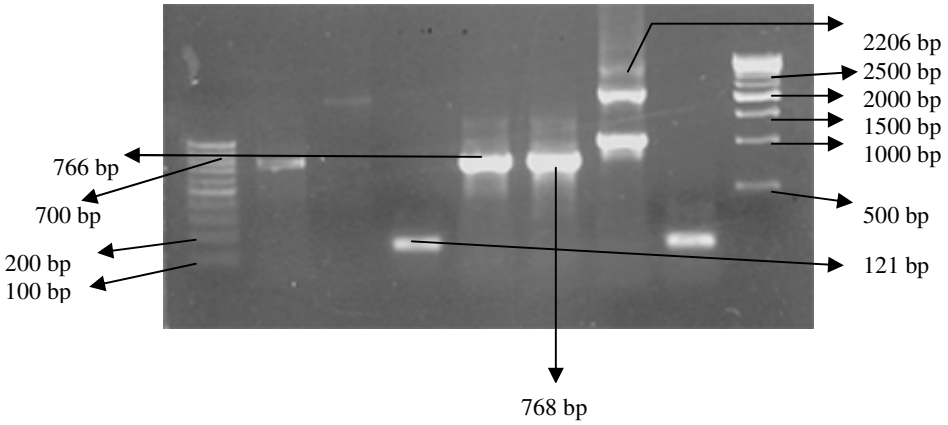


Figure 3.21. Confirmation of the generation of pMEP-IRES-rev-tat by PCR-based detection
Lanes: 1. 100 bp DNA Ladder; 2. pMEP-IRES-rev amplified by primers REV-NHE3 and IRES; 3. pMEP-IRES-rev amplified by primers REV-NHE5 and IRES; 4. pMEP-IRES-rev amplified by primers REV-NHE5 and TAT-NHE3-1; 5. pMEP-IRES-rev-tat amplified by primers REV-NHE3 and IRES; 6. pMEP-IRES-rev-tat amplified by primers TAT-NHE5-1 and ires2; 7. pMEP-IRES-rev-tat amplified by primers IRES and ires2; 8. pMEP-IRES-rev-tat amplified by primers REV-NHE5 and TAT-NHE3-1; 9. 500 bp DNA Ladder

3.4. Construction of An Episomal Plasmid Vector That Contains A Toxic Gene Whose Expression Will Be Dependent Upon HIV-1 'Rev' and 'Tat' Proteins

An expression vector that contains the conditional toxic gene under the control of the HIV-1 promoter LTR was generated with the purpose of when this plasmid is co-transfected with expression plasmids containing HIV-1 tat and rev cDNA sequences, the presumed expression of this toxic gene may be achieved since LTR needs to be transactivated by tat to abandon its transcriptionally silent situation. The mentality summarized briefly above led us to construct an episomal vector in which some cis-acting signals required for the effect of rev were subcloned as well with the expectation of increased gene expression.

3.4.1. Construction of pREP-LTR Vector

The 748-bp length fragment containing the 5' LTR and splice donor 1 sequences from HIV-1 molecular clone pNL4-3 (NIH AIDS reagent program) were PCR-amplified using 5' primer LTR5 with a unique XbaI site and 3' primer LTR3 with a unique KpnI restriction site. Following double digestion with these enzymes, the excised fragment was cloned into the pREP9 digested with the same enzymes to employ a positional cloning. The resulting plasmid was called pREP-LTR (Fig. 3.22).

As a result of transformation 12 ampicillin-resistant colonies were obtained and the plasmids of these colonies were subjected to the two restriction analysis to confirm the cloning. First, these transformants were digested with SacI restriction enzyme and 5 of them (no: 5, 7, 9, 10, 12) yielded the correct sizes of 7107 bp and 3531 bp (Fig. 3.23). Then, 3 of these 5 (no: 9, 10, 12) were subjected to the a second restriction analysis with Bgl II which in the case of the formation of the recombinant molecule yielded 3 fragments of 7844, 2588, and 206-bp lengths (Fig. 3.24). Excluding sample numbered 12, the two other yielded 7844 and 2588-bp length fragments, but not 206-bp length fragment. However that was a tolerable case considering the differences of fragment sizes and their EtBr binding capacities.

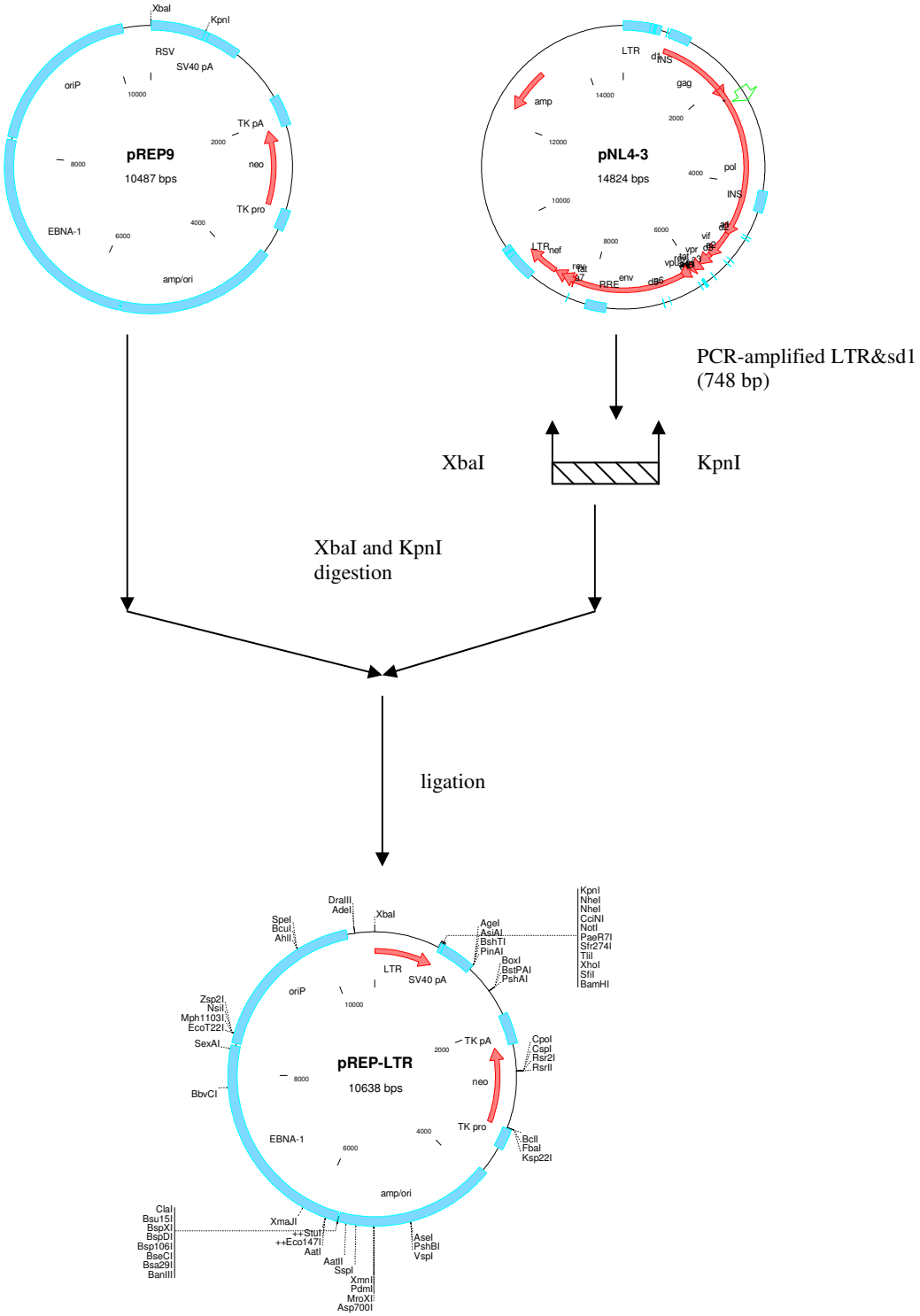


Figure 3.22. Construction of pREP-LTR vector

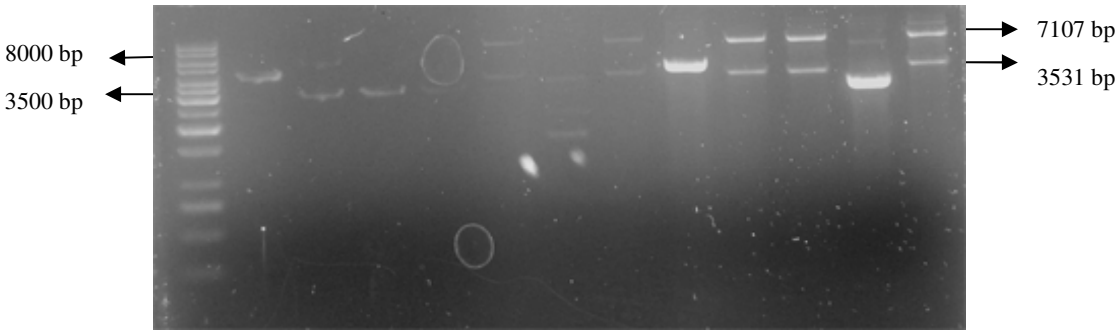


Figure 3.23. Restriction analysis of Ap-resistant colonies by SacI
Lane 1: 10 kb DNA Ladder; Lanes 2-13: samples tested

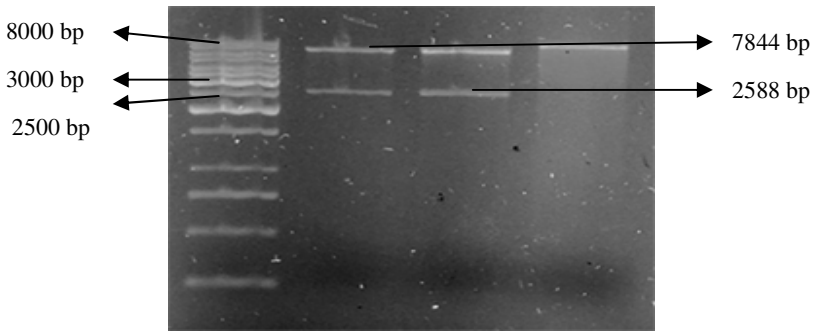


Figure 3.24. Restriction analysis of 3 transformants by Bgl II
Lanes: 1. 10 kb DNA Ladder; 2. transformant 9 digested with Bgl II; 3. tranformant 10 digested with Bgl II; 4. transformant 12 digested with Bgl II

3.4.2. Construction of pREP-LTR-INS-RRE Vector

Cis-acting INS and RRE sequences were used to render the suicide plasmid vector generated dependent upon not only Tat protein but also Rev protein of HIV-1. INS (CRS) sequences do not affect transcriptional activity of a gene but rather inhibit gene expression posttranscriptionally by preventing the export of the transcripts from nucleus to cytoplasm (Maldarelli et al., 1991). RNA containing an INS element can be entrapped and spliced in the nucleus in the absence of Rev (Cochrane et al., 1991). This barrier is overcome when the RRE is present in cis and the rev gene product is in trans (Maldarelli et al., 1991).

The construction of pREP-LTR-INS-RRE plasmid vector was generated with 2 steps, which of first HIV-1 molecular clone pNL4-3 plasmid was digested with MunI and of the 3 fragments of different sizes released, 9147-bp length fragment was gel isolated and self-ligated to generate pNL4-3-MunI that will be employed as the template to amplify HIV-1 INS, RRE and splice acceptor 7 sequences by primers INS5 and RRE3 which contain XhoI and BamHI restriction sites respectively. The 2007-bp XhoI-BamHI PCR product was then subcloned into the same restriction sites of plasmid pREP-LTR for the generation of pREP-LTR-INS-RRE vector (Fig. 3.25).

Generation of pNL4-3-MunI was confirmed with the yielded 2 fragments of 5165 and 3982-bp lengths when the recombinant molecule was excised with Eco81l (Fig. 3.26) For the generation of pREP-LTR-INS-RRE, of the many colonies obtained for the transformation of XhoI-BamHI excised fragments ligated together, 9 of them was double digested with BamHI and XhoI restriction enzymes which were so diagnostic when employed together since this digestion reaction yielded with the cloned insert of correct 2007-bp length (Fig. 3.27).

