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DEVELOPMENT OF TECHNIQUES FOR TESTING THE EFFECT OF THE E1 REGION ON ADENOVIRUS INTEGRATION

By

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ABSTRACT

Adenoviral vectors are currently the most widely used gene therapeutic vectors, but their inability to integrate into host chromosomal DNA shortened their transgene expression and limited their use in clinical trials.

In this project, we initially planned to develop a technique to test the effect of the early region 1 (E1) on adenovirus integration by comparing the integration efficiencies between an E1-deleted adenoviral vector (SubE1) and an E1-containing vector (SubE3). However, we did not harvest any SubE3 virus, even if we repeated the transfection and successfully rescued the SubE1 virus (2/4 transfections generated viruses) and positive control virus (6/6). The failure of rescuing SubE3 could be caused by the instability of the genomic plasmid pFG173, as it had frequent internal deletions when we were purifying it.

Therefore, we developed techniques to test the effect of E1 on homologous recombination (HR) since literature suggested that adenovirus integration is initiated by HR. We attempted to silence the E1 in 293 cells by transfecting E1A/B-specific small interfering RNA (siRNA). However, no silenced phenotype was observed, even if we varied the concentrations of E1A/B siRNA (from 30 nM to 270 nM) and checked the silencing effects at different time points (48, 72, 96 h). One possible explanation would be that the E1A/B siRNA sequences are not potent enough to induce the silenced phenotype.

For evaluating HR efficiencies, an HR assay system based on bacterial transformation was designed. We constructed two plasmids (designated as pUC19-dl1 and pUC19-dl2) containing different defective *lacZα* cassettes

(forming white colonies after transformation) that can generate a functional *lacZα* cassette (forming blue colonies) through HR after transfecting into 293 cells. The HR efficiencies would be expressed as the percentages of the blue colonies among all the colonies. Unfortunately, after transformation of plasmid isolated from 293 cells, no colony was found, even at a transformation efficiency of 1.8×10^7 colonies/μg pUC19, suggesting the sensitivity of this system was low.

To enhance the sensitivity, PCR was used. We designed a set of primers that can only amplify the recombinant plasmid formed through HR. Therefore, the HR efficiencies among different treatments can be evaluated by the amplification results, and this system could be used to test the effect of E1 region on adenovirus integration. In addition, to our knowledge there was no previous studies using PCR/ Realtime PCR to evaluate HR efficiency, so this system also provides a PCR-based method to carry out the HR assays.

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ABBREVIATIONS

AAV - Adeno-associated virus
Ad - Adenovirus/ Adenoviral
$CaCl_2$ - Calcium chloride
CAR - Coxsackie and adenovirus receptor
cDNA - Complementary DNA
CFTR - Cystic fibrosis transmembrane regulator
CMV - Cytomegalovirus
C_T - Cycle of threshold
CTL - Cytotoxic T lymphocytes
DEPC - Diethylpyrocarbonate
DMSO - Dimethylsulfoxide
DNA - Deoxyribose nucleic acid
DNase - Deoxyribonuclease
dNTP - Deoxyribose nucleoside triphophate
dsRNA - Double stranded RNA
DTT - Dithiothreitol
EDTA - Ethylenediaminetetraacetic acid
EtBr - Ethidium bromide
HEK 293 - Human embryonic kidney 293
HeLa - Henrietta Lacks cervical carcinoma cell line
HEPES - 1-Piperazineethane sulfonic acid
HSV - Herpes simplex virus
HSV-tk - Herpes simplex virus thymidine kinase
ITR - Invert terminal repeat
KCl - Potassium chloride
LacZ - β-galactosidase
LB - Luria broth
MHC - Major histocompatibility complex
MOPS - 3-(N-Morpholino) propanesulfonic acid

mRNA	-	Messenger RNA
NaCl	-	Sodium chloride
NPC	-	Nuclear pore complex
OD	-	Optical density
ORF	-	Open reading frame
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
Retro	-	Retrovirus/ retroviral
RISC	-	RNA-induced silencing complex
RNA	-	Ribonucleic acid
RNAi	-	RNA interference
RNase	-	Ribonuclease
SCID	-	Severe combined immunodeficiency
SCID-XI	-	X-linked severe combined immunodeficiency
SDS	-	Sodium dodecyl sulphate
siRNA	-	Small interfering RNA
SV40	-	Simian virus 40
TAE	-	Tris-acetate EDTA
TE buffer	-	Tris-EDTA buffer
UV	-	Ultraviolet
X-gal	-	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

1. INTRODUCTION

The adenovirus was first isolated in 1953 (Rowe et al., 1953; Hillemann & Werner, 1954) from human adenoidal tissue, and was hence named. Since then, it has been widely used in molecular biology research. For example, the adenovirus "E1" region has been intensively studied because its protein products have vast regulatory functions, such as *trans*-activation/ repression of many host and virus genes, change of the physiological status of host cells, and oncogenic transformation of rodent cells.

Many papers about gene therapy using adenoviral vectors can be found on the National Center for Biotechnology Information (NCBI) website. Adenoviral vectors are the most extensively used vectors in gene therapy. They can be used in cancer gene therapy, vaccination against infectious diseases, and treatment of monogenic disorders. However, the transient expression of adenoviral vectors limited their use in clinical trials. Scientists developed many strategies to prolong the expression of adenoviral vectors, but so far there is no success. Nonetheless, if an adenoviral vector could integrate into the host genome, the long-term expression of a transgene can be achieved and gene therapy can be successful.

In this study, our goal is to develop techniques for testing the effect of the E1 region on adenovirus integration. Here, I will first introduce the overview of adenoviruses and then elaborate on the E1 region. Next, I will explain the strategies to prolong the expression of adenoviral vectors and finally, I will present a literature review on adenovirus integration.

1.1 Adenovirus Overview

1.1.1 Classification

Adenovirus belongs to the family *Adenoviridae*, which can be further divided into 4 different genera: Atadenovirus, Aviadenovirus, Mastadenovirus, and Siadenovirus. Human adenovirus belongs to Mastadenovirus; it contains 6 subgroups (from A to F) and 51 serotypes (Figure 1.1), the division of which is mainly based on immunological criteria (Benko *et al*., 1999).

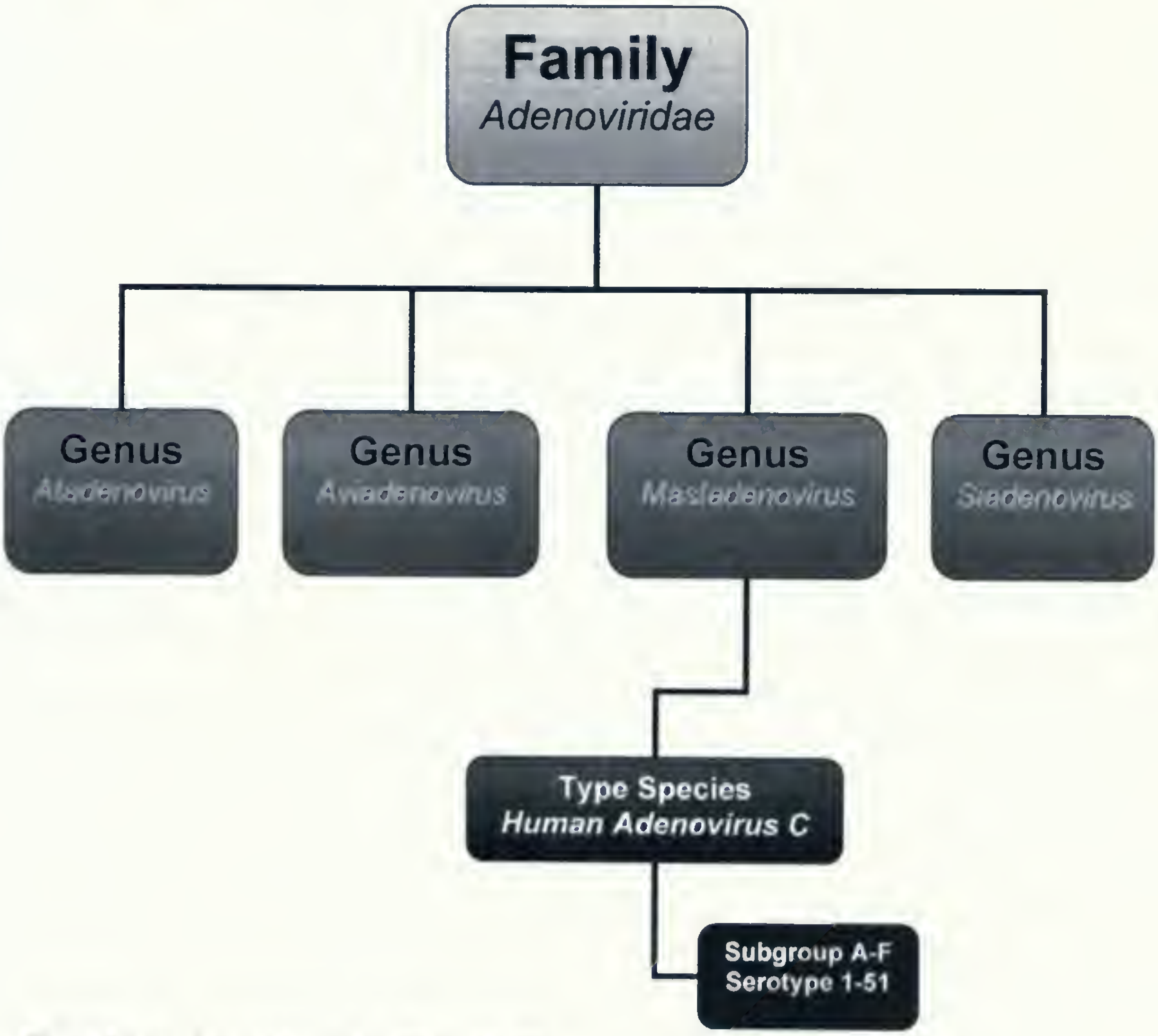

Family
Adenoviridae
Genus
Atadenovirus
Genus
Aviadenovirus
Genus
Mastadenovirus
Genus
Siadenovirus
Type Species
Human Adenovirus C
Subgroup A-F
Serotype 1-51

Figure 1.1. Taxonomy of *Adenoviridae*.

The classification of human adenoviruses with different immunological properties is also shown in Table 1.1. One index in this table, the percentage of GC in adenoviral DNA, is thought to be negatively correlated to the oncogenic potential of adenovirus (Green, 1970).

Table1.1. Classification of human adenoviruses (modified from Baum, 1984).

Subgroup	Serotypes	Hemagglutination groups	Oncogenic potential		GC%
			Tumors in animals	Transformation in tissue culture	
A	12, 18, 31	Little or no agglutination	High	+	48-49
B	3, 7, 11, 14, 16, 21, 34, 35	Complete agglutination of monkey erythrocytes	Moderate	+	50-52
C	1, 2, 5, 6	Partial agglutination of rat erythrocytes	Low or none	+	57-59
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42, 47	Complete agglutination of rat erythrocytes	Low or none	+	57-61
E	4		Low or none	+	57-59
F	40, 41		Unknown		

Adenoviridae is speculated to share a common ancestor with the enterobacterium bacteriophages of the family *Tectiviridae* (Davidson *et al.*, 2003).

1.1.2 Structure

Adenoviruses are non-enveloped, icosahedral particles (Horne *et al.*, 1959; Stewart *et al.*, 1991). The sizes of these particles are around 70-100 nm in diameter. The viral particles contain 13% of DNA and 87% of protein (percentages are based on mass ratios). The structure of an adenovirus is shown in Figure 1.2, and the designations, locations and functions of different adenoviral proteins are listed in Table 1.2.

The capsid of an adenovirus virion is composed of 252 capsomeres including 240 hexon capsomeres and 12 penton capsomeres; 12 knobbed fibers are protruded from the penton capsomeres (Khilko *et al.*, 1990). One hexon capsomere consists of three hexon monomers (protein II) (Horwitz *et al.*, 1970). One penton capsomere is composed of a trimeric fiber (peptide IV) and five penton monomers (peptide III) (van Oostrum & Burnett, 1985). These three major proteins (II, III, and IV) make up the outer shell of the adenoviral capsid.

Inside the capsid, several proteins are closely related to the viral DNA. The terminal proteins (TPs) are covalently attached to the 5' termini of adenovirus DNA (Rekosh *et al.*, 1977). The proteins V, VII, and mu are intimately associated with the viral DNA (Anderson *et al.*, 1989), and they probably have histone-like functions. There are also some minor proteins, such as VI, VIII, IX, IIIa and IVa2; which are important in linking the major proteins and keeping the capsid structure stable (Everitt *et al.*, 1973; 1975).

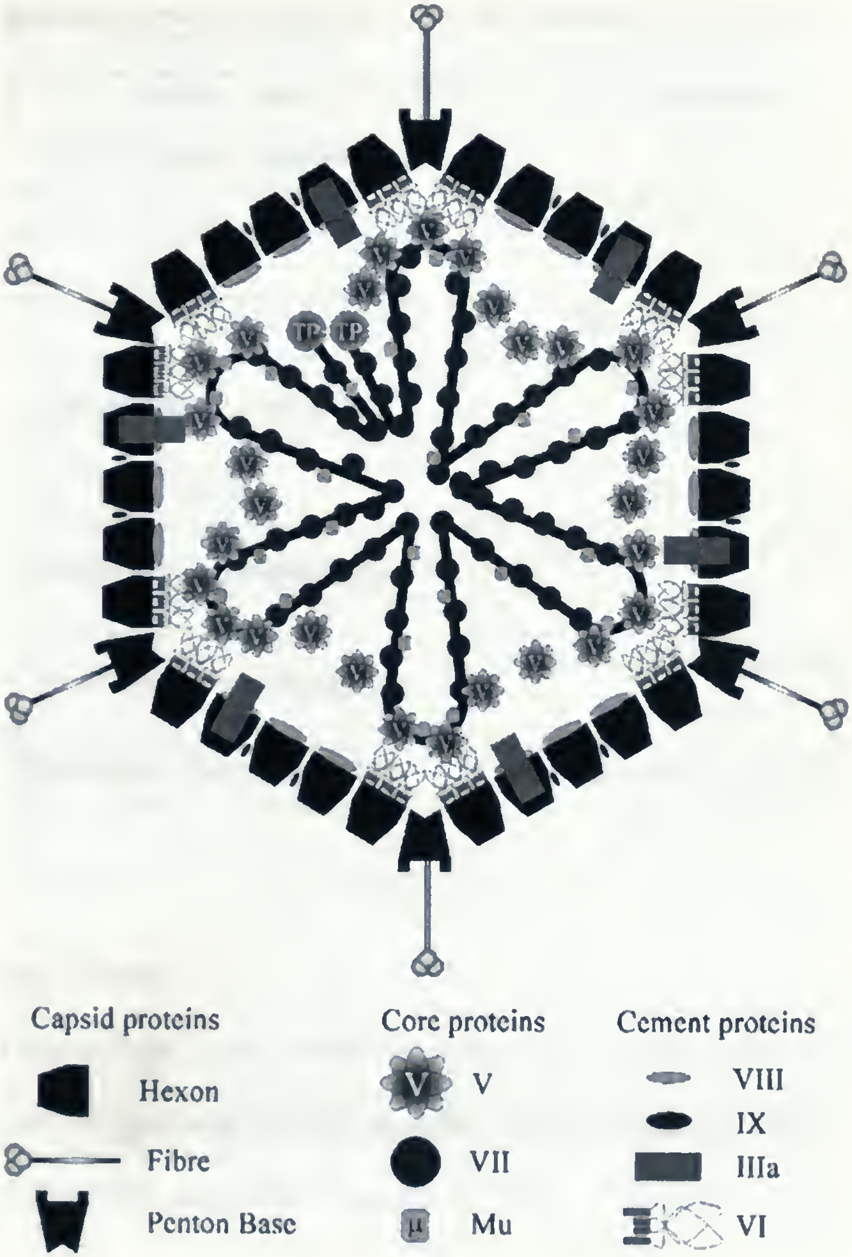

TP
TP
V
Capsid proteins
Hexon
Fibre
Penton Base
Core proteins
V
VII
μ
Mu
Cement proteins
VIII
IX
IIIa
VI

Figure 1.2. Structure of an adenovirus particle (from Russell, 2000).

Table 1.2. Adenoviral proteins and their functions (modified from Shenk, 2001).

Name	Location	Functions
II	Hexon monomer	Structural
III	Penton base	Penetration
IIIa	Penton base-associated	Penetration
IV	Fiber	Receptor binding; haemagglutination
V	Core	Histone-like; packaging
VI	Hexon minor polypeptide	Stabilization/ assembly of particle
VII	Core	Histone-like
VIII	Hexon minor polypeptide	Stabilization/ assembly of particle
IX	Hexon minor polypeptide	Stabilization/ assembly of particle
TP	Genome – Terminal protein	Genome replication

1.1.3 Genetic Content

Adenovirus has linear, double-stranded DNA, with a size of 35-38 kbp. Both ends of the genome contain inverted terminal repeats (ITRs) which are essential in viral DNA replication (Hay *et al*., 1995). Terminal proteins (TPs) are covalently attached to the 5' termini (Rekosh *et al*., 1977) and serve as a primer for DNA replication. The genome contains many coding regions, and the genome structure of Ad5 is shown in Figure 1.3.

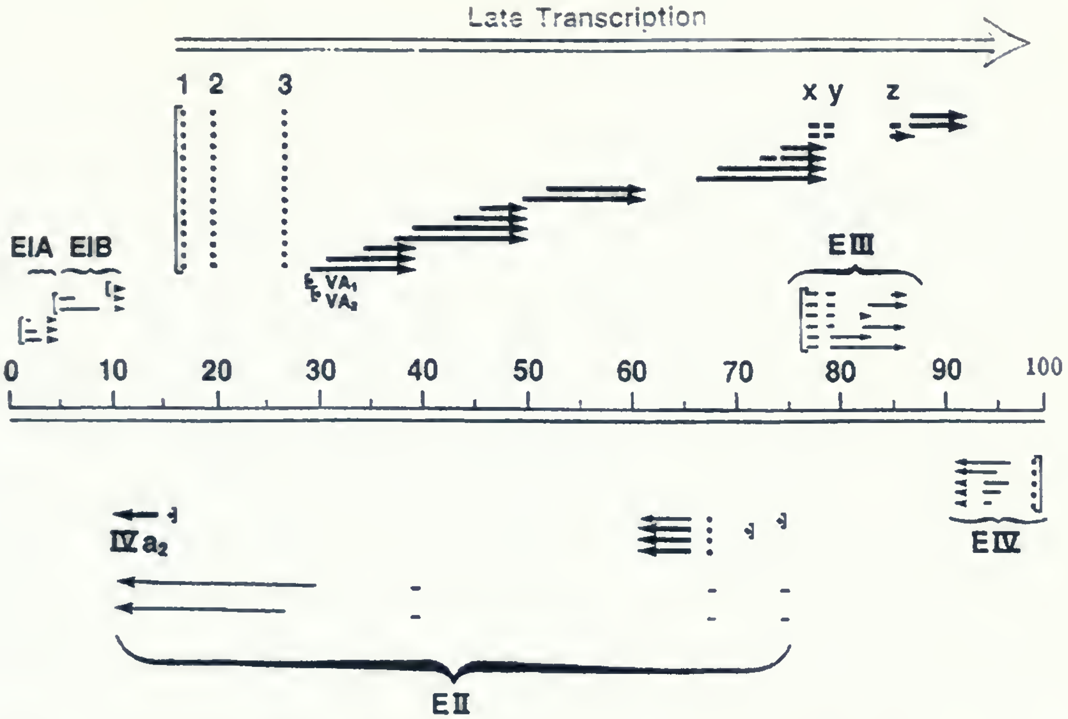


Figure 1.3. Structure of adenovirus serotype 5 genome (Hitt *et al.*, 1998). Messages from the early regions are indicated as light lines and late messages are indicated in bold. Major late transcription unit is indicated by the open arrow.

These transcription units can be grossly divided into two categories, early transcription units (early regions) and late transcription units (late regions), based on the time of these units to be transcribed. There are four early regions in total: E1 (early region 1), E2, E3 and E4. E1 can be further divided into E1A and E1B. E1A is the first gene to be expressed, and its proteins can interact with cellular proteins and *trans*-activate other early genes (i.e. E1B, E2, E3 and E4) (Jones & Shenk, 1979). E1B proteins cooperate with E1A to transform the host cells and to provide optimal viral replication conditions (Rao *et al.*, 1992; Sarnow *et al.*,

1982). The detailed description of adenovirus E1A and E1B functions will be described in section 1.2 “Adenovirus Early Region 1”.

E2 region encodes three enzymes involved in viral DNA replication. They are DNA-binding protein (DBP), viral polymerase (pol) and pTP (precursor of terminal protein), and these provide the machinery for replication of virus DNA (Temperley & Hay, 1992; Hay *et al.*, 1995).

E3 encodes several proteins important in combating the host immune system and one protein to lyse the infected cell. One of these products, the E3 gp19kDa, retains the class I major histocompatibility molecules (MHC I) in the endoplasmic reticulum (Burget & Kvist, 1987), and therefore the cytotoxic T lymphocytes (CTLs) cannot recognize and kill the infected cells. Another protein, the receptor internalization and degradation protein (RID), can internalize and degrade the Fas receptors (Shisler *et al.*, 1997); thus no Fas (apoptotic signal) will be taken by the host cell and apoptosis blocked. In addition, one protein named adenovirus death protein (ADP) can facilitate the lysis of the host cell to release progeny virus efficiently (Tollefson *et al.*, 1996).

E4 functions like a miscellaneous tool box to cooperate with other early genes. Its functions include regulating mRNA accumulation (Weigel & Dobbelstein, 2000), resisting lysis by CTLs (Kaplan *et al.*, 1999), modulating virus replication and host protein synthesis (Halbert *et al.*, 1985) among others.

Adenovirus late regions (including L1-L5) encode structural proteins, which are the components of the adenoviral capsids.

1.1.4 Life cycle

The adenovirus life cycle can be clearly divided into two phases, an early phase and a late phase, which happen respectively before and after the start of viral DNA replication. Its life cycle has several major events including: adsorption, internalization, entering into nucleus, expression of early genes, DNA replication, expression of late genes, virion assembly, disruption of cell membrane and release of progeny virus.

The adsorption of adenovirus to target cells is mediated by the high-affinity interaction between the fiber knob and a cellular receptor. The primary receptor for human adenovirus was shown to be identical to that for coxsakie B virus (Bergelson *et al.*, 1997; Roelvink *et al.*, 1998) and has therefore been termed the coxsakie/ adenovirus receptor (CAR).

Once an adenovirus particle attaches to the cell surface through the primary receptor, it will interact with a secondary receptor and become internalized. The secondary receptors for adenovirus are shown mainly to be αv integrins (Wickham *et al.*, 1993), which can recognize an RGD motif that is exposed on the penton base (Steward *et al.*, 1997). After the binding of integrin to the RGD motif, the attached adenovirus will enter the host cell by receptor-mediated endocytosis (FitzGerald *et al.*, 1983). The diagrams for the adsorption and internalization processes are shown in Figure 1.4.

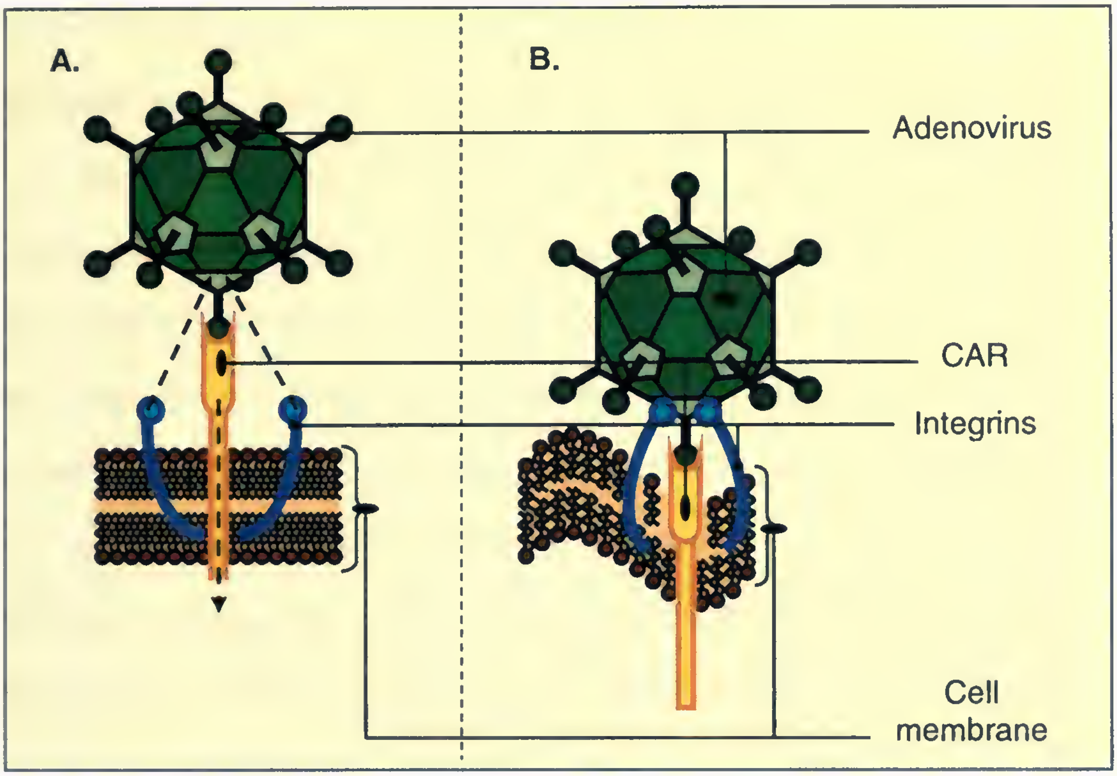


Figure 1.4. Diagram presenting the adsorption and internalization processes of an adenovirus particle. A) The adsorption of adenovirus through the primary receptor CAR. B) Adenovirus internalization mediated by αv integrins. The diagram is adapted from a figure on the website of Krackeler Scientific, Inc. (website: http://www.krackeler.com/products/fid/2862).

The adenovirus enters the host cell plasma through the clathrin-mediated coated-pit pathway, during which clathrin will form a coat to wrap the adenovirus and merge the virus with an endosome (Varga *et al.*, 1991). Then there is a sequential disassembly of the virion (Greber *et al.*, 1993) (Figure 1.5). The virion disruption occurs by selective dissociation and proteolytic degradation of virion constituents, which is mainly mediated by the virus-coded protease (Greber *et al.*,

1994). The disruption of the adenovirus particle helps to lyse the endosome before the virus merges with a lysosome and gets degraded (Greber *et al.*, 1993).

Next, the partially disassembled adenovirus will be transferred to the nucleus region by the dynein and microtubule (Leopold et al., 2000) (Figure 1.5). The dynein functions as a motor protein while the microtubule functions as the framework and superhighway for viral delivery into the nucleus (reviewed in Greber & Way, 2006). Once it arrives at the nuclear pore complex (NPC), the virion will dock to the NPC-filament protein CAN/nup214, and then the histone H1 will form a complex with the hexons and the viral DNA will be imported via such a hexon-histone complex (Trotman *et al.*, 2001). Therefore, by interacting with cellular components, the adenovirus genome can enter into the nucleus efficiently.

Once inside the nucleus, the genome is connected to the nuclear matrix through the TP (Angeletti & Engler, 1998; Fredman & Engler, 1993), and then the transcription of early genes starts. One hour post-infection, the adenovirus-specific transcripts can be detected (Nevins *et al.*, 1979). The first gene to be transcribed is the E1A, and the protein products will *trans*-activate the expression of the other early regions E1B, E2, E3, E4 (Jones & Shenk, 1979). As discussed earlier, the E1A and E1B proteins modify the cell physiological status and facilitate the viral replication; the E2 produces proteins involved in viral DNA synthesis; the E3 gene products work on fighting host immune system; and E4 proteins provide supplementary functions to help completing the virus life cycle. After the expression of all the early proteins, the viral DNA replication starts.

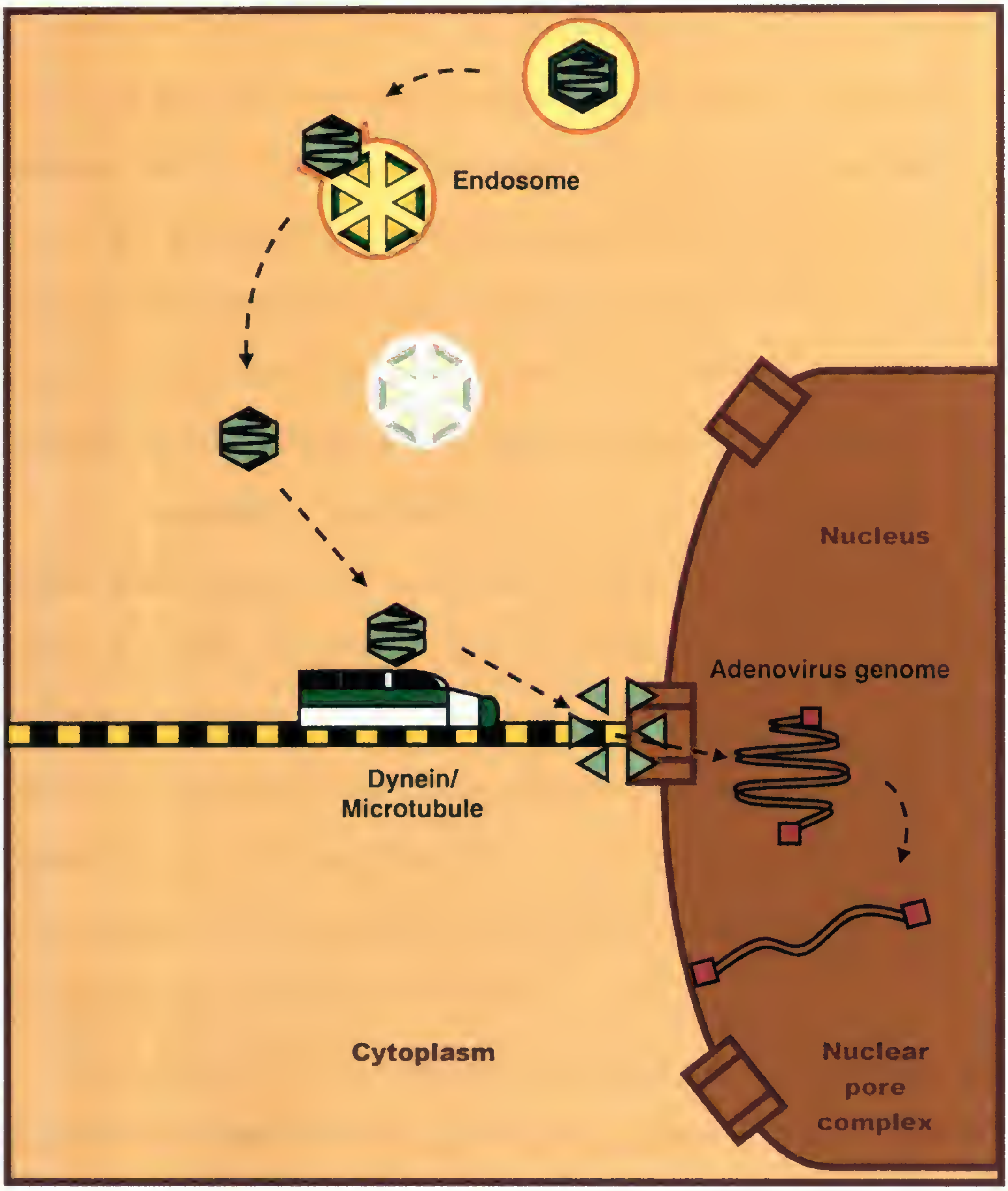

Endosome
Nucleus
Adenovirus genome
Dynein/
Microtubule
Cytoplasm
Nuclear
pore
complex

Figure 1.5. The transportation of the adenoviral genome into the nucleus of a host cell. After the internalization, the adenovirus will be wrapped inside an endosome. The adenovirus disrupts the endosome and then its genome will be transferred into the nucleus by a cellular protein p32. This figure is inspired from Leopold *et al.*, (2000), Trotman *et al.*, (2001) and the virology tutorial of University of Calgary (website: http://www.mcb.uct.ac.za/tutorial/calgary_files/).

New viral DNA is synthesized by the precursor of TP (pTP) (Challberg *et al.*, 1982) and the viral polymerase (Temperley & Hay, 1992). The new strand is synthesized from 5' to 3' and the old strand is displaced. The displaced DNA strand is then circularized through base-pairing of viral left and right ITRs to form a partial double-stranded structure (Lechner and Kelly, 1977). Then pTP and polymerase will synthesize its complementary strand through the partial double-stranded structure (Temperley & Hay, 1992) to replicate more adenoviral DNA.

The expression of adenovirus late genes begins when the viral DNA synthesis starts (Figure 1.6). All the late proteins are controlled by the same promoter, the major late promoter (MLP) (Ziff & Evans, 1978). It has been reported that a cellular transcription factor, designated major late transcription factor (MLTF), can activate the transcription of late genes (Miyamoto *et al.*, 1985). In addition, a virally encoded protein IVa2, which can bind to the binding site of MLP and trigger the transcription of late genes, can also promote the production of late proteins (Lutz & Kendinger, 1996).

During the late phase of the adenovirus life cycle, its E1B 55kDa and E4 orf6 protein can form a complex (Sarnow *et al.*, 1984) to shut off host protein synthesis and enhance viral protein synthesis (Babiss & Ginsberg, 1984; Halbert *et al.*, 1985). The synthesis and processing of cellular transcripts is still active, but the cellular mRNAs cannot accumulate in the cytoplasm, suggesting that their transport is blocked (Beltz & Flint, 1979). Further studies suggested that the E1B-E4 complex interacts with cellular proteins such as AP5 to control the transportation of the viral or host cellular mRNAs (Gabler *et al.*, 1998).