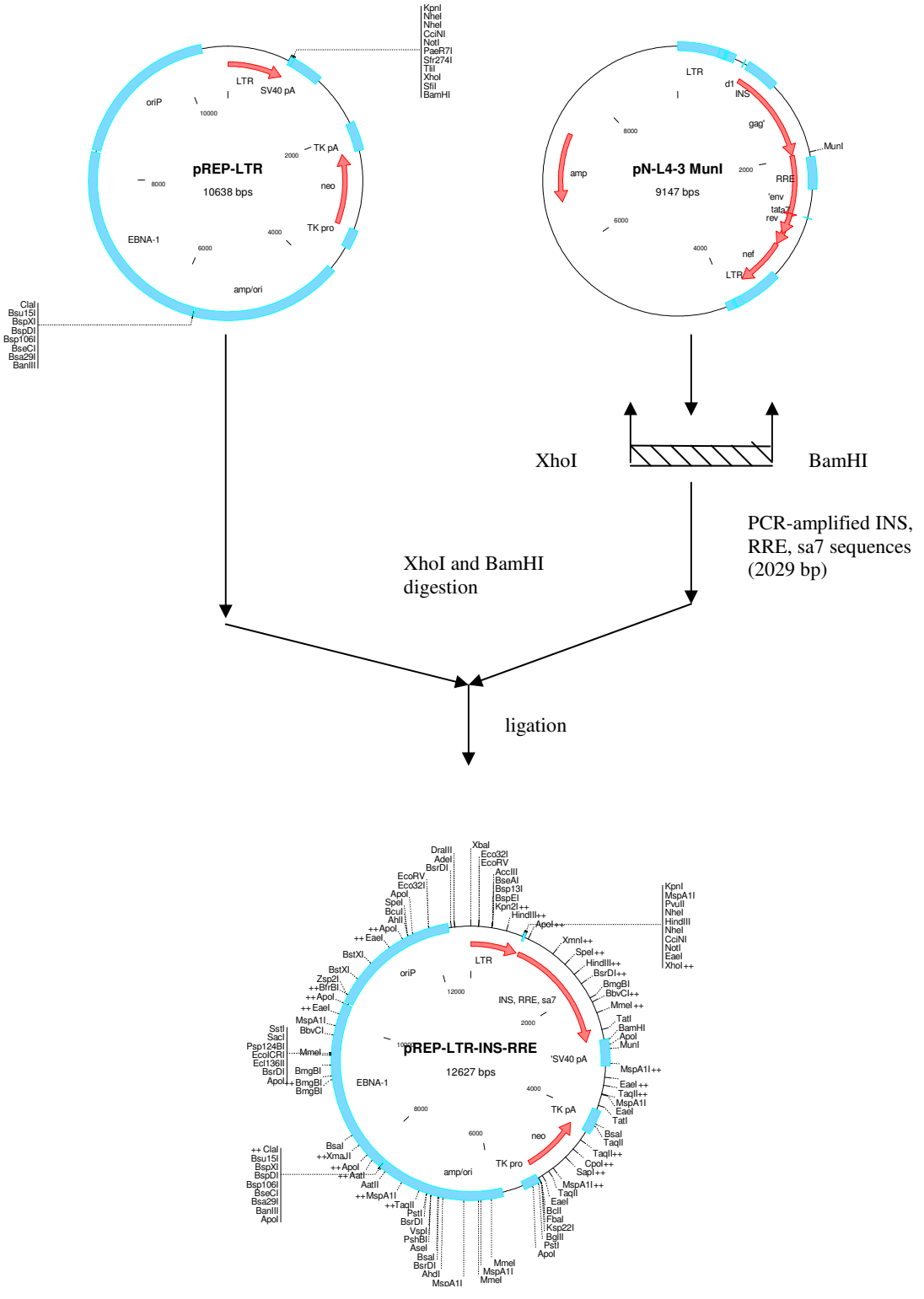


Figure 3.25. Construction of pREP-LTR-INS-RRE vector

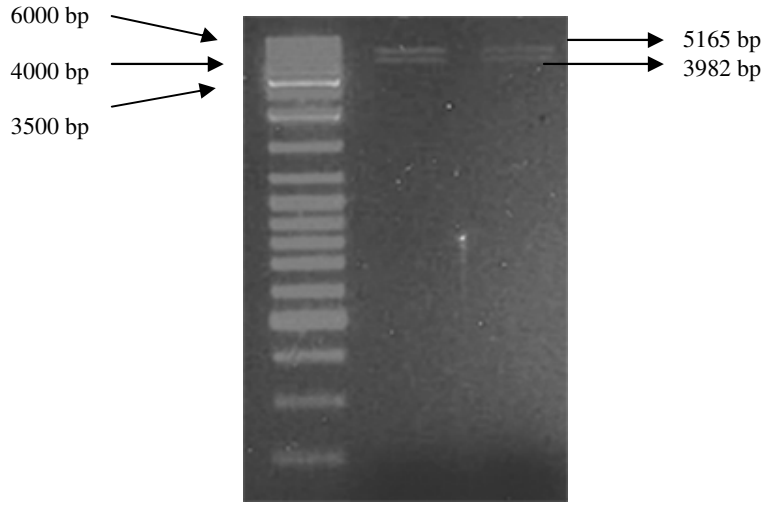


Figure 3.26. Confirmation of pNL4-3-MunI with Eco81l restriction
Lane 1: 10 kb DNA Ladder
Lanes 2-3: samples tested

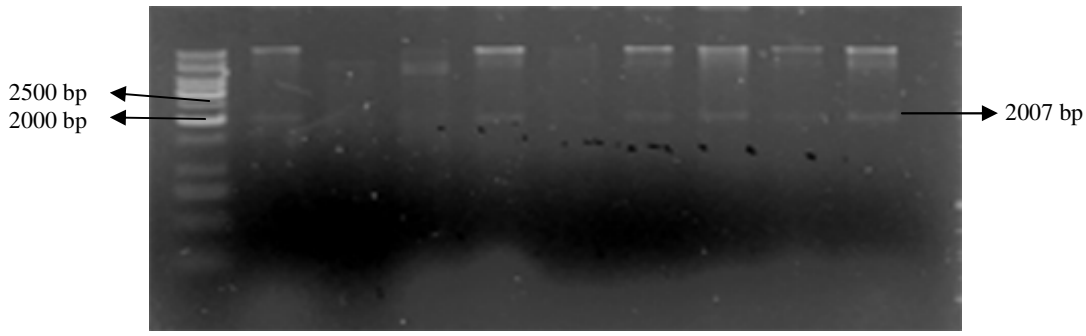


Figure 3.27. Confirmation of the generation of PREP-LTR-INS-RRE with restriction analysis
Lanes: 1. 10 kb DNA Ladder; 2-10: 9 plasmids of Ap-resistant colonies digested with BamHI and XhoI

3.4.3. Construction of pREP-LTR-INS-RRE-TK Suicide Vector

HSV-TK (Herpes Simplex Virus, Thymidine Kinase) gene was cloned into the plasmid vector pREP-LTR-INS-RRE to construct pREP-LTR-INS-RRE-TK. The Herpes simplex virus-1 thymidine kinase, the product of which confers sensitivity to the prodrug ganciclovir, remains the prototypic suicide gene (Klatzmann et al., 1999) By the generation of this suicide vector, the expression of the toxic gene could be achieved when Tat and Rev expressing cells transfected with this vector were grown in medium supported with ganciclovir, which then results with cell death.

The construction of this suicide vector was carried out by two steps. First, the thymidine kinase gene was obtained from HSV genomic DNA by PCR amplification using the TK5 and TK3 primers that contain the KpnI and HindIII restriction sites, respectively and the 1141 bp-length KpnI-HindIII excised amplified fragment was cloned into the same restriction sites of pREP9 to generate pREP-TK (Fig. 3.28) As the second step, the constructed pREP-TK was digested with KpnI and NotI restriction enzymes to yield thymidine kinase gene which were cloned into the same restriction sites of plasmid vector pREP-LTR-INS-RRE to construct pREP-LTR-INS-RRE-TK suicide vector (Fig. 3.29).

The confirmation of the generation of pREP-TK was made by PCR detection for the amplification of TK gene. Of the 56 Ap-resistant colonies obtained, 22 colonies were tested and 6 of them were positive for TK amplification ensuring the generation of pREP-TK (Fig. 3.30). One of these recombinants were chosen to be employed for the generation of pREP-LTR-INS-RRE-TK. The transformation of KpnI-NotI excised and ligated fragments resulted with a pretty number of colonies of which the 22 were first subjected to the colony PCR to detect the amplification of TK gene (Fig. 3.31). They were all positive for TK amplification and one of them was employed for further confirmation analysis with restriction analysis by KpnI and NotI together with PCR amplification . The double digestion of the recombinant by KpnI and NotI resulted with the correct size of 1144-bp length insert TK (Fig. 3.32).

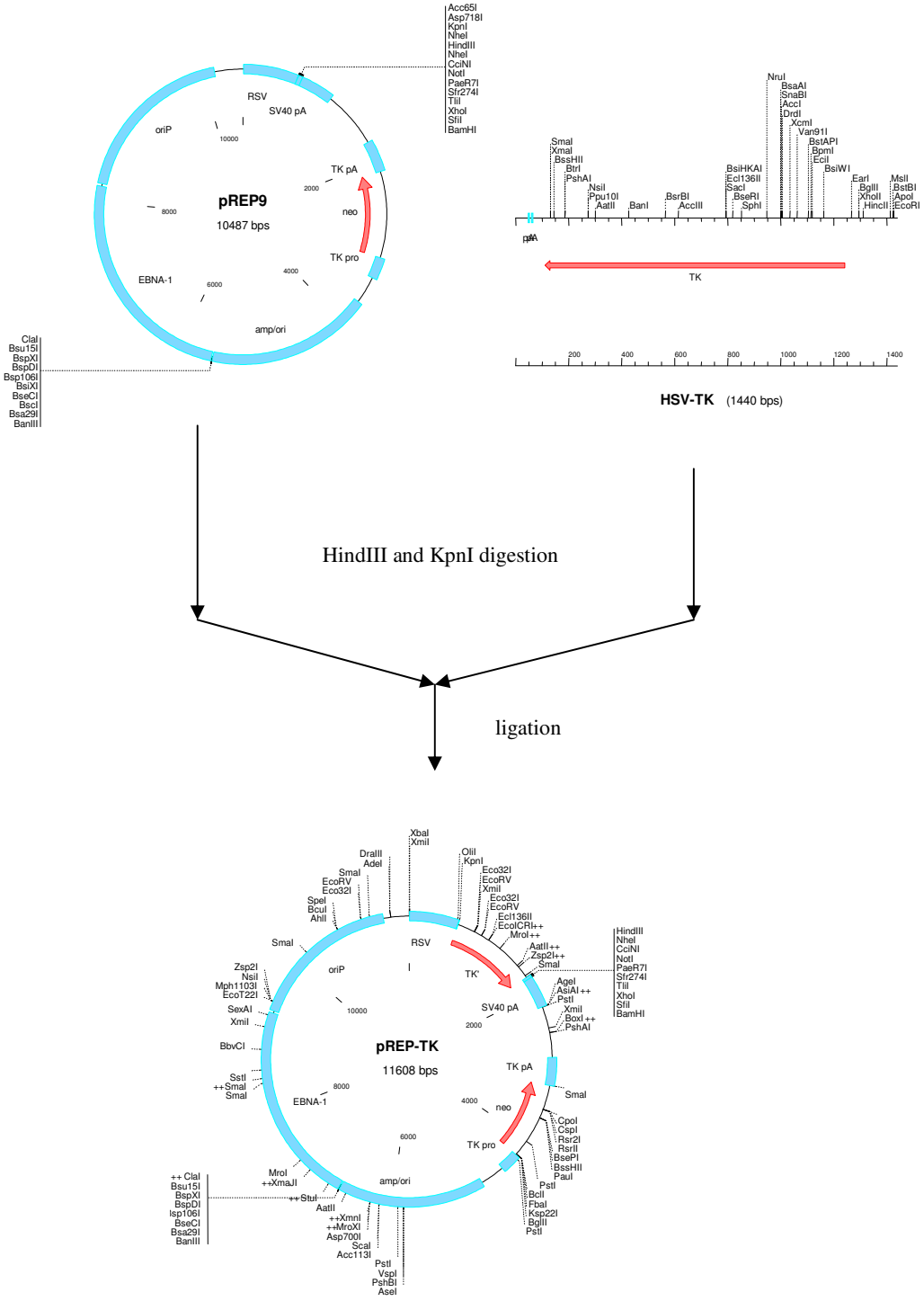


Figure 3.28. Construction of pREP-TK vector

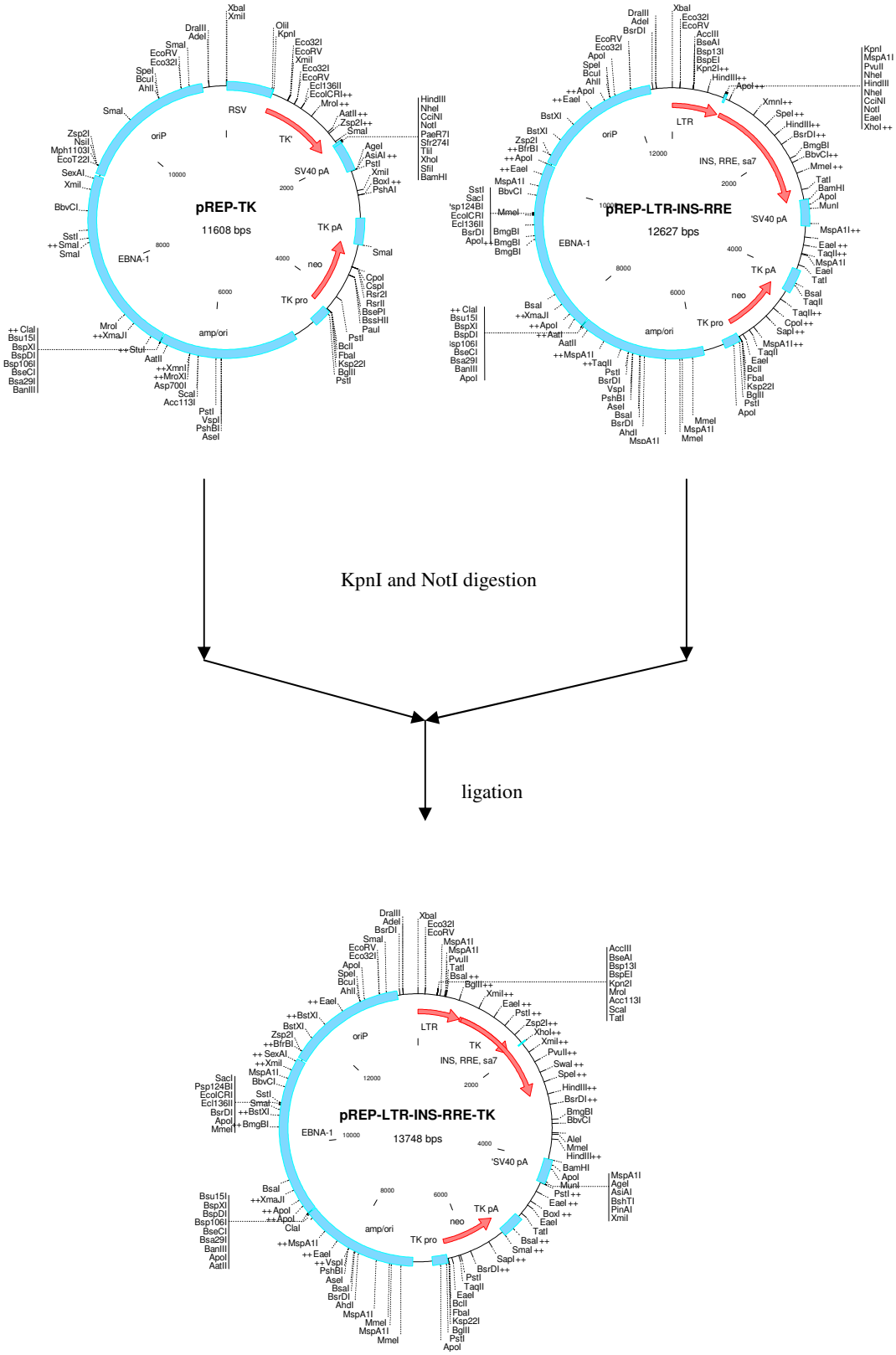


Figure 3.29. Construction of pREP-LTR-INS-RRE-TK suicide vector

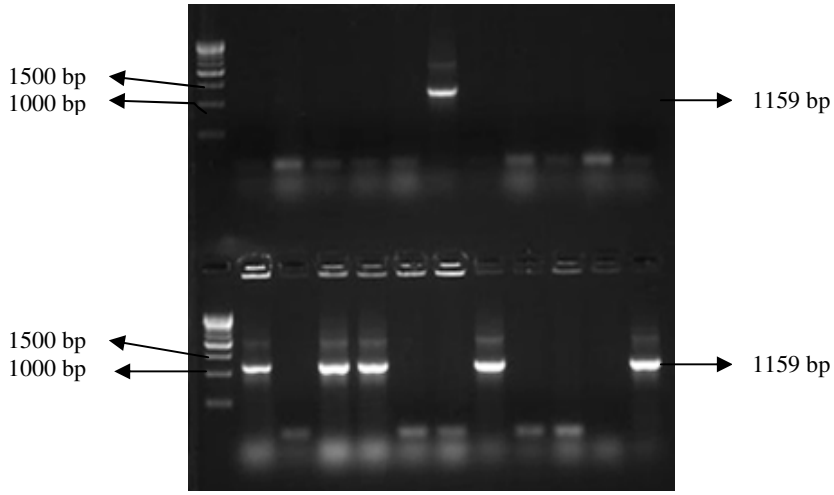


Figure 3.30. Confirmation of the generation of pREP-TK by PCR
Lanes above: 1. 0.5-5.0 kb DNA marker; 2-12: colonies (no: 1-11) amplified by primers TK5 and TK3
Lanes below: 1. 0.5-5.0 kb DNA marker; 2-12: colonies (no: 12-22) amplified by primers TK5 and TK3

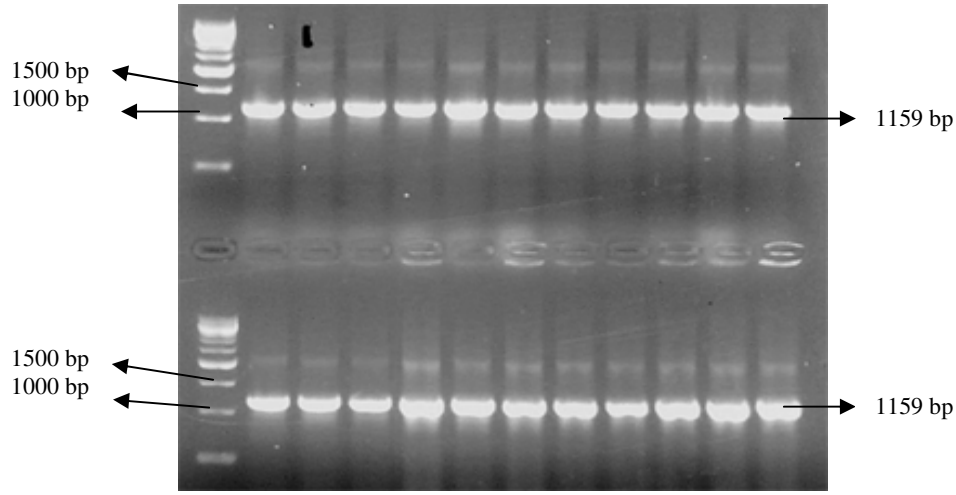


Figure 3.31. PCR analysis of Ap-resistant colonies for pREP-LTR-INS-RRE-TK
Lanes above: 1. 0.5-5.0 kb DNA marker; 2-12: colonies (no: 1-11) amplified by TK5 and TK3
Lanes below: 1. 0.5-5.0 kb DNA marker; 2-12: colonies (no:12-22) amplified by TK5 and TK3

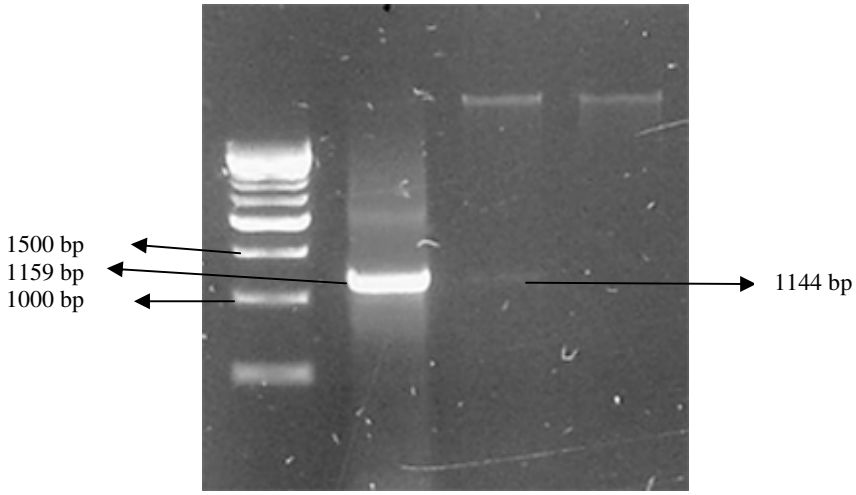


Figure 3.32. Confirmation of the construction of pREP-LTR-INS-RRE-TK
Lanes: 1. 0.5-5.0 kb DNA marker; 2. pREP-LTR-INS-RRE-TK amplified by primers TK5 and TK3; 3. pREP-LTR-INS-RRE-TK digested with KpnI-NotI; 4. Control: pREP-LTR-INS-RRE digested with KpnI-NotI

3.5. Monitoring Gene Expression via Reporter Genes

Two reporter genes (CAT and lacZ) were employed to construct pREP-LTR-INS-RRE-CAT, pREP-LTR-INS-RRE-CAT-lac and pREP-LTR-INS-RRE-TK-lac plasmid vectors. Reporter genes are very important in gene cloning since they guide us to monitor the expression profiles of specific sequences with the mostly simple histochemical tests. LacZ gene encodes the enzyme beta-galactosidase and the binding of this enzyme to its substrate X-gal results with a visible blue color (Emerman&Kimpton, 1992). To exploit this case for our purpose, lacZ gene was cloned into pREP-LTR-INS-RRE-TK suicide vector. So that, when eukaryotic cells expressing Rev and Tat were transfected with this vector and grown in medium supplemented with X-gal together with the appropriate antibiotics for selection, the resulting blue color could be a diagnostic signal for the gene expression. Deeper cases were also considered for future use hence pREP-LTR-INS-RRE-CAT and pREP-LTR-INS-RRE-CAT-lac vectors were constructed. The beta-galactosidase assay provides a powerful way of determining gene expression but in the case of obtaining a result that does not emphasize a specific type of cell death for cells co-transfected with pREP-LTR-INS-RRE-TK-lac and vectors expressing tat and rev, there may be some possibilities to be evaluated. This may be related with the toxin gene itself, if the expression of thymidine kinase does not carry out, it makes no sense to anticipate a specific cell death. On the other hand, it may sign to some problems regarding our constructs, for instance, if a mutation occurred in the promoter LTR sequences, certainly the genes cloned downstream of LTR can not be expected to be expressed. In pREP-LTR-INS-RRE-CAT vector, CAT replaced the suicide gene TK. Therefore, when transmitted into the host organism, gene expression profile of CAT will mimic the expression profile of the TK gene since CAT gene will be under the influence of the same control sequences as the TK . Therefore, CAT RNA containing the INS sequences will accumulate in the nucleus of transfected cells like TK. Since addition of Rev in trans and the RRE in cis is anticipated to rescue CAT expression, the relative CAT expression on protein level will be a determinative measurement of rev expression. Thus, verifying CAT protein expression would be a diagnostic signal to assure that the suicide plasmid vector generated is dependent upon not only Tat protein but also Rev protein.

LacZ will be expressed under circumstances depending on the action of Rev. In the case there is no Rev participation, intron containing RNA is forced to be spliced and the excision of the intervening regions results with the occurrence of lacZ mature mRNA which is then translated in the cytoplasm to yield lacZ protein. On the other hand, a different pathway will be followed when Rev participation is in question. Rev interaction with INS and RRE sequences will counteract the splicing pressure forced in its absence thus will enable the export of intron containing RNA (herein, TK and INS-RRE sequences) from the nucleus. Since there is neither IRES for simultaneous translation of these two genes as it was the case for the previously constructed recombinant molecules in the study nor a heterologous promoter to employ the transcription of the second gene, which refers to lacZ in that case, lacZ expression can not be anticipated. What happens is that TK transcripts normally entrapped and spliced in the nucleus in the absence of Rev can be exported from the nucleus to cytoplasm where they will be translated and produce toxic protein (Fig. 3.33).

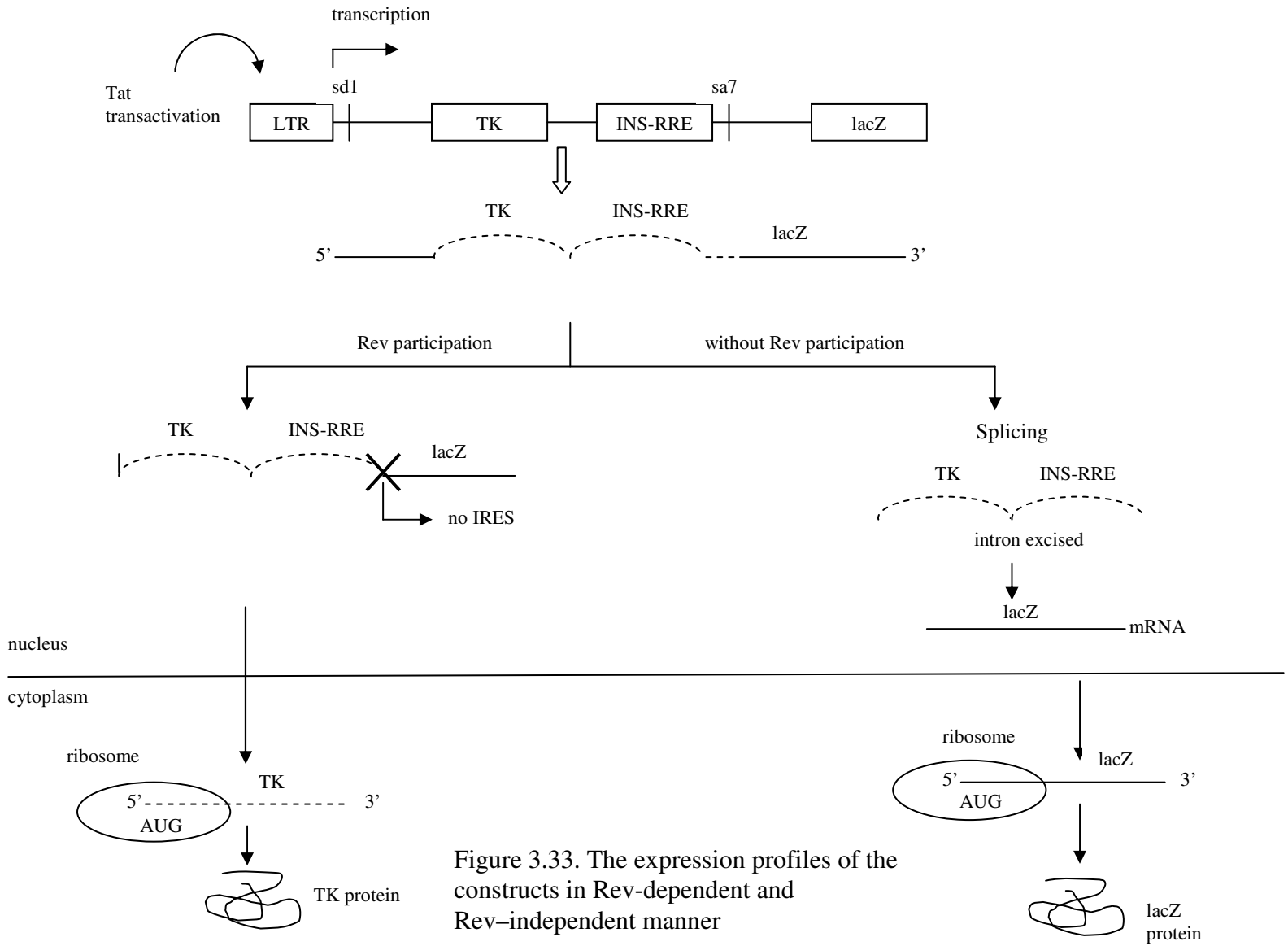


Figure 3.33. The expression profiles of the constructs in Rev-dependent and Rev–independent manner