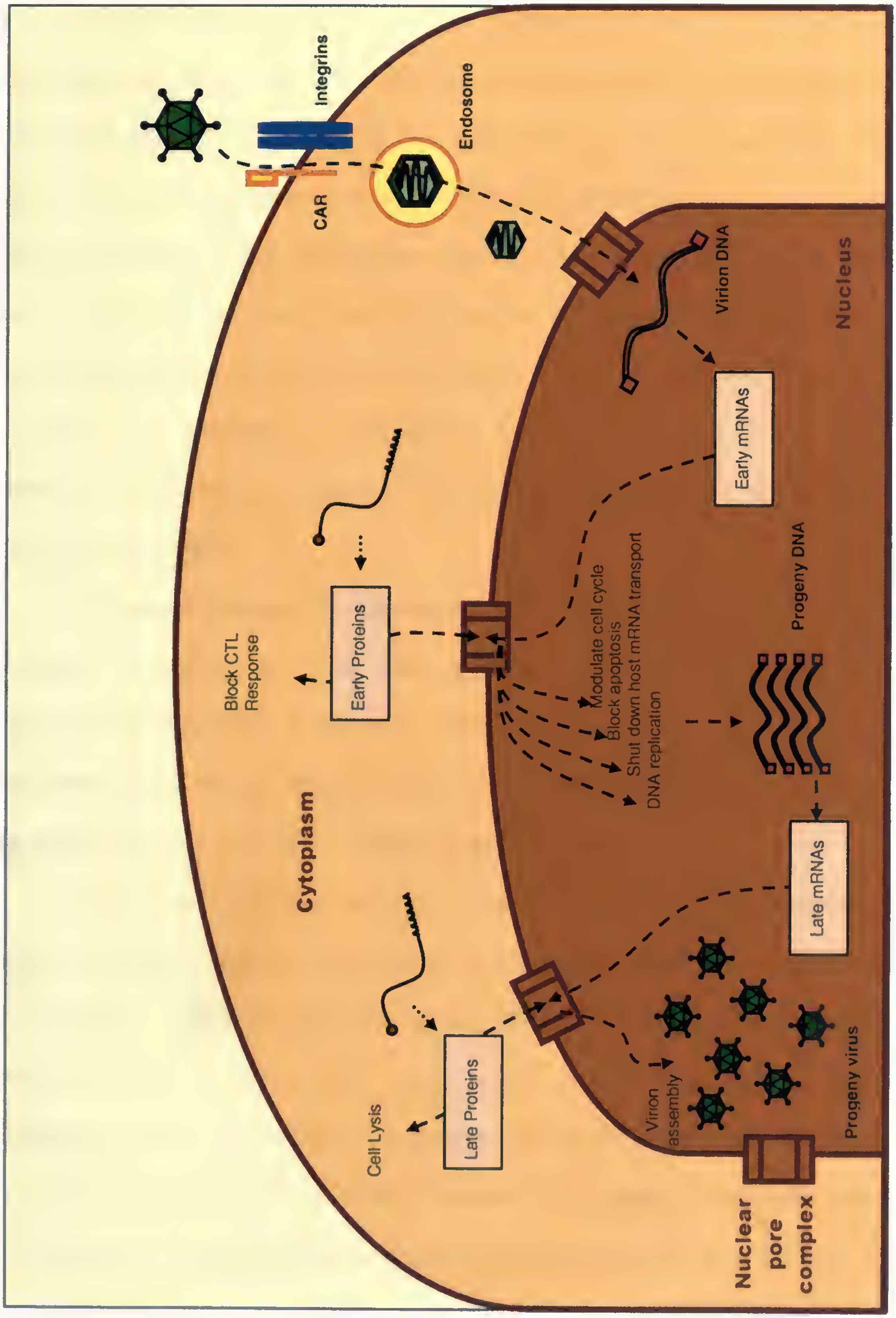

Integrins
CAR
Endosome
Virion DNA
Nucleus
Early mRNAs
Early Proteins
Block CTL Response
Cytoplasm
Modulate cell cycle
Block apoptosis
Shut down host mRNA transport
DNA replication
Progeny DNA
Late mRNAs
Late Proteins
Cell Lysis
Virion assembly
Progeny virus
Nuclear pore complex

Figure 1.6. Adenovirus life cycle overview (reproduced from Shenk, 2001).

Finally the virus will be assembled, the host cell lysed, and the progeny virus released. Once the adenoviral structural proteins are accumulated, the adenovirus assembly begins. The assembly of trimeric hexon capsomers starts after the hexon monomer is synthesized in the cytoplasm (Liebowitz and Horwitz, 1975) and it requires the participation of another late viral protein, the L4 100-kd protein, which functions as a scaffolding protein (Hong *et al.*, 2005). Next is the assembly of penton capsomeres (the complex of penton base and fiber), which are built in the cytoplasm and transferred back to the nucleus (Horwitz *et al.*, 1969). Then the hexon and penton capsomeres will aggregate in the nucleus to form the viral capsids.

The assembled capsids will encapsidate the viral genomic DNA, and this process is governed by a viral DNA packaging signal at the left end of the genome (Hammarskjold & Winberg, 1980). The packaging signal is a DNA sequence about 260 bp long and viral DNA missing this sequence cannot be packaged (Hearing *et al.*, 1987; Grable & Hearing, 1992).

When assembling the new virion, the L3-encoded protease cleaves the cellular proteins to prevent the formation of intermediate filaments and to disrupt the integrity of infected cells (Chen *et al.*, 1993). In addition, an E3-11.6 kDa protein, named the adenovirus death protein (ADP), changes the nuclear infrastructure and permeabilizes the nuclear membrane so the virus can egress into the cytoplasm (Rao *et al.*, 1996; Tollefson *et al.*, 1996). Finally, the plasma membrane will be disintegrated and the virus will be released from the cell. The adenovirus life cycle in an overview is shown in Figure 1.6.

1.2 Adenovirus Early Region 1

Adenovirus early region 1 (E1) regulates multiple cellular and viral genes to facilitate viral replication. It can be subdivided into E1A and E1B. In some extensively studied molecular pathways, E1A can interact with p53-TBP to activate most viral genes, modulate pRb-E2F to induce the infected cells to enter S phase, and regulate p300/CBP to repress the transcription of certain genes such as p53. By these interactions, E1A exerts a profound physiological change in host cells such as oncogenic transformation. While E1A is necessary for transformation of infected cells, it is not enough for a complete transformation, thus E1B is needed to cooperate with E1A to fully transform host cells.

In view that E1A and E1B interact with multiple important cellular proteins, it is possible that the adenovirus E1 region is related to the adenovirus integration process. Because I will carry out experiments with cultured cells only, I will focus on the molecular interactions between E1A/B and cellular proteins, rather than their effects on transformation and oncogenesis in animals.

1.2.1 Early Region 1A

The E1A region of Ad5 is located at 1.3~4.5 map units (related to the adenovirus genome map and one map unit=365 bp). It is the first transcription unit to be expressed after the viral DNA reaches the nucleus, and its transcription is controlled by a constitutively active promoter (Hearing & Shenk, 1986).

The Ad5 E1A region has five mRNA species: respectively 13S, 12S, 11S, 10S, and 9S (Stephens & Harlow, 1987) (Figure 1.7), which were named by their mRNA sedimentation coefficients. Among these five species, the 13S and 12S

mRNA are the major species and are expressed in a large amount during the early phase of infection (Chow *et al.*, 1979). The 11S and 10S mRNA species are minor species and are expressed mainly in the late phase of infection (Stephens & Harlow, 1987; Ulfendahl *et al.*, 1987), and the smallest transcript 9S mRNA is most abundant in late phase (Spector *et al.*, 1980).

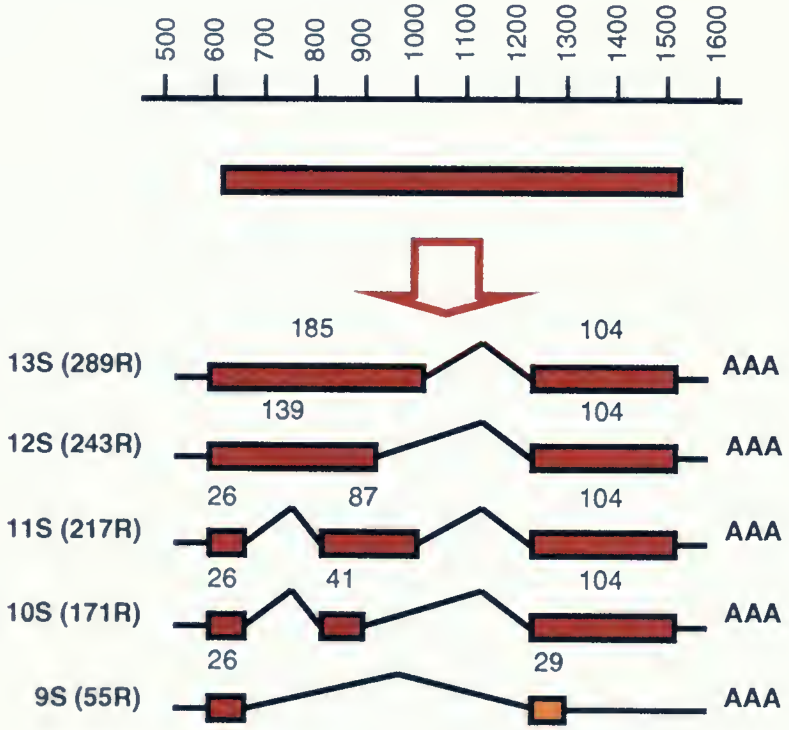

500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
13S (289R)
185
104
AAA
12S (243R)
139
104
AAA
11S (217R)
26
87
104
AAA
10S (171R)
26
41
104
AAA
9S (55R)
26
29
AAA

Figure 1.7. Splicing patterns of Ad5 E1A (adapted from Stephen & Harlow, 1987). The exons are represented as red rectangles, the introns as carets, and the poly (A) signal shown as "AAA". The C-terminus of the smallest protein product (9S) is not identical to other products and is illustrated as an orange rectangle. The numbers of amino acids of the protein fragments are indicated above the red rectangles.

Among these five transcripts, the functions of 13S (289R) and 12S (243R) products are most extensively characterized. Domains in the proteins that are conserved among adenovirus serotypes are identified as conserved regions 1 to 4 (Avvakumov, 2002). The major functions of E1A proteins are carried out by these domains. These conserved regions are shown in Figure 1.8. Among these conserved regions, CR3 is exclusive to the 13S proteins, and has the ability to regulate the basal transcription (Horikoshi *et al.*, 1991).

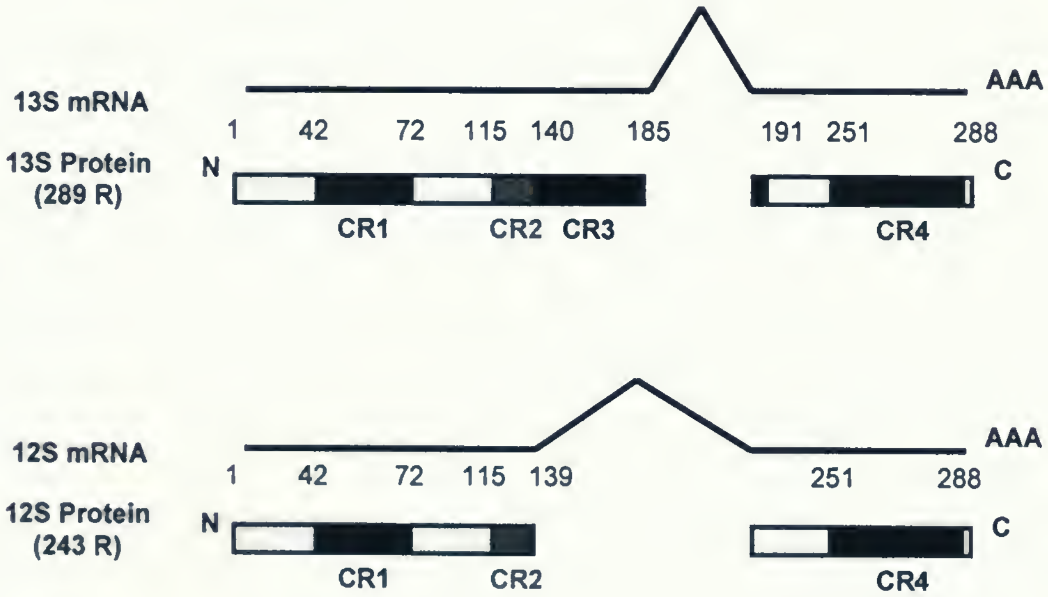

13S mRNA
AAA
1
42
72
115
140
185
191
251
288
13S Protein
(289 R)
N
C
CR1
CR2
CR3
CR4
12S mRNA
AAA
1
42
72
115
139
251
288
12S Protein
(243 R)
N
C
CR1
CR2
CR4

Figure 1.8. Conserved regions of adenovirus E1A 13S and 12S proteins (adapted from Shenk, 2001 and Avvakumov, 2002). The 13S and 12S mRNA exons are represented as lines, introns as carets, and poly (A) signal as "AAA". The polypeptides encoded by the 13S and 12S are illustrated as rectangles. The conserved protein domains are designated as conserved regions from 1 to 4 (CR1, CR2, CR3, and CR4), and the amino acids at the boundaries of conserved regions are indicated above the rectangles.

Adenovirus E1A proteins can activate other viral early genes and increase their transcription about 50-100 fold (Jones & Shenk, 1979), and they can promote quiescent cells to enter S phase (Hiebert *et al.*, 1989; 1991). They can also repress certain genes, such as p53 (Somasundaram & El-Deiry, 1997). However, this transcriptional activation or repression is not performed by direct binding of E1A proteins to the specific viral gene promoters (Ferguson *et al.*, 1985). Rather, it is believed that E1A proteins exert their functions by interacting with various cellular transcription factors and regulatory proteins. Indeed, immuno-precipitation of E1A proteins isolated from [^{35}S]-methionine labelled Ad5 infected cells shows that E1A interacts with a number of cellular proteins, they are 400 kDa, 300 kDa, 130 kDa, 107 kDa, 105 kDa (pRb), 60 kDa and 33 kDa, respectively (Yee & Branton, 1985; Harlow *et al.*, 1986). E1A proteins can interact with at least these cellular proteins to activate or repress other viral genes or cellular genes *in trans*.

The typical E1A-related molecular pathways include the interactions of E1A proteins with p53-TBP, pRb-E2F and p300/ CBP. The interaction with p53-TBP and pRb- E2F demonstrated their transcription activating effects and that with p300/CBP complex showed their transcription repressing impacts.

First, E1A protein can activate the other adenoviral genes through the TATA motif by binding to TBP (TATA binding protein) (Lee *et al.*, 1991), displacing p53, and regulating basal transcription. This is carried out by the E1A 13S CR3 domain, a strong trans-activation domain, and most viral regions and many cellular genes are activated by this region (Lillie *et al*, 1987; Lillie & Green,

1989). E1A 13S CR3 can bind to TBP which is the DNA-binding subunit of transcription factor II D (TFIID) (Boyer & Berk, 1993). Because the TATA motif is ubiquitous in mammalian expression systems, the binding of E1A 13S CR3 to the TBP can regulate the basal transcription condition (Horikoshi *et al.*, 1991).

As is shown in Figure 1.9. A, both p53 and E1A 13S can bind to TBP (Liu *et al.*, 1993; Boyer & Berk, 1993). Once p53 binds to TBP, the transcription will be repressed (Seto *et al.*, 1992), but E1A 13S can replace p53 and relieve the p53-mediated repression (Horikoshi *et al.*, 1995) thus starting the transcription of other adenoviral genes and certain cellular genes.

Second, E1A proteins can activate the cellular S phase-related genes and adenovirus E2 genes. This is carried out by binding of E1A CR1/CR2 domain to the cellular retinoblastoma tumor suppressor (pRb) and freeing the transcription factor E2F (Chellappan *et al.*, 1992) (Figure 1.9. B). The transcription factor E2F regulates the expression of genes at the G_1/S boundary and adenovirus E2 gene (and hence the name) (Babiss, 1989). However, in the G_0/G_1 phase, E2F forms a complex with pRb and is inactivated by pRb, and thus the transcription of S phase genes are repressed (Chellappan *et al.*, 1991). E1A proteins can bind to pRb and free E2F. By liberating E2F, E1A activates a series of genes important for S phase, such as c-myc (Hiebert *et al.*, 1989) and dihydrofolate reductase (Hiebert *et al.*, 1991), as well as the adenovirus E2 early promoter (Bagchi *et al.*, 1990). As a result, the quiescent host cells will be induced to enter the S phase to support enough substrates for viral DNA replication and the E2 proteins will be produced to provide viral DNA replicating enzymes.

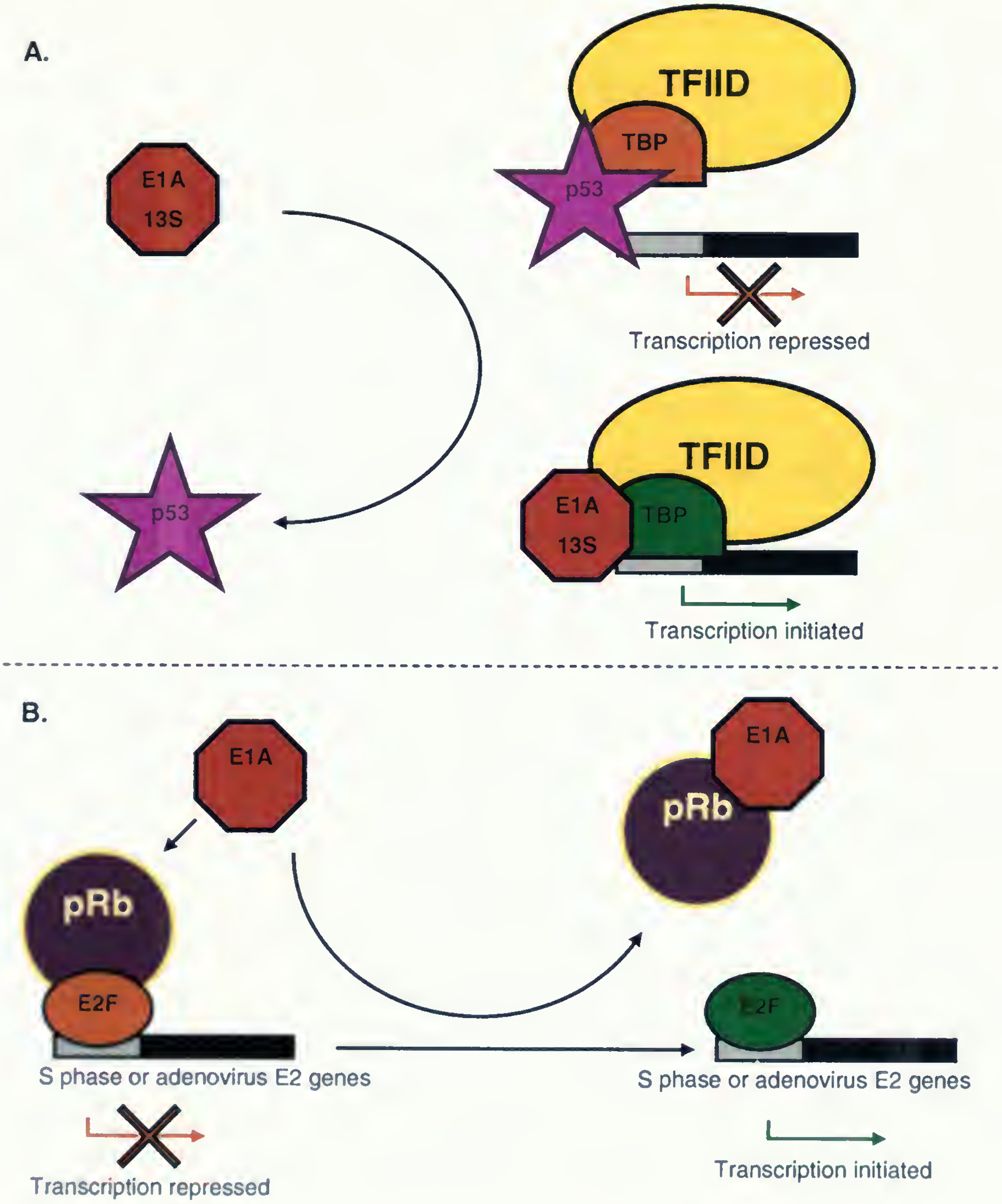

A.
E1A
13S
TFIID
TBP
p53
Transcription repressed
p53
TFIID
E1A
13S
TBP
Transcription initiated
B.
E1A
pRb
E2F
S phase or adenovirus E2 genes
Transcription repressed
E1A
pRb
E2F
S phase or adenovirus E2 genes
Transcription initiated

Figure 1.9. Adenovirus E1A protein-mediated *trans*-activation. A) E1A 13S protein replaces p53 and binds to TBP, thus ablating the repression of p53 and initiating the transcription of other viral early genes. B) E1A proteins transcriptionally activate E2 or S phase genes.

Last but not least, E1A proteins can also *trans*-repress certain genes by interacting with p300/ CBP. The *trans*-repression effect was found mainly in the N-terminus/ CR1 portion of E1A proteins (Stein *et al.*, 1990), which is required for E1A proteins to bind to the transcription factors p300 and CBP (CREB-binding protein) (Lundblad *et al.*, 1995).

The proteins p300 and CBP are transcriptional co-activators for many transcription factors, and they were also reported to be histone acetyltransferases (HAT) (Ogryzko *et al.*, 1996). By binding to sequence-specific transcription factors, they can perform promoter-specific histone acetylation (Figure 1.10). Such acetylation events can loose the association between histones and DNA, facilitate the transcription factors to bind to the promoter, and therefore enhance the transcription activity.

However, the E1A proteins could bind to p300/ CBP to repress transcription (Yang *et al.*, 1996) (Figure 1.10). The interaction of E1A with p300/ CBP has been reported to inhibit their HAT activity (Chakravarti *et al.*, 1999; Hamamori *et al.*, 1999), so they are unable to transcriptionally activate the related genes (Reid *et al.*, 1998). Such E1A-induced *trans*-repression affects the transcription of some important genes, such as p53 gene (Somasundaram & El-Deiry, 1997), suggesting the vastly regulatory activity of E1A.

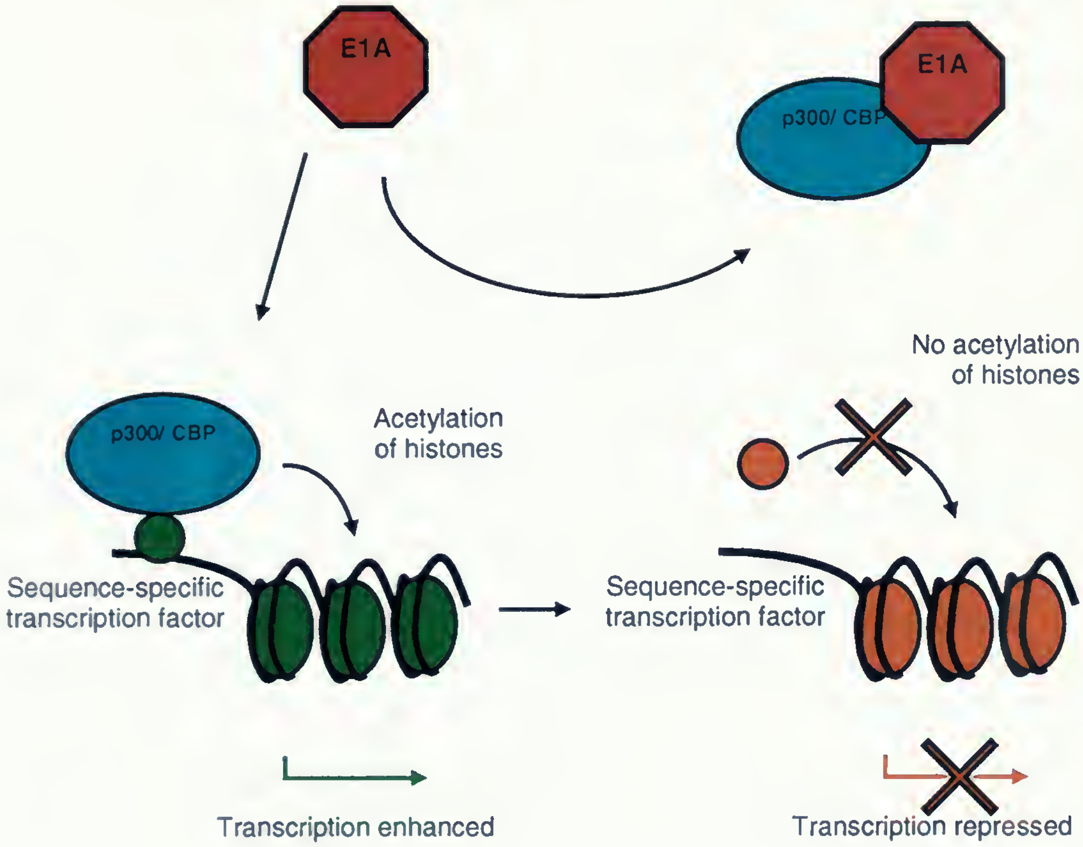

E1A
E1A
p300/ CBP
No acetylation
of histones
p300/ CBP
Acetylation
of histones
Sequence-specific
transcription factor
Sequence-specific
transcription factor
Transcription enhanced
Transcription repressed

Figure 1.10. The mechanism of transcriptional repression induced by adenovirus E1A proteins (reproduced from PhD thesis of Michael Sheun, Western Ontario University, ON).

Of course, E1A proteins are involved in many more pathways in addition to these three. However, in essence, E1A can make the infected cells suitable for adenovirus replication by binding to one protein and freeing the other. Based on this protein-protein interacting mechanism, E1A exerts many physiological changes of infected cells and even the host organisms, for example, E1A can transform the host cells together with E1B, as discussed below.

1.2.2 Early Region 1B

Adenovirus serotype 5 E1B region ranges from 4.6~11.3 map units, and is located after that of the E1A region. It mainly codes for two proteins: E1B 19kDa and E1B 55kDa. These two products share the same N-terminus and C-terminus, and are only different in their splicing sites. The splicing pattern of Ad5 E1B is shown in Figure 1.11.

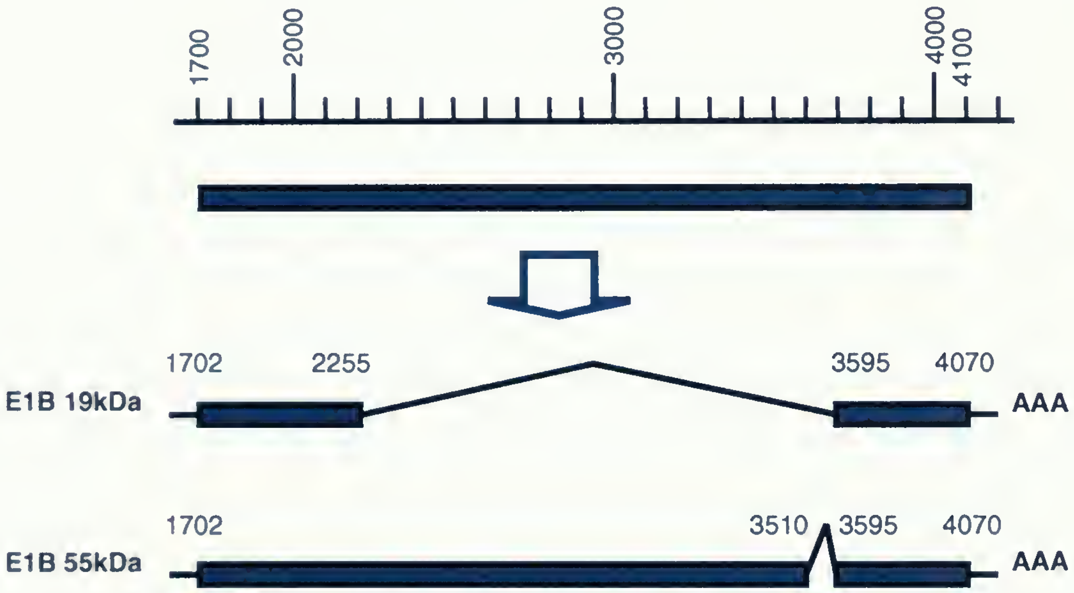


Figure 1.11. Splicing patterns of Ad5 E1B (adapted from Spector, 1982). The exons are represented as blue rectangles, the introns as carets, and the poly (A) signal shown as "AAA". All numbers shown are nucleotides as numbered from the left end of the Ad5 genome.

Both two proteins of E1B are concerned with anti-apoptotic effects. As discussed above, adenovirus E1A proteins can bind to TBP, displace p53 and make it accumulate in the cytoplasm. The high concentration of p53 in the cytoplasm will induce apoptosis because concentrated p53 can activate

apoptosis-related genes such as the *Bax* family genes. Once activated and expressed, the *Bax* family proteins can permeabilize the mitochondria and release cytochrome c (cyt c), which will in turn activate caspase-9 and induce apoptosis (Imazu *et al.*, 1999).

However, adenovirus E1B 19kDa protein can counteract this p53-mediated apoptotic event. Adenovirus E1B 19kDa is analogous to cellular Bcl-2 protein (Rao *et al.*, 1992), both of which can bind to the *Bax* family proteins, prevent the permeabilization of mitochondrial membrane, maintain the cyt c within mitochondria, and therefore block the apoptosis (Han *et al.*, 1996) (Figure 1.12. A). Indeed, when infecting mammalian cells with adenoviruses with mutations in E1B 19kDa, there was degradation of cellular/ viral DNA and increased cytopathic effect (White *et al.*, 1984), showing the sign of apoptosis.

Therefore, the E1B 19kDa protein blocks apoptosis by ablating the functions of the *Bax* family proteins rather than directly interacting with p53. On the other hand, adenovirus E1B 55kDa protein can directly bind to p53 and affect its activities (Sarnow *et al.*, 1982). Once it binds to p53, E1B 55kDa can suppress the transcriptional activation of p53, and consequently block p53-induced apoptotic pathways, such as the *Bax* proteins – cyt c – caspase-9 pathway (Imazu *et al.*, 1999) (Figure 1.12. B). Thus, like E1A, the E1B 55kDa protein binds to a tumor suppressor protein, antagonizes its normal activity, and regulates cell cycle progression. Both E1A and E1B proteins are referred to as oncoproteins, because they can disrupt the functions of tumor suppressor proteins and induce tumors (reviewed in Shenk, 2001).

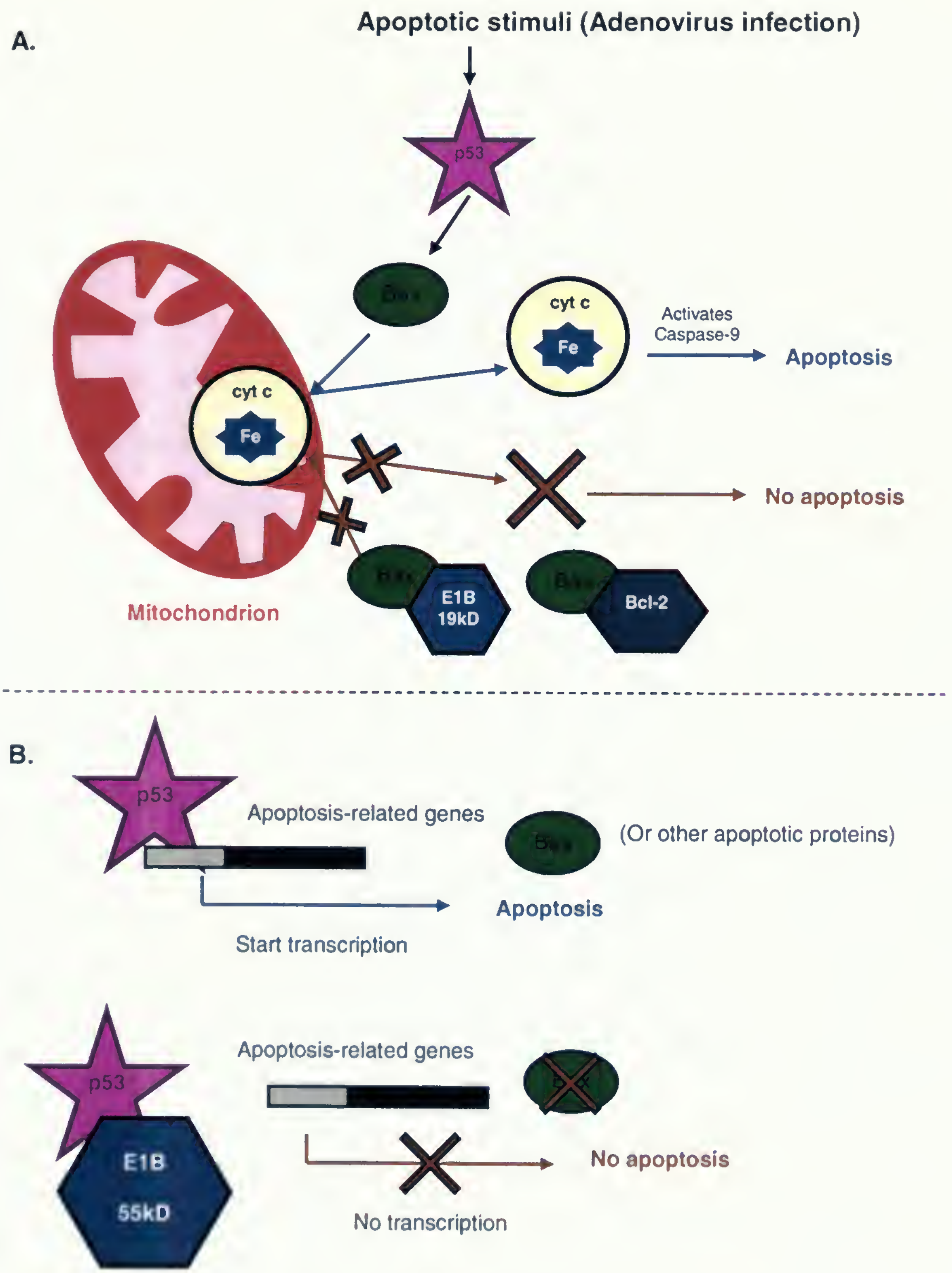

A.
Apoptotic stimuli (Adenovirus infection)
p53
cyt c
Fe
Activates
Caspase-9
Apoptosis
cyt c
Fe
No apoptosis
E1B
19kD
Bcl-2
Mitochondrion
B.
p53
Apoptosis-related genes
(Or other apoptotic proteins)
Apoptosis
Start transcription
p53
Apoptosis-related genes
E1B
55kD
No apoptosis
No transcription

Figure 1.12. Functions of E1B 19kDa and 55kDa. A) E1B 19kDa protein in blocking apoptosis. B) E1B 55 kDa protein in blocking apoptosis.