3.5.1. Construction of pREP-LTR-INS-RRE-CAT Plasmid Vector

The same strategy was employed to construct pREP-LTR-INS-RRE-CAT vector as in the case of the construction of pREP-LTR-INS-RRE-TK suicide vector. First, the reporter gene CAT (chloramphenicol acetyl transferase) in pCI-neo plasmid was amplified by the forward primer CAT-5 and the reverse primer CAT-3 which contain restriction sites for KpnI and HindIII respectively. The 685-bp length PCR-amplified fragment was double digested with KpnI and HindIII and the resulting 659-bp length fragment was cloned into the same restriction sites exist in the multiple cloning site of pREP9 to construct pREP-CAT (Fig. 3.34). pREP-CAT plasmid was employed to yield 670-bp length CAT fragment which then subcloned as a KpnI-NotI fragment into pREP-LTR-INS-RRE to construct pREP-LTR-INS-RRE-CAT (Fig. 3.35).

Upon the transformation of ligated KpnI-HindIII excised fragments from pREP9 and pCI-neo for the generation of pREP-CAT, 73 Ap-resistant colonies were obtained and 11 colonies were screened for the amplification of CAT gene. The generation of pREP-CAT vector was confirmed since one colony (no:2) gave the size of correct amplicon (Fig. 3.36). Upon the transformation employed for the generation of pREP-LTR-INS-RRE-CAT, many Ap-resistant colonies (approximately 150) obtained and 22 of them were subjected to the PCR analysis for CAT amplification. Excluding one colony (no:7) the colonies were all positive for the correct size of amplified CAT (Fig. 3.37) No: 1 transformant was chosen for further analysis including the double digestion of its plasmid DNA with KpnI and NotI together with PCR analysis repeated once more (Fig. 3.38).

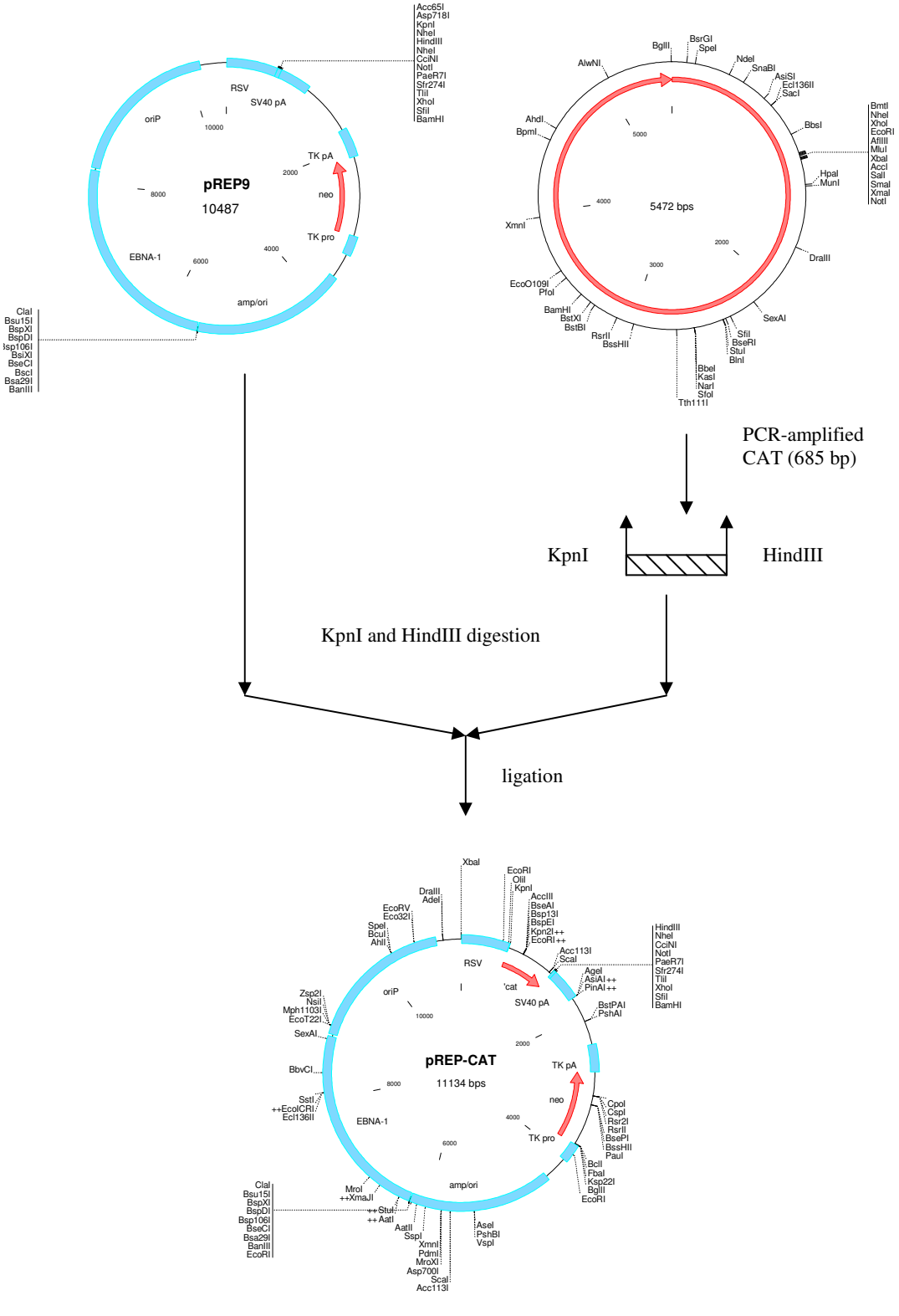


Figure 3.34. Construction of pREP-CAT

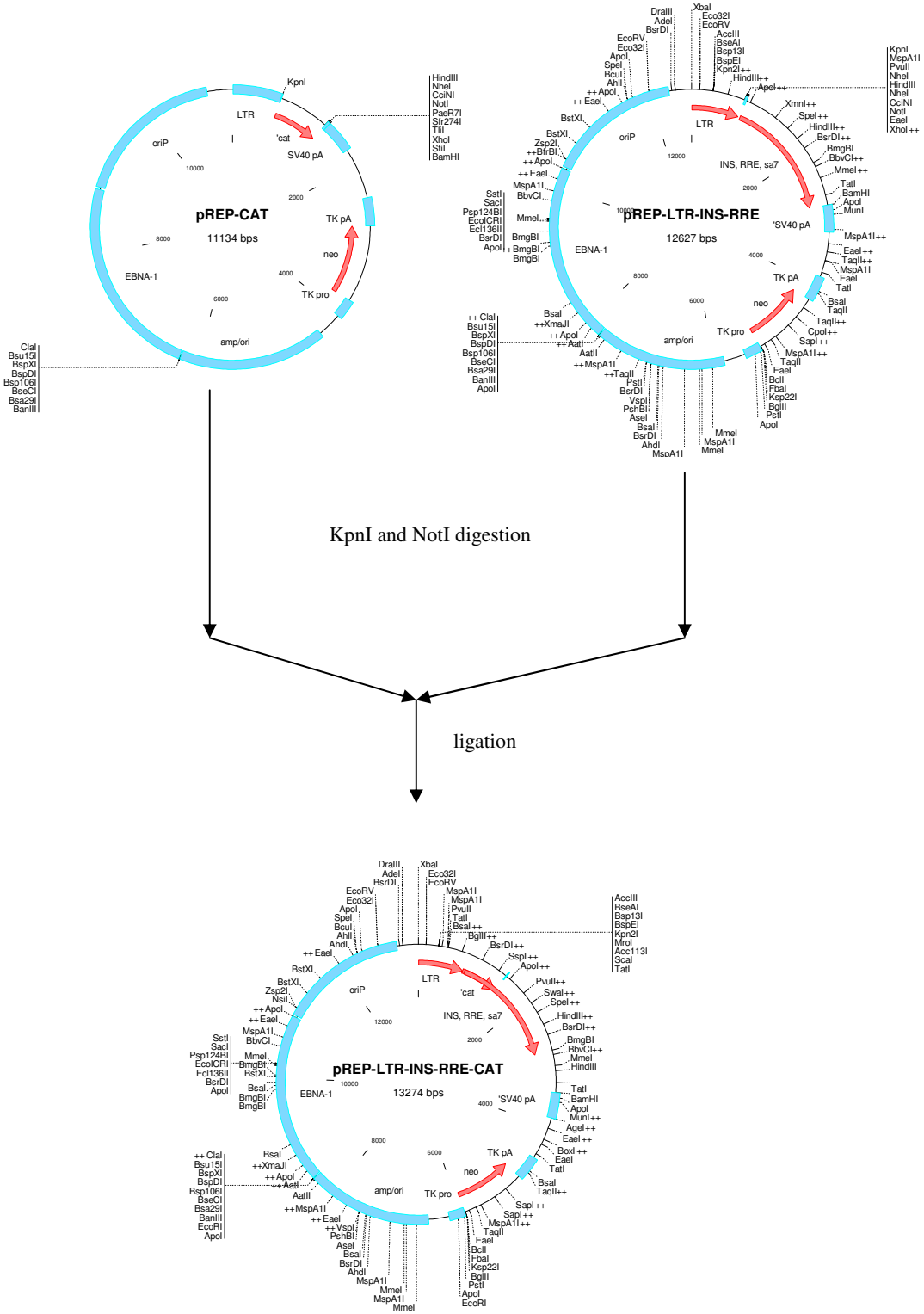


Figure 3.35. Construction of pREP-LTR-INS-RRE-CAT

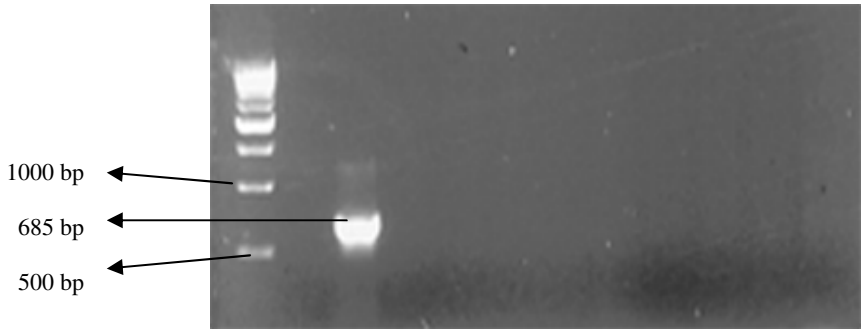


Figure 3.36. Confirmation of the generation of pREP-CAT by PCR
Lanes: 1. 0.5-5.0 kb DNA marker; 2-12: 11 colonies amplified by CAT5 and CAT3

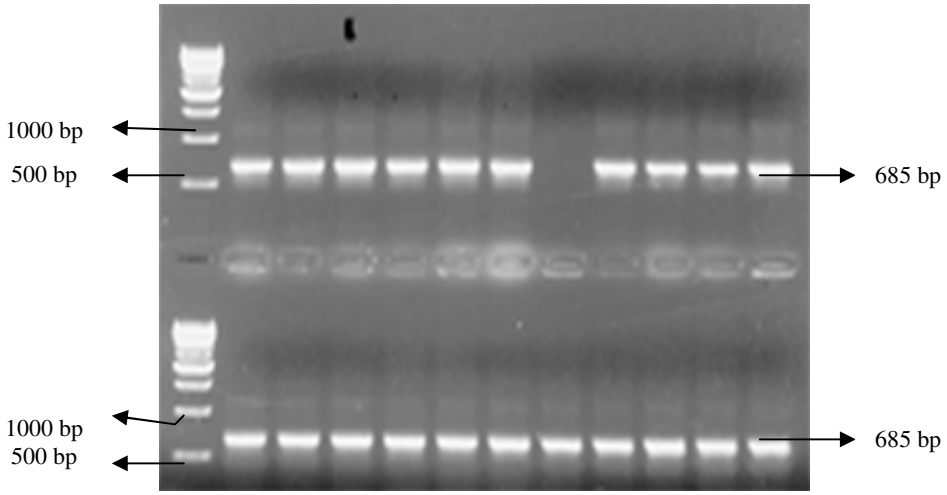


Figure 3.37. PCR amplification of the Ap-resistant colonies for pREP-LTR-INS-RRE-CAT
Lanes above: 1. 0.5-5.0 kb DNA marker; 2-12: colonies (no: 1-11) tested
Lanes below: 0.5-5.0 kb DNA marker; 2-12: colonies (no: 12-22) tested

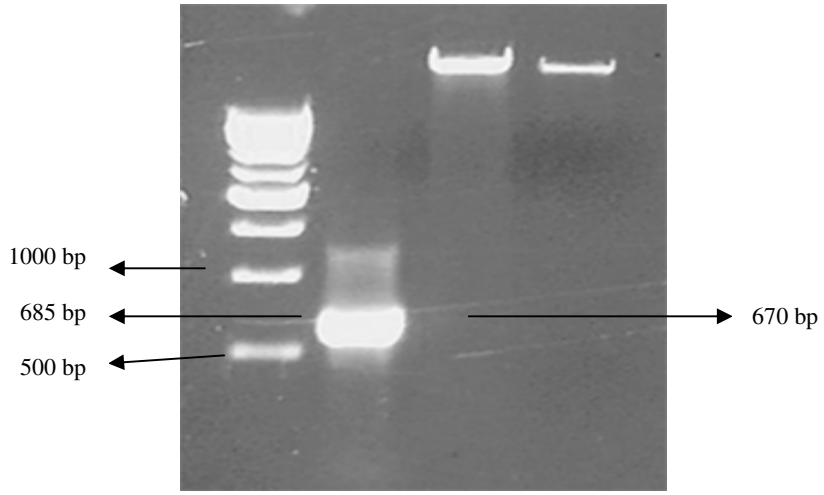


Figure 3.38. Confirmation of the construction of pREP-LTR-INS-RRE-CAT
Lanes: 1. 0.5-5.0 kb DNA marker; 2. pREP-LTR-INS-RRE-CAT amplified by primers CAT5 and CAT3; 3. pREP-LTR-INS-RRE-CAT digested with KpnI-NotI; 4. control: pREP-LTR-INS-RRE digested with KpnI-NotI

3.5.2 Construction of pREP-LTR-INS-RRE-CAT-lac Vector

The plasmid vector pREP-LTR-INS-RRE-CAT-lac that will allow the constitutive expression of lacZ gene was constructed by amplifying lacZ from pcDNA 3.1 /HisB/lacZ plasmid by primer pair lac5 and lac3 which carry BamHI restriction sites at both ends and the resulting excised amplicon of 3195-bp lenth was cloned into the BamHI site of pREP-LTR-INS-RRE-CAT (Fig. 3. 39) Since this is not a positional cloning, the orientation of the insert was verified by the correct choice of restriction enzyme that will allow to the discrimination of the recombinants of whether lacZ gene was cloned in the desired or reversed orientation.

Upon transformation of ligated BamHI-excised fragments for the generation of pREP-LTR-INS-RRE-CAT-lac, 13 colonies were obtained and half of these colonies were employed for the amplification of lacZ. Plasmids of 2 colonies in which amplification carried out (no: 3 and no: 7) were used for the confirmation tests that were managed by both PCR analysis to proove the presence of the insert and restriction analysis to analyze the orientation of lacZ gene. With PCR analysis, resulting amplicon of correct size demonstrated the generation of the recombinant molecule (Fig. 3. 40). The second step needs to be controlled was the orientation of lacZ gene in the resulting recombinant molecule and according to the assesment of plasmid maps of possible 2 recombinants, NheI restriction enzyme was found convenient to be used for this purpose. Digestion of the desired recombinant molecule with NheI results with 2 fragments of 14406-bp and 2063-bp lengths. On the other hand, 2 fragments of 11301-bp and 5168-bp lengths are generated upon digestion of the recombinant molecule where lacZ was cloned in the reversed orientation. So, the clear discrimination between 2063-bp lenth fragment and 5168-bp lenth fragment is informative in terms of the determining the orientation of lacZ gene (Fig. 3.41)

The evaluation of the results obtained with both PCR analysis and restriction analysis proved the construction of pREP-LTR-INS-RRE-CAT-lac plasmid vector.

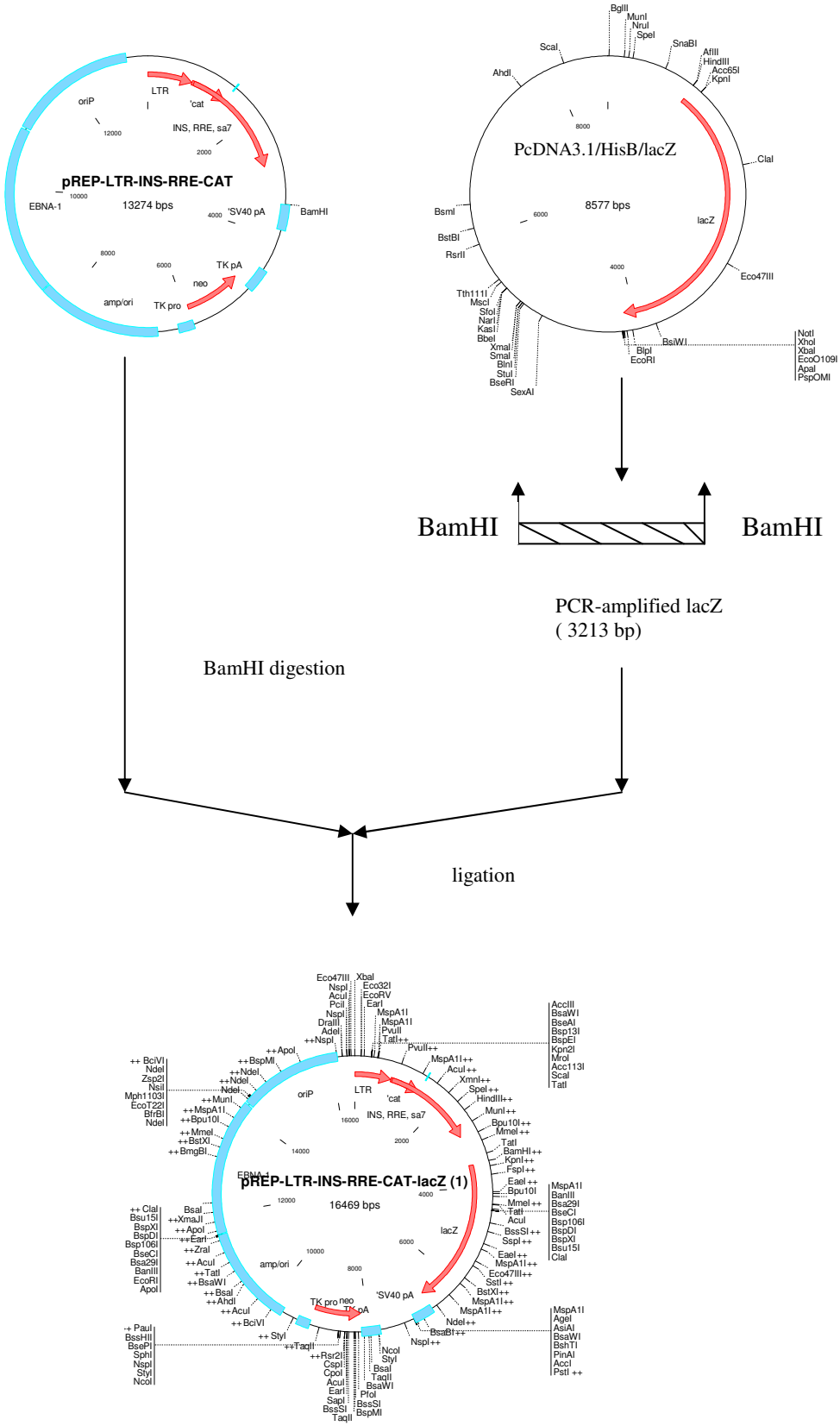


Figure 3.39. Construction of pREP-LTR-INS-RRE-CAT-lac

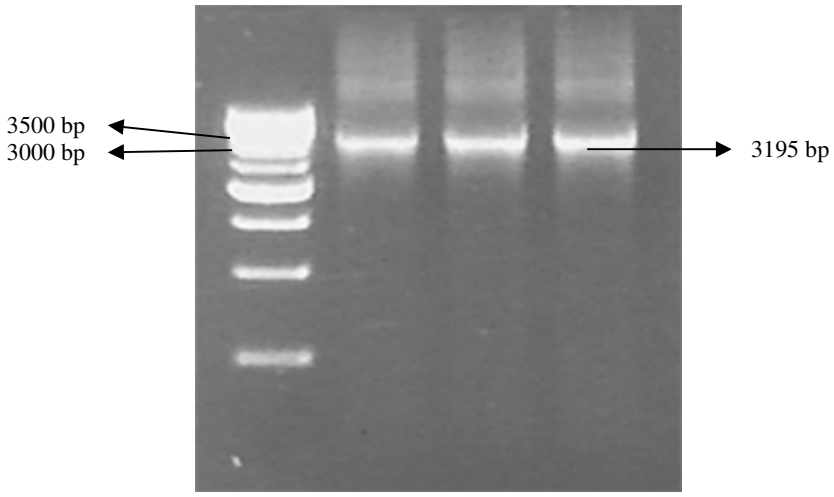


Figure 3.40. PCR analysis of Ap-resistant transformants by primers lac5 and lac3
Lanes: 1. 0.5-5.0 kb DNA marker; 2. positive control: pcDNA3.1/HisB/lacZ amplified; 3. transformant 3 amplified; 4. transformant 7 amplified; 5. negative control: pREP-LTR-INS-RRE-CAT amplified

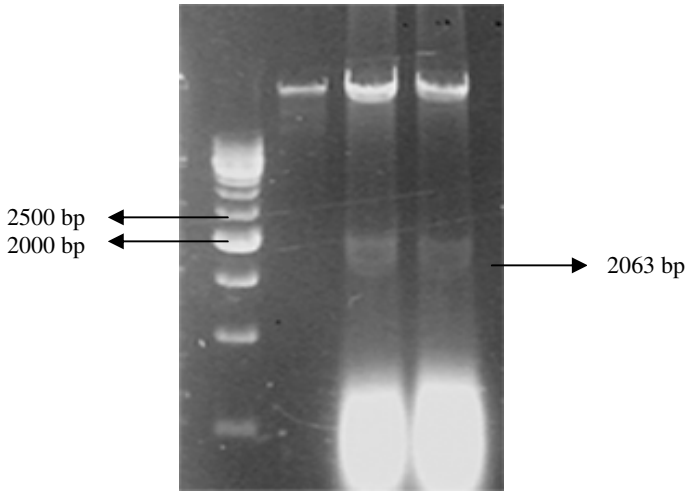


Figure 3.41. Restriction analysis to confirm the orientation of pREP-LTR-INS-RRE-CAT-lac
Lanes: 1. 0.5-5.0 kb DNA marker; 2. control: pREP-LTR-INS-RRE-CAT digested with NheI; 3. transformant 3 digested with NheI; 4. transformant 7 digested with NheI

3.5.3. Construction of pREP-LTR-INS-RRE-TK-lac Vector

Firstly, the cloning route followed for the construction of pREP-LTR-INS-RRE-TK-lac was the same as the construction of pREP-LTR-INS-RRE-CAT-lac vector. Using the plasmid pcDNA.3.1/HisB/lacZ as the template, lacZ gene was amplified by primers lac5 and lac3 that have BamHI sites at both ends and the excised amplicon was cloned into the BamHI site of the suicide vector pREP-LTR-INS-RRE-TK. The presence and the orientation of the lacZ was assessed by both PCR analysis and restriction analysis again. What we determined was the likelihood of a deletion was in question since the amplification reaction for TK gene in pREP-LTR-INS-RRE-TK-lac did not result with the correct size of amplicon. In order to search this deletion we considered the positions of restriction enzymes' sites in pREP-LTR-INS-RRE-TK-lac and managed some control digestions according to these calculations. The digestions that were carried out and the interpretations of the bands seen in Fig. 3. 42 are as below:

- The double digestion of pREP-LTR-INS-RRE-TK-lac with XbaI and KpnI yields 3 fragments of 795-bp, 3262-bp, and 12886-bp lengths. Since these sites do not interfere with the open reading frame of TK, only some estimations may be made, and according to our estimations the band expected to be 3262-bp length was approximately 2800-bp length.
- The double digestion of pREP-LTR-INS-RRE-TK-lac with XbaI and NheI yields 3 fragments of 1934-bp, 2063-bp, and 12946-bp lengths. The situation above is valid for the digestions employed with these enzymes as well, the restriction sites do not interfere with orf of Tk gene. On the other hand, the 1934-bp length fragment was estimated as 1500-bp length and when these data considered together our opinion of a possible deletion was stronger.
- SacI digestion of pREP-LTR-INS-RRE-TK-lac yields 4 fragments of 748-bp, 4782-bp, 7882-bp, and 3531-bp lengths. What could be diagnostic was that one of the SacI sites was at position 1241 and this interferes with the orf which is between 798 and 1927 of TK. Therefore, if a deletion occurred, this SacI site would have been deleted as well and this will change the lengths of fragments yielded. With the deletion of SacI site in the orf of TK, 3 fragments of 5530-bp, 7882-bp and 3531-bp are yielded. These sites can be seen in Fig. 3.42.