One can imagine how E1A and E1B oncoproteins regulate the cell cycle progress from G_0/G_1 to S phase safely through their interactions with cellular proteins such as p53, pRb, and p300/CBP. E1A proteins induce quiescent cells to enter the S phase but they also cause p53 to accumulate and may induce apoptosis, therefore E1B must prevent p53-mediated apoptosis. Thus the two subsets of proteins create an environment optimal for viral replication.

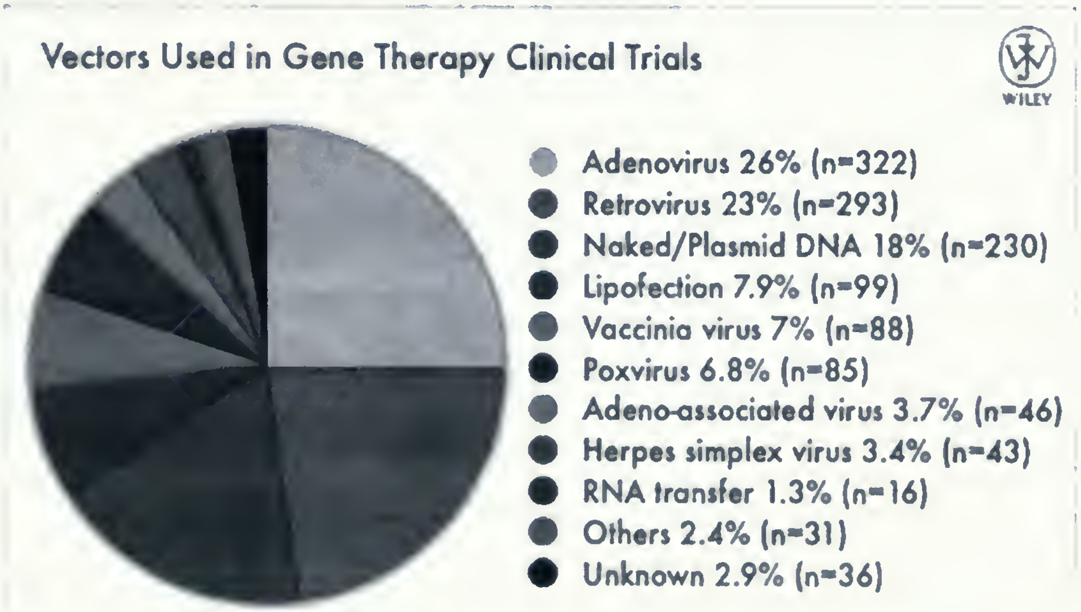
In the context of a cultured rodent cell, these same events lead to oncogenic transformation. The oncogenically transformed cells exhibited changes in morphology, growth in reduced serum, and development without anchorage-dependence (summarized in Shenk, 2001). It has been reported that only the adenovirus E1 region is sufficient to oncogenically transform cultured rodent cells (Graham *et al.*, 1975; van der Eb & Houweling, 1977). However, E1A alone cannot transform rodent cells; E1B must be present to cooperate with adenovirus E1A in transforming host cells (Yew & Berk, 1992). Although the exact mechanism of oncogenic transformation is not completely revealed, a complex series of molecular interactions and cell signaling traffics regulated by E1A and E1B proteins must have happened.

With regards to their multiple functions, adenovirus E1A and E1B proteins would probably have an impact on virus integration. There are very few papers discussing the relationship between E1A and E1B proteins and virus integration (such as van Doren *et al.*, 1984). These papers will be discussed below in section 1.3.5: "Literature Review on Adenovirus Integration".

1.3 Adenoviral Vectors in Gene Therapy

1.3.1 Gene Therapy Overview

Gene therapy can be simply defined as replacing a defective gene with a functional one by means of gene delivery. To achieve this goal, various gene delivery methods have been developed. These methods include viral vector-mediated methods and non-viral methods. Currently, viral vectors are more extensively used because they can deliver a transgene more effectively (reviewed in Nuno-Gonzalez *et al.*, 2005). Adenoviral vectors are chief among all the gene therapy methods; they were used in 26% of all clinical trails as of May, 2007 (Figure 1.13). On the other hand, non-viral methods have a much lower efficiency, and only naked plasmid is widely applied. Different transferring methods have their respective advantages and disadvantages (Table 1.3).



The Journal of Gene Medicine, © 2007 John Wiley and Sons Ltd www.wiley.co.uk/genmed/clinical

Figure 1.13. Vectors used in gene therapy clinical trials (from website: http://www.wiley.co.uk/genmed/clinical/).

Table 1.3. Characteristics of most commonly used gene transfer vectors in gene therapy clinical trials (modified from Worgall, 2005).

Vector type	Transgene capacity	Advantages	Disadvantages
Plasmid	<10 kb	Easy to manipulate. Non-immunogenic. Target cell not specific.	Poor transduction efficiency. Limited persistence.
Adenovirus	7.5~36 kb	Easy to produce at high titers. High expression levels. Almost all cell types transducible. Infects replicating and non-replicating cells. Cell targeting possible.	Host immune and inflammatory response. Transient expression.
Adeno-associated virus	4.5 kb	Almost no immunogenicity. Non-pathogenic in humans. Infects replicating and non-replicating cells. Long term expression.	Difficult to produce at high titers. Small transgenes. Late onset of expression.
Retrovirus	8 kb	Non-immunogenic. Stable integration in dividing cells. Cell type retargeting possible.	Insertional mutagenesis. Low titers. Infects only proliferating cells.

1.3.2 Designing of Adenoviral Vectors

It is generally recognized that there are three generations of adenoviral vectors (reviewed in Russell, 2000). The first generation has the E1 region deleted, and was termed E1-deleted adenoviral vector (ΔE1). The second generation has the other early genes (such as E2) deleted and the third generation has nearly all the genome deleted.

The first generation adenoviral vectors are the most extensively used vectors (reviewed in Russell, 2000; Goncalves & de Vries, 2006). These vectors are deleted in the E1A and E1B transcription units. Traditionally, the ΔE1 adenoviral vectors are generated by transfecting two types of DNA molecules into E1-complementing human cell lines such as human embryonic kidney (HEK) 293 cells (Graham *et al.*, 1977) or other E1-complementing cells (reviewed in Goncalves & de Vries, 2006). These two types of DNA molecules are: a shuttle plasmid containing the transgene cassette in place of E1, and a genomic DNA plasmid consisting of most of the adenoviral genome except for the E1 region which is replaced with an antibiotic-resistance gene. Therefore, there are two homologous regions shared by the two molecules (at both ends of the transgene) (Figure 1.14), and homologous recombination would occur through the two homologous regions. After homologous recombination, a ΔE1 adenoviral vector is generated, as is shown in Figure 1.14.

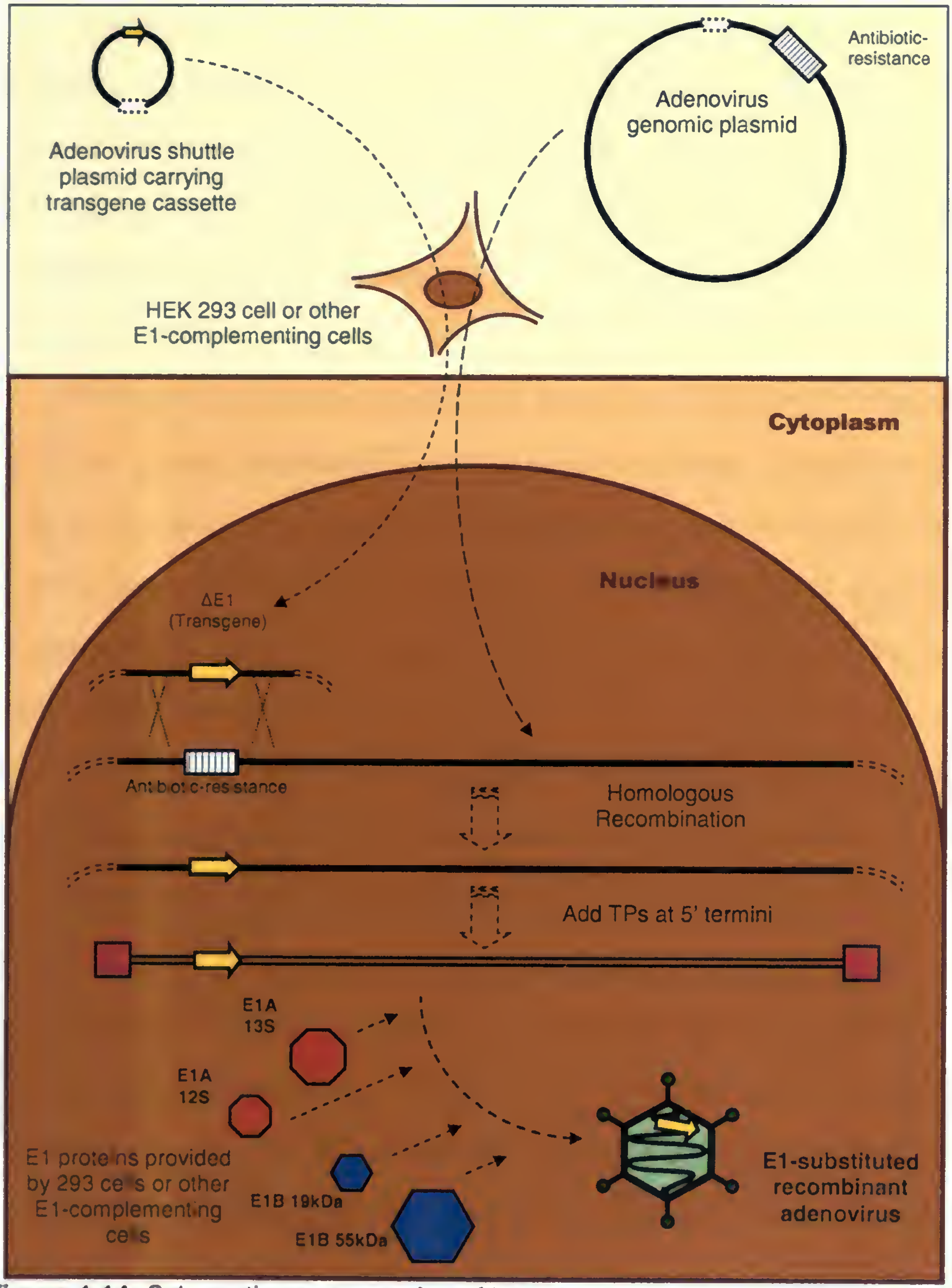

Antibiotic-resistance
Adenovirus genomic plasmid
Adenovirus shuttle plasmid carrying transgene cassette
HEK 293 cell or other E1-complementing cells
Cytoplasm
Nucleus
ΔE1 (Transgene)
Antibiotic-resistance
Homologous Recombination
Add TPs at 5' termini
E1A 13S
E1A 12S
E1 proteins provided by 293 cells or other E1-complementing cells
E1B 19kDa
E1B 55kDa
E1-substituted recombinant adenovirus

Figure 1.14. Schematic representation of homologous recombination to generate recombinant adenovirus. The generation of the E1-deleted adenoviral vector requires the E1 proteins provided by the host cells.

However, the generated ΔE1 adenoviral vectors often contain replication-competent adenovirus (RCA) contamination (Lochmueller *et al.*, 1994). The RCAs can replicate inside the human body and cause a strong immune response and inflammation. The generation of RCAs might be caused by the recombination between the vectors and the E1 sequence present in 293 cells (Louis *et al.*, 1997).

The second generation of adenoviral vector was constructed to eliminate the RCAs. It was constructed by excising some or all of the E2 genes, which encodes the proteins for viral DNA replication, and was propagated using suitable complementing cell lines (Lusky *et al.*, 1998; Moorhead *et al.*, 1999). Therefore, RCAs cannot be generated with these vectors. However, the host immune response was still a major problem.

The third generation adenoviral vector is constructed by deleting all of the viral genes except for the ITRs and packaging signal (Morsy *et al.*, 1998; Steinwaerder *et al.*, 1999). This vector is also referred to as a "helper-dependent adenoviral vector" (HDAd) because it cannot replicate by itself and depends on a helper virus for propagation. However, the contaminating helper virus, which is replicable, might cause troubles in clinical trials. Therefore, some strategies were attempted to use the Cre-loxP recombinase system to prevent the packaging of the helper virus (Ng *et al.*, 1999; Tashiro *et al.*, 1999). The major advantages of the HDAd vectors are their large transgene capacity (up to 36 kb), low immunogenicity and longer expression duration compared to ΔE1 vectors;

however, the deletion of the whole genome also makes this vector unstable (Harui *et al.*, 1999; reviewed in Russell, 2000; Goncalves & de Vries, 2006).

1.3.3 Applications of Adenoviral Vectors

Adenoviral vectors can be used for various types of gene therapy clinical trials such as treatment of cancers, vaccination against infectious diseases, and gene therapy for monogenic diseases.

First, adenoviral vectors can transfer genes such as the suicide gene *HSV-tk* (herpes simplex virus-thymidine kinase) (Haj-Ahmad & Graham, 1986) and tumor suppressor gene *p53* into cancer cells, so these vectors were used in cancer gene therapy trials (such as Herman *et al.*, 1999; Lang *et al.*, 2003). There is a report suggesting that adenoviruses with a mutation in E1B 55kDa can only replicate in p53-defective cells (which are normally tumor cells), and once they replicate, they can lyse the cancer cells (Bischoff *et al.*, 1996). This mutant was also used in cancer gene therapy trials (such as Makower *et al.*, 2003).

Second, adenoviral vectors can be modified to be recombinant vaccines. For example, it was one of the most widely tested vectors for developing potential HIV vaccines, (such as Shiver & Emini, 2004; reviewed in Girard *et al.*, 2006), in addition to pox viral vector vaccines and DNA vaccines.

Last but not least, adenoviral vectors are well known for treating cystic fibrosis, a monogenic disease that is caused by a defect in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (NCBI OMIM: 219700). The adenoviral vectors carrying *CFTR* gene were used in clinical trials (Knowles *et al.*, 1995), however, the expression duration of the vector was very short and

the symptom of cystic fibrosis was barely corrected (such as the cases in Knowles *et al.*, 1995; Zuckerman *et al.*, 1999). To some extent, the transient expression of adenoviral vectors limited their use in gene therapy of monogenic diseases. Therefore maneuvers must be taken to extend the expression duration of adenoviral vectors.

1.3.4 Strategies to Prolong the Expression of Adenoviral Vectors

Although adenoviral vectors are used in various types of gene therapy trials because they boast high titer and excellent delivery efficiency (reviewed in Nuno-Gonzalez *et al.*, 2005), their major problem is the inability to express the transgene persistently. These properties are good enough for cancer gene therapy and recombinant vector vaccination, which do not require long term therapeutic effects. However, for the authentic and traditional "gene therapy", namely, to permanently replace a defective gene with an intact gene by means of transgene delivery, they are far from ideal.

The inability of adenoviral vectors to continuously express the transgene is caused by two main problems. One is that these vectors have strong immunogenicity which will induce host immune cells to eliminate the infected host as well as adenoviral vectors (reviewed in Russell, 2000). The other is that they are deemed to be lacking the necessary integrating elements to insert their genomes into host chromosome DNA (reviewed in Russell, 2000; Mitani & Kubo, 2002).

As to the first problem, a variety of strategies were developed to decrease Ad vector-induced strong immune response, such as use of hexon-modified Ad

vectors (Roy *et al.*, 1998), the use of cationic liposomes- encapsulated Ad vectors (Yotnda *et al.*, 2002), the use of rare human Ad serotypes (Shiver & Emini, 2004), and the use of HDAd (helper-dependent Ad) vectors (reviewed in Jozkowicz & Dulak, 2005) to reduce the host immune response (reviewed in Bangari & Mittal, 2006) and prolong the transgene expression. Some of these modifications showed longer expression duration. For example, the use of HDAd was reported to achieve much longer gene expression than the E1-deleted control vector (Kim *et al.*, 2001; Schiedner *et al.*, 2002). The reason for the longer expression of HDAd might be that the deletion of other viral genes removes leaky expression and prevents CTLs from killing the infected cells (reviewed in Mitani & Kubo, 2002).

As to the second problem, various methods were attempted to make Ad vectors into "integrating" vectors. This is carried out by combining certain integrating elements (such as that of retrovirus and adeno-associated virus) with Ad vectors to generate a hybrid virus.

One of these hybrid vectors is the adenovirus – retrovirus (Ad-Retro) hybrid vector. The typical Ad-Retro vector is constructed by inserting a transgene flanked by retroviral long terminal repeat (LTR) sequences and genes encoding other retroviral proteins into an adenoviral vector (Yoshida *et al.*, 1997; Zheng *et al.*, 2000; Zheng *et al.*, 2003) (Figure 1.15). This hybrid vector, in essence, is a retroviral vector which is delivered by an adenoviral vector. After infecting the cells with the Ad-Retro vector, the transgene flanked by LTRs will be transcribed and will form a retroviral vector. The retroviral vector will then infect a

second host cell, reverse-transcribe the transgene together with LTRs, and incorporate the transgene into the host genomic DNA (retroviral vector is reviewed in Kurian *et al.*, 2000).

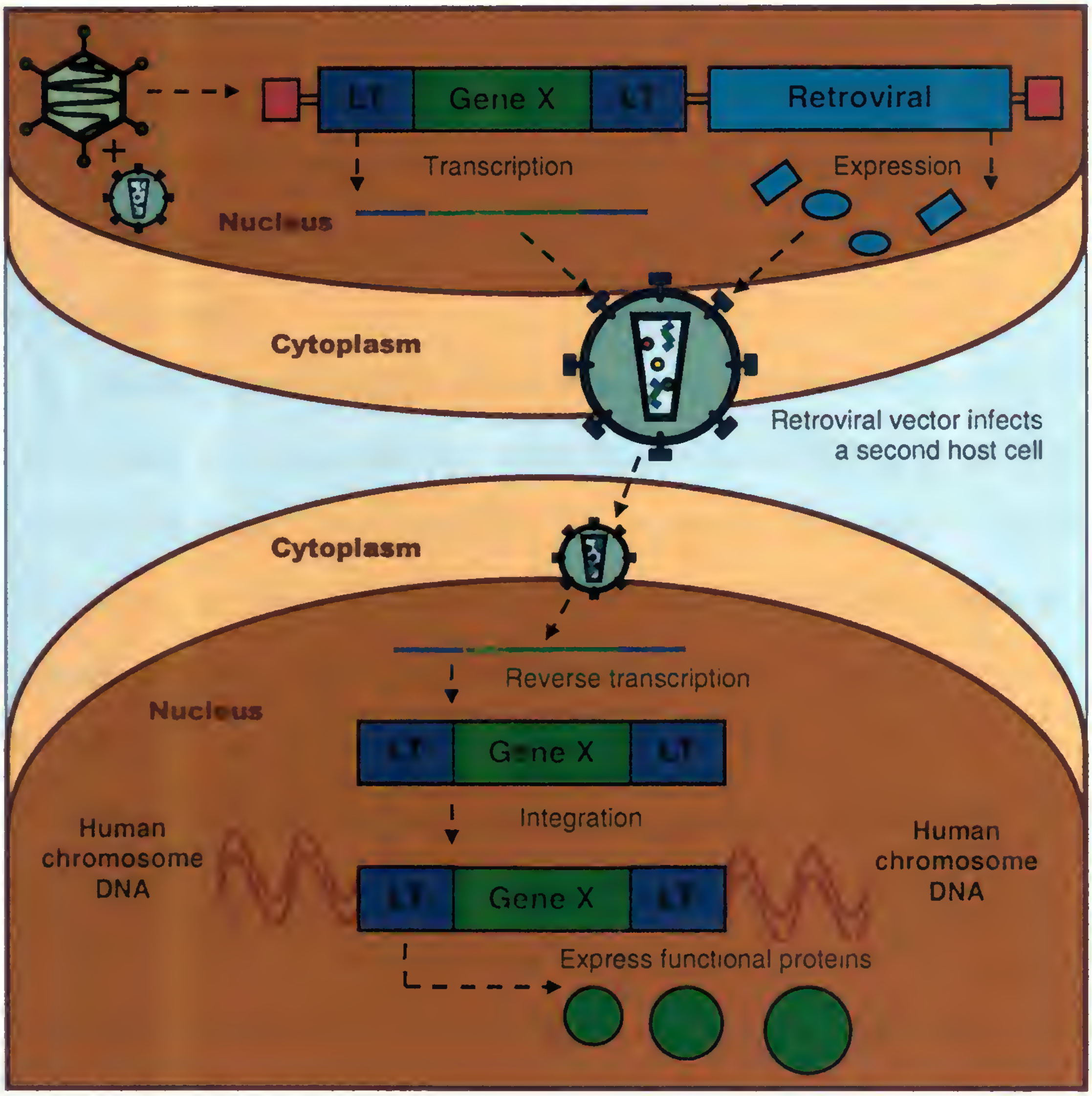

LT
Gene X
LT
Retroviral
Transcription
Expression
Nucleus
Cytoplasm
Retroviral vector infects a second host cell
Cytoplasm
Reverse transcription
Nucleus
LT
Gene X
LT
Integration
Human chromosome DNA
LT
Gene X
LT
Human chromosome DNA
Express functional proteins

Figure 1.15. Working mechanisms of Ad-Retro hybrid vector (reproduced from Mitani & Kubo, 2002).

However, there is a danger. Because the retroviral vectors can induce insertional mutations in the patients' genomes, they may activate an oncogene or inactivate a tumor suppressor gene (reviewed in Nuno-Gonzalez *et al.*, 2005;

Worgall, 2005). This occurred in a gene therapy trial of SCID-XI (X-linked severe-combined immunodeficiency), in which 3 out of 11 children in this trial developed leukemia (Cavazzana-Calvo *et al.*, 2000; Hacein-Bey-Abina *et al.*, 2003; Check, 2005). A follow-up study showed that this was caused by the retroviral vector-induced insertional mutation of the gene (MLO2) which controls the T cell leukemia genesis (McCormak *et al.*, 2003). Considering the above information, intensive care must be paid to make sure that this type of Ad-Retro hybrid vector is safe.

The other hybrid vector is adenovirus – adeno-associated virus (Ad-AAV) hybrid vector. It is supposed to preferentially integrate into a unique site on human chromosome 19 and therefore reduce the risk of insertional mutations that Ad-Retro (or retroviral) vectors have. This is carried out by inserting the transgene flanked by the inverted terminal repeats of AAV (AAV-ITRs) and an AAV *REP* gene cassette into an HDAd vector (Figure 1.16).

This strategy was inspired by the unique property of AAV. The AAV is the only eukaryotic site-specific integrating virus that can integrate into one site on human chromosome 19, designated as *AAVS1* (Kotin *et al.*, 1991). Currently, it is generally believed that its site-specific integration requires only two components: the AAV-ITR and the AAV-Rep protein (encoded by *REP*). The AAV-ITR serves as the substrate for integration, while the AAV Rep protein functions as a switch regulating the integration or excision of AAV (reviewed in Goncalves, 2005). However, the AAV vector cannot site-specifically integrate because the AAV genome is too small (4.7 kb) to accommodate both the

transgene and AAV *REP*. Therefore, the Ad-AAV hybrid vector boasting both large transgene capacity and site-specific integrating ability was developed.

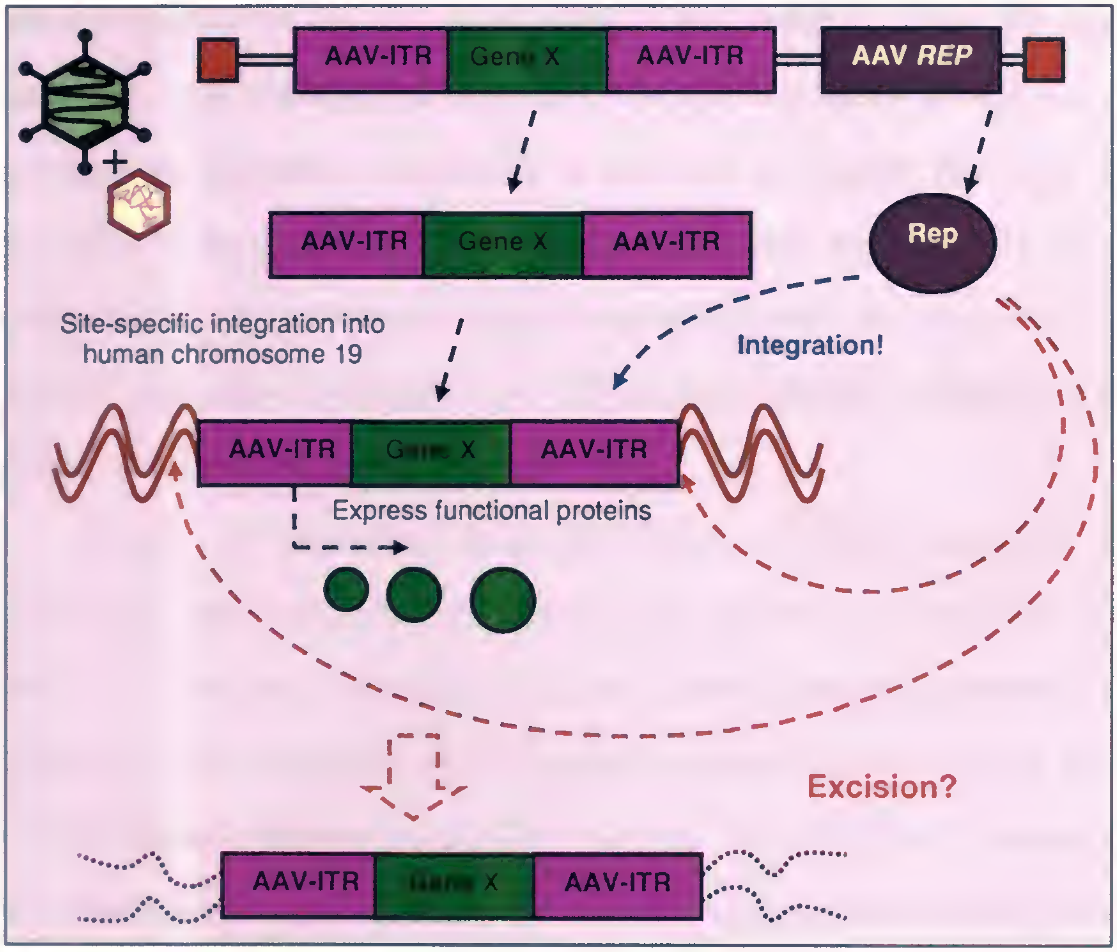

AAV-ITR
Gene X
AAV-ITR
AAV *REP*
AAV-ITR
Gene X
AAV-ITR
Rep
Site-specific integration into human chromosome 19
Integration!
AAV-ITR
Gene X
AAV-ITR
Express functional proteins
Excision?
AAV-ITR
Gene X
AAV-ITR

Figure 1.16. Illustration of Ad-AAV hybrid vectors (inspired and modified from Mitani & Kubo, 2002). Ad-AAV vector is supposed to site-specifically integrate into a site on human chromosome 19 because it is armed with the AAV-ITR and AAV Rep protein. However, the presence of Rep protein in the infected cells may easily excise the integrated "ITR-transgene-ITR" cassette.

There are two teams currently carrying out the research on Ad-AAV vector-based gene delivery *in vivo* (Recchia *et al*., 2004; Goncalves *et al*., 2006). It was reported in both papers that the site-specific integration of the transgene

was found in transgenic mice carrying the human *AAVS1* site, suggesting that this Ad-AAV hybrid vector did integrate into the human genome site-specifically. However, neither of these papers reported the duration of the transgene expression. The transgene expression of this Ad-AAV vector is likely not as persistent as expected. Actually, it is not hard to imagine that, after the integration of the transgene into the host chromosome, the transgene will be excised easily in the presence of Rep proteins and therefore the integrant is very unstable (also shown in Figure 1.16). Thus, more delicate modifications are required if a stable integrant is needed.

Except for bestowing integrating ability onto the adenovirus by incorporating other viral elements, there is another strategy: gene targeting (GT), which can make gene exchange in a defined region of host chromosome. This strategy is performed by flanking the transgene cassette with two regions which are homologous with that in host chromosome. Therefore, once homologous recombination occurs, the defective genes will be replaced by the wildtype genes, and *vice versa* (Capecchi *et al.*, 1989). A gene targeting strategy mediated by adenoviral vector is illustrated in Figure1.17.

However, GT performed by homologous recombination often has a frustratingly low efficiency (typical frequencies lie below 10^{-6} as stated in Goncalves, 2005). A recent study used an HDAd vector to correct a mutation in the hypoxanthine phosphoribosyl transferase (Hprt) locus in mouse embryonic stem cells, but the GT efficiency was not ideal (Ohbayashi *et al.*, 2005). In this study, although the GT efficiency reached as high as 0.2% per transduced cells,

the random integration (RI) efficiency was 5%, which is much higher than the GT efficiency. Therefore, the side-effects of random integration, such as insertional mutation, might overwhelm the benefit from the GT, and more consideration should be taken before clinical trials.

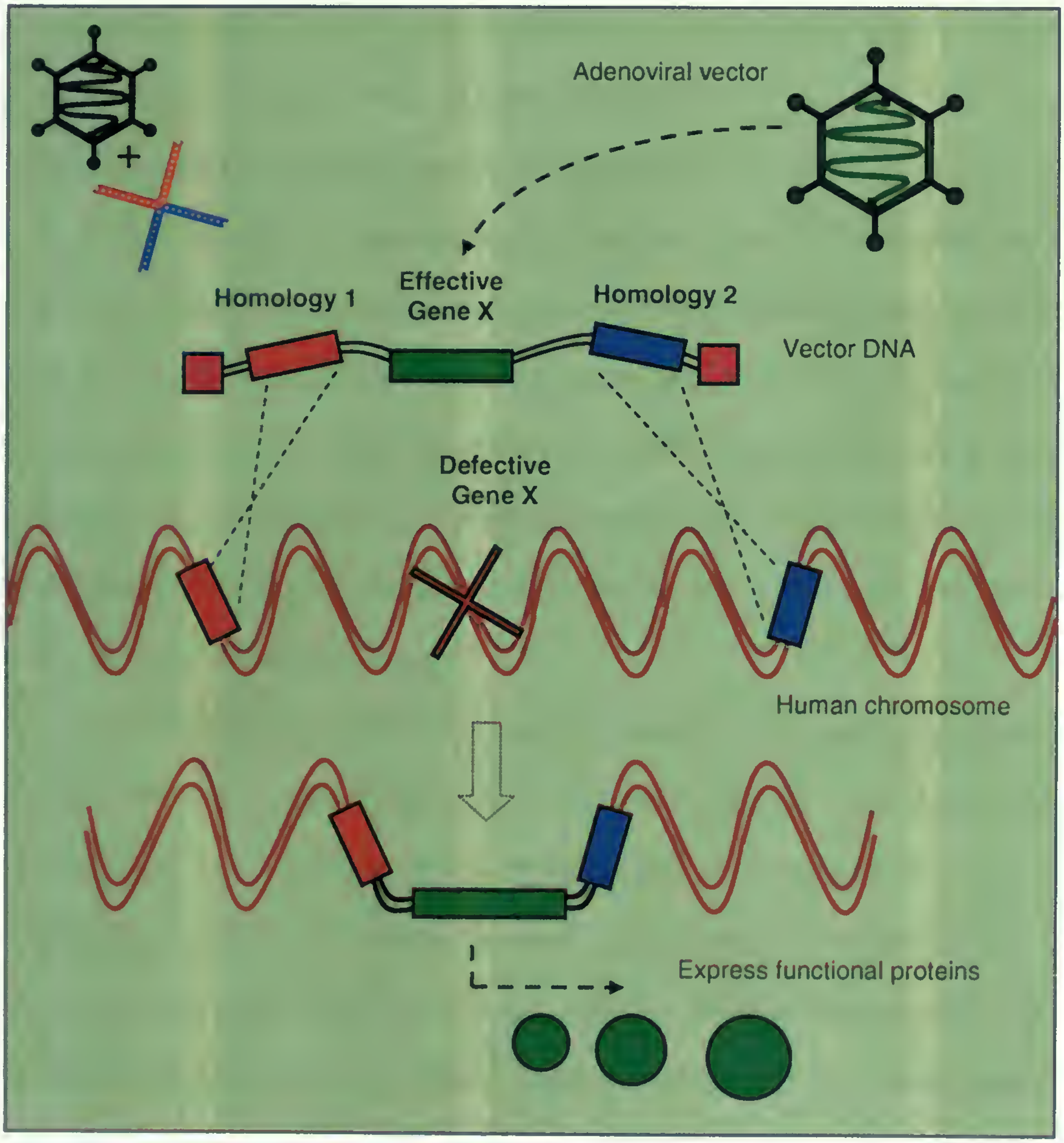

Adenoviral vector
+
Homology 1
Effective Gene X
Homology 2
Vector DNA
Defective Gene X
Human chromosome
Express functional proteins

Figure 1.17. Gene targeting (GT) through homologous recombination by using an adenoviral vector (adapted from Mitani & Kubo, 2002).

To sum up, many strategies has been tested to overcome the limitation of transient expression of adenoviral vectors, but so far the problems have not been solved. However, if we could investigate the properties of the virus, and understand whether there are virus-encoded proteins that can mediate integration, we could modify the adenoviral vectors accordingly. Considering the above, we decided to perform a literature review to see whether there is research on the molecular mechanisms of adenovirus integration.

1.3.5 Literature Review on Adenovirus Integration

In the last section "Adenovirus Early Region 1", it was explained that the adenovirus E1 region has multiple functions such as modulating the physiological status of infected cells and transforming rodent cells into oncogenic phenotypes. Actually oncogenic transformation of rodent cells induced by E1 is generally believed to be related with adenovirus integration into rodent genomic DNA. Such belief is rooted from the results that the sequence of E1 can be found in nearly all of the transformed cells.

To explain this, it is better to introduce the idea that adenovirus life cycle changes when infecting different host cells. Some cells are the natural host of adenoviruses, and they support the materials for the viral replication and are termed "permissive cells" (have maximal virus yields). In these cells, the adenovirus will finally lyse the infected cells to release progeny adenovirus. However, other cells (such as rodent cells) cannot provide the adenovirus with sufficient replication materials. Adenoviruses can enter these cells but they cannot complete a life cycle (reviewed in Shenk, 2001). These cells are referred

as "semi-permissive cells" (have less than maximal virus yields). It was found that human adenovirus occasionally integrates into semi-permissive cells. For example, upon the infection of wildtype human Ad12, up to 50% of the cell-associated Ad12 DNA is integrated into the chromatin of semi- permissive baby hamster kidney cells (Doerfler, 1970).

The research of adenovirus integration started from mid 1960s. Different adenovirus serotypes (such as Ad2, Ad5 and Ad12) are able to oncogenically transform rodent cells *in vitro* (Freeman *et al.*, 1967; Doerfler, 1968; Williams, 1973). Later it was found out that the adenovirus DNA sequences can recombine with that of the host and are then passed on to the cell's descendents like any other part of the cellular genome (Doerfler, 1970), suggesting that the adenovirus genomic DNA was integrated into host chromosomes. Many studies were then carried out to survey the integrated viral segments present in different cell lines and their general organization (Graham, 1975; McDougall *et al.*, 1975; Sambrook *et al.*, 1976; Mayer & Ginsberg, 1977).

Next, the research of adenovirus integration had a small peak from 1979 to 1984. At that time, there were many investigations concerned with the integration of Ad2/ Ad5 and SV40 in rodent cells. These papers examined various aspects of adenovirus integration, including the sites of adenoviral integration (Visser *et al.*, 1980; Visser *et al.*, 1981; Sambrook *et al.*, 1980; Doerfler *et al.*, 1980; Neumann & Doerfler, 1981; Gahlmann *et al.*, 1982; Spector, 1983), the integration efficiency (Doerfler, 1970; Casto *et al.*, 1979;), the inner structure of the integrants (Fujinaga *et al.*, 1980; Visser *et al.*, 1980; Visser *et al.*,

1981; Fisher *et al.*, 1982), and the transcription and expression profiles of viral genes after the integration events (Fujinaga *et al.*, 1980; Ruben *et al.*, 1982). Nearly all of these papers suggested that adenovirus integrates at random sites and did not show whether the virus has its own integrating elements.

However, there was one exception: Doerfler *et al.* (1980) studied adenovirus integration using Ad12 to infect rodent cells since 1968 (Doerfler, 1968), and they suggested that adenoviruses can selectively integrate into host chromosome DNA. The integration might be carried out by a recombination event (Neumann & Doerfler, 1981) between short homologous sequences (which they named as "patch homologies") shared by the host and the virus (Gahlmann *et al.*, 1982).

By applying Southern hybridization, they found that the viral DNA is inserted into limited numbers of sites in the host DNA since three lines of Ad12-induced rat brain tumor cells exhibit identical integration sites (Doerfler *et al.*, 1980). In another experiment with *Eco*RI/ *Bam*HI digestion and Southern blot, they found that the recombination between host DNA and adenoviral DNA is a frequent event and the recombinants involve at least the adenovirus ITR and packaging signal (Neumann & Doerfler, 1981). In addition, by cloning and sequencing the virus-host junction DNA and by comparing its sequences with the host and virus DNA, they showed that the junction DNA sequences are identical to virus and host genomic DNA. The length of these identical sequences ranged from 8 to 12 nucleotides, as small as a "patch" (Gahlmann *et al.*, 1982). Therefore, they proposed that the integration of adenovirus should be mediated

by recombination events at the "patch homologies" between the host and the virus DNA (Gahlmann *et al.*, 1982).

Presuming that this hypothesis is correct, however, the exact molecular mechanisms that mediate adenovirus integration remained to be investigated. Scientists believed that the adenovirus E1 region should be involved in adenovirus integration events because the E1 region can always be found in the chromosome DNA of oncogenically transformed cells (Dijkema *et al.*, 1979; Graham *et al.*, 1975; Houweling *et al.*, 1980; van der Eb & Houweling, 1977; van der Eb *et al.*, 1977). Graham *et al.* used mechanically sheared Ad5 genomic DNA to generate the human embryonic kidney (HEK) 293 cell line (Graham *et al.*, 1977), and the integrated DNA sequence turned out to be exactly the E1 region (from sequence 1 to 4344) (Spector, 1983; Louis *et al.*, 1997).

Therefore, adenovirus integration events were considered to be related to the adenovirus E1 region. Under such background, van Doren and his colleagues developed an assay to test whether the adenovirus E1 region is obligatory for the integration of viral DNA (van Doren *et al.*, 1984), but their methods and results cannot demonstrate whether E1 region has an effect on adenovirus integration. In this experiment, the E1 region of the Ad5 genome was replaced with a neomycin-resistance gene, so the cells infected with this vector could survive the G418 selection. Then the cultured cells were infected with this vector and cultured in G418-containing medium for twenty-one days. After the selection, the genomic DNA was extracted from the surviving cells and analyzed by Southern blot and sequencing. The results showed that the adenovirus

genomic DNA was indeed inserted into the host chromosomes. Based on such methods and results, they claimed that E1 is not obligatory for adenovirus integration. However, one can imagine that the integration of adenovirus DNA might be caused by the selection pressure of G418, rather than the deletion of E1 region. Although their results suggested that E1 is not necessary for adenovirus integration, the lack of an E1-containing (or wildtype) adenovirus as a positive control makes the results inconclusive to explain whether the E1 region makes a difference for adenovirus integration. Therefore, whether E1 can affect adenovirus integration still remains unanswered.

Recent studies on adenovirus integration are mainly concerned with the integration of adenoviral vectors (Harui *et al.*, 1999; Hillgenberg *et al.*, 2001, Wang *et al.*, 2005, Ohbayashi *et al.*, 2005), rather than the integration molecular mechanisms. In addition, the integration of multiple hybrid virus vectors (such as retrovirus and AAV) was also tested, but the results were not ideal, as discussed above (Yoshida *et al.*, 1997; Zheng *et al.*, 2000; Recchia *et al.*, 2004; Goncalves *et al.*, 2006). Noticeably, in all of these papers, the E1 region was deleted.

Doerfler and his colleagues continued their investigations on Ad12 integration in rodent cells. However, these studies focused on the localization and inner structure of the integrants (such as Doerfler *et al.*, 1995; Knoblauch *et al.*, 1996; Wronka *et al.*, 2004), oncogenic transformation of Ad12 (such as Pfeffer *et al.*, 1999), and the *de novo* methylation after the integration events (reviewed in Doerfler, 2005).

In sum, there are few papers that investigated the molecular mechanisms of adenovirus integration; many of them tested the integration sites, expression profiles and inner structures of the integrants. None of these papers demonstrated the relationship between the adenovirus E1 region and the virus integration, and none of them provided a system to carry out such research. Therefore, in this study we decided to develop a system to evaluate the effect of the E1 region on adenovirus integration, so the relationship between the E1 region and adenovirus integration can be determined in the long run.

1.4 Objectives of this Project

The objectives of this project were to develop techniques for testing the effect of the E1 region on adenovirus integration.

2. MATERIALS AND METHODS

2.1 Bacterial Culture

2.1.1 Bacterial Strain

Bacterial strain *Escherichia coli* (*E. coli*) DH5α (Gibco, BRL) was used as hosts for DNA cloning. This strain has the following genotype: F^-, Φ80, δ*lac*Z, ΔM15, Δ(*lac*ZYA-*arg*F), U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(rk^-, mk^+), *pho*A, *sup*E44, *thi*-1, λ^-, *gry*A96, *rel*A1) (Woodcock *et al.*, 1989).

2.1.2 Maintenance of Bacterial Strain

The bacterial strain was generally maintained in sterile Luria-Bertani broth (LB) at 37°C. The LB solution contains 1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 1% (w/v) NaCl and was sterilized by autoclaving.

For LB agar plates, 2% (w/v) agar was added to the LB solution prior to autoclaving. The autoclaved LB agar was allowed to cool to 45°C, and an appropriate amount of antibiotics, such as 100 μg/mL ampicilin or 50 μg/mL kanamycin, was added for the selection of transformed bacterial cells. When blue/ white screening is needed, 20 μg/mL 5-bromo-4-chloro- 3-indolyl-bD-galactoside (X-gal) was mixed with the LB agar solution, and the solution was distributed into Petri dishes before solidification.

For cryopreservation, bacterial cells in LB were mixed with 8% (v/v) sterile glycerol and stored at -70°C. To revive the frozen cells they were thawed, diluted with the proper amount of LB, and incubated at 37°C overnight.

2.1.3 Preparation and Transformation of Competent Cells using Calcium Chloride

The preparation and transformation of competent *E. coli* using calcium chloride is performed according to standard protocols (Cohen *et al.*, 1972; Sambrook *et al.*, 1989).

2.1.4 Small-Scale Extraction and Purification of Plasmid DNA

Small-scale plasmid DNA extraction and purification (plasmid mini-preparation) were carried out using an alkaline lysis method (Birnboim & Doly, 1979).

2.1.5 Intermediate-Scale Extraction and Purification of Plasmid DNA

Intermediate-scale plasmid DNA isolation was carried out with Plasmid DNA MaxiPrep Kit (Endotoxin Free) (Norgen Biotek Corp.) according to the manufacturer's instruction. The isolated plasmid DNA was then confirmed by restriction enzyme digestion analysis.

2.1.6 Large-Scale Extraction and Purification of Plasmid DNA

For large-scale plasmid DNA preparation, a candidate colony was inoculated into 50 mL of LB with appropriate antibiotics and incubated at 37°C overnight. The next day, the culture was transferred into 500 mL LB with antibiotics and incubated at 37°C for 6 h. The culture was then poured into a 1 L Beckman centrifuge bottle and centrifuged at 4°C at 4560 x g for 1 h. The

supernatant was decanted and the centrifuge bottle placed on a paper towel to dry the remaining LB medium. The bacterial cell pellet was resuspended with 50 mL of resuspension solution (50 mM Tris-HCl, 10 mM EDTA, 100 µg/ mL RNase A), and the resuspension was incubated at room temperature for 1 h. To break down the cell wall, 50 mL of lysis solution (0.2 M NaOH, and 1.0% w/v SDS) was added. The centrifuge bottle was inverted several times and incubated at room temperature for less than 5 min and the lysate was then mixed with 50 mL of neutralization solution (2 M potassium acetate, pH 4.8) and incubated at 4°C for 2 h.

The neutralized lysate was transferred into a 250 mL Beckman centrifuge bottle and centrifuged at 4°C at 30,100 x g for 20 min. The supernatant was poured onto a funnel covered with an eight-micron filter paper (Whatman), and the filtrate was collected in a Beckman 1000 mL centrifuge bottle. The filtrate was further treated with 5 units/mL RNase T1 (Cedarlane Laboratories Ltd., Worthington) and incubated at 55°C for 30 min. Then the filtrate was mixed with 2.5 volumes of ice-cold 95% ethanol and left in a -20°C freezer overnight.

The next day the plasmid DNA precipitate formed and was mixed by gently inverting the centrifuge bottle. The precipitate was then distributed into several 250 mL Beckman centrifuge bottles and centrifuged at 30,100 x g for 20 min to pellet the plasmid DNA. The supernatant was discarded and the bottles dried on paper towel to remove residual ethanol. Once the bottles were completely dried, 5 mL of TE buffer (10 mM Tris-HCl, and 1 mM EDTA, pH 7.5) was added into each centrifuge bottle and the DNA pellet dissolved. The dissolved DNA was

transferred into a 15 mL centrifuge tube. Another 5 mL of TE buffer was used for rinsing the 250 mL bottles to collect remnant plasmid DNA. The collected plasmid DNA was confirmed by restriction enzyme digestion analysis prior to cesium chloride banding.

2.1.7 Cesium Chloride Banding and Dialysis

The crude large-scale plasmid DNA preparation was further purified by cesium chloride (CsCl) banding and subsequent dialysis. Exactly 1.01 g CsCl was added for every 1 mL of dissolved plasmid DNA and the final density of the DNA solution was at 1.55-1.59 g/mL. For every liter of bacterial culture harvested, 200 µL of ethidium bromide (EtBr) (10 mg/mL) was added, and the DNA/EtBr mixture was transferred into Beckman OptiSeal ultracentrifuge tubes. These ultracentrifuge tubes were sealed with proper stoppers and assembled in a type 70.1 Ti rotor that was then placed in the Beckman L8-80M ultracentrifuge and centrifuged at 22°C at 246,960 x g for 22 h.

After ultracentrifugation, the lower band that contains supercoiled plasmid DNA was extracted by a 1 mL syringe with an 18-gauge needle and transferred to a 15 mL centrifuge tube. The EtBr was extracted repeatedly with CsCl-saturated isoamyl alcohol until no pink color was left in the plasmid DNA solution. The DNA solution was then transferred into Spectra/Por 7 dialysis tubing (Spectrum Laboratories, Inc.) and dialyzed against 10 L of dialysis buffer (autoclaved dH_2O or TE buffer). The dialysis buffer was changed every 24 h for

3 d. After dialysis, the DNA was filter-sterilized with a 0.2 µm syringe filter and stored at -20°C.

2.2 DNA Cloning

2.2.1 DNA Quantification

Plasmid DNA isolated from bacterial culture was quantified by spectrophotometry. The DNA sample was diluted in dH_2O with a certain dilution factor (normally 1:50). The OD (optical density) values of the sample were then determined by the spectrometer at the absorbance wavelengths of 260 nm (A_{260}) and 280 nm (A_{280}). After acquiring the OD value, the DNA concentration was calculated according to the following formula (Sambrook *et al.*, 1989):

$$[\text{DNA}]\ (\mu\text{g}/\ \mu\text{L}) = \frac{(A_{260}) \times (\text{dilution factor}) \times (50\ \text{ng}/\ \mu\text{L})}{1000}$$

When the purity of DNA sample needs to be examined (such as plasmid DNA for transfection), the relative purity was determined by the ratio of A_{260} to A_{280}. The sample was considered to be pure if the A_{260}/A_{280} was between 1.8 and 1.9.

2.2.2 Restriction Enzyme Digestion

Restriction enzyme digestion was carried out according to the manufacturer's instructions (New England Biolabs or Fermentas). Approximately 1 to 10 units of restriction enzyme were used in a 25 µL reaction system.

2.2.3 Inactivation of Enzymes

The inactivation of enzymes was done by heating or by adding DNA loading dye. Under circumstances when multiple restriction enzymes with different buffer requirements were needed, heat inactivation was used. The plasmid digested with the first restriction enzyme was heated at 65-80°C for 15 min to inactivate the restriction enzyme. Then the other enzyme together with proper buffer was added to carry on the digestion. On the other hand, if the digestion needed to be confirmed by agarose electrophoresis, the enzymes were inactivated by adding 5 µL of 6X loading dye containing SDS (0.05% bromophenol blue, 20% glycerol, 2% SDS).

2.2.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out according to regular procedure (Sambrook *et al.*, 1989). The gel was then viewed under ultraviolet (UV) light, and the image was captured and documented using AlphaImager 2200 (Alpha Innotech).

2.2.5 Gene Clean using Norgen Gel Extraction Kit

The extraction of certain DNA bands was done using the DNA Gel Extraction Kit (Norgen Biotek Corp.). The gene clean procedures were carried out according to the manufacturer's instruction.

2.2.6 DNA Ligation

Bacteriophage T4 DNA ligase (NEB) was used to ligate the DNA fragments. To make a 25 µL reaction volume, proper amount of vector and insert DNA, 1 µL of T4 DNA ligase (400 units) and 2.5 µL of T4 DNA ligase buffer (10 mM $MgCl_2$, 20 mM dithiothreitol (DTT), 50 mM Tris, 1 mM ATP, pH 7.5) were added into the mixture. Distilled water was then used to bring the volume to 25 µL. The ligation reaction was incubated at room temperature for 2 h for cohesive end ligation and overnight for blunt end ligation.

2.3 Cell Culture

2.3.1 Cell Lines

Human Embryonic Kidney 293 cell line (HEK 293) (Graham *et al.*, 1977) and Henrietta Lacks cervical carcinoma cell line (HeLa) (Gey *et al.*, 1952) were used in this experiment. Both cell lines were maintained as monolayer cultures and incubated in a water-jacketed incubator (Fisher Scientific, Pittsburgh PA) at 37°C with 96% humidity and 5% CO_2.

2.3.2 Maintenance of Cell Culture

The cell culture medium contains Minimum Essential Medium (MEM) (Invitrogen Corp., Gibco), 1% (v/v) Antibiotic-Antimycotic (10,000 units/mL penicillin G sodium, 10,000 ug/mL streptomycin sulfate and 25 µg/mL amphotericin B, Invitrogen Corp., Gibco), 0.43mg/mL GLUTAMAX^{TM}-1 (Invitrogen Corp., Gibco), 0.225% (w/v) sodium bicarbonate (Invitrogen Corp.,

Gibco), and 5~10% (v/v) Fetal Bovine Serum (FBS, PAA Laboratories Inc.) for 293 cells or 5~10% (v/v) Donor Horse Serum (DHS, PAA Laboratories Inc.) for HeLa cells.

The HEK 293 cells were generally maintained in a 150-mm petri dish. Once the cell monolayer reached 90% confluency, the cells were passaged. Four mL of saline citrate (15 mM sodium citrate, 135 mM potassium chloride) was added to the cell monolayer, and the dish was incubated at 37°C for 5 min. The cells were detached from the plates by gently tapping the side of the dish and the cell suspension was distributed evenly in 2 petri dishes. Then the medium was added to make a final volume of 20 mL and the cells were incubated.

The maintenance of HeLa cells is the same as 293 cells except for the detachment step. To dislodge HeLa cells, 0.25% trypsin (Invitrogen Corp., Gibco) in 4 mL of versene (for 1 L: 1.0g glucose, 0.4g KCl, 8.0g NaCl, 0.58g $NaHCO_3$ and 0.2g EDTA) was used instead of saline citrate.

2.3.3 Cell Freezing and Thawing

Cell freezing was carried out when the cells grew to the late log phase (the cell monolayer is at 90% confluency). The cells were detached, counted and centrifuged at 670 x g for 5 min and then resuspended in FBS/ DHS containing 10% dimethyl sulfoxide (DMSO). The cell suspension was then transferred to plastic ampoules with screw caps. The ampoules were packaged with an

insulating tissue towel and placed in a -20°C freezer overnight. The next day, the ampoules were stored at -70°C.

The thawing procedure was carried out as quickly as possible to maximize the cell viability. Cells were thawed by immersing the ampoule in a 37°C water bath. Once the cells were completely thawed, they were poured in a 100-mm tissue culture dish and supplemented with 10 mL of culture medium. The cell culture medium containing DMSO was changed 24 h after the thawing.

2.3.4 Mammalian Cell Counting

The cells were lifted and transferred to a 50 mL polypropylene centrifuge tube. Eighteen µL of the cell suspension was mixed with 2 µL of 0.04% trypan blue (Sigma) and the mixture was incubated at room temperature for less than 3 min. The dead cells took up the trypan blue and showed blue color under the light microscope whereas the living cells showed no color.

A hemocytometer (Improved Neubauer) (Hausser Scientific) was covered with a cover slip and 15 µL of the cell-trypan blue mixture was loaded on the notch of the hemocytometer. The hemocytometer was then placed on the light microscope and the number of the living cells (no blue color) was counted.

2.3.5 Mammalian Cell Transfection

Two types of transfection methods were used in this experiment: a calcium phosphate-mediated method and a liposome-mediated method. The transfection experiments were generally performed in 6-well plates.

2.3.5.1 Transfection Mediated by Calcium Phosphate

Calcium phosphate transfection was originally developed by Graham and van der Eb (1973) and optimized by Jordan *et al.* (1996). Two hours before transfection, the cell culture medium was replaced with 2 mL of fresh medium. Five µg of plasmid DNA (25 µg DNA/ mL cell culture medium) was made up to 90 µL with TE buffer (pH 8.0) and mixed with 10 µL of 2.5 M $CaCl_2$. A 15 mL centrifuge tube containing 100 µL of 2XHEPES (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4{\cdot}2H_2O$, 12 mM dextrose, 50 mM HEPES, pH 7.05, filter sterilized) was vigorously shaken on a vortex and the DNA-TE-$CaCl_2$ mixture was added dropwise into the HEPES solution. The DNA-$CaPO_4$ mixture was then distributed evenly into the cell culture medium. The medium was changed at 6 h post-transfection to minimize cytotoxicity. One day after transfection, the transfection efficiency was tested.

2.3.5.2 Transfection Mediated by Lipofectamine^{TM} 2000

Lipofectamine^{TM} 2000 (Invitrogen) was used as transfection reagent for both plasmid DNA and siRNA. For the transfection of plasmid DNA, 5 µL of Lipofectamine^{TM} 2000 per well was used. At the time of transfection, the cell monolayer should be no less than 70% confluent; otherwise, the transfected cells would show cytotoxicity. The Lipofectamine^{TM} 2000 was mixed with OptiMEM I Reduced Serum Medium (Invitrogen Corp., Gibco) to 250 µL, and the mixture was incubated at room temperature for 5 min. The desired amount of plasmid DNA (usually 2.5 or 5.0 µg per well) was also made up to 250 µL using OptiMEM I. After 5 min incubation, the Lipofectamine^{TM} 2000 and plasmid DNA were

mixed and left at room temperature for 20 min. Before adding the liposome and plasmid DNA, the culture medium that contained 1% antibiotics was replaced with 2 mL of medium that does not contain antibiotics (antibiotics-free) to reduce the cytotoxicity. Then the 500 µL of liposome-DNA mixture was added in the wells. The transfected cells were placed at 37°C and incubated for less than 6 h, and the medium was replaced with medium without antibiotics.

For siRNA transfection, 2.5 µL of Lipofectamine^{TM} 2000 per well was used. The Lipofectamine^{TM} 2000 was first mixed with 247.5 µL of OptiMEM I and incubated at room temperature for 5 min. The appropriate amount of siRNA (30-90 nM) was mixed with OptiMEM I in a final volume of 250 µL. The liposome and siRNA were then mixed and incubated at room temperature for 15 min. Meanwhile, the cells were lifted, counted and added into the plates, and then supplemented with medium without antibiotics to a final volume of 2.0 mL. Then the liposome-siRNA complex was added to the plate and incubated at 37°C. The fresh antibiotics-free medium was changed 24 h post-transfection to maximize the transfection efficiency while minimizing the cytotoxicity.

2.3.6 Assay for β-Gal Activities

The transfection efficiency of plasmid DNA was evaluated by a β-gal activity assay. The plasmid pCMVβ, which carries a β-galactosidase (*lacZ*) gene, was used as a reporter. The procedure was carried out according to standard protocols (Sambrook *et al.*, 1989).

2.3.7 Total RNA and Plasmid DNA Isolation from Mammalian Cells

The total RNA and genomic DNA were purified from cultured HEK 293 and HeLa cells using RNA/ DNA/ Protein Purification Kit (Norgen Biotek Corp.). The isolation was performed according to the manufacturer's instruction.

2.4 Adenoviral Culture

2.4.1 Cotransfection of Plasmids to Rescue Recombinant Adenovirus

The recombinant adenovirus was rescued by cotransfecting the shuttle plasmid and viral genomic plasmid DNA into 293 cells. The transfection protocol is explained in section "Cell Culture". The amount of plasmid DNA was 5.0 μg per well in a 6-well plate (2.5 μg for each plasmid).

2.4.2 Viral DNA Extraction and Confirmation

The cytopathic effect (cpe) normally appeared at 7~15 d post-transfection. When the cpe was nearly complete (i.e. the majority of cells were rounded but still attached to the plates), the plate was left in the cell culture hood without disturbing for 30 min to settle down the floating cells. The medium was then transferred into a 4 mL-glass vial, supplemented with sterile glycerol (final concentration 10%, v/v) and stored at -70°C.

Most of the infected cells should remain on the surface of the plate. Next, 1 mL of lysis solution (10 mM Tris-HCl, 100 mM EDTA, 0.4% (w/v) SDS, 0.5 mg/mL pronase) was used to lyse the cells. The plate was incubated at 37°C for 4-10 h. Five hundred μL of 1:1 phenol-chloroform was added to 500 μL of the

lysate. The mixture was shaken vigorously to homogenize the organic and aqueous phase. Then the tube was centrifuged at 16,000 x g for 2 min to separate the two phases. The upper phase was carefully transferred to a fresh tube and mixed thoroughly with 500 µL of chloroform. The tube was centrifuged at 16,000 x g for 2 min. The upper phase was transferred to a new tube, and 600 µL of 95% ice-cold ethanol was added and the tube was placed at -20°C for 30 min to precipitate the viral and host genomic DNA. The tube was then spun at 16,000 x g for 20 min and the supernatant was discarded. The DNA pellet was washed again with 900 µL of 70% ethanol, incubated at -20°C for 30 min, and centrifuged at 16,000 x g for 20 min. The supernatant was decanted. The tube was air-dried at 37°C for 5 min and the DNA pellet dissolved in 50 µL of TE buffer. The extracted viral DNA was then digested and confirmed with *Hin*dIII.

2.5 RNA Interference, RNA Isolation and RT-PCR

2.5.1 RNA Interference

The RNA interference was carried out by transfecting chemically synthesized small interfering RNA (siRNA) (Ambion). The transfection protocol is described in section “Cell Culture”.

2.5.2 Total RNA Isolation from Mammalian Cells

The total RNA was isolated from mammalian cells using the Total RNA Purification Kit (Norgen Biotek) according to the manufacturer’s instruction. The integrity of isolated RNA was further analyzed by running an RNA gel.

2.5.3 RNA Gel

Formaldehyde agarose gel electrophoresis was used to examine the integrity of the RNA samples. The RNA gel was prepared using standard protocols (Sambrook *et al.*, 1989). After the electrophoresis, the gel was viewed under ultraviolet (UV) light and the image captured using the AlphaImager 2200 (Alpha Innotech) AlphaEaseFC software.

2.5.4 RNA Quantification

RNA quantification using spectrophotometry was carried out using a similar procedure to DNA spectrophotometric quantification, but the formulation was changed as shown below (Sambrook *et al.*, 1989).

$$\underset{(\mu g/\ \mu L)}{[RNA]} = \frac{(A_{260}) \times (\text{dilution factor}) \times (40\ ng/\ \mu L)}{1000}$$

2.5.5 Reverse Transcription

The volume of RNA sample required in reverse transcription (RT) was calculated according to the OD_{260} reading. Then the calculated amount of sample was mixed with 0.5 μL of 0.5μg/ μL Oligo $(dT)_{18}$ Primer (Fermentas) and made up with RNase-free water to 5 μL. During RT reactions, the mixture was heated to 70°C for 5 min to denature the secondary structure of the RNA, and held at 4°C. Then the RT reaction solution containing 4 μL 5X First Strand Buffer (250 mM Tris-HCl [pH 8.3 at RT], 375 mM KCl, 15 mM $MgCl_2$), 2 μL of 0.1 M DTT, 1 μL of 10 mM dNTPs, 0.5 μL Superscript II reverse transcriptase (Invitrogen) and 7.5 μL RNase-free water was added. The cDNA was

synthesized at 25°C for 5 min, 42°C for 90 min and 70°C for 15 min, followed by a holding at 4°C. Finally the synthesized first strand cDNA was diluted to 50 µL with dH_2O.

2.5.6 PCR and Realtime PCR:

Polymerase chain reaction (PCR) (Mullis *et al.*, 1986) was performed according to the polymerase manufacturer (BioTherm). Most of the PCR primers used in this experiment were designed using the Primer3 program (Rozen & Skaletsky, 2000).

The sequence of the Ad5 E1A forward primer is 5'-ACACCG-GGACTGAAAATGAG-3'; the sequence of the Ad5 E1A reverse primer is 5'-AAGGACCGGAGTCACAGCTA-3'. The sequence of the Ad5 E1B forward primer is 5'-AGACACAAGAATCGCCTGCT-3'; the sequence of the Ad5 E1B reverse primer is 5'-CACAATGCTTCCATCAAACG-3'. The sequence of the GAPDH forward primer is 5'-AAGGCTGGGG-CTCATTTGCAG-3'; the sequence of the GAPDH reverse primer is 5'-CCAAATTCGTTGTCATACCAGG-3' (the amplification will generate an 895 bp band from gDNA and a 638 bp from cDNA). The sequence of the β-actin forward primer is 5'-TGGACATCCGCAAAGACCTG-3'; the sequence of the β-actin reverse primer is 5'-CCGATCCACACGGAGTACTT-3' (166 bp). The UCONLY forward primer is located at 20-39 bp of pUC19 and the sequence is 5'-CGGTGAA-AACCTCTGACACA-3'; the UCONLY reverse primer is located at 419-439 bp of pUC19, and the sequence is 5'-TGCAGGTCG-ACTCTAGAGGAT-3'.

All the PCR reactions were performed using the iCycler PCR machine (Bio-Rad). For a 20 µL PCR reaction system, 1 µL of DNA/ cDNA template, 0.12 µL of each primer (50 mM stock), 0.2 µL of BioTherm Taq DNA polymerase (5 units/ µL), 2 µL of 10X PCR buffer (750 mM Tris-HCl [pH 8.8], 200 mM $(NH_4)_2SO_4$, 0.1% v/v Tween 20), 1 µL of 10 mM dNTPs and 15.6 µL of dH_2O were added in a PCR tube and placed in the iCycler. The DNA/ complementary DNA template was amplified at 94°C for 3 min to dissociate the DNA double strands. Then the heat cycles were carried out at the annealing temperature (5°C lower than the primer melting temperature) for 30 sec, at the extension temperature of 72°C for 90 sec, and at the denaturation temperature of 94°C for 30 sec. The total cycling numbers varied between 20 and 35 cycles. The reaction was held at 72°C for 5 min followed by incubation at 4°C.

Realtime PCR was used to evaluate homologous recombination efficiency. The reaction mixture of Realtime PCR contained 50 ng of template DNA with known concentrations or 2 µL of genomic DNA isolated from HEK 293 cells, 1x SYBR GREEN master mix (Eurogentec), and 0.12 µL of each primer (50 mM stock) in a 15 µl volume of reaction. The PCR mixture was heated at 95°C for 15 min to activate the Hotstart enzyme. The cycling temperature and time were set as 95°C for 15 sec, 58°C for 30 sec, and 72°C for 1 min, and repeated for 40 cycles. Then the temperature returned to 58°C for 1 min, and the melt curve survey was carried out with an escalating temperature of 0.5°C per 10 sec. The melt curve reaction was performed with 80 rounds.

3. RESULTS

3.1 Construction of Plasmids for Generating Recombinant Adenoviruses

The long term aim of this project was to test whether the E1 region affects adenovirus integration. This can be carried out by comparing the integration efficiencies between an E1-deleted adenoviral vector and an E1-containing vector. To achieve this goal, two types of adenoviral vectors with proper reporters were designed. In the E1-deleted adenoviral vector, the E1 region will be substituted with a green fluorescent protein (GFP) cassette, and was designated as SubE1. In the E1-containing adenoviral vector, the E1 region will be maintained, while the E3 region will be replaced with the red fluorescent protein (RFP); it was named SubE3.

3.1.1 Overall Strategies of Rescuing Recombinant Adenoviruses

The AdMax (Microbix) adenoviral vector system was used in this experiment. This system is modified from adenovirus serotype 5 (Ad5) genomic DNA. It requires both a shuttle plasmid carrying a transgene cassette and a genomic plasmid containing adenoviral genetic content. These two plasmids would be co-transfected into host cells. Once inside the host cell, homologous recombination would occur at the homologies shared by the shuttle and genomic plasmids, and thus the transgene will be shifted into the viral genome to generate recombinant adenovirus. The schematic representations of rescuing the SubE1 and SubE3 viruses are illustrated in Figure 3.1 and 3.2, respectively.

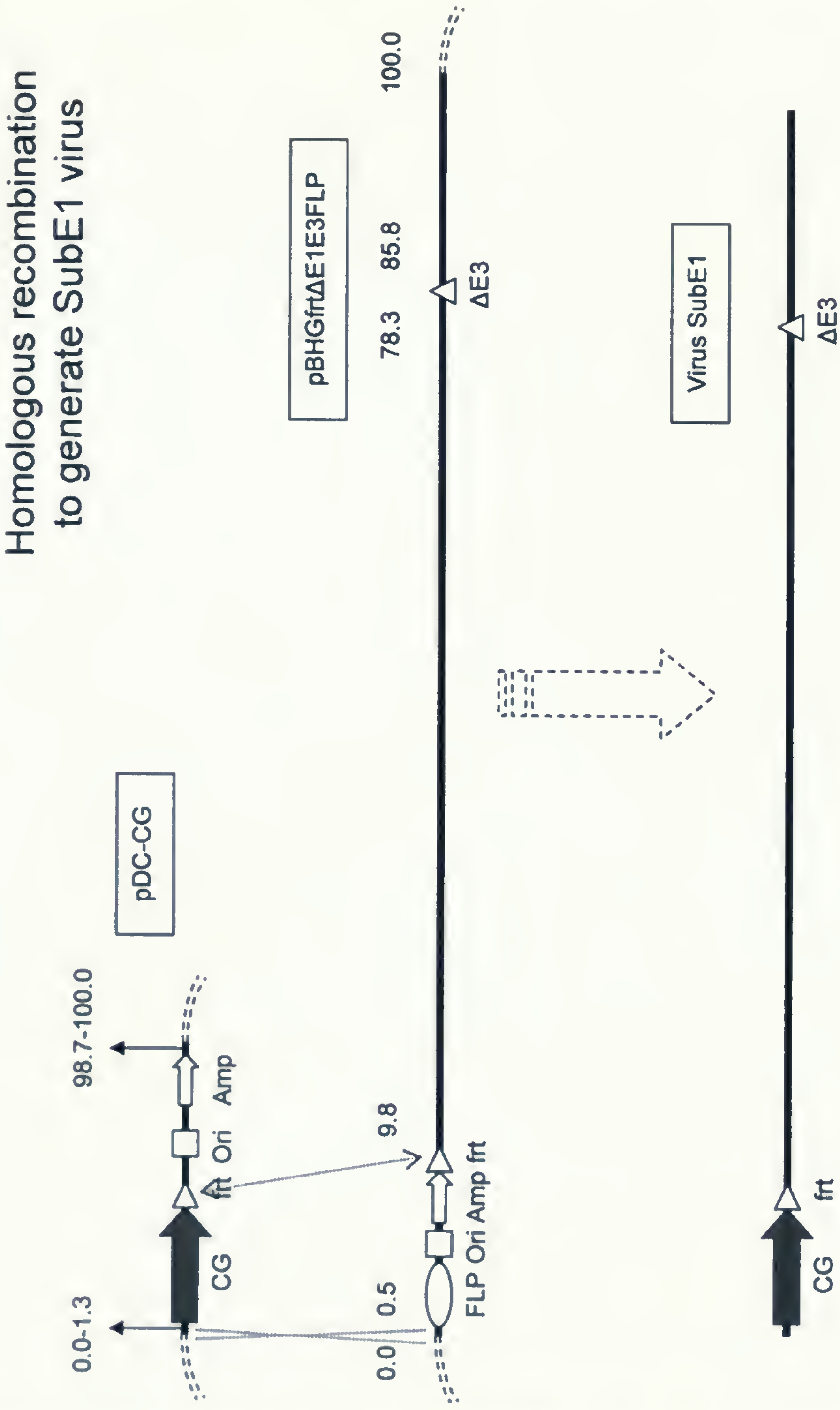

Homologous recombination to generate SubE1 virus
pDC-CG
0.0-1.3
98.7-100.0
CG
frt
Ori
Amp
pBHGfrtΔE1E3FLP
0.0
0.5
9.8
78.3
85.8
100.0
FLP Ori Amp frt
ΔE3
Virus SubE1
CG
frt
ΔE3

Figure 3.1. Homologous recombination to generate SubE1 adenovirus. The thick black line represents the genome of Ad5. The numbers above the thick black line denote the percentages of the virus genome map unit (m.u.).