- Lastly, pREP-LTR-INS-RRE-TK-lac plasmid vector was digested with AccI enzyme and 7 fragments expected to be at lengths of 1092-bp, 719-bp, 4007-bp, 697-bp, 285-bp, 6616-bp, and 3527-bp could not be seen. AccI was a diagnostic enzyme too since one of the AccI sites (at position 1092) interferes with the orf of TK and the deletion of this site results with 6 fragments at lengths of 4619-bp, 719-bp, 4007-bp, 697-bp, 285-bp, and 6616-bp. However, only 3 fragments was observed on the gel and this is not an extraordinary situation if the close lengths of certain bands are taken into account. Thus, the first band seen on the gel refers to 6616-bp length, the second band refers to 4619-bp length and 4007-bp length fragments altogether. Finally, the third band on the gel refers to fragments of 719-bp and 697-bp lengths. The 285-bp length fragment that would generate a very weak signal could not be observed as expected.

  The assesment of all these control digestions emphasized a deletion occurred in TK gene after cloning lacZ. Since a possible deletion was out of question in CAT gene after cloning of lacZ in pREP-LTR-INS-RRE-Cat-lac plasmid vector, it may be attributed to the interaction between sequences of TK and lacZ genes.

Since the construction of pREP-LTR-INS-RRE-TK-lac vector was not possible by this route followed a new strategy was developed.

In the newly developed strategy, the constructed pREP-LTR-INS-RRE-CAT-lac plasmid vector was taken advantage and both pREP-LTR-INS-RRE-CAT-lac and pREP-LTR-INS-RRE-TK suicide vector were digested with XbaI and XhoI restriction enzymes. Then, the 1945-bp length small fragment also containing TK gene together with LTR and INS-RRE sequences were cloned as a XbaI-XhoI fragment into the plasmid vector pREP-LTR-INS-RRE-CAT-lac by replacing the 1471-bp length small fragment including CAT. Thus, with this simple strategy replacing CAT with TK by leaving all control sequences remain the same as before, pREP-LTR-INS-RRE-TK-lac plasmid vector was constructed (Fig. 3.43).

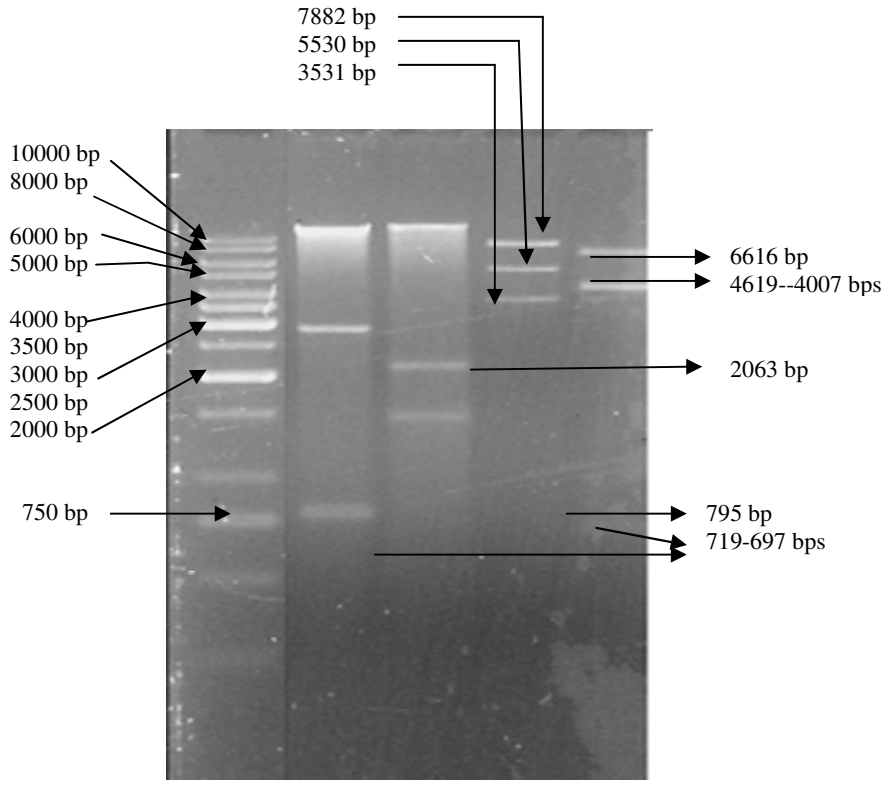


Figure 3.42. Confirmation of the deletion in TK gene in pREP-LTR-INS-RRE-TK-lac Lanes: 1. 10 kb DNA Ladder; 2. pREP-LTR-INS-RRE-TK-lac digested with XbaI and KpnI; 3. pREP-LTR-INS-RRE-TK-lac digested with XbaI and NheI; 4. pREP-LTR-INS-RRE-TK-lac digested with SacI; 5. pREP-LTR-INS-RRE-TK-lac digested with AccI

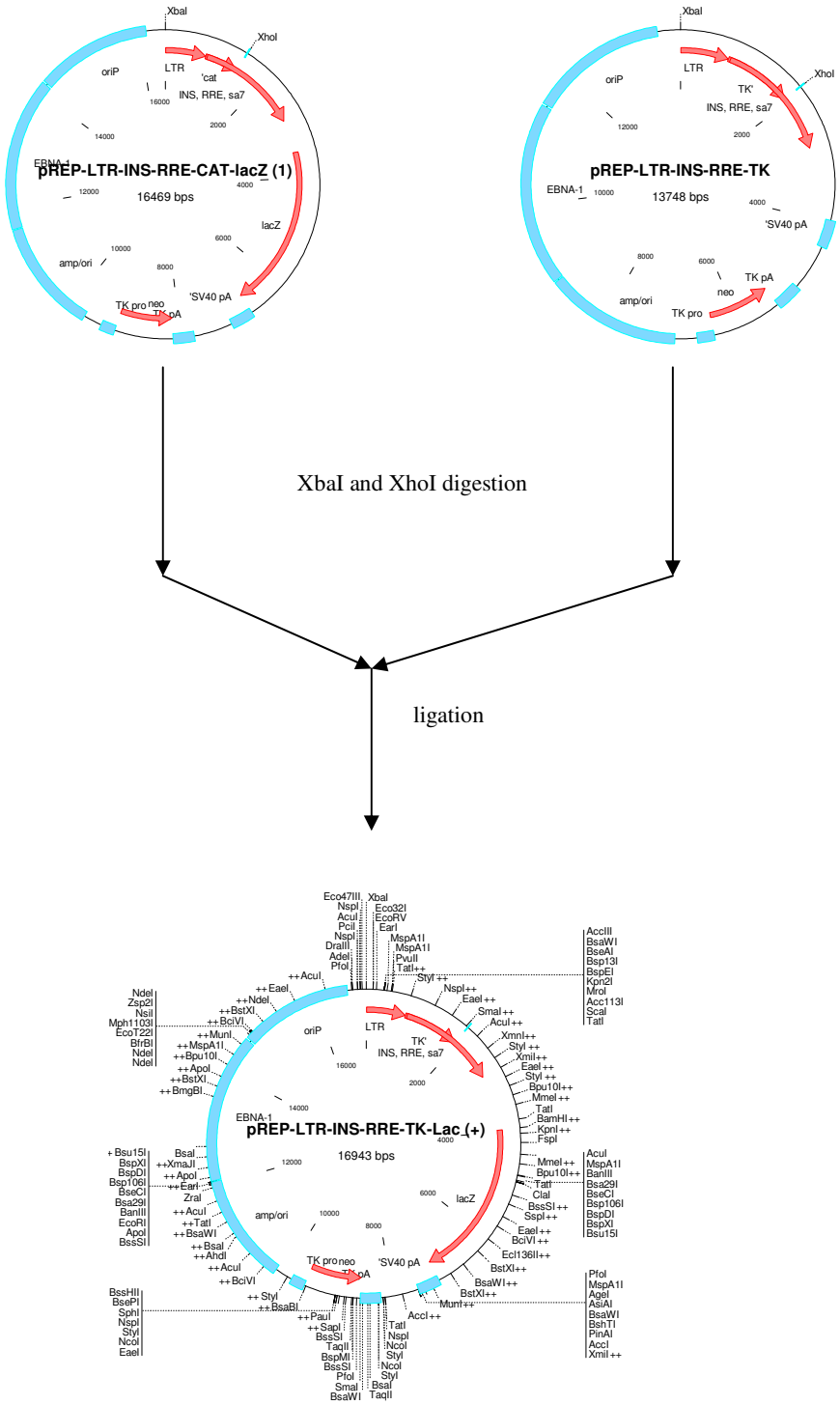


Figure 3.43. Construction of pREP-LTR-INS-RRE-TK-lac

After XbaI-XhoI excised fragments were ligated together, a small volume of this ligation mixture was transformed into competent E. coli cells and 2 Ap-resistant colonies were obtained. These colonies were firstly controlled for the amplification of TK gene by primers TK5 and TK3 in order to have an early idea of whether a deletion was in question this time or not. Both of the colonies gave the correct length corresponding to the amplified TK (Fig. 3.44) Then, the other half of one of these colonies (no:2) were employed for plasmid isolation and all the required control tests were managed with the plasmid DNA. So that, the construction of pREP-LTR-INS-RRE-TK-lac plasmid vector was demonstrated (Fig. 3.45).

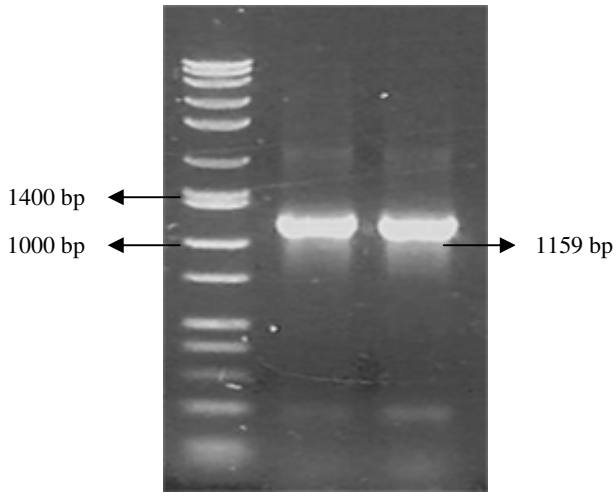


Figure 3.44. PCR analysis of Ap-resistant colonies for pREP-LTR-INS-RRE-TK-lac
Lanes: 1. 10 kb marker; 2. sample 1 amplified by TK5 and TK3; 3. sample 2 amplified by TK5 and TK3

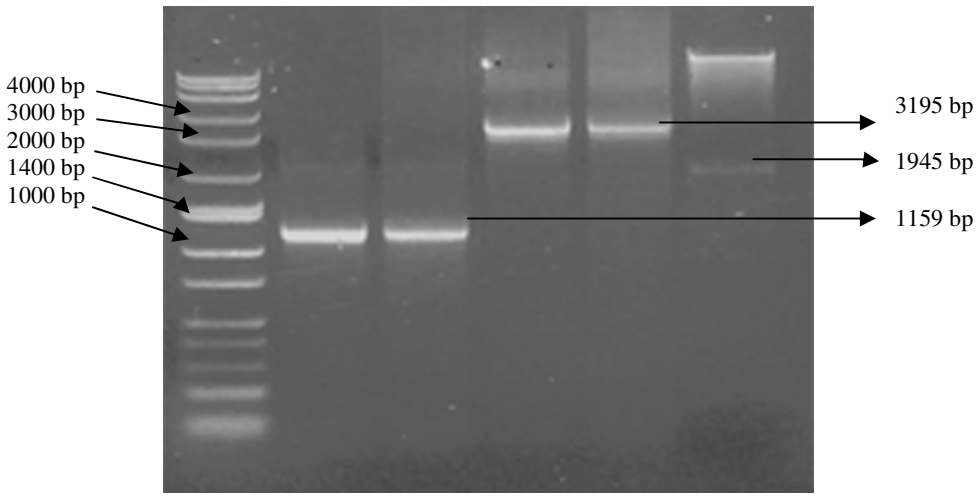


Figure 3.45. Confirmation of the construction of pREP-LTR-INS-RRE-TK-lac
Lanes: 1. 10 kb marker; 2. positive control: pREP-LTR-INS-RRE-TK amplified by primers TK5 and TK3; 3. pREP-LTR-INS-RRE-TK-lac amplified by primers TK5 and TK3; 4. positive control: pcDNA 3.1/HisB/lacZ amplified by primers lac5 and lac3; 5. pREP-LTR-INS-RRE-TK-lac amplified by primers lac5 and lac3; 6. pREP-LTR-INS-RRE-TK-lac digested with XbaI and XhoI

3.6. Shuttle Vectors: From Prokaryotic to Eukaryotic Host Cells

Up to now, all of the studies were conducted in the bacterial host *E. coli*. Since the convenient way for the continuation of required manipulations that prokaryotic cells offer, they are highly preferred in the recombinant DNA technology. After the desired recombinant molecules were generated, the effect of specific genes or regulatory sequences can be investigated upon transfer of these DNA molecules into eukaryotic cells. Shuttle vectors thus enable the transfer of DNA molecules between organisms even if they are not so close to each other evolutionally. Since the plasmid vectors constructed in this study have both replication origins of *E.coli* and mammalian cells enabling the maintenance of DNA in two systems, these constructs could be transferred to eukaryotic host cell with the aim of studying the effects and interactions of specific sequences cloned. Transmission of DNA into a eukaryotic cell is somehow similar to bacterial tranformation with the difference of the term used for this transfer (transfection).