The shuttle plasmid for SubE1 virus is designated as pDC-CG. It was cloned by inserting a GFP cassette into the plasmid pDC511. The resulting plasmid pDC-CG contains the left end (0.0-1.3 m.u.) and right end (98.7-100.0 m.u.) of Ad5 genome. It also contains an *frt* site, which is essential for FLP (a yeast recombinase)-mediated site-specific recombination.

The SubE1 genomic plasmid pBHGfrtΔE1E3FLP contains almost all of the adenovirus genetic content except for two deletions: one is deleted from 0.5 to 9.8 m.u. of Ad5 genome, the other from 78.3 to 85.8. In addition, it contains an *FLP* gene cassette that expresses the recombinase FLP; this recombinase mediates the site-specific recombination at the *frt* site.

Once these two plasmids are co-transfected into HEK 293 cells, the homologous recombination should occur at 0.0-0.5 m.u. (the left ends of the two plasmids). In addition, a site-specific recombination should take place through FLP-mediated DNA sequence exchange at the *frt* site. Upon these two recombination events, the SubE1 virus was generated (Figure 3.1).

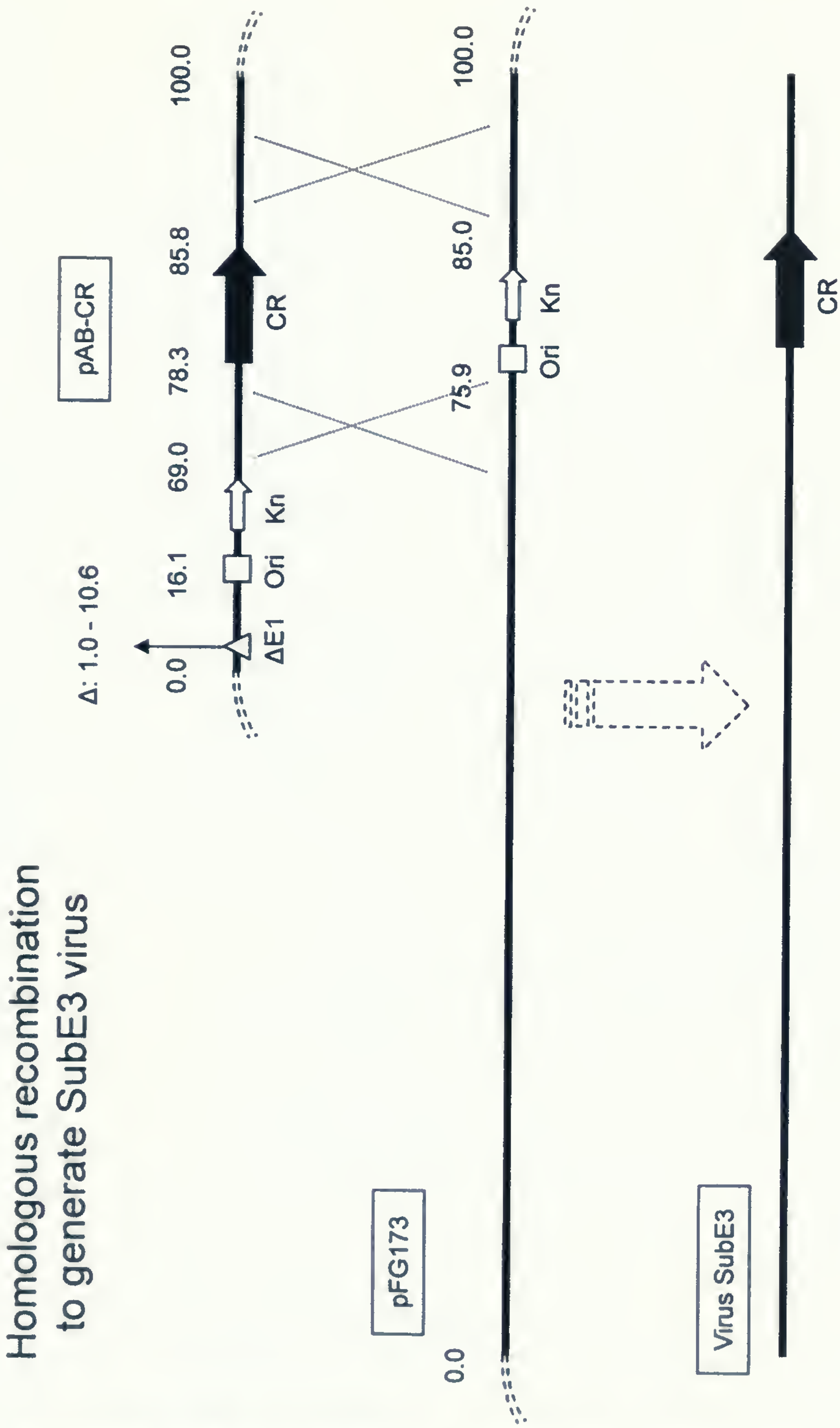

Homologous recombination
to generate SubE3 virus
pAB-CR
Δ: 1.0 - 10.6
0.0
16.1
69.0
78.3
85.8
100.0
ΔE1
Ori
Kn
CR
pFG173
0.0
75.9
85.0
100.0
Ori
Kn
Virus SubE3
CR

Figure 3.2. Homologous recombination to generate SubE3 adenovirus. The thick black line represents the genome of Ad5. The numbers above the thick black line denote the percentage of the virus genome map unit (m.u.).

Similarly, SubE3 virus could also be generated through *in vivo* homologous recombination. As is shown in Figure 3.2, the homologous recombination would occur near the right end of the Ad5 genome (between 69.0-78.3 and 85.9-100.0). The resulting DNA molecule will contain most of the Ad5 genome except for a RFP-substitution in E3 region.

During the preparation of plasmids for generating recombinant adenoviruses, only the shuttle plasmids are required to be designed and constructed. The construction of the shuttle plasmids is shown in the following sections. The first two plasmids to be constructed are the reporter plasmids for SubE1/ E3 viruses, respectively pCMV-GFP and pCMV-RFP. The construction was carried out by placing the GFP/ RFP cassettes under the control of cytomegalovirus (CMV) promoter. The details of the construction are shown in sections 3.1.2 and 3.1.4.

The next step was to insert the CMV-GFP/ CMV-RFP into the shuttle plasmid backbones. For the SubE1 virus, the CMV-GFP cassette was inserted into the plasmid pDC-511 to form pDC-CG, as described in section 3.1.3. For the SubE3 virus, the CMV-RFP cassette was ligated into pAB26 to form pAB-CR, as depicted in section 3.1.5.

3.1.2 Construction and Confirmation of pCMV-GFP

The plasmid pCMV-GFP was the reporter plasmid for the SubE1 virus. It was constructed by placing the GFP cassette from pAcGFP1 under the control of the immediate early cytomegalovirus promoter ($P_{\text{CMV IE}}$) from pCMVβ. The

schematic representation for constructing the plasmid pCMV-GFP is shown in Figure 3.3.

In the plasmid pAcGFP1, the GFP cassette is under the control of the prokaryotic *lac* promoter (P_{lac}). Therefore, by placing the GFP cassette under the control of $P_{CMV\ IE}$, the GFP can be expressed in mammalian cells. The GFP cassette was obtained by digesting pAcGFP1 with *Sma*I and *Not*I.

The $P_{CMV\ IE}$ backbone was generated by digesting pCMVβ with *Stu*I and *Not*I. The digestion with these two enzymes resulted in two fragments with similar length, 3524 and 3640 bp, which are difficult for the electrophoresis separation and subsequent gel extraction. Therefore, pCMVβ was first digested with *Eco*RV, and then digested with *Stu*I and *Not*I. The digestion generated three fragments with distinct lengths, 1300, 2224 and 3640 bp, which were easily separated on an agarose gel.

After the digestion and gel extraction, either the GFP cassette or pCMVβ backbone contains a blunt end and a *Not*I overhang. Therefore, the two fragments are complementary to each other, the ligation was facilitated and the screening procedures simplified.

The generated plasmid was then confirmed by restriction enzyme digestion and agarose gel electrophoresis. The restriction enzymes and the expected restriction lengths are shown in Table 3.1. The agarose gel of the digestion result is shown in Figure 3.4.

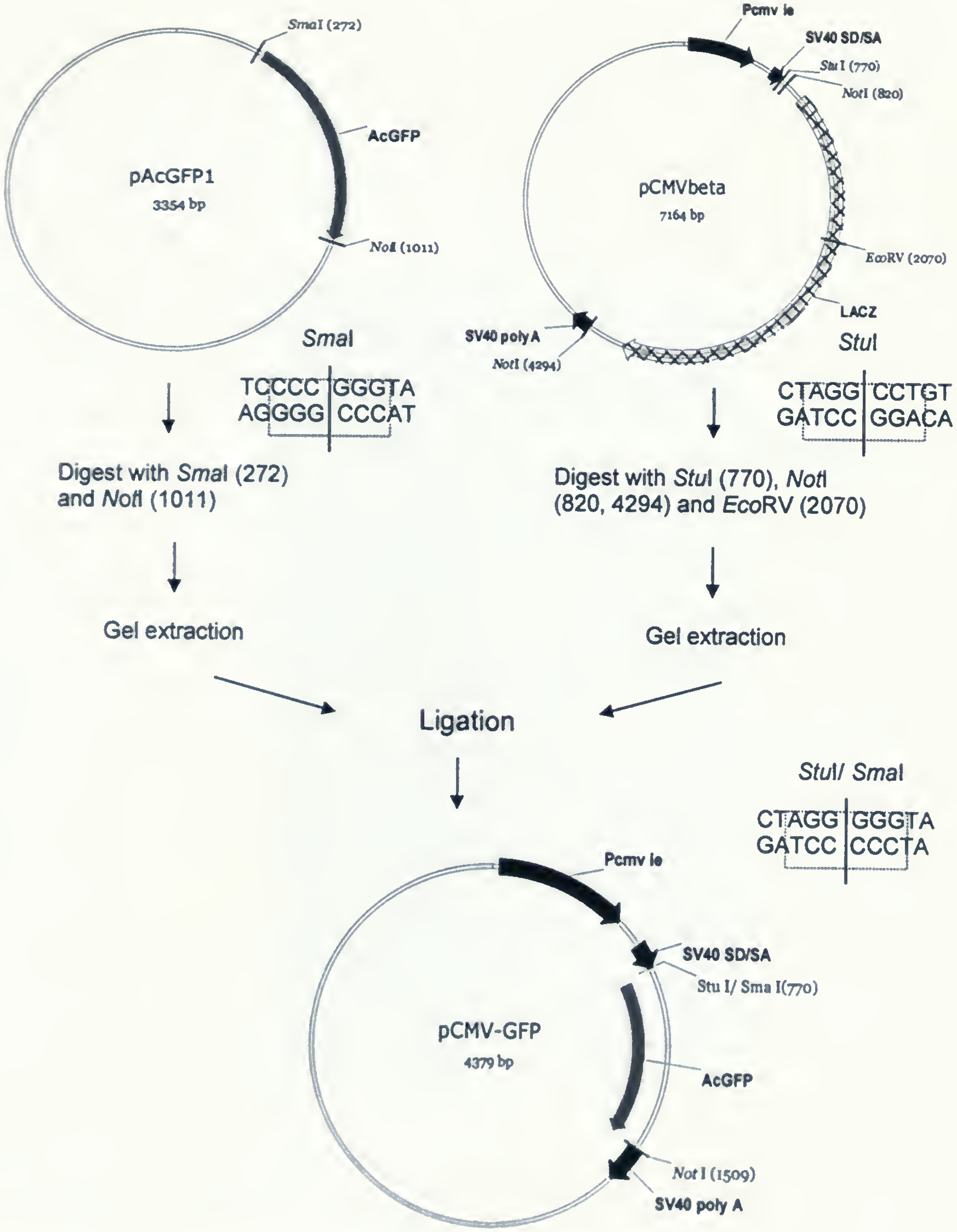
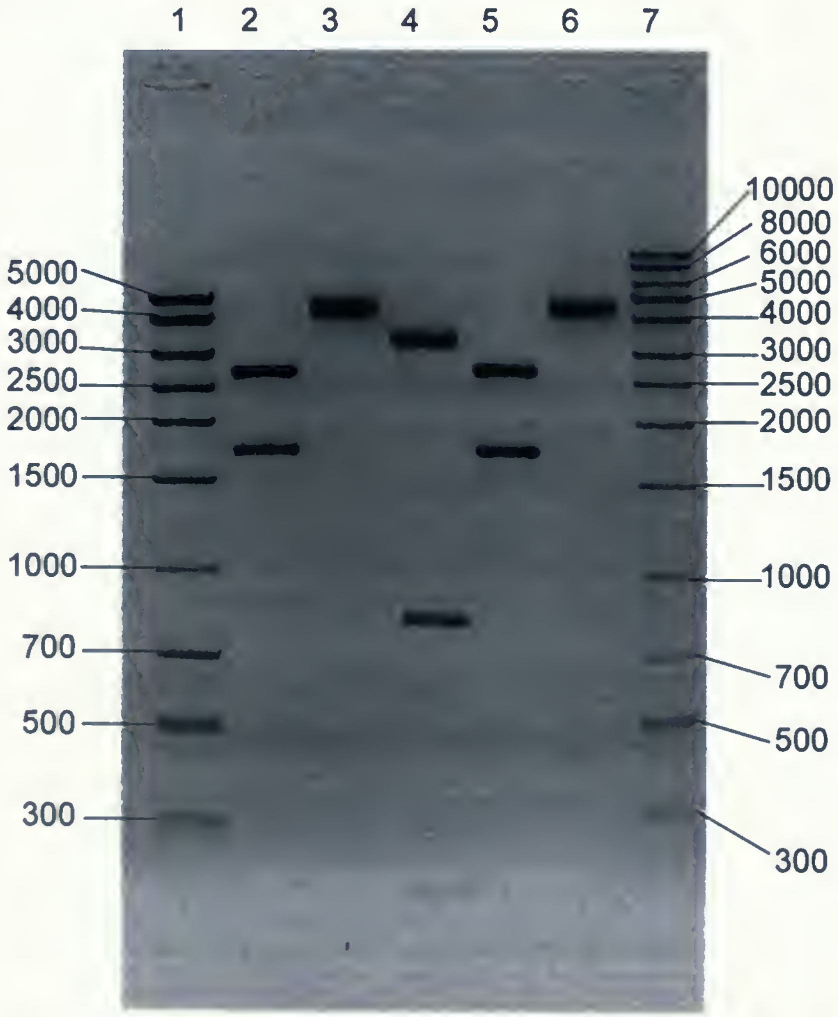

SmaI (272)
AcGFP
pAcGFP1
3354 bp
NotI (1011)
SmaI
TCCCC GGGTA
AGGGG CCCAT
Digest with SmaI (272) and NotI (1011)
Gel extraction
Pcmv ie
SV40 SD/SA
StuI (770)
NotI (820)
pCMVbeta
7164 bp
EcoRV (2070)
LACZ
SV40 polyA
NotI (4294)
StuI
CTAGG CCTGT
GATCC GGACA
Digest with StuI (770), NotI (820, 4294) and EcoRV (2070)
Gel extraction
Ligation
StuI/ SmaI
CTAGG GGGTA
GATCC CCCTA
Pcmv ie
SV40 SD/SA
Stu I/ Sma I(770)
pCMV-GFP
4379 bp
AcGFP
Not I (1509)
SV40 poly A

Figure 3.3. The strategy for placing the GFP cassette under the control of CMV promoter to generate plasmid pCMV-GFP.

Table 3.1. Restriction enzyme analysis of pCMV-GFP.

Enzyme	*Eco*RI *Sal*I	*Not*I	*Bam*HI	*Pst*I	*Ava*I
Fragment size (bp)	2653 1726	4379	3294 809 198 78	2665 1714	4379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5000
4000
3000
2500
2000
1500
1000
700
500
300

Figure 3.4. Restriction enzyme digestion of pCMV-GFP on agarose gel. Lane 1: Norgen MidRanger marker; Lane 7: Norgen HighRanger marker; Lane 2-6: plasmid DNA digested by restriction enzymes: *Eco*RI and *Sal*I; *Not*I; *Bam*HI; *Pst*I; *Ava*I. Numbers on the sides refer to marker sizes in basepairs.

3.1.3 Construction and Confirmation of pDC-CG

The plasmid pDC-CG was the shuttle plasmid for SubE1 virus. It was constructed by placing the CMV-GFP cassette from pCMV-GFP into the backbone of plasmid pDC511. The strategy for constructing pDC-CG is shown in Figure 3.5.

The plasmid pDC511 was the backbone for the virus shuttle plasmid. It contains left end (0.0%-1.3%) and right end (98.7-100.0%) of Ad5 genome. It also contains an *frt* site for FLP-mediated site-specific recombination. The left end of the Ad5 genome and the *frt* site are the substrates for recombination events to generate the recombinant adenovirus. The backbone of pDC511 was obtained by digesting the plasmid with *Eco*RI and *Sal*I.

The plasmid pCMV-GFP was also digested with *Eco*RI and *Sal*I. Then the CMV-GFP cassette and the backbone of pDC511 were ligated and transformed into bacterial cells. The recombinant plasmid DNA was isolated and confirmed with various restriction enzymes. The restriction enzymes used for confirmation and the expected digestion patterns are shown in Table 3.2. The agarose gel picture is shown in Figure 3.6.

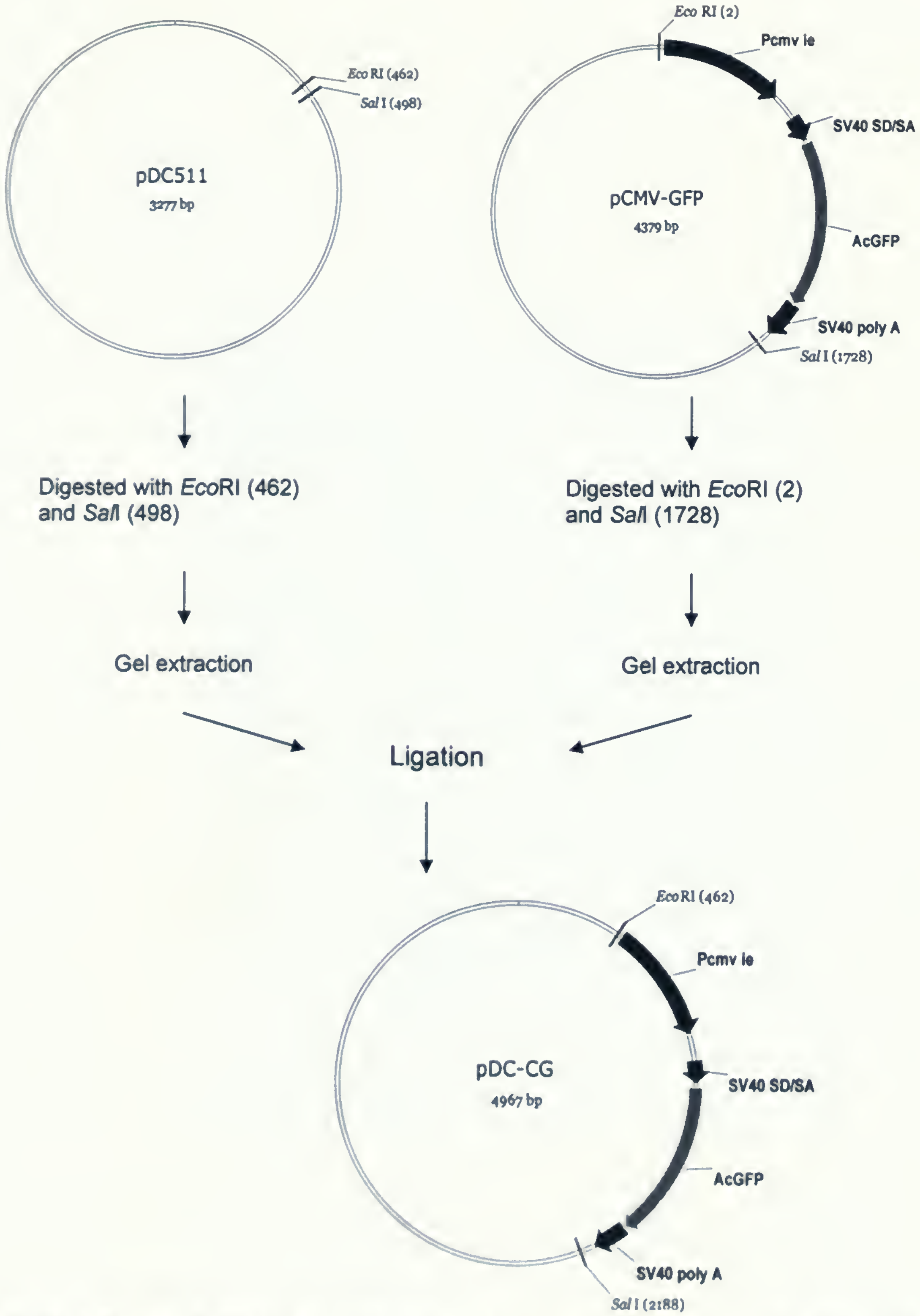
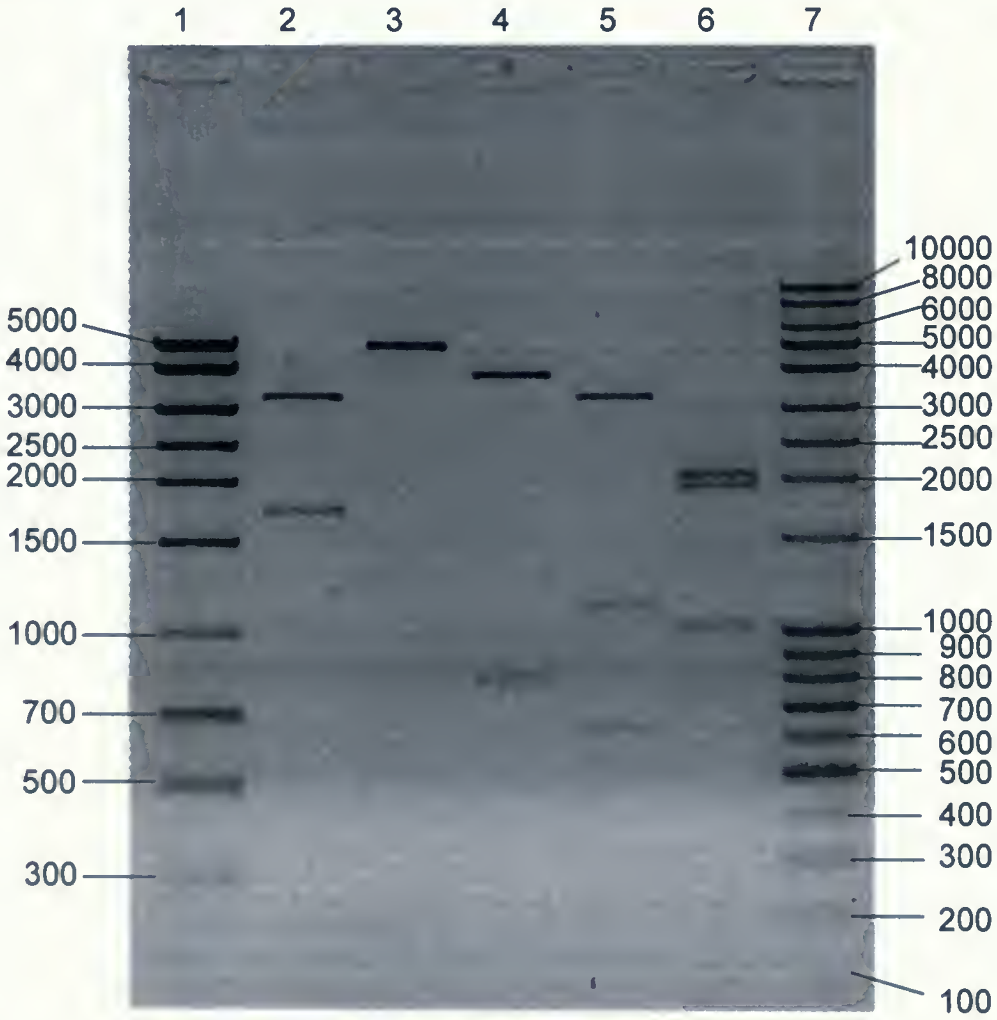

Eco RI (462)
Sal I (498)
pDC511
3277 bp
Eco RI (2)
Pcmv ie
SV40 SD/SA
pCMV-GFP
4379 bp
AcGFP
SV40 poly A
Sal I (1728)
Digested with *Eco*RI (462) and *Sal*I (498)
Digested with *Eco*RI (2) and *Sal*I (1728)
Gel extraction
Gel extraction
Ligation
EcoRI (462)
Pcmv ie
pDC-CG
4967 bp
SV40 SD/SA
AcGFP
SV40 poly A
Sal I (2188)

Figure 3.5. The strategy for inserting CMV-GFP cassette into the backbone of pDC511 to generate plasmid pDC-CG.

Table 3.2. Restriction enzyme analysis of pDC-CG.

Enzyme	*Eco*RI *Sal*I	*Not*I	*Bam*HI	*Xba*I	*Hinc*II
Fragment size (bp)	3241 1726	4967	3864 809 198 78 18	3241 1097 629	2019 1885 986 77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900
800
700
600
500
400
300
200
100

Figure 3.6. Restriction enzyme digestion of pDC-CG on agarose gel. Lane 1: Norgen MidRanger marker; Lane 7: Norgen HighRangerPlus marker; Lane 2-6: plasmid DNA digested by restriction enzymes: *Eco*RI and *Sal*I; *Not*I; *Bam*HI; *Xba*I; *Hinc*II. Numbers on the sides refer to marker sizes in basepairs.

3.1.4 Construction and Confirmation of pCMV-RFP

The plasmid pCMV-RFP was the reporter plasmid for SubE3 virus. To create pCMV-RFP, the RFP fragment from pDsRed-Monomer (under the control of the P*lac* promoter) was taken out and inserted into pCMVβ backbone. The schematic presentation for constructing pCMV-RFP is shown in Figure 3.7.

The procedure of making pCMV-RFP was identical to that of generating pCMV-GFP. The plasmid pCMVβ was cut with the enzyme *Stu*I, *Not*I and *Eco*RV. The plasmid pDsRed-Monomer was cut with the enzyme *Sma*I and *Not*I. Both the RFP fragment and CMV backbone have a blunt end and a *Not*I cohesive end, so the ligation was facilitated. The ligation was then transformed into bacterial cells and the recombinant plasmid purified. The enzymes used for the confirmation and expected restriction lengths are shown in Table 3.3. The agarose gel for the digestion result is shown in Figure 3.8.

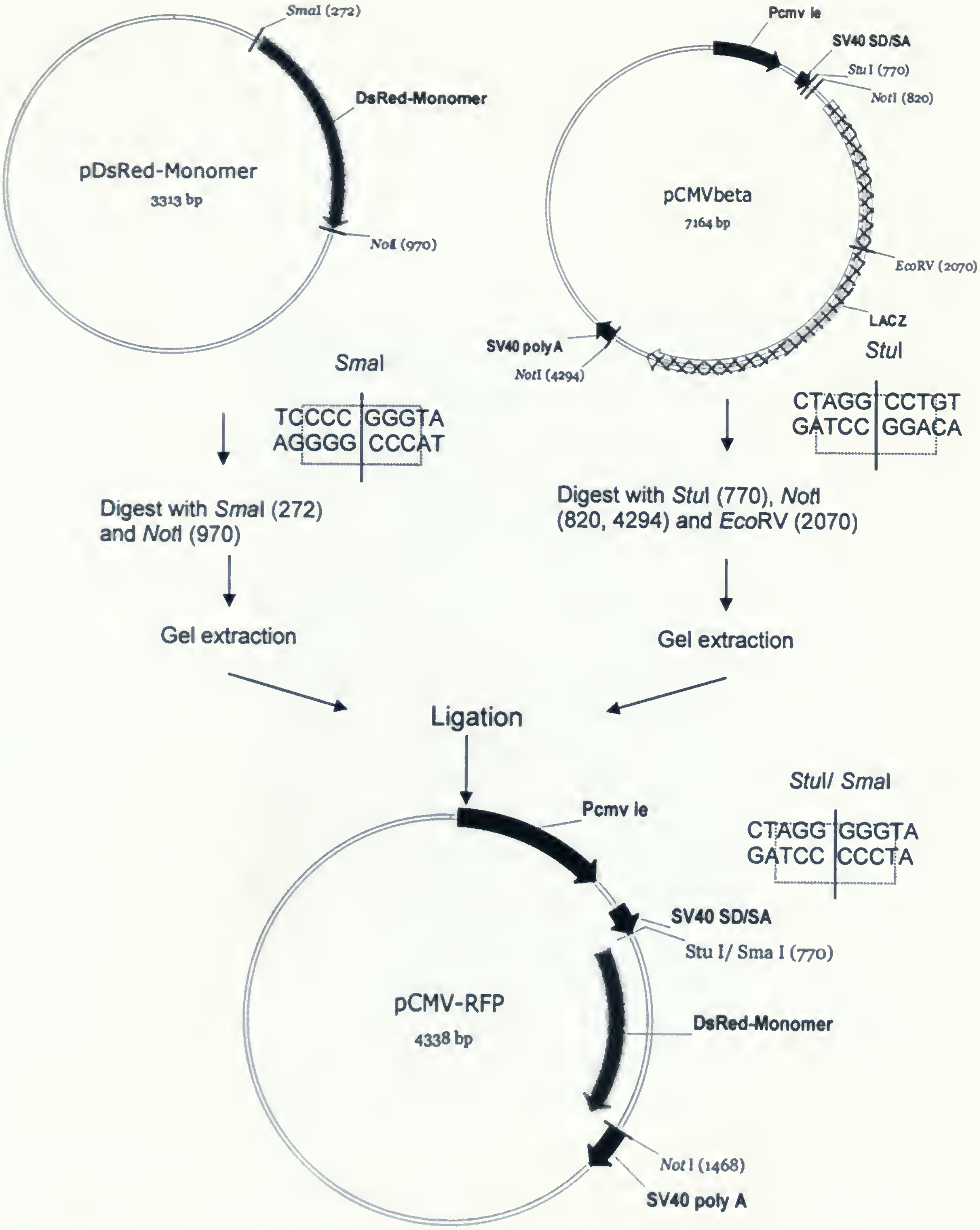
SmaI (272)
DsRed-Monomer
pDsRed-Monomer
3313 bp
NotI (970)
Pcmv Ie
SV40 SD/SA
StuI (770)
NotI (820)
pCMVbeta
7164 bp
EcoRV (2070)
LACZ
SV40 polyA
NotI (4294)
SmaI
TCCCC GGGTA
AGGGG CCCAT
StuI
CTAGG CCTGT
GATCC GGACA
Digest with SmaI (272) and NotI (970)
Digest with StuI (770), NotI (820, 4294) and EcoRV (2070)
Gel extraction
Gel extraction
Ligation
Pcmv Ie
StuI/ SmaI
CTAGG GGGTA
GATCC CCCTA
SV40 SD/SA
Stu I/ Sma I (770)
pCMV-RFP
4338 bp
DsRed-Monomer
NotI (1468)
SV40 poly A

Figure 3.7. The strategy for placing the RFP cassette under the control of CMV promoter to generate plasmid pCMV-RFP.

Table 3.3. Restriction enzyme analysis of pCMV-RFP.

Enzyme	*Eco*RI *Sal*I	*Not*I	*Bam*HI	*Pst*I	*Ava*I
Fragment size (bp)	2653 1685	4338	3294 768 198 78	2665 903 569 201	3930 408

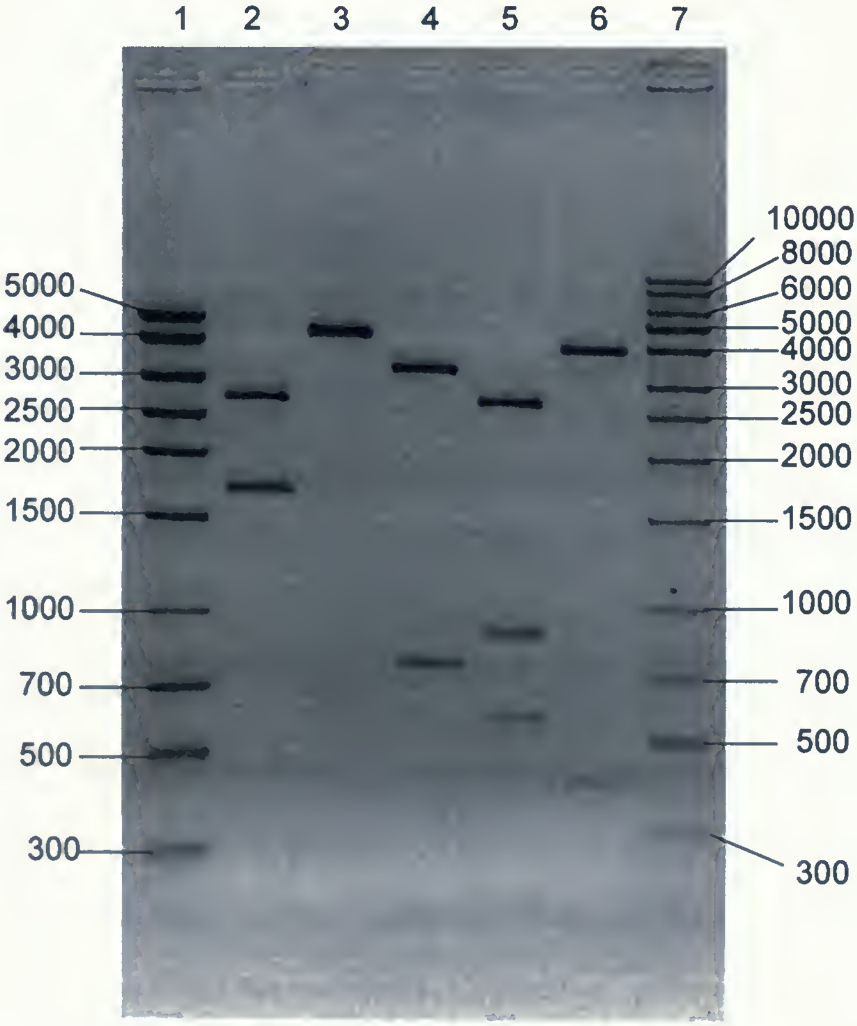
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Figure 3.8. Restriction enzyme digestion of pCMV-RFP on agarose gel. Lane 1: Norgen MidRanger marker; Lane 7: Norgen HighRangerPlus marker; Lane 2-6: plasmid DNA digested by restriction enzymes: *Eco*RI and *Sal*I; *Not*I; *Bam*HI; *Pst*I; *Ava*I. Numbers on the sides refer to marker sizes in basepairs.

3.1.5 Construction and Confirmation of pAB-CR

The plasmid pAB-CR was the shuttle plasmid for generating SubE3 virus. To generate pAB-CR, the CMV-RFP cassette from pCMV-RFP was inserted into the backbone of plasmid pAB26. The strategy for constructing pAB-CR is shown in Figure 3.9.

The plasmid pAB26 contains the 69.0%-78.3% and 85.8%-100.0% of the adenovirus genome, which are the homologous recombination substrates for generating SubE3 adenovirus. The transgene cassette is supposed to be flanked inside. The plasmid was digested with *Sal*I and *Sca*I, and *Sca*I cleaved the plasmid with a blunt end.

The plasmid pCMV-RFP was cut with *Sal*I and *Sfo*I, and the digestion of *Sfo*I also left the plasmid with a blunt end. The DNA fragments for the transgene and the backbone were then ligated and transformed into bacterial cells.

Notably, when transforming the ligation for pAB-CR, it was hard to harvest any candidate colonies. It is probably because the size of the ligation (~15.6kb) is large and the ligation efficiency was low. Therefore, when transforming bacterial cells, the incubation time (after heat shock) was increased from 1 h to 2 h, and the desired colonies were obtained. Restriction enzymes were used to confirm the plasmid and the expected lengths are shown in Table 3.4. The agarose gel picture is shown in Figure 3.10.

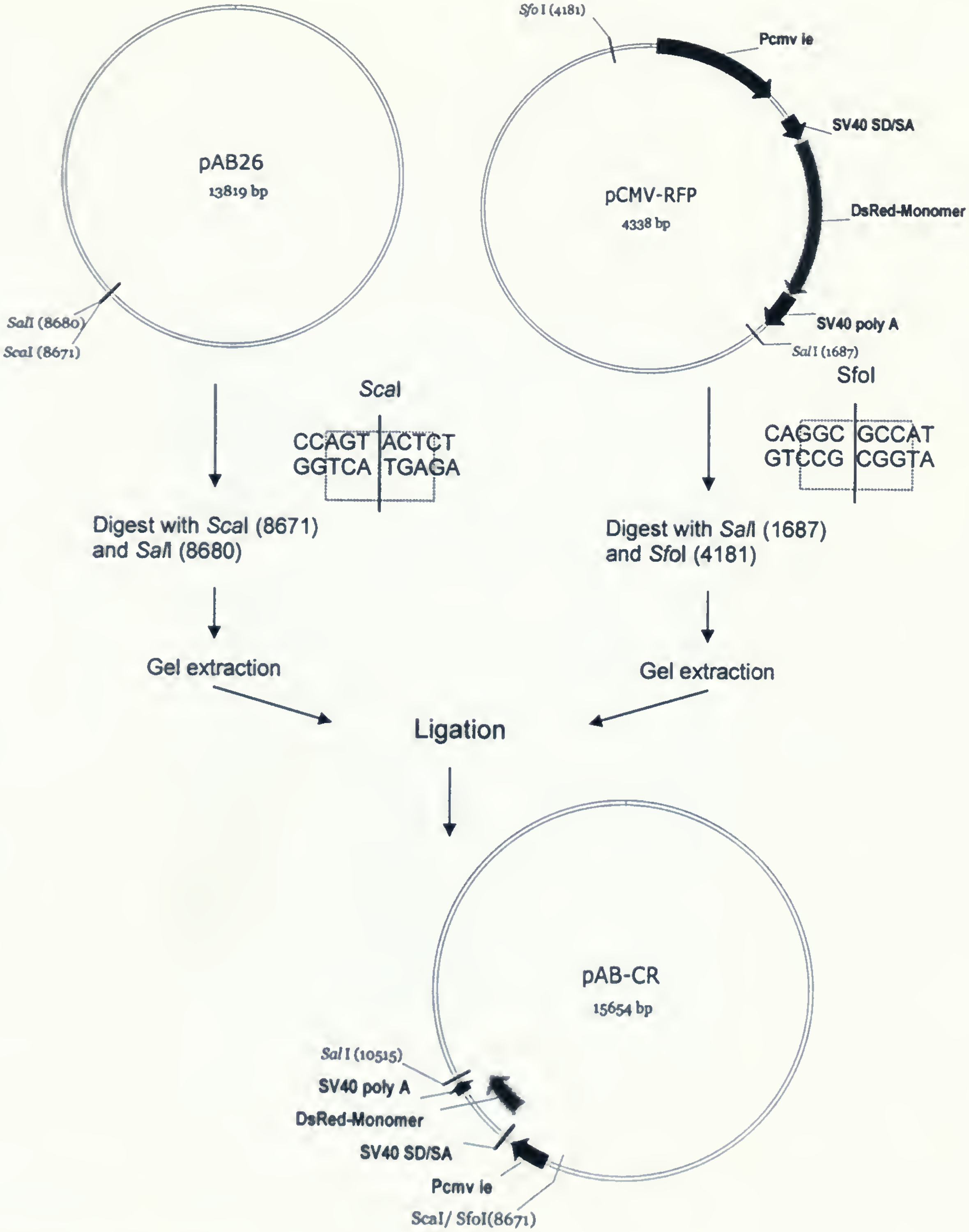
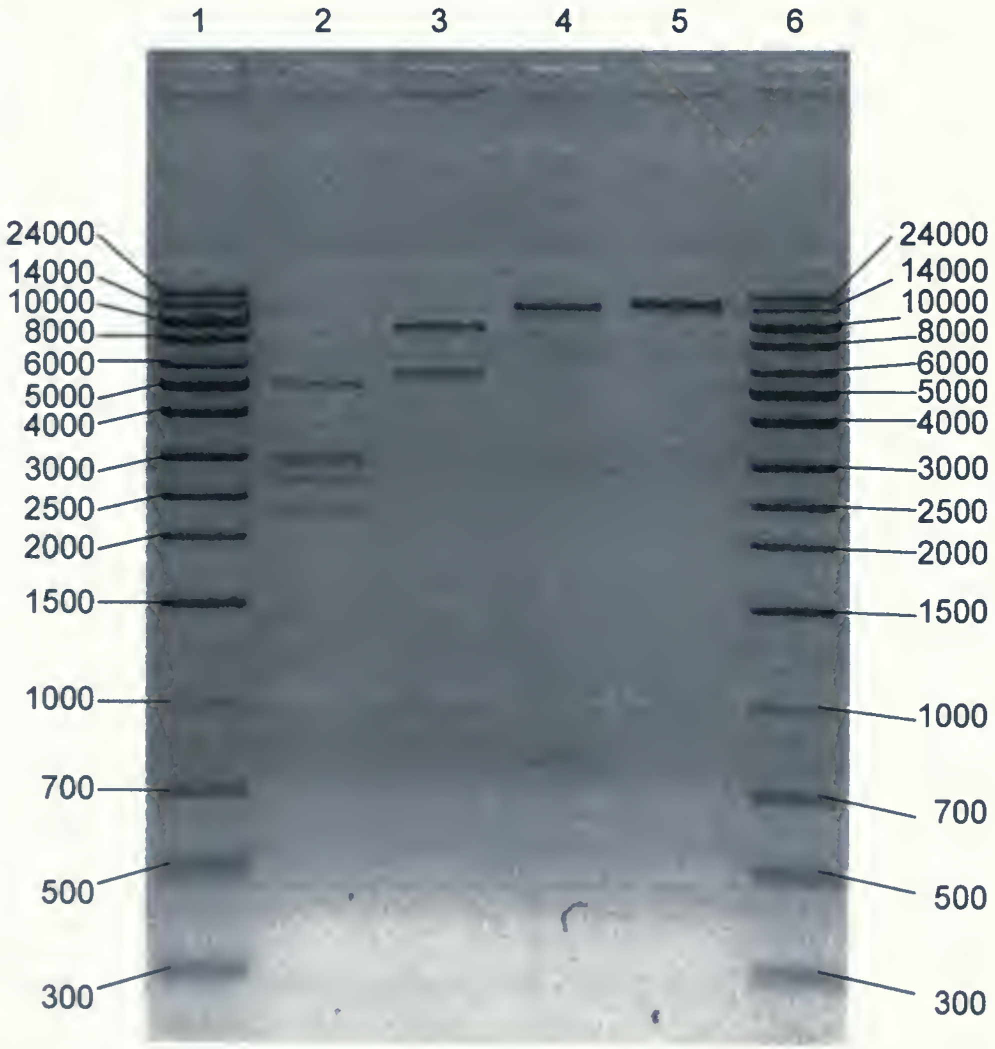

pAB26
13819 bp
SalI (8680)
ScaI (8671)
SfoI (4181)
Pcmv ie
SV40 SD/SA
pCMV-RFP
4338 bp
DsRed-Monomer
SV40 poly A
SalI (1687)
ScaI
CCAGT ACTCT
GGTCA TGAGA
SfoI
CAGGC GCCAT
GTCCG CGGTA
Digest with ScaI (8671) and SalI (8680)
Digest with SalI (1687) and SfoI (4181)
Gel extraction
Gel extraction
Ligation
pAB-CR
15654 bp
SalI (10515)
SV40 poly A
DsRed-Monomer
SV40 SD/SA
Pcmv ie
ScaI/ SfoI(8671)

Figure 3.9. The strategy for inserting CMV-RFP cassette into the backbone of pAB26 to generate plasmid pAB-CR.

Table 3.4. Restriction enzyme analysis of pAB-CR.

Enzyme	*Xho*I *Hind*III	*Eco*RI	*Xba*I	*Sal*I *Xho*I
Fragment size (bp)	4974 2937 2873 2623 2247	9148 5525 981	13802 1056 796	14608 1046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500
300

Figure 3.10. Restriction enzyme digestion of pAB-CR on agarose gel. Lane 1 and 6: Norgen UltraRanger marker; Lane 2-5: plasmid DNA digested by restriction enzymes: *Xho*I and *Hind*III; *Eco*RI; *Xba*I; *Sal*I & *Xho*I. Numbers on the sides refer to marker sizes in basepairs.

3.2 Attempts to Rescue SubE3 Recombinant Adenovirus

As explained above, we expected to generate SubE1 and SubE3 viruses, so the effect of the E1 region on adenovirus integration can be evaluated by comparing the percentages of cells showing the GFP/ RFP signal. However, we did not get SubE3 virus even after various optimization and trouble-shooting attempts. Therefore, we could only describe the process of trouble-shooting and explain the possible reasons for the failure of rescuing SubE3 virus.

3.2.1 Attempts to Rescue Virus using Calcium Phosphate-Mediated Transfection

Initially, we used the traditional calcium phosphate-mediated transfection method to rescue the recombinant adenovirus. All the transfection experiments were carried out in 6-well plates (the diameter of each well is 34 mm).

In calcium phosphate-mediated transfection, assuming that the concentration of Ca^{2+} is fixed (the optimal Ca^{2+} concentration is suggested to be 250 mM) (Jordan *et al.*, 1996), only three variables are adjustable to optimize the transfection efficiency. These variables are: the confluency of cell monolayer at the time of transfection, the amount of DNA used for transfection, and the exposure time of the cell monolayer to the $CaPO_4$-DNA complex.

Firstly, the confluency of the cell monolayer is suggested to be 70-80% (Hitt *et al.*, 1998). It was empirically determined that the confluency of the cell monolayer would be around 70% if 1×10^6 cells were plated 24 h prior to

transfection. Therefore, 1×10^6 cells were seeded on the plates one day prior to transfection.

Secondly, the exposure time of the cell monolayer to the $CaPO_4$-DNA complex was determined to be 6 h because shorter exposure time reduced the transfection efficiency while longer time induced cell morbidity (most of the cells became sickle-shaped and many were dead).

Thirdly, various amounts of shuttle plasmid and genomic plasmid DNA were used in the attempts to rescue the viruses. According to Jordan *et al.*, (1996) the optimal plasmid DNA concentration is 2.5 µg of DNA per mL of culture medium, and higher DNA concentrations will reduce the transfection efficiency. Therefore, no more than 5.0 µg of plasmid DNA was used for cotransfection (each well contains 2 mL of culture medium). The positive control plasmid pFG140 was transfected with two different concentrations to evaluate the transfection efficiency. In addition, pUC19 was used as a negative control. The amounts of plasmid DNA used for cotransfection are shown in Table 3.5.

Table 3.5. Different amounts of plasmid DNA used for cotransfection.

SubE1 virus		**SubE3 virus**		**pFG140**	**pUC19**
pDC-CG	**pBHGfrtΔE1E3FLP**	**pAB-CR**	**pFG173**		
1.25 µg	1.25 µg	1.25 µg	1.25 µg	2.5 µg	2.5 µg
1.25 µg	2.50 µg	1.25 µg	2.50 µg	5.0 µg	2.5 µg
2.50 µg	1.25 µg	2.50 µg	1.25 µg	2.5 µg	2.5 µg
2.50 µg	2.50 µg	2.50 µg	2.50 µg	5.0 µg	2.5 µg

In addition, there is a nonadjustable factor – the physiological status of the cells. Because the cell physiological status changes from time to time, the experiment designed in Table 3.5 was carried out in triplicate on three different days.

However, neither the SubE1/ SubE3 recombinant viruses nor the positive control virus was successfully rescued. We tried several more times using the same amounts of DNA, but no single virus was generated. Therefore, we investigated three factors that may cause this problem: the viability of the E1 region in 293 cells; the transfection methods and the quality of the plasmid DNA, as discussed below.

3.2.2 Amplification of E1 mRNA and gDNA of 293 Cells

The generation of both the positive control virus and SubE1 virus requires active E1 proteins. This is because the plasmids for SubE1 virus do not contain the E1 region and the positive control plasmid has an insertional mutation downstream of the E1A region (Graham, 1984). As introduced in 1.3.2 "Designing of Adenoviral Vectors", HEK 293 cells persistently express the E1 region, so the failure of expression of E1 products may result in a problem with harvesting viruses. In order to verify the viability of the E1 region in 293 cells, we examined the messenger RNA (mRNA) and genomic DNA (gDNA) that were isolated from 293 cells.

The 293 E1 mRNA and gDNA were examined by RT-PCR. The total RNA from 293 cells was isolated and the integrity of the RNA sample is demonstrated

in Figure 3.11 A. The RNA was then reverse-transcribed and amplified by PCR with E1A primers or E1B primers. The expected lengths of the cDNA (mRNA) amplification are shown in Table 3.6.

In addition, because the isolated RNA contains trace amounts of gDNA from 293 cells, the amplification of minus RT (RT reaction without the reverse transcriptase SuperScript II) should generate the bands of E1A and E1B gDNA. As a positive control, the genomic DNA of wildtype Ad5 (dl309) was amplified as well. The expected lengths of the gDNA amplification are shown in Table 3.6. The amplification results on an agarose gel are shown in Figure 3.11 B.

As is shown in Figure 3.11, the amplification results were as expected. Noticeably however, the negative control of E1A amplification (Figure 3.11B, Lane 3) has a band identical to that of E1A genomic DNA amplification, although much fainter. The reason for this phenomenon is not known, but the amplification of E1A negative control always had a faint band. On the contrary, the negative controls for the E1B of 293 did not have any amplification, even if we increased the exposure time of the gel under UV light.

Table 3.6. The expected lengths of amplification products of Ad5 E1A and E1B mRNA (cDNA) and gDNA.

E1A		E1B	
mRNA (cDNA)	gDNA	mRNA (cDNA)	gDNA
724 bp (13S) 586 bp (12S)	840 bp	1463 bp (55kd) 208 bp (19kd)	1546 bp

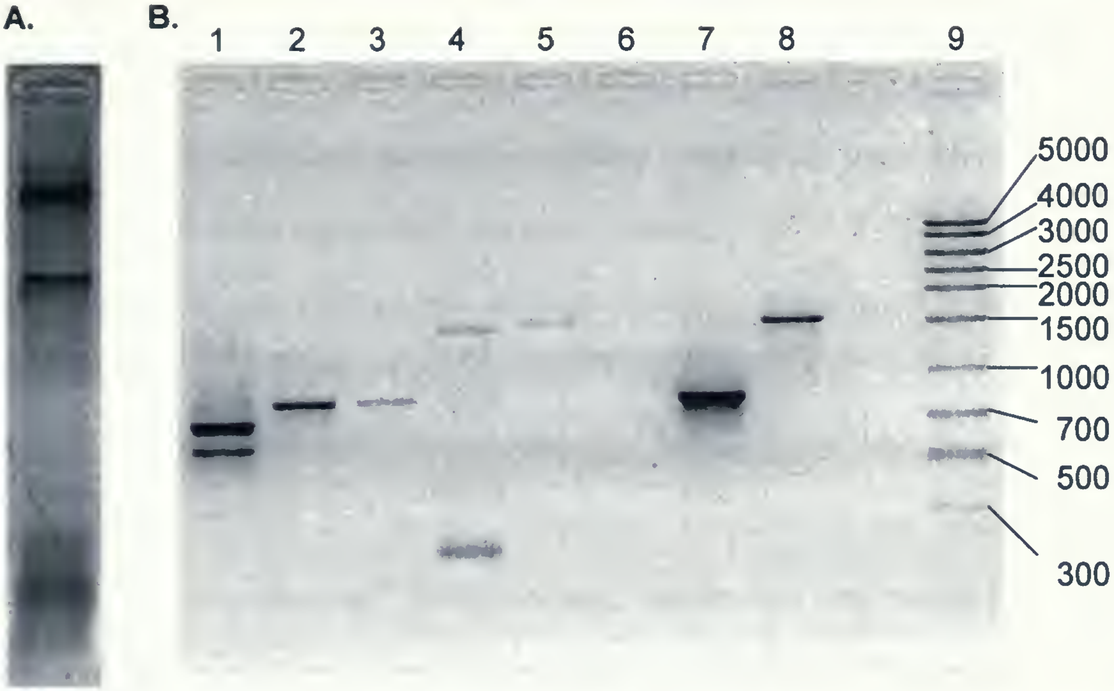

Figure 3.11. RT-PCR amplification of the Ad5 E1A/ E1B on agarose gel. A) The RNA isolation control. B) PCR amplification of cDNA and gDNA of E1A/ E1B of 293 cells. Lanes 1-3, 7: amplification with E1A primers; Lanes 4-6, 8: amplification with E1B primers. Lanes 1 & 4: (+) RT amplification; Lanes 2 & 5: (-) RT amplification; Lanes 3 & 6: amplification without templates; Lanes 7 & 8: amplification with banded Ad5 dl309 as positive control. Lane 9: Norgen MidRanger marker. Numbers on the sides refer to marker sizes in basepairs.

3.2.3 Attempts to Rescue Virus using Liposome-Mediated Transfection and Rescue of SubE1 Virus

Considering that the positive control plasmid pFG140 did not generate virus, the plasmid pCMVβ was chosen instead and the β-gal activity assay was used as the indicator of transfection efficiency.

To ensure that the liposome-mediated method is applicable for transfecting 293 cells, we compared the transfection efficiency between calcium phosphate-mediated transfection and liposome-mediated transfection using pCMVβ. In a triplicate transfection experiment, the same amount of pCMVβ DNA was used for both methods (2.5 μg DNA per mL of culture medium); the amount of $CaPO_4$ was constant, and the amount of Lipofectamine^{TM} 2000 was 5.0 μL.

As shown in Figure 3.12, the difference between the calcium phosphate-mediated transfection and liposome-mediated transfection was apparent: the efficiency of Lipofectamine^{TM} 2000-mediated transfection was nearly 5 times as much as that of calcium phosphate-mediated transfection. This result was not replicable: the percentages of the blue cells differed from the experiments carried out on different days, probably due to the different physiological conditions of the cells (Jordan *et al.*, 1996). However, the liposome-mediated method generally had higher transfection efficiencies than calcium phosphate-mediated method. We therefore chose liposome-mediated method in the following experiments.

Next, we optimized the transfection condition of Lipofectamine^{TM} 2000-mediated transfection. As suggested by Invitrogen, 2.5, 5.0 and 10.0 μL of Lipofectamine^{TM} 2000 were used. In addition, various amounts of plasmid DNA, respectively 0.25, 0.50, 1.0, 2.5, 5.0, and 10.0 μg were used for each well; among these amounts, the smaller ones (0.25, 0.50, and 1.0 μg) were used to observe the potential cytotoxicity of liposome. As shown in Figure 3.13, transfection efficiencies were generally higher when higher amounts of liposome and/ or higher amounts of plasmid DNA were used.

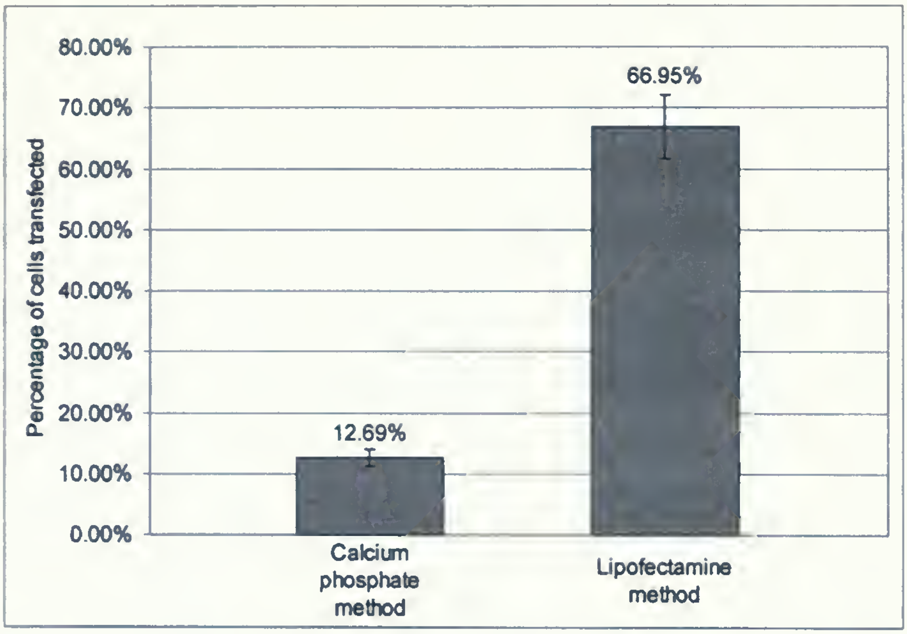

Figure 3.12. Comparison of transfection efficiencies between the calcium phosphate method and the Lipofectamine^{TM} 2000 method. The percentages of transfected cells were obtained by calculating the ratio between the number of X-gal stained blue cells and the total number of cells (mean ± s.d.; n=3).

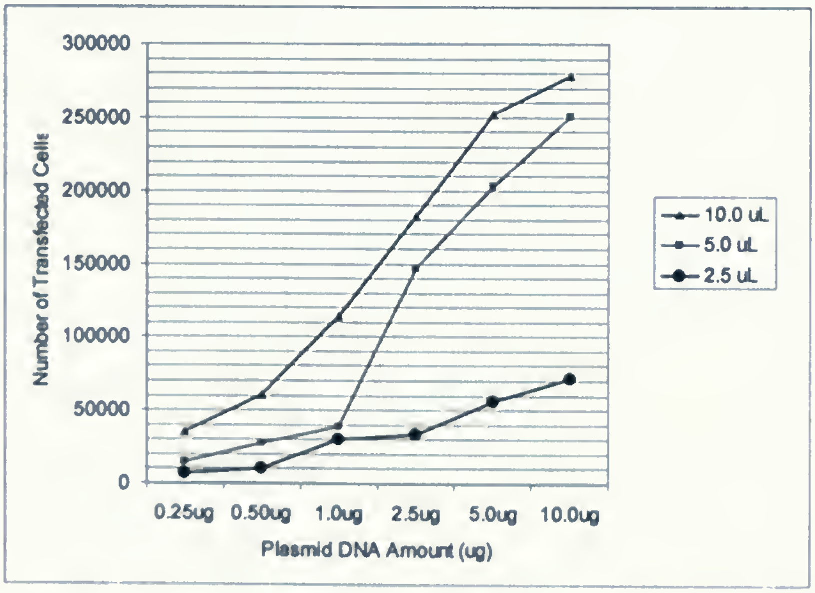

Figure 3.13. The liposome-mediated transfection efficiency using different amounts of Lipofectamine^{TM} 2000 and plasmid DNA.

However, using 10.0 µL of Lipofectamine™ 2000 also induced greater cytotoxicity (many cells were floating and the outline of the cells had changed from smooth into rough and irregular). Moreover, using 10.0 µg of plasmid DNA was generally toxic to the cell monolayers, while the use of smaller amounts of plasmid DNA (5.0 µg or less) caused little or no cytotoxicity. The toxic effects of different amounts of Lipofectamine™ 2000 and plasmid DNA on cell monolayers are shown in Table 3.7.

Table 3.7. The cytotoxic effects of different amounts of Lipofectamine™ 2000 and plasmid DNA on cell monolayers. -: Essentially no cell showed toxicity. +: Low toxicity, approximately 10-25% of transfected cells showed morbidity. ++: Medium toxicity, approximately 25-50% of cells showed morbidity. +++: High toxicity, approximately 50-75% of cells showed morbidity.

Plasmid DNA / Liposome	0.25 µg	0.50 µg	1.0 µg	2.5 µg	5.0 µg	10.0 µg
10.0 µL	+	+	+	++	++	+++
5.0 µL	-	-	-	-	-	++
2.5 µL	-	-	-	-	-	+

Finally, the optimal transfection condition, with which we can obtain the minimal cytotoxicity while maintaining the maximal transfection efficiency, was determined to be 5.0 µL of Lipofectamine™ 2000, 5.0 µg of plasmid DNA, cell

monolayer at 70% confluency at the time of transfection, and 6 h of incubation of liposome-DNA on cultured cells.

After the optimization, the experiment designed in Table 3.5 was carried out using this optimized liposome-mediated transfection condition. Approximately 15 d later, SubE1 virus and positive control virus were successfully rescued (2 out of 4 wells of SubE1 virus and 2 out of 2 wells of positive control viruses were rescued in a single experiment). However, none of the SubE3 virus was rescued (0 out of 4).

The genomic DNA of SubE1 virus or positive control virus was isolated using phenol-chloroform extraction method and digested with *Hin*dIII. The expected restriction lengths of the two viruses are shown in Table 3.8. The gel picture for the digestion is illustrated in Figure 3.14.

Table 3.8. Restriction analysis of *Hin*dIII digestion of SubE1 virus and positive control virus.

*Hin*dIII digestion	SubE1 virus	Positive control virus
Fragment size (bp)	8010 5322 4942 4597 3012 2937 2081 1004 75	8010 5555 5322 5013 4597 3437 2937 2081 933 75

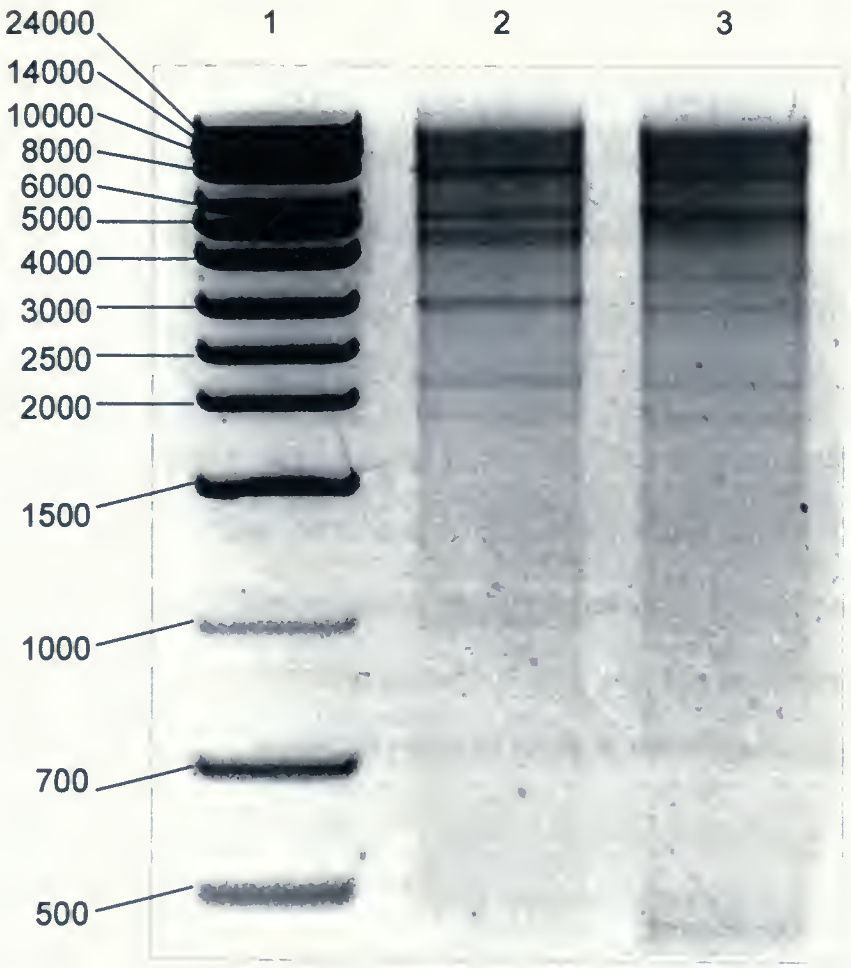
24000
14000
10000
8000
6000
5000
4000
3000
2500
2000
1500
1000
700
500
1
2
3

Figure 3.14. Restriction enzyme digestion of SubE1 virus and positive control virus on an agarose gel. Lane 1: Norgen UltraRanger marker; Lanes 2 & 3: SubE1 and positive control recombinant adenovirus genomic DNA digested by restriction enzyme *Hin*dIII.

However, the SubE3 virus was not harvested, even if we repeated the experiment designed in Table 3.5 on several different days. Therefore, the final possibility – the quality of the plasmid DNA – was examined.

3.2.4 Transfection with Different Batches of Plasmid DNA Preparation

Finally, we proposed that the failure of rescuing SubE3 virus was due to problems with the plasmid DNA. This might be caused by either the problem of the CsCl-banding of plasmid DNA or the problem of the plasmid's own properties (for example, the unstable property of the SubE3 genomic plasmid pFG173 might cause this failure).

The first problem was examined by transfecting different batches of plasmid DNA preparations. One extra batch of CsCl-banded pAB-CR, pFG173 and pFG140 plasmid DNA was prepared, and then both the old and new pAB-CR and pFG173 were cotransfected into six-well plates with the amounts listed in Table 3.5. The old and new batches of pFG140 plasmid DNA were also transfected as the positive controls to evaluate the quality of CsCl banding preparation. This experiment was carried out in duplicate and repeated on three different days. However, none of the SubE3 cotransfection (0/32) generated SubE3 virus, even though all wells (6/6) of the positive control virus were harvested. We kept on repeating the experiment with the optimized transfection condition, but no SubE3 virus was generated.

Therefore, it might be the problem of the plasmid DNA that prevents us from harvesting any SubE3 virus. In the virus systems that we used (AdMax from Microbix), the SubE3 virus system does not contain a recombinase system, without which the generation of an adenoviral vector would be difficult; besides, the genomic plasmid pFG173 is extremely unstable according to the literature (Hitt *et al.*, 1998) and our observations. These problems above may contribute

together to the failure of rescuing SubE3 virus, and they will be elaborated in "DISCUSSION".

Because of the failure of rescuing SubE3 virus, the original strategy was abandoned. We have to come up with an alternative way to evaluate the effect of Ad5 E1 on adenovirus integration. Considering that adenovirus integration may require homologous recombination events, we tried to measure the effect of Ad5 E1 on homologous recombination.

3.3 Construction of Plasmids for Homologous Recombination Assay

3.3.1 Overall Strategies for Homologous Recombination Assays

As introduced in literature review, Gahlmann *et al.*, (1982) believed that adenovirus integration is mediated by homologous recombination through the "patch homologies" between the virus and its host. Therefore, we decided to test the impact of E1 on the cellular homologous recombination system, so its effect on virus integration can be indirectly assessed.