As eukaryotic cells, HeLa (name origins from the name of the patient which these cells were first taken and then distributed to different regions of the world, the abbreviation for Henrietta Lacks) cells were employed. The story of HeLa cells was tissue from the epidermoid carcinoma of the human cervix was placed in roller tube cultures on February 8, 1951, which then results in a strain of malignant epithelial cells described as strain HeLa. After the several passages of these cells from this year on, the stock cultures of HeLa cells were established (George Gey, 1953) In this study, HeLa cells were a matter of choice because of the potential advantages such as rapid growth, high plating efficiency and transfection efficiency they offer.

3.6.1. Transfection of HeLa Cells with Expression Vectors Carrying HIV-1 Regulatory Genes

HeLa parental cells were cultured in DMEM supplemented with 10% FBS and the confluency of the cells was assessed with an inverted microscobe and when they were approximately 50% confluent, they were transfected with plasmid vectors carrying HIV-1 regulatory genes with changing concentrations. Before transfections

experiments, DNAs to be used in transfections were further purified. 3 different transfections were employed: HeLa cells transfected with pMEP-IRES-tat (13 μg), HeLa cells transfected with pMEP-IRES-rev (6.5 μg) and HeLa cells transfected pMEP-IRES-rev-tat (1.8 μg). 2 days after transfection, the cells were selected with 250 μg/ml hygromycin until clones appeared. The untransfected HeLa cells and untranfected but exposed to hygromycin pressure HeLa cells served as controls. All of the untransfected HeLa cells grown in the presence of hygromycin died in 1-2 days as expected. Parental HeLa cells remained the same and they were continued to be passaged. The situations of transfected HeLa cells were controlled every 1-2 days and a few healthy cells present for each transfected HeLa cell did not remain the same. While hygromycin-resistant colonies were generated for Hela cells transfected with pMEP-IRES-tat in approximately one month period, the few healthy cells for Hela transfected with pMEP-IRES-rev and HeLa transfected with pMEP-IRES-rev-tat could not resist no more and did not survive. It is likely that the concentrations of DNAs used in the transfection effected the transfection efficiency. Transfections for pMEP-IRES-rev and pMEP-IRES-rev-tat were repeated again with DNA concentrations ranging between 10-15 μg. It is emphasized that the optimum amount of DNA may vary from 10 to 30 μg depending on the cell line and the preperation of DNA (Okayama et. al., 1987). In the following one month, hyromycin-resistant clones of HeLa cells harboring pMEP-IRES-rev and pMEP-IRES-rev-tat plasmids were generated.

It is important to emphasize that HeLa-rev, HeLa-tat, and HeLa-rev-tat are the names given to cell lines in which the expression of the cloned genes were demonstrated. However, these names were used in the study to correspond to hygromycin-resistant clones of HeLa cells transfected with either pMEP-IRES-rev, pMEP-IRES-tat, or pMEP-IRES-rev-tat plasmid vectors, for the sake of simplicity.

3.6.2. Western Blot Analysis of Cell Lysates

Since hygromycin-resistant clones of HeLa-tat were the ones that were generated lastly, they could not be employed for the first Western Blotting analysis. On the other hand, two other, HeLa-rev and HeLa-rev-tat cell lines were subjected to the Western blotting analysis for the detection of both target proteins rev and tat, which are 19-kD and 15 kD respectively. Western blotting was established by using monoclonal

antibodies for rev (cat no: 7376) and tat (cat no: 1974) (supplied through NIH AIDS Research and Reference Reagent Program), and secondary antibody (Anti-Mouse IgG Alkaline Phosphatase Conjugate) (Sigma) which was used with its substrate BCIP/NBT. Untransfected HeLa cells served as negative control in Western blotting analysis.

Although any band was not observed corresponding to 15kD or 19 kD for HeLa cell lysates employed for Western blotting analysis, the detection of the band corresponding to the molecular mass of rev in HeLa-tat and also the detection of the band corresponding to the molecular mass of tat in HeLa-rev were confusing results which directed us to repeat blotting but this time by using only one monoclonal antibody at the period of the blotting establishment.

HeLa, HeLa-rev, HeLa-tat, HeLa-rev-tat cell lysates were employed for Western blotting for the detection of 15-kD Tat with the monoclonal antibody for Tat different from the previous one (cat no: 4138).

Nonspecific bands corresponding to molecular mass of Tat was obtained even for untransfected HeLa cells and somehow these bands were observed in the emty lanes as well.

Though monoclonal antibodies have advantage over polyclonal antibodies in terms of the specificity they present since they target only one epitope, it does not every time prevent the generation of false positive signals. The interpretations of the results obtained with Western blotting were cumbersome since they could be related with the cross-reactions among proteins searched and proteins present in HeLa, as well as with the deteriorations in the antigenic structures of proteins stemming from either SDS-PAGE or transfer process. Bacause of these reasons, these results were attributed as indeterminate.

3.6.3. Genomic DNA Isolation from Transfected HeLa Cell Lines

When the complexity of structural and functional relationships among macromolecules considered, together with the technical problems stem from the very sensitive detection methods for gene expression, it does not make sense to speak unfavorably of expression for the searched genes. Therefore, two steps back were targeted with the idea of demonstrating the presence of searched genes in cell lines presumed to be generated. This was quite important because if the presence of searched

genes could be detected in eukaryotic cells, then new techniques could be developed for the detection of gene expression whether at RNA or protein level.

With the purpose mentioned above, HeLa to serve as negative control, HeLa-rev, HeLa-tat and HeLa-rev-tat cell lines were employed for genomic DNA isolation. After the confirmation of the presence of genomic DNA in all cell lines, these DNAs were used as templates for PCR analysis. Tat amplification was observed only in HeLa-tat cells. Rev amplification did not carry out in HeLa-rev and HeLa-rev-tat cell lines. It seemed that PCR conditions were in need to be optimized for the detection of rev amplification (Fig. 3.46).

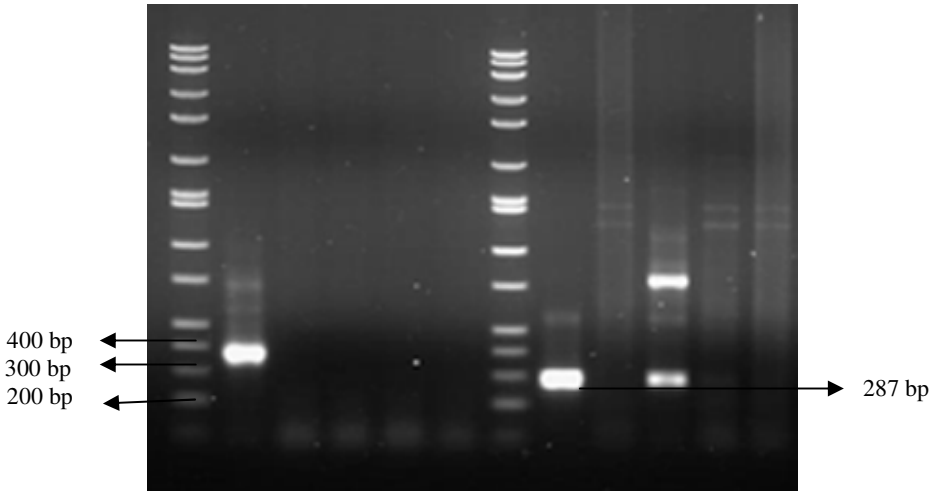


Figure 3.46. PCR analysis of genomic DNAs isolated from transfected cell lines for the amplification of rev and tat
Lanes: 1. 10 kb marker DNA; 2. positive control: pCV1 amplified by primers REV-NHE5 and REV-NHE3; 3. HeLa-rev amplified by primers REV-NHE5 and REV-NHE3; 4. HeLa-tat amplified by primers REV-NHE5 and REV-NHE3; 5. HeLa-rev-tat amplified by primers REV-NHE5 and REV-NHE3; 6. negative control: HeLa amplified by primers REV-NHE5 and REV-NHE3; 7. 10 kb marker DNA; 8. positive control: pCV1 amplified by primers TAT-NHE5 and TAT-NHE3; 9. HeLa-rev amplified by primers TAT-NHE5 and TAT-NHE3; 10. HeLa-tat amplified by primers TAT-NHE5 and TAT-NHE3; 11. HeLa-rev-tat amplified by primers TAT-NHE5 and TAT-NHE3; 12. negative control: HeLa amplified by primers TAT-NHE5 and TAT-NHE3

3.6.4. Total RNA Isolation from HeLa Cell Lines

HeLa, HeLa-rev, HeLa-tat and HeLa-rev-tat cell lines were employed for RNA isolation and the quantity of the RNAs to be used for the RT-PCR reaction was standardized to 2.5 µg. G3PDH housekeeping gene was employed in a separate PCR cocktail to confirm the conversion of the transcripts into complementary DNA. cDNAs of HeLa, HeLa-rev, HeLa-tat and HeLa-rev-tat were screened for the amplification of tat and rev genes (Figure 3.47).

RNA isolation was carried out for 2 times for HeLa-tat cell line, called as HeLa-tat1 for the initially isolated RNA and HeLa-tat2 for the one later isolated.

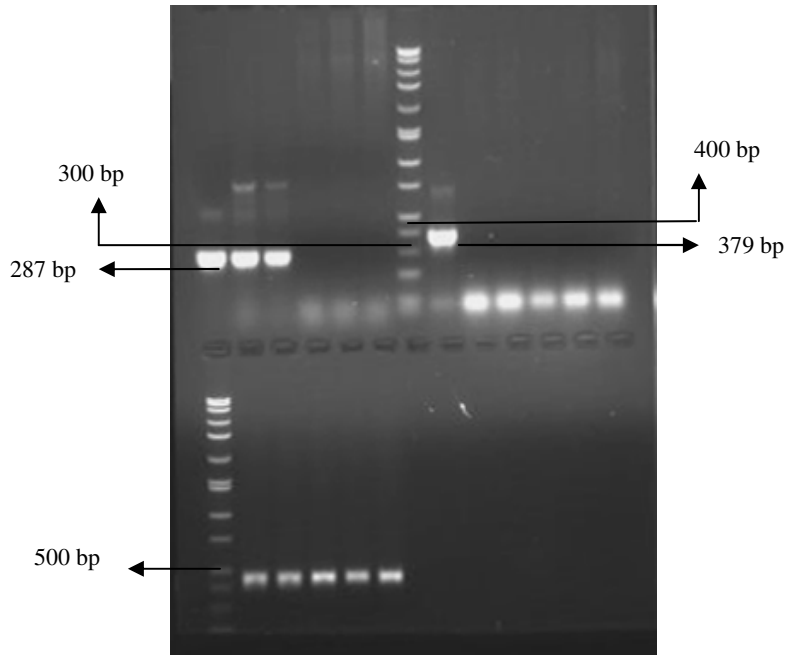


Figure 3.47. Amplification patterns of tat and rev genes from cDNAs of cell lines
Lanes above: 1. positive control for PCR: pCV1 amplified by primers TAT-NHE5 and TAT-NHE3, 2. HeLa-tat1 cDNA amplified by primers TAT-NHE5 and TAT-NHE3; 3. HeLa-tat2 cDNA amplified by primers TAT-NHE5 and TAT-NHE3; 4. HeLa-rev cDNA amplified by primers TAT-NHE5 and TAT-NHE3; 5. HeLa-rev-tat cDNA amplified by primers TAT-NHE5 and TAT-NHE3; 6. HeLa cDNA amplified by primers TAT-NHE5 and TAT-NHE3; 7. 10 kb marker; 8. positive control for PCR: pCV1 rev amplified by primers REV-NHE5 and REV-NHE3; 9. HeLa-rev cDNA amplified by primers REV-NHE5 and REV-NHE3; 10. HeLa-tat1 cDNA amplified by primers REV-NHE5 and REV-NHE3; 11. HeLa-tat2 cDNA amplified by primers REV-NHE5 and REV-NHE3; 12. HeLa-rev-tat cDNA amplified by primers REV-NHE5 and REV-NHE3; 13. HeLa cDNA amplified by primers REV-NHE5 and REV-NHE3
Lanes below: 1. 10 kb marker; 2-6: internal controls: 2. HeLa cDNA amplified by primers G3PDH5 and G3PDH3; 3. HeLa-rev cDNA amplified by primers G3PDH5 and G3PDH3; 4. HeLa-tat1 cDNA amplified by primers G3PDH5 and G3PDH3; 5. HeLa-tat2 cDNA amplified by primers G3PDH5 and G3PDH3; 6. HeLa-rev-tat cDNA amplified by primers G3PDH5 and G3PDH3

CHAPTER 4

DISCUSSION

In this study, the comparable expression profiles of the toxic gene HSV-1 TK on parental HeLa cells and HeLa cells expressing HIV-1 regulatory genes was desired to be assessed. However, since the generation of stable cell lines expressing Tat and Rev proteins could not be achieved, the possibility of working with the suicide vector pREP-LTR-INS-RRE-TK-lac constructed for this purpose was out of question. What could be done was the several optimizations together with some repeated trials, but these trials were interrupted under the pressure of time constrations.