In order to test the effect of E1 on homologous recombination efficiency, we planned to take a "reverse" strategy. Because the HEK 293 cells persistently express the E1 region, the difference that E1 could make can be evaluated by comparing 293 cells with the E1 gene knocked-down and normal 293 cells. Therefore, later we attempted to knock down the expression of the E1 region by RNA interference, as explained below in 3.4.1.

For the homologous recombination efficiency, it can be assessed by a homologous recombination assay. The overall strategy for this assay is shown in Figure 3.15. We mutated the *lacZα* coding region of plasmid pUC19 and generated two plasmids with different mutations. Therefore, the two plasmids containing different defective *lacZα* cassettes can form a functional *lacZα* cassette through intermolecular homologous recombination. The two defective plasmids, once transformed into bacterial cells, will form white colonies; while only the plasmid that has undergone homologous recombination and generated the functional *lacZα* can form blue colonies. The homologous recombination efficiencies can be determined by calculating the percentages of the blue colonies among all the blue and white colonies.

The construction of these two plasmids, designated as pUC19-dl1 and pUC19-dl2, are shown in the following sections 3.3.2 and 3.3.3.

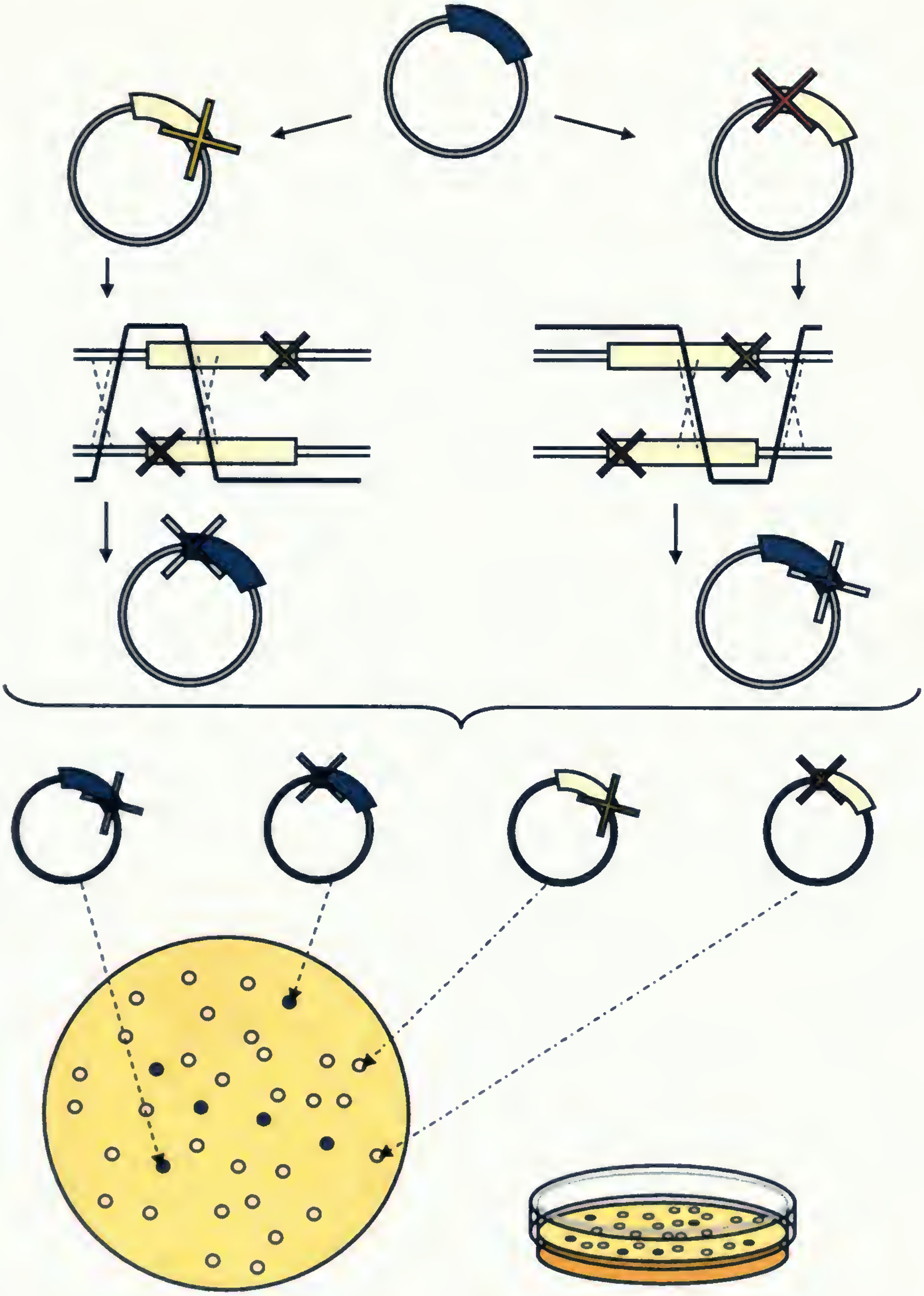

Figure 3.15. Schematic representation of homologous recombination assays. The homologous recombination efficiencies will be interpreted by the percentages of the blue colonies in total colonies.

3.3.2 Construction and Confirmation of pUC19-dl1

The plasmid pUC19-dl1 was constructed by deleting a 17 bp fragment from pUC19. The purified pUC19 was digested with *Sma*I and *Hinc*II and then self-ligated. Because this 17bp fragment was inside the *LacZα* gene of pUC19, the *LacZα* had a frame-shift mutation and was therefore inactivated. The strategy for generating pUC19-dl1 is illustrated in Figure 3.16. The restriction enzymes and the anticipated digestion patterns are shown in Table 3.9. The electrophoresis gel picture is shown in Figure 3.17.

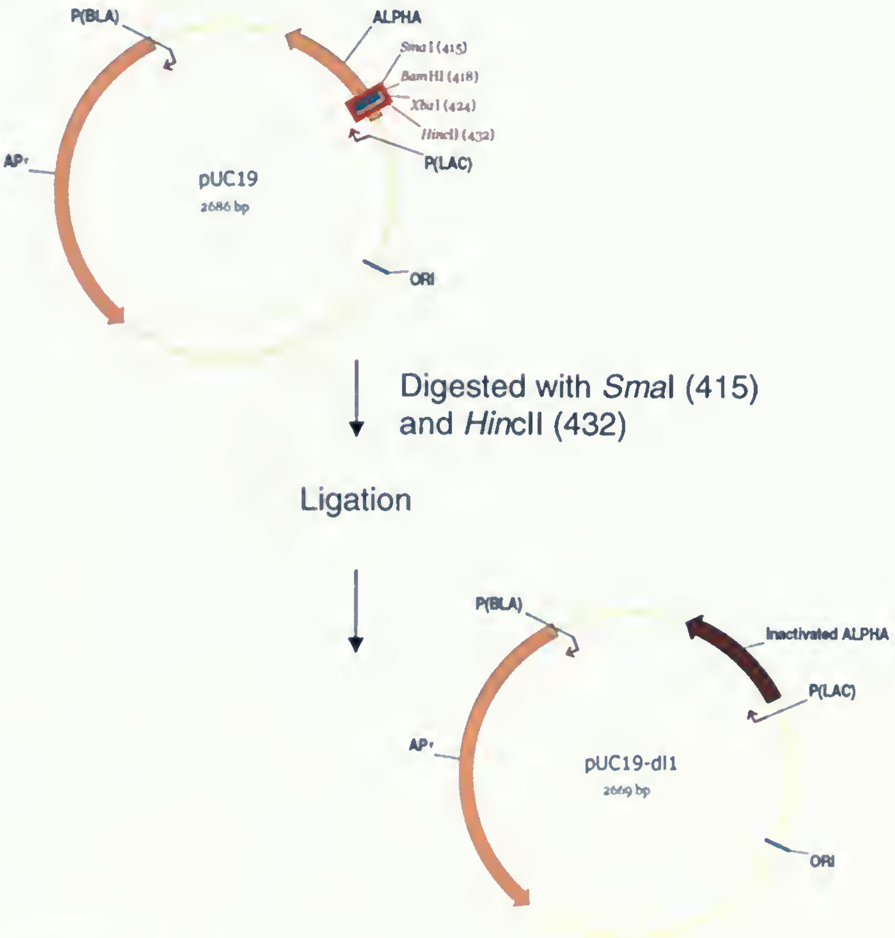
P(BLA)
ALPHA
SmaI (415)
BamHI (418)
XbaI (424)
HincII (432)
P(LAC)
AP^r
pUC19
2686 bp
ORI
Digested with SmaI (415)
and HincII (432)
Ligation
P(BLA)
Inactivated ALPHA
P(LAC)
AP^r
pUC19-dl1
2669 bp
ORI

Figure 3.16. The strategy for constructing pUC19-dl1.

Table 3.9. Restriction enzyme analysis of pUC19-dl1.

Enzyme	*Bam*HI	*Sma*I	*Hinc*II	*Nde*I	*Drd*I
Fragment size (bp)	-	-	-	2669	1869 800

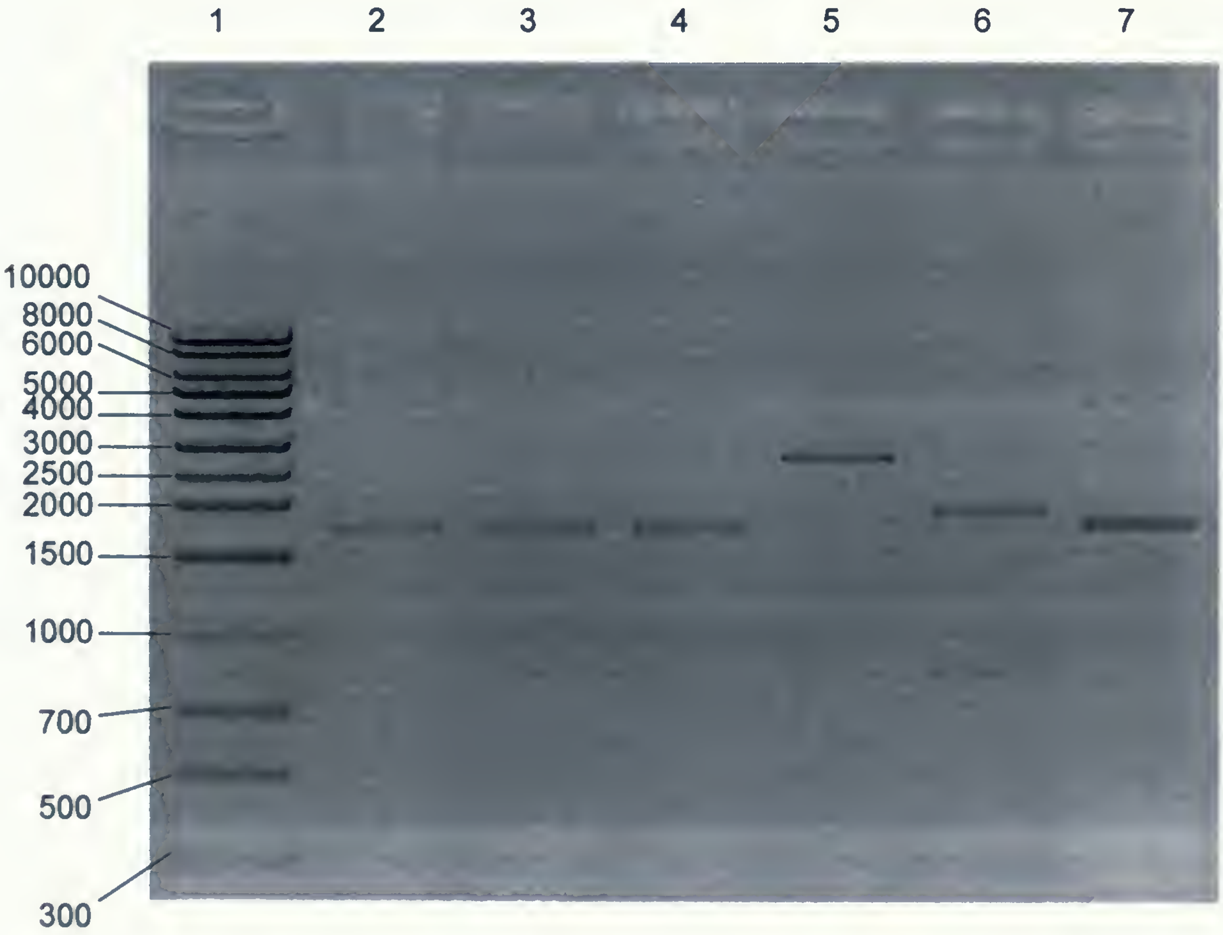
1
2
3
4
5
6
7
10000
8000
6000
5000
4000
3000
2500
2000
1500
1000
700
500
300

Figure 3.17. Restriction enzyme digestion of pUC19-dl1 on an agarose gel. Lane 1: Norgen HighRanger marker; Lane 2-6: plasmid DNA digested by restriction enzymes: *Bam*HI; *Sma*I; *Hinc*II; *Nde*I; *Drd*I; Lane 7: undigested pUC19-dl1 plasmid DNA. Numbers on the sides refer to marker sizes in basepairs.

3.3.3 Construction and Confirmation of pUC19-dl2

Similar to the creation of pUC19-dl1, the plasmid pUC19-dl2 was constructed by removing 3' end of the *LacZα* gene from pUC19. To generate pUC19-dl2, the plasmid pUC19 was digested with *Sfo*I and *Zra*I and then self-ligated. The strategy for constructing pUC19-dl2 is shown in Figure 3.18. The plasmid with inactivated *LacZα* was then isolated from white colonies and digested with restriction enzymes. The restriction enzymes and expected digestion patterns are shown in Table 3.10. The digestion picture is shown in Figure 3.19.

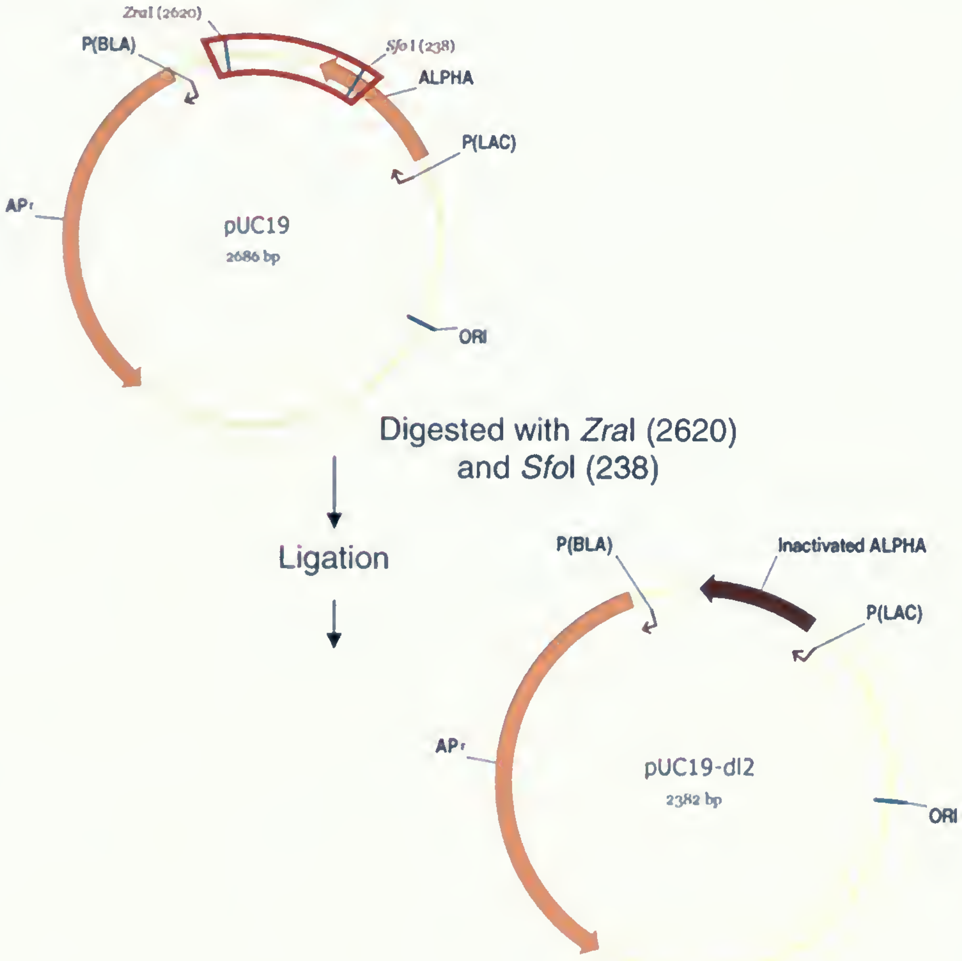
ZraI (2620)
P(BLA)
SfoI (238)
ALPHA
P(LAC)
APr
pUC19
2686 bp
ORI
Digested with ZraI (2620) and SfoI (238)
Ligation
P(BLA)
Inactivated ALPHA
P(LAC)
APr
pUC19-dl2
2382 bp
ORI

Figure 3.18. The strategy for constructing pUC19-dl2.

Table 3.10. Restriction enzyme analysis of pUC19-dl2.

Enzyme	*Bam*HI	*Sma*I	*Hinc*II	*Nde*I	*Drd*I
Fragment size (bp)	2382	2382	2382	-	2382

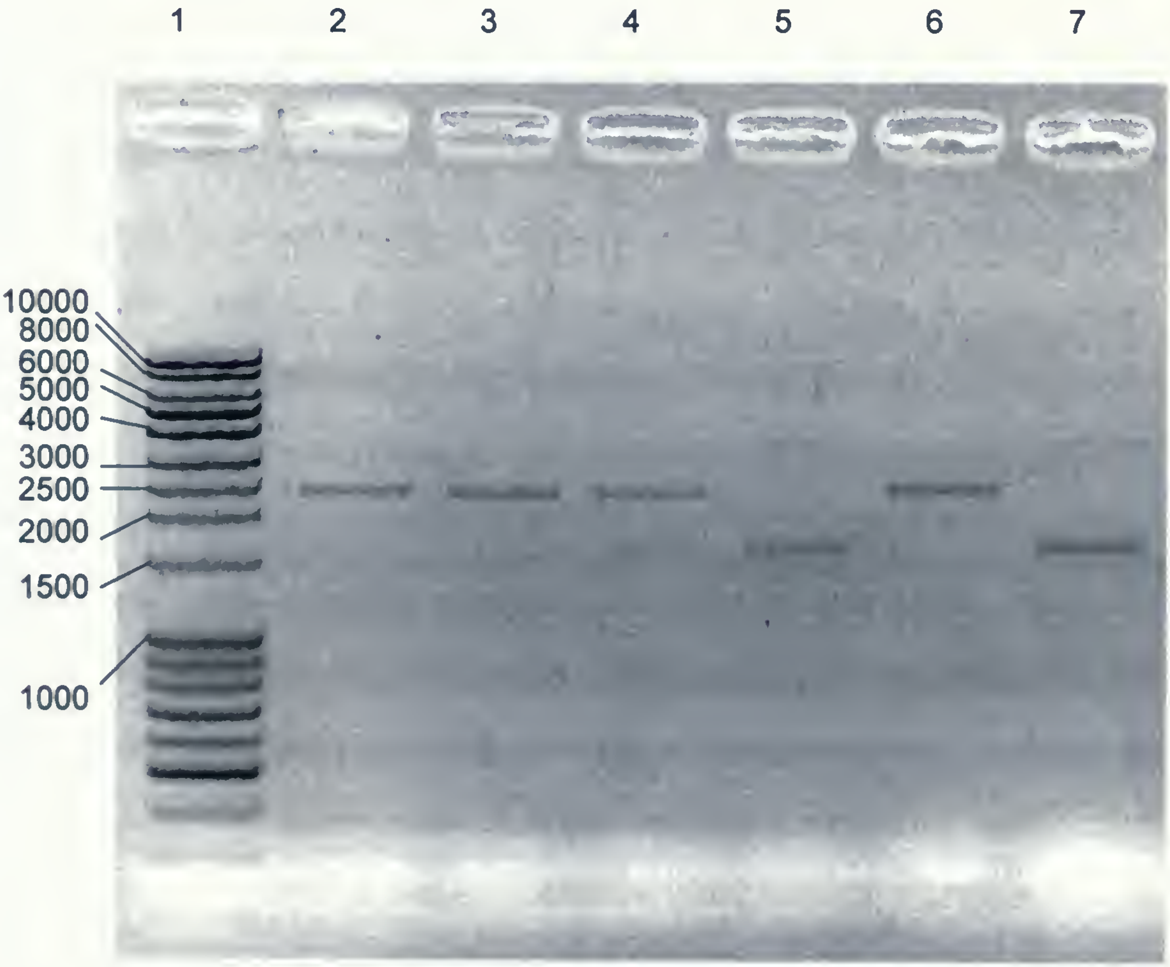
1
2
3
4
5
6
7
10000
8000
6000
5000
4000
3000
2500
2000
1500
1000

Figure 3.19. Restriction enzyme digestion of pUC19-dl2 on an agarose gel. Lane 1: Norgen HighRanger marker; Lane 2-6: plasmid DNA digested by restriction enzymes: *Bam*HI; *Sma*I; *Hinc*II; *Nde*I; *Drd*I; Lane 7: undigested pUC19-dl2 plasmid DNA. Numbers on the sides refer to marker sizes in basepairs.

3.4 Evaluation of the Effect of Ad5 E1 on Homologous Recombination

3.4.1 Screening for siRNA Target Sequences

RNA interference (RNAi) technology was used to silence the expression of adenovirus E1 region in HEK 293 cells. In our experiment, the chemically synthesized siRNAs ordered from Ambion were used, and the siRNAs were transfected with Lipofectamine^{TM} 2000. In order to obtain efficient silencing, the selection of the target sequences is crucial. The siRNA target sequence of Ad5 E1A was obtained from a previous paper (Hacker *et al.*, 2004) (AAGATCCCAACGAGGAGGCGG; nucleotides 723-743 of Ad5).

However, the sequence of E1B is not available in publications and other resources; therefore, a screening for the E1B siRNA target sequence was performed. The Ad5 E1B sequence was obtained from Entrez Nucleotide (Website: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide) and the accession number is [AC_000008]. The target sequence was determined using Ambion's siRNA design tools (website: http://www.ambion.com/techlib/misc/siRNA_tools.html) and the sequence AAGAATCGCCTGCTACTGTTG in the E1B region was chosen for the RNA interference experiment. This sequence is equal to the 2101-2121 bp in the Ad5 genome.

3.4.2 Optimization of siRNA Transfection

Except for the effectiveness of the target sequences, a successful RNAi experiment is largely dependent on the transfection efficiency. The approach to testing the siRNA transfection efficiency is to transfect a positive control siRNA

against a certain gene and to observe whether this gene is successfully silenced. Therefore, the positive control GAPDH siRNA (Ambion) was transfected into HEK 293 cells to monitor the silencing efficiency on the GAPDH gene.

The siRNA transfection conditions suggested by Ambion are: 3-6 μL of transfection reagent, 75 pmol (30 nM) of positive control GAPDH siRNA, 30-50% confluency at the time of transfection, and 24 h of incubation time of liposome-siRNA complex with cultured cells.

In the initial experiment, approximately 4×10^5 cells were plated in each well of a 6-well plate, so the confluency of the cell monolayer would reach 30-40% at the time of transfection. Then 5.0 μL of Lipofectamine^{TM} 2000 together with 0 or 30 nM GAPDH siRNA (in triplicate) were transfected into the 293 cells; the 0 nM condition was a mock transfection used to exclude the possibility of non-specific silencing effects. The medium was changed after 24 h because longer exposure of cells to liposome induced cytotoxicity. The silencing efficiency was examined 72 h post transfection by endpoint RT-PCR using GAPDH primers.

However, the silencing effect was unobserved and inconsistent: the difference of expression levels between the transfection with 0 nM and 30 nM GAPDH siRNA were barely visible, and among the cells treated with the same amount of siRNA, the expression levels were shown to be variable (the amplified bands had different intensities).

Actually, it was observed that the cell monolayers were over-confluent at the time of isolating total RNA. We presumed the over-confluency might change

the cell physiological status and cause this inconsistency, so the input cell number should be determined to avoid the over-confluency. Therefore, we plated different cell numbers and observed the confluency at different time points. Respectively 1, 2, 4, and 8×10^5 cells were seeded on the plates, and the confluency changes were observed every 24 h until 96 h. The result is shown in Table 3.11.

Table 3.11. The correlation between time and the cell monolayer confluency with different input cell numbers. The percentages of confluency were roughly determined by the area occupied by the cell monolayer in one well. OC: over-confluent.

	Initial cell input number			
	1×10^5	**2×10^5**	**4×10^5**	**8×10^5**
24 h	10%	20%	30%	70%
48 h	30%	40%	70%	OC
72 h	50%	70%	95%	OC
96 h	70%	95%	OC	OC

Among these four different cell input numbers, 2×10^5 is most suitable for this experiment, because 72 h is needed for the maximal silence of the gene (suggested by the literature such as Hacker *et al.*, 2004; Yun *et al.*, 2004). The confluency at 72 h will be too low (50%) with 1×10^5 cells, and too high (95%, OC) with 4×10^5 and 8×10^5 cells. With 2×10^5 input cells, the monolayers would not become over-confluent and meantime provide the most abundant RNA samples.

The studies on RNAi generally suggested that the siRNA-mediated silencing takes a long time (normally 72 h) (Dalby *et al.*, 2004; Yun *et al.*, 2004); therefore, we attempted to develop a faster transfection protocol. A "reverse transfection" protocol was adopted in this experiment, because it was suggested to be time-saving (Dalby *et al.*, 2004). In this "reverse" method, the cell-plating and transfection procedures were carried out at the same time, rather than plating the cells and transfecting them 24 h later.

Thereafter, the siRNA transfection experiment was carried out by reverse-transfecting 2×10^5 cells/ well, together with 5.0 µL of Lipofectamine^{TM} 2000 and 0 or 30 nM GAPDH siRNA. However, 5.0 µL of Lipofectamine^{TM} 2000 was cytotoxic – approximately 20-30% of the cells were rounded and the outline of many cells had changed from smooth into irregular and jagged – probably due to the decrease of cell input number. In addition, the RT-PCR results also showed inconsistency among several replicates transfected with the same amount of siRNA.

Such toxicity may be due to either the overly high amount of Lipofectamine^{TM} 2000, or the problem of siRNA. We first tested the cytotoxic effects induced only by Lipofectamine^{TM} 2000. As is shown in Table 3.12, with different amounts of Lipofectamine^{TM} 2000, the cells showed different degrees of cytotoxicity. However, when the amounts of the Lipofectamine^{TM} 2000 were at 2.5 µL/ well or below, the cells appeared to be healthy, as compared to the no-liposome negative control. Therefore 2.5 µL/ well of Lipofectamine^{TM} 2000 was used in the following experiments.

Table 3.12. The cytotoxicity induced by various amounts of Lipofectamine™ 2000 on 2×10^5 cells. The cells were incubated in Lipofectamine™ 2000 for 24 h. . -: Essentially no cell showed toxicity. +: Minor toxicity, approximately 10-25% of transfected cells showed morbidity. ++: Medium toxicity, approximately 25-50% of cells showed morbidity. +++: High toxicity, approximately 50-75% of cells showed morbidity.

	Replicate 1	Replicate 2	Replicate 3
10.0 μL	+++	+++	+++
7.5 μL	++	+++	+
5.0 μL	+	++	+
2.5 μL	-	-	-
1.0 μL	-	-	-
No liposome control	-	-	-

After fixing the amount of Lipofectamine™ 2000, the silencing effect of GAPDH siRNA was found. Upon transfecting with both 0 and 30 nM siRNA, no apparent cytotoxicity was observed, so the previous cytotoxicity might be solely induced by Lipofectamine™ 2000. Seventy-two hours later, the total RNA was isolated. The endpoint RT-PCR results showed that the silencing effect was significant and the effects among the same treatments were consistent. The amplification result is shown in Figure 3.20.

Therefore, the optimal siRNA transfection conditions were set to be 2×10^5 cells, 2.5 μL of Lipofectamine™ 2000, 30 nM siRNA, and testing the silencing effects at 72 h post transfection. With these conditions above, only the concentrations of E1A and E1B siRNAs need to be adjusted.

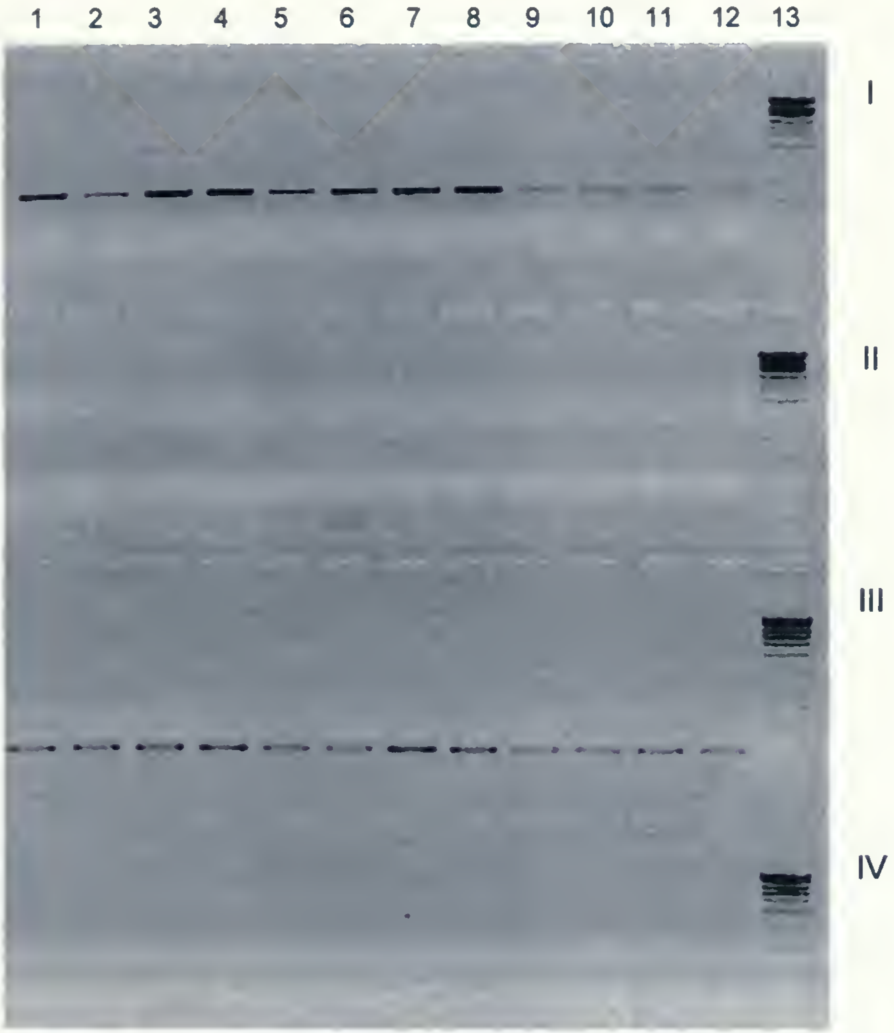

Figure 3.20. RT-PCR amplification of total RNA isolated from GAPDH siRNA-transfected HEK 293 cells on an agarose gel. The amplification was carried out in 25 cycles. Lanes 1-4: cell monolayer without any treatment; Lanes 5-8: cell monolayer treated with 2.5 µL of Lipofectamine^{TM} 2000; Lanes 9-12: cell monolayer transfected with 30 nM GAPDH siRNA in 2.5 µL of Lipofectamine^{TM} 2000; Lane 13: Norgen MidRanger marker. Row I: (+) RT amplification of total RNA with GAPDH primers; Row II: (-) RT amplification of total RNA with GAPDH primers; Row III: (+) RT amplification of total RNA with β-Actin primers; Row IV: (-) RT amplification of total RNA with β-Actin primers.

3.4.3 Attempts to Silence E1A and E1B Gene in 293 Cells

Next, the 293 cells were transfected with 0 nM and 30 nM of E1A and E1B siRNA, but there was no significant reduction in E1A/E1B mRNA amount (results not shown). Such problem might be caused by an insufficient amount of siRNA. Therefore, we increased the concentration of the siRNA to 30, 60 and 90 nM, respectively, to observe whether there was silencing effect. However, the silencing effect was still not observed, as shown in Figure 3.21 & 3.22.

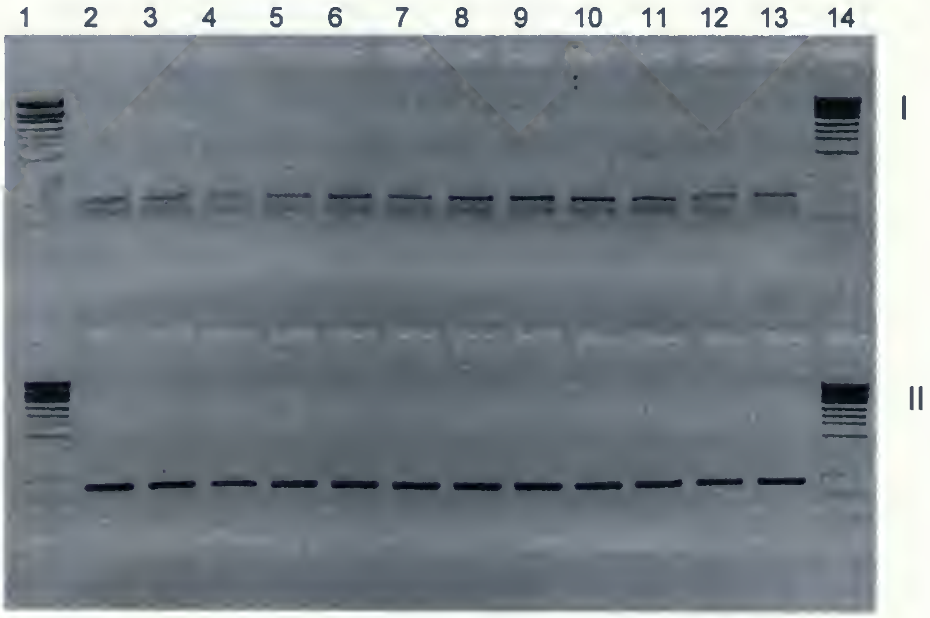

Figure 3.21. Test of E1A silencing effect with various siRNA concentrations (30-60-90 nM). Lanes 1 & 14: Norgen HighRanger Marker. Lanes 2-4: 293 cells with mock transfection. Lanes 5-7: 293 cells transfected with 30 nM E1A siRNA. Lanes 8-10: 293 cells transfected with 60 nM E1A siRNA. Lanes 11-13: 293 cells transfected with 90 nM E1A siRNA. Row I: cDNA amplified with E1A primers. Row II: cDNA amplified with GAPDH primers.

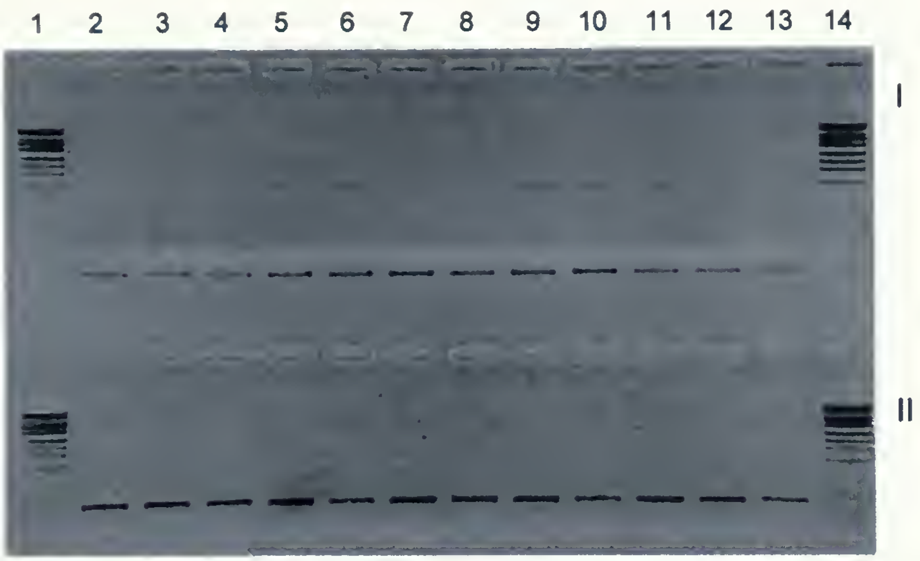

Figure 3.22. Test of E1B silencing effect with various siRNA concentrations (30-60-90 nM). Lanes 1 & 14: Norgen HighRanger Marker. Lanes 2-4: 293 cells with mock transfection. Lanes 5-7: 293 cells transfected with 30 nM E1B siRNA. Lanes 8-10: 293 cells transfected with 60 nM E1B siRNA. Lanes 11-13: 293 cells transfected with 90 nM E1B siRNA. Row I: cDNA amplified with E1B primers. Row II: cDNA amplified with GAPDH primers.

The above experiment was repeated twice, but no significant reduction of E1A/B mRNA was observed. Therefore, we further increased the concentration of E1A/B siRNA to 90, 180 and 270 nM, but the results were still negative, as the gel pictures were similar to Figure 3.21 & 3.22 (data not shown).

In addition, to exclude the possibility that the silencing time (72 h) is not too long or too short to display the silencing effect, an experiment concerning the silencing effect at different time points was also carried out. The 293 cells were treated with 0 or 90 nM siRNA, and the total RNA samples at 48, 72 and 96 h post transfection were isolated (the cell number at 0 and 24 h post transfection

were too few that the concentrations of the isolated RNA could not be determined using spectrophotometry). Nonetheless, there was no evident silencing effect at different time points, as shown in Figure 3.23 & 3.24.

Considering that the GAPDH can be silenced efficiently, the experiments above suggested that the E1A/E1B siRNA target sequences might not be potent enough to induce the reduced expression of E1A and E1B in HEK 293 cells.

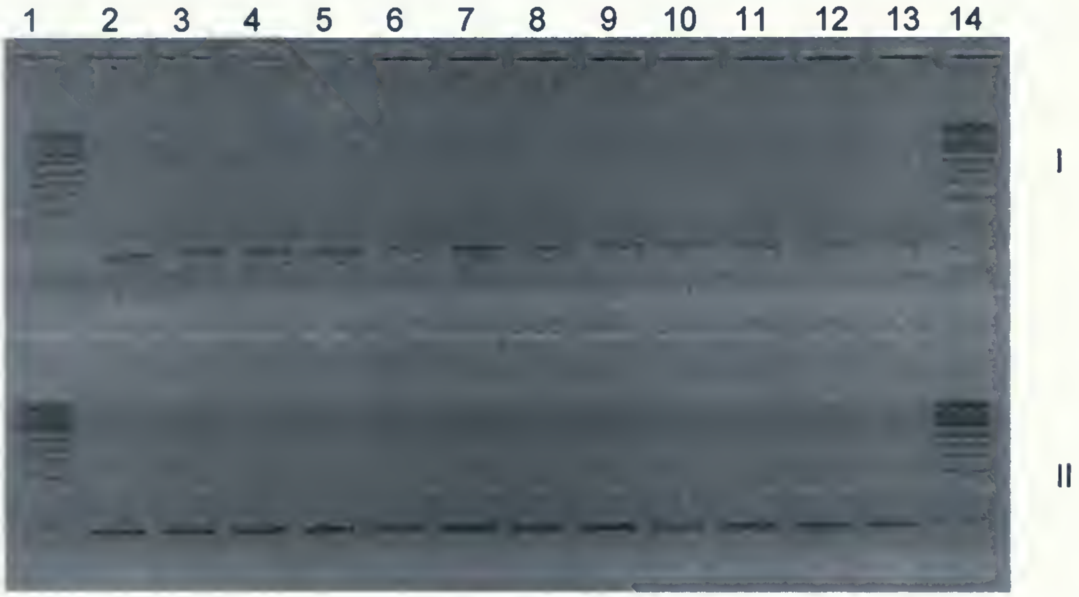

Figure 3.23. Silencing effect of 90 nM E1A siRNA at different time points. The experiment was carried out in triplicate but only two replicates were loaded. Lanes 1 & 14: Norgen HighRanger marker. Lanes 2 & 3: 293 cells with mock transfection at 48 h post transfection. Lanes 4 & 5: 293 cells transfected with 90 nM E1A siRNA at 48 h post transfection. Lanes 6 & 7: mock transfection at 72 h. Lanes 8 & 9: cells transfected with 90 nM E1A siRNA at 72 h. Lanes 10 & 11: mock transfection at 96 h. Lanes 12 & 13: cells transfected with 90 nM E1A siRNA at 96 h. Row I: cDNA amplified with E1A primers. Row II: cDNA amplified with GAPDH primers.

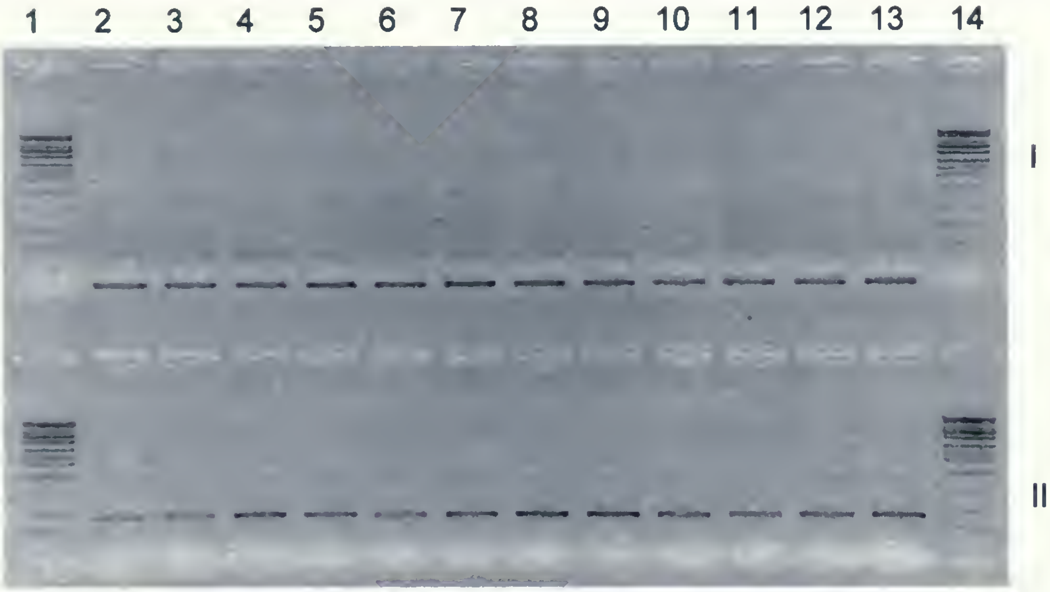

Figure 3.24. Silencing effect of 90 nM E1B siRNA at different time points. The experiment was carried out in triplicate but only two replicates were loaded. Lanes 1 & 14: Norgen HighRanger marker. Lanes 2 & 3: 293 cells with mock transfection at 48 h post transfection. Lanes 4 & 5: 293 cells transfected with 90 nM E1B siRNA at 48 h post transfection. Lanes 6 & 7: mock transfection at 72 h. Lanes 8 & 9: cells transfected with 90 nM E1B siRNA at 72 h. Lanes 10 & 11: mock transfection at 96 h. Lanes 12 & 13: cells transfected with 90 nM E1B siRNA at 96 h. Row I: cDNA amplified with E1B primers. Row II: cDNA amplified with GAPDH primers.

3.4.4 Attempts to Test Homologous Recombination Efficiency by Bacterial Transformation

Even though there was no significant reduction of E1A/E1B mRNA, we tried to test the homologous recombination efficiency with our recombination assay – by using bacterial transformation to calculate the percentages of blue

colonies in total colonies. Unexpectedly, the transformation efficiency was very low as no colonies were observed.

In this homologous recombination assay, we treated 293 cells with non-transfected control, 90 nM E1A siRNA, 90 nM E1B siRNA, and 90 nM negative control (NegC) siRNA (the sequence of which has no significant homology with known human genes); the experiment was performed in triplicate. HeLa cells as the control cell line were treated in the same way. Twelve hours after the transfection of plasmid DNA, the genomic DNA isolated from 293 cells and HeLa cells was then transformed into bacterial cells. However, no colonies were observed in all treatments and the results are shown in Table 3.13.

Except for the gDNA isolated from different treatments, 50 ng of pUC19 was transformed at the same time as the indicator of transformation efficiency, and the efficiency was 4.5×10^4 colonies per µg of pUC19. Such transformation efficiency was low and is not sensitive enough to detect a plasmid DNA input of less than 22 pg.

Therefore, the purchased High Efficiency GC5^{TM} Competent Cells (GeneChoice) were transformed with different gDNA samples to test the homologous recombination efficiency. However, no colonies were observed, even if the pUC19 transformation showed that the transformation efficiency was 1.8×10^7 colonies per µg of pUC19; such transformation efficiency can detect plasmid DNA more than 0.056 pg.

Table 3.13. Transformation efficiency of gDNA isolated from HEK 293 cells and HeLa cells. HR%: percentage of homologous recombination.

293 Cells	**Replicate 1**			**Replicate 2**			**Replicate 3**		
	white	Blue	HR%	white	Blue	HR%	white	Blue	HR%
Mock 0nM	0	0	-	0	0	-	0	0	-
E1A 90nM	0	0	-	0	0	-	0	0	-
E1B 90nM	0	0	-	0	0	-	0	0	-
NegC 90nM	0	0	-	0	0	-	0	0	-

HeLa Cells	**Replicate 1**			**Replicate 2**			**Replicate 3**		
	white	Blue	HR%	white	Blue	HR%	white	Blue	HR%
Mock 0nM	0	0	-	0	0	-	0	0	-
E1A 90nM	0	0	-	0	0	-	0	0	-
E1B 90nM	0	0	-	0	0	-	0	0	-
NegC 90nM	0	0	-	0	0	-	0	0	-

3.4.5 Testing Homologous Recombination Efficiency by PCR

In order to test the homologous recombination efficiency, we developed a new evaluation strategy based on PCR. Because pUC19 has extra fragments compared to pUC19-dl1 and pUC19-dl2, we designed the primers (designated as "UCONLY" primers) that can only amplify pUC19. As shown in Figure 3.25, the binding site of the UCONLY forward primer is located in the deleted fragment of pUC19-dl2, so it cannot bind to pUC19-dl2; while the reverse primer is in the deleted part of pUC19-dl1 and it cannot anneal to pUC19-dl1.

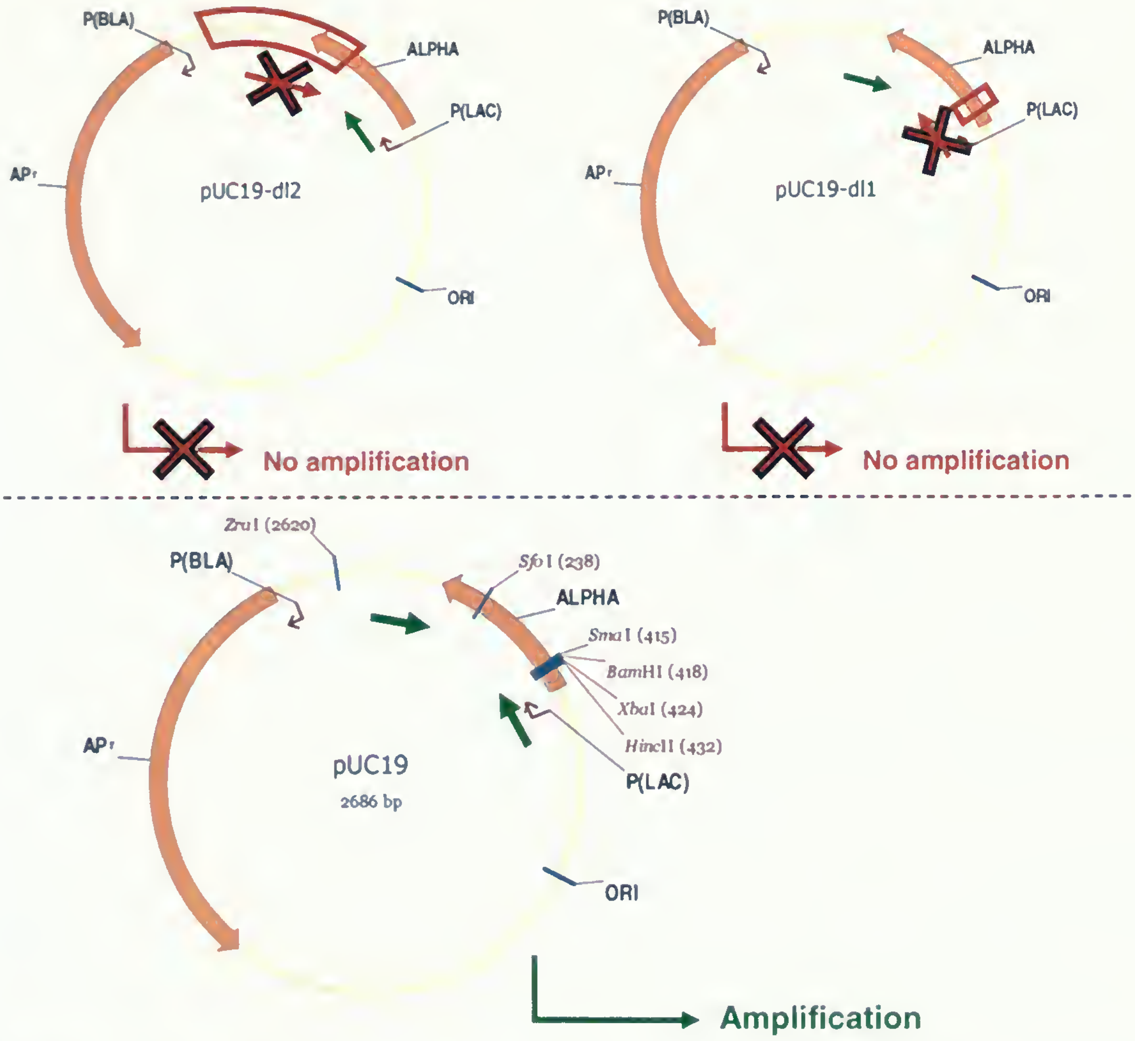
P(BLA)
ALPHA
P(LAC)
APr
pUC19-dl2
ORI
No amplification
P(BLA)
ALPHA
P(LAC)
APr
pUC19-dl1
ORI
No amplification
Zru I (2620)
P(BLA)
Sfo I (238)
ALPHA
Sma I (415)
BamHI (418)
XbaI (424)
HincII (432)
P(LAC)
APr
pUC19
2686 bp
ORI
Amplification

Figure 3.25. The illustration of primer design for testing homologous recombination efficiency. This set of primers can only anneal to and amplify pUC19, rather than the two plasmids with deletions.

Therefore, only the recombinant version of the transfected plasmid will result in a PCR product. In addition, because the UCONLY primers generate a single band, Realtime PCR can be applied to compare or quantify the homologous recombination efficiencies. A typical Realtime PCR amplification result is shown in Figure 3.26. According to the Realtime PCR result, the 50 ng

of CsCl-banded pUC19 sample was amplified at first, but the increase of the amplification stopped later, probably due to the inhibition caused by a high amount of plasmid DNA. The pUC19 which was isolated from transfected 293 cells appeared next and the amplification kept on increasing. The gDNA isolated from 293 cells with 12 different treatments (as designed in Table 3.13) showed an increase in the C_T value around 28 cycles, suggesting the homologous recombination indeed happened, albeit at a low level. The negative controls were pUC19-dl1 and pUC19-dl2 which were isolated from transfected 293 cells, as well as dH_2O; these controls showed no amplification at all. Therefore, by comparing the C_T values of different samples, we can evaluate the amounts of recombinant plasmid and consequently homologous recombination efficiencies.

The gel picture for Realtime PCR is shown in Figure 3.27. The bands of pUC19 amplification (Lanes 2 and 3) were stronger than the recombinant plasmid generated by intermolecular recombination between pUC19-dl1 and pUC19-dl2 (Lanes 9-20), suggesting that the homologous recombination happened at a low level; the negative controls had no amplification, indicating that this amplification was specific to pUC19. The experiments above suggested that this PCR-based homologous recombination assay system can be used to evaluate the homologous recombination efficiency and has a much higher sensitivity than the bacterial transformation method.

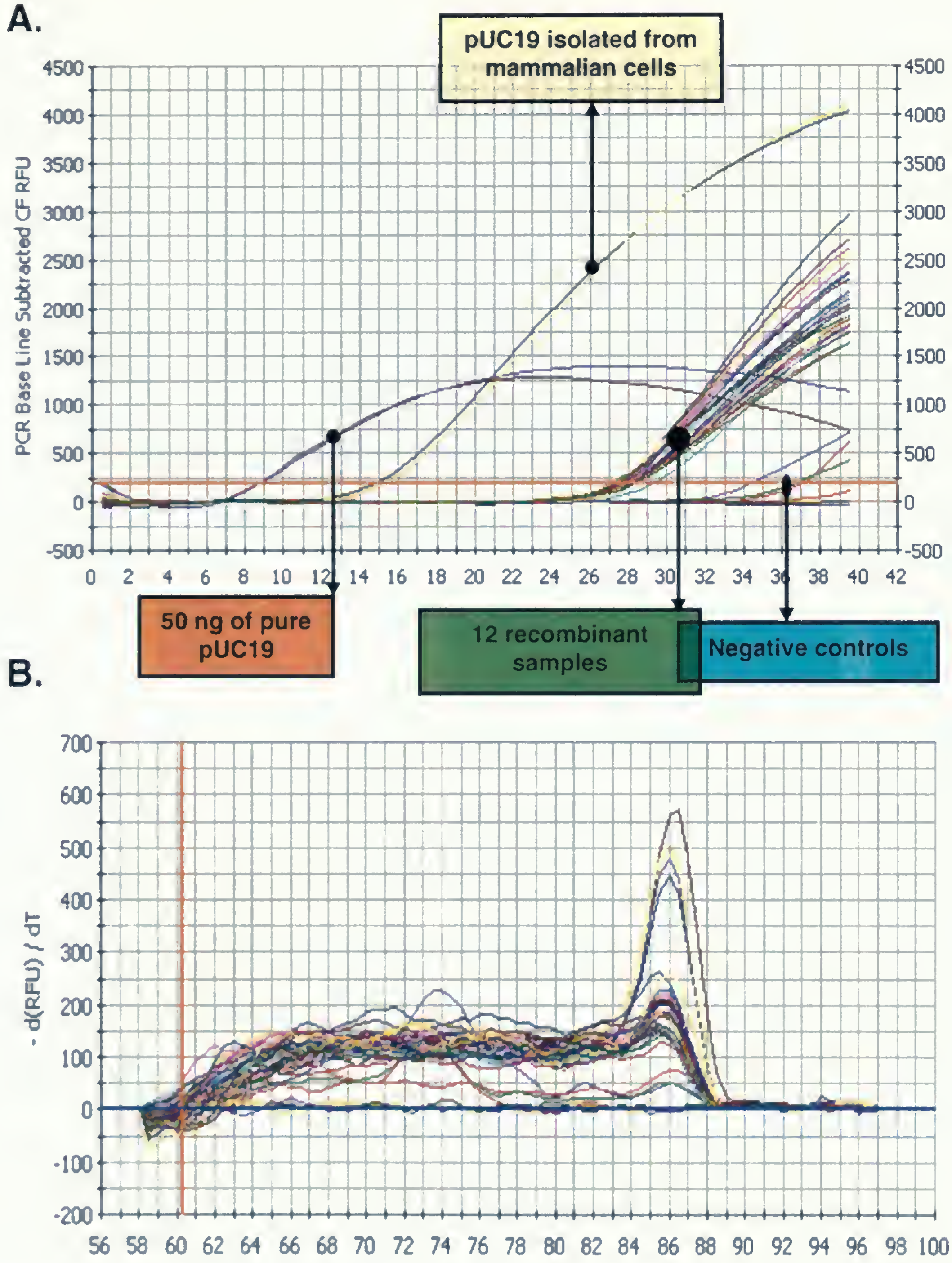

Figure 3.26. Realtime PCR amplification of plasmid DNA isolated from 293 cells. A) PCR quantification curve. B) Melt curve. The experiment was carried out in duplicate to avoid the reading error of the PCR machine.

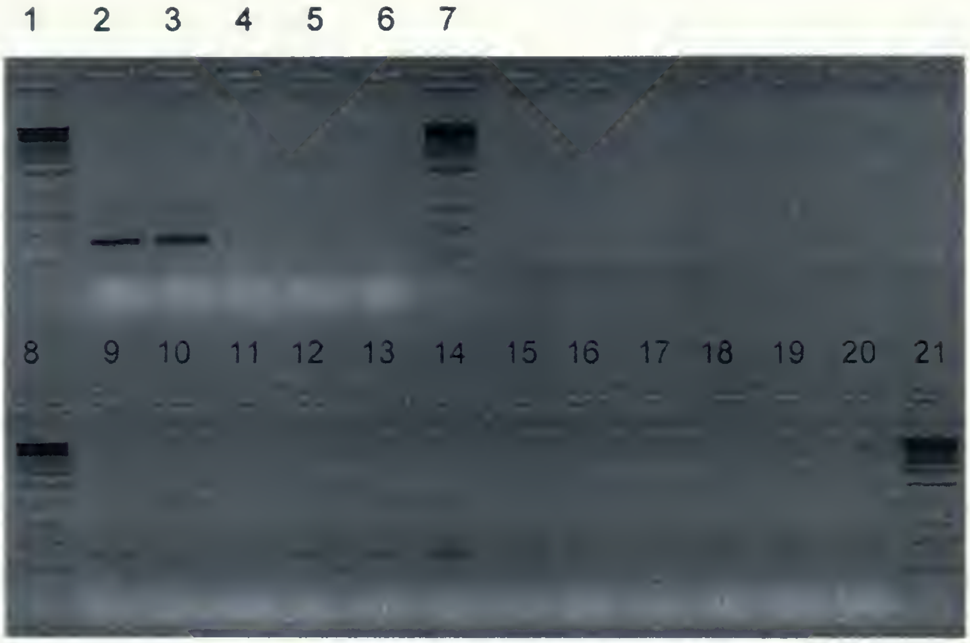

Figure 3.27. Gel picture for Realtime PCR results of gDNA isolated from 293 cells. Lanes 1, 7, 8, and 21: Norgen HighRanger marker. Lanes 2-6: Amplification of 50 ng of banded pUC19, pUC19 transfected and isolated from mammalian cells, pUC19-dl1 and pUC19-dl2 transfected and isolated from mammalian cells, and dH_2O, respectively. Lanes 9-11: Amplification of gDNA isolated from mock siRNA transfected cells. Lanes 12-14: 90 nM E1A siRNA transfected cells. Lanes 15-17: 90 nM E1B siRNA transfected cells. Lanes 18-20: 90 nM NegC siRNA transfected cells.

4. DISCUSSION

The objective of this project is to test the effect of the E1 region on adenovirus integration. Originally, we planned to carry this out by comparing the integration efficiencies between an E1-deleted and an E1-containing adenoviral vector. The fluorescent proteins GFP and RFP were chosen as reporters to facilitate the assessment of the integration efficiencies.

Unfortunately, we did not generate SubE3 virus even after numerous attempts at optimization and trouble-shooting. At the beginning, we used the traditional calcium phosphate-mediated transfection method (Jordan *et al.*, 1996), but neither the SubE1/ SubE3 recombinant viruses nor the positive control virus were successfully rescued. This failure could be caused by three problems: 1) the 293 cells were not healthy enough to provide E1 protein products; 2) the transfection efficiency was too poor to transport the plasmids into the nucleus; 3) the plasmid DNA was not good enough for generating recombinant adenoviruses.

To investigate these problems, we first tested the viability of the E1 region in 293 cells using PCR and RT-PCR and the results showed that the E1 region was viable. Then we tried to enhance the transfection efficiency by using Lipofectamine^{TM} 2000. Although using Lipofectamine^{TM} 2000 we were able to rescue the SubE1 virus and the positive control virus, however, we still could not rescue the SubE3 virus. Finally, we tested the quality of our CsCl-banded preparation. None of the SubE3 virus (0/32) was generated even if all wells (6/6) of the positive control virus were harvested, suggesting that the CsCl banding

preparation was qualified enough, and it might be the problems with the plasmid DNA that prevents us from harvesting SubE3 virus.

There might be two problems with the plasmid DNA. One possibility is that the plasmid system used for generating the SubE3 virus is not as efficient as that used for generating the SubE1 virus and the positive control virus. Unlike the *frt*/FLP-containing SubE1 virus system which can generate recombinant virus more efficiently through FLP-mediated site-specific recombination (Ng *et al.*, 2001), the SubE3 virus system does not have such elements to facilitate the rescue (Bett *et* al., 1994). Similarly, the generation of the positive control virus does not require recombination (Graham, 1984). Therefore, the rescue of the SubE3 virus is much more challenging than that of the SubE1 virus and the positive control virus.

The other possibility is that pFG173 is an unstable plasmid which can prevent the SubE3 virus from being rescued. It has been documented that some adenovirus genomic plasmids such as pFG173 are unstable in *E. coli* and often have internal deletions (Ghosh-Choudhury *et al.*, 1986; Hitt *et al.*, 1998). Our results were concordant to these findings, as we observed that after plating pFG173-transformed DH5α cells some of the colonies were smaller than the others. Knowing that bacterial cells transformed with larger plasmids will grow more slowly, we isolated plasmid DNA from the smaller colonies which turned out to contain pFG173 (by *Hin*dIII digestion analysis). Plasmid DNA was also isolated from the larger colonies, and it was found that these larger colonies contained self-deleted pFG173 (data not shown). Therefore, the instability of

pFG173 could lead to critical deletions/ mutations that prevented the generation of the SubE3 virus.

The difficulties associated with generating the SubE3 recombinant adenovirus revealed some of the problems of the adenovirus culture. Currently, the first generation of adenovirus vectors (Haj-Ahmad & Graham, 1986; Ghosh-Choudhury *et al.*, 1986) is most widely used. However, the rescue of the first generation of vectors is time-consuming because the homologous recombination (HR) efficiency in cultured cells is low (typically below 10^{-6}) (reviewed in Goncalves, 2005), and thus the rescue of a recombinant virus generally requires 10 to 15 days (based on literature and our observations). In addition, the generation of recombinant adenoviral vectors is dependent on the transfection efficiency, but the transfection efficiencies of the plasmid into the 293 cell line (Graham *et al.*, 1977) and other complementing cell lines (reviewed in Goncalves & de Vries, 2006) are difficult to estimate, and thus the generation of adenoviral vectors is thus highly unpredictable. Taken together, a simple, quick and predictable system for the rescuing adenoviral vectors remains to be developed.

Due to the problem of rescuing the SubE3 virus, we had designed an alternative strategy to test the effect of the E1 region on adenovirus integration. We decided to measure the effect of Ad5 E1 on HR because literature suggests that adenovirus integration might be initiated by HR events (Gahlmann *et al.*, 1982).

In this new strategy, we planned to take a reversed way to evaluate the effect of the E1 region. Since HEK 293 cells persistently express the E1 region,

the impact of E1 on HR can be assessed by comparing the recombination frequencies between normal 293 cells and E1 gene knocked-down 293 cells. The expression of the E1 region in 293 cells can be knocked down using RNA interference (RNAi) technology.

RNAi is a sequence-specific, post-transcriptional gene silencing process, and it is initiated by a double-stranded RNA (dsRNA) that is homologous with the target gene. In mammalian cells, chemically synthesized small interfering RNA (siRNA) is commonly used (Elbashir *et al.*, 2001; Reynolds *et al.*, 2004). Small interfering RNAs are short dsRNAs that are 21 to 25 nucleotides in length. After transfecting the siRNA into mammalian cells, the siRNA will be assembled into a ribonuclease-containing complex known as the RNA-induced silencing complex (RISC). The siRNAs then guide the RISC to the target mRNAs so that the RISC-ribonuclease degrades these mRNAs and thus silence the gene.

In our experiment chemically synthesized siRNAs ordered from Ambion were used. However, the transfection of E1A and E1B siRNAs did not show evident silencing. The unsuccessful silencing could be caused by three reasons: 1) the transfection efficiency was poor; 2) the 293 cells were not good for the silencing; 3) the target sequences used in this experiment are not potent so that they cannot trigger an efficient silencing effect.

To overcome these problems, we first examined the transfection efficiency by transfecting 30 nM GAPDH siRNA and observed a silencing effect. After transfecting 30 nM GAPDH siRNA, the RT-PCR results suggested that the expression of GAPDH had significant reduction, while the amplification of the

house-keeping gene β-actin showed the consistent expression. Thus the silencing of GAPDH was effective and specific, and the first two possibilities for unsuccessful silencing of the E1A and E1B can be excluded.

Next, we retested the silencing effect of E1A and E1B using the conditions developed above, but there was no significant reduction in their expression. We transfected 30 nM E1A and E1B siRNAs into 293 cells and did not observe the silenced phenotype, although this concentration was found to be enough to silence the GAPDH gene. We further examined the transfection with higher concentrations of siRNA (30-60-90 nM and 90-180-270 nM), but the silencing effect was still not observed. Lastly, we tried to find out whether the examining time (72 h post transfection) was too long or too short to observe the silenced phenotypes, but the RT-PCR results demonstrated that there were no differences at varying time points post transfection (48, 72, 96 h, respectively).

Therefore, it is likely that the siRNA sequences that we chose are not strong enough to induce the silencing effects in the 293 cells. As for the E1A siRNA sequence, although it was acquired from an article (Hacker *et al.*, 2004) and was supposed to be effective, it gave poor results in our experiment. One possibility could be that the researchers used an siRNA-expressing vector rather than a chemically synthesized siRNA. Once the siRNA-expressing vector is transferred into the nucleus, it will synthesize a short hairpin RNA (shRNA), which will then be cut into the active siRNA (Sui *et al.*, 2002). By cloning the sequence into an siRNA-expressing vector, we could achieve the silencing

effects. However, our early concern was that such plasmids could form white colonies and interfere with our calculation of HR efficiency.

As for the E1B siRNA, we could not locate any literature showing validated target sequences, therefore a screening for the E1B siRNA sequence was performed. The E1B target sequence was chosen based on four basic principles (Reynolds *et al.*, 2004): 1) the siRNA sequence should begin with "AA"; 2) the resulting 21 nucleotide should have a GC content of 30-50%; 3) the target RNA sequence should not contain extensive secondary structure; 4) the sequence should not have significant homology to any human gene.

Except for these four principles, it is also recommended to generate multiple siRNAs with various sequences of the target mRNA to increase the chances of silencing (Reynolds *et al.*, 2004). We did not use multiple siRNAs due to the high price of the chemically synthesized siRNA, and this lack of multiple siRNAs might be the reason for the ineffectiveness of the E1B siRNA.

Therefore, extra efforts are needed to obtain the successful silencing of the E1A/B genes, and there are at least three approaches that could be taken: 1) ordering multiple E1A- and E1B-specific siRNAs, rather than purchasing only one; 2) cloning more siRNA sequences into siRNA-expressing vectors to deliver different E1A and E1B siRNAs; 3) purchasing a long dsRNA and preparing an "siRNA cocktail".

First, purchasing more chemically synthesized siRNAs is the most convenient way, as it only requires testing the purchased siRNAs and the data can be acquired. However, the chemically synthesized siRNAs are expensive

(approximately $600 CAD for each siRNA), so the budget of the investigation must be carefully planned before acquiring multiple synthesized siRNAs.

Second, cloning multiple siRNA sequences into a plasmid will be much cheaper compared to ordering multiple synthesized siRNAs. As mentioned earlier, once the siRNA plasmid is delivered into the nucleus the siRNA will be expressed and the target mRNA degraded (Sui *et al.*, 2002), but our original concern was that if we used bacterial transformation to evaluate HR then these plasmids will give white colonies, which could interfere with the calculation of HR efficiency.

Third, successful silencing can also be achieved by transfecting an "siRNA cocktail" produced by the digestion of a long dsRNA. The long dsRNA