After hyg-resistant clones of transfected cell lines occurred, the presence of Tat and Rev proteins was tested with Western Blotting. As explained in detail in 'Results' section, the interpretation of the results was far from being accepted as positive. Surely, the universality of the genetic code implies to the translation of protein coding sequences in heterologous systems. On the other hand, there are numerous factors to be considered when the nuclear export of mRNA is in question. With the idea of that the translational activity of a gene would be linked into its history in the nucleus and this could determine the fate of that gene, the research was concentrated at DNA and RNA level as well.

PCR reactions carried out with genomic DNA templates of hyg-resistant transfected cell lines revealed the presence of tat amplicon only in cells transfected with pMEP-IRES-tat. Rev amplicon was observed neither in the cells transfected with pMEP-IRES-rev nor in the ones transfected with pMEP-IRES-rev-tat. This result brings together the question of then how the parental cells managed to cope with the antibiotic selection pressure maintained in the medium during all the passages if they did not uptake the plasmid vectors. I must confess that this situation is quite open to be discussed, on the other hand I may put forward some speculations. İf no hyg-resistant clones were generated, this could be explained by the possibility of that DNA which entered the cell was a target for nucleases since this is occasionally the fate of foreign DNA entering the cell. The transfection procedure itself may sometimes cause to the inefficient entry of the lipid-DNA complex into the cell as explained by Düzgüneş et

al. since the precise mechanisms by which lipid-DNA complexes interact with the cell membrane are unknown. What is known is that the most of DNA is taken up through endocytosis. Since fusion of the endocytotic vesicles with lysosomes can lead to the degradation of DNA, the foreign DNA must enter the cytoplasm before this fusion event for a successful transfection (Düzgüneş et al., 1996).

Leaving this possibility aside by considering the generation of hyg-resistant cells, according to my idea, this situation may be explained with the nature of the plasmids. The plasmid vectors constructed in the study for transfections origin from pMEP4 which is an EBV-based plasmid vector. Recombinant DNA plasmids containing oriP and a dominant selectable marker gene, such as G418 resistance or hygromycin resistance can be maintained in an extrachromosomal form in cells, provided that EBNA-1 gene which permits episomal maintenance in cells not expressing other EBV-encoded proteins was incorporated into such plasmids (Lupton et al., 1985; Yates et al., 1985). There are two EBV genetic elements constituting oriP, that are required in cis for the extrachoromosomal maintenance of plasmids: a 30-bp sequence repeated 20 times and a 65-bp sequence arranged in a dyad (Lupton et al., 1985; Reisman et al., 1985). What is interesting is that some tolerable deletions in these regions may still give rise to stimulated numbers of drug-resistant colonies, though not being capable of extrachromosomal plasmid maintenance (Lupton et al., 1985). The same effect may also be observed with EBNA-1 gene when some specific kinds of deletions are in question. If the introduced plasmid vector presumed to be maintained extrachromosomal does not behave in that way, one may contemplate about then what happens. It is a probability that it contacts with the chromosomal DNA. In fact, it was demonstrated that oriP could confer mitotic stability in cells containing EBNA-1 and cause the artificial plasmid to associate with condensed human chromosomes during mitosis (Simpson et al., 1996) and EBNA-1 itself could associate with human metaphase chromosomes (Grogan et al., 1983). This may be a mechanism developed by the cell to keep plasmids during mitosis in order to prevent their loss to the cytoplasm. Certainly, the given information does still not explain the PCR result obtained with genomic DNA templates, on the other hand it may be the explanation of drug-resistant cells. Any picture can not be drawn explicitly unless the repeated transfection trials are made. In the case that though repeated transfections with standardized conditions and DNA concentrations, the presence of searched genes will have not been verified, it was only then I might put forward my hypothesis that if there is a probability for episomal

DNA to be in contact with the chromosomal DNA of the cell, then it may also be in contact with the same factors that chromosomal DNA does, such as histones, or histone-like proteins. If this interaction is in question especially in the target sequences being searched for, this may retain the sequence-specific primers from attaching to their target sites.

As the later search topic, total cytoplasmic RNAs of hyg-resistant transfected cell lines were isolated and subjected to RT-PCR. The only positive result was the amplicon corresponding to tat cDNA in cells transfected with pMEP-IRES-tat. This result is important in terms of demonstrating that the promoter that directed the expression of the first gene in either of the constructs was active. On the other hand, since no band was observed corresponding to tat and rev cDNA in cells transfected with pMEP-IRES-rev-tat, an approximate comparison between the first gene expression and second gene expression which is dependent upon the promoter IRES could not be made.

The demonstration of the expression of tat at RNA level but not at protein level could be related with the lack of splice sites. Splicing is not essential for nuclear export, on the other hand it may facilitate the recruitment of mRNAs for translation. According to Luo and Reed, mRNA splicing can result not only in the removal of nuclear retention factors but also in the selective recruitment of nuclear RNA export factors (Luo and Reed, 1999). Presuming that the contradictory result obtained with Western Blotting was ignored, the very poor bands observed could be explained with the insufficient entry of mRNAs into the cytoplasm.

After the experienced handicaps mentioned are overwhelmed in the future, the model created will be able to prove its potency strongly.

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APPENDIX A

Compositions of Buffers and Stock Solutions

1. LB broth, per liter

10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 ml 1 N NaOH

2. LB agar, per liter

When LB broth was saturated with ~ 14-15 g agar, LB agar was obtained.

3. dNTP (10X)

10 µl of each 100 mM dATP, dCTP, dGTP and dTTP were taken. After they were mixed briefly, 460 µl sterile dH_2O was added and mixed again. Hence, 2 mM concentration was obtained for each of them.

4. 50X TAE Electrophoresis Buffer

242 g Tris base and 37.2 g Na_2EDTA ($2H_2O$) was dissolved in 900 ml deionized water. After adding 57.1 ml glacial acetic acid, the volume was adjusted to 1 liter with water.

5. Solutions Used in Alkali-lysis Plasmid Isolation Procedure:

Solution I:

- 50 mM glucose
- 25 mM Tris-HCl pH 8.0
- 10 mM EDTA pH 8.0

Storage: 4°C

Solution II:

- 0.2 N NaOH (stock: 10N, 5N or 1 N)
- 1% SDS (stock: 10%)

Storage: RT

Solution III, per 0.1 liter

- 5 M potassium acetate (KAc) (60 ml)
- glacial acetic acid (11.5 ml)

- H_2O (28.5 ml)

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.
Storage: 4°C

6. Versene Solution (in mM) pH 7.2

- 0.537 EDTA
- 136.8 NaCl
- 2.68 KCl
- 8.1 Na_2HPO_4
- 1.47 KH_2PO_4

7. CMF-PBS (Calcium-magnesium free-phosphate buffered saline), per liter

- 0.2 g KCl
- 0.2 g KH_2PO_4
- 8.0 g NaCl
- 1.15 g Na_2HPO_4

8. Acrylamide/ bisacrylamide solution

30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide

9. Ammonium persulfate, 10%

0.1 g ammonium persulfate was dissolved in 1 ml water. It was discarded after use since it was freshly prepared every time.

10. 100 mM $CaCl_2$ solution (for competent cells)

By dissolving 11.1 g $CaCl_2$ in 100 ml ultra pure H_2O, 100 ml 1 M $CaCl_2$ solution was obtained. It was filter sterilized and divided to 10 ml aliquots, then stored at -20°C. When required for the preperation of competent cells, one aliquot was taken and let to be thawn. Then, 90 ml ultra pure dH_2O was added and the resulting mix was filter sterilized again. So that, freshly prepared 100 mM 100 ml $CaCl_2$ was obtained.

11. EDTA (ethylenediamine tetraacetic acid) (0.5 M)

186.1 g EDTA was dissolved in 800 ml of deionized water and pH was adjusted to 8.0 with 10 N NaOH. Volume was brought to 1000 ml with deionized water.

12. Ethidium bromide (10 mg/ml)

0.2 g ethidium bromide (EtBr) was dissolved in 20 ml dH_2O. It was mixed well and stored at 4°C in dark.

13. PCR mixture (50 µl)

- Mg free Taq DNA polymerase buffer 5 µl
- $MgCl_2$ (25 mM) 4 µl
- Sterile dH_2O 33.5 µl
- Forward primer (10 picomole/µl) 1 µl
- Reverse primer (10 picomole/µl) 1 µl
- dNTP (2 mM) 5 µl
- Taq DNA polymerase 0.5 µl (2.5 U)

14. Freezing mixture

90% FBS, 10% DMSO (filter-sterilized)

15. Protein loading buffer (2X)

- 200 mM DTT*
- 50 mM Tris-HCL pH 6.8
- 50% glycerol
- 4% SDS
- 0.01% bromophenol blue
    - DTT was added at the stage of sample loading from a 1 M stock solution.

16. Transfer buffer (5X) pH 8.3

- 195 mM glycine
- 240 mM Tris base
- 0.185% SDS

For 1X working concentration; 100 ml 5X transfer buffer, 100 ml methanol and 300 ml dH_2O were mixed to obtain the required 500 ml 1X solution.

17. Tris-glycine electrophoresis buffer (5X)

15.1 g Tris base and 94 g glycine were dissolved in 900 ml dH_2O. Then, 50 ml 10% (w/v) stock solution of SDS was added and the volume was adjusted to 1000 ml with dH_2O.

18. Blocking solution

5% non-fat dried milk * in PBS

- added at the stage of usage

19. Phosphate-free blocking solution

- 5% (w/v) non-fat dried milk *
- 150 mM NaCl
- 50 mM Tris-Cl pH 7.5
    - added at the stage of usage

20. Wash solution (1X)

1% (v/v) Tween 20 in PBS

21. Phosphate-free wash solution (1X)

- 150 mM NaCl
- 50 mM Tris-Cl pH 7.5

22. RIPA buffer

- 150 mM NaCl
- 10 mM Tris-HCl pH 7.6
- 1% Triton X-100
- 1% Nadeoxycholate
- 0.1% SDS

23. PMSF (phenylmethylsulfonylfluoride)

100 mM PMSF was dissolved in isopropanol and filter-sterilized. It was stored at -20°C to be used at 2mM working concentration when required.

24. Antibiotic stock solutions

Antibiotic	Stock solution conc.	Working concentration
Ampicillin	50 mg/ml in dH_2O	50 µg/ml
Tetracyclin	5 mg/ml in dH_2O	10 µg/ml
Hygromycin B	48 mg/ml in dH_2O	250 µg/ml
G418	50 mg/ml in dH_2O	300 µg/ml

APPENDIX B

Primers (Restriction sites are underlined)

Primer	Sequence
TAT-NHE5	5'-CCT TCT CGG CTA GCA TGG AGC CAG TAG ATC CTA G-'
TAT-NHE3	5'-CCT TCT CGG CTA GCC TAT TCC TTC GGG CCT GTC GG-3'
TAT-NHE5-1	5'-CCT TCT CGG CGG CCG CAT GGA CCC AGT AGA TCC TAG-3'
TAT-NHE3-1	5'-CCT TCT CGG CGG CCG CCT ATT CCT TCG GGC CTG TCG-3'
REV-NHE5	5'-CCT TCT CGG CTA GCA TGG CAG GAA GAA GCG GAG AC-3'
REV-NHE3	5'-CCT TCT CGG CTA GCC TAT TCT TTA GCT CCT GAC TCC-3'
IRES	5'-GTT GAC GCA AAT GGG CGG TA-3'
ires2	5'-ACA CGA ACA CCG GGC GTC TG-3'
LTR-5	5'-CCT TCT CGT CTA GAT GGA AGG GCT AAT TTG GTC CC-3'
LTR-3	5'-CCT TCT CGG GTA CCC TCC TTC TAG CCT CCG CTA G-3'
INS-5	5'-CCT TCT CGC TCG AGG AGA TGG GTG CGA GAG CGT C-3'
RRE-3	5'-AGT GCT AAG GAT CCG TTC ACT AAT CG-3'
TK-5	5'-CCT TCT CGG GTA CCA TGG CTT CGT ACC CCT GCC ATC-3'
TK-3	5'-CCT TCT CGA AGC TTT CAG TTA GCC TCC CCC ATC TCC-3'
CAT-5	5'-CCT TCT CGG GTA CCA TGG AGA AAA AAA TCA CTG G-3'
CAT-3	5'-CCT TCT CGA AGC TTC GCC CCG CCC TGC CAC-3'
lac5	5'-TTC TCG GGA TCC ACC ATG GGG GGT TCT CAT-3'
lac3	5'-TTC TCG GGA TCC TGC AGA ATT CGG CTT TAT TA-3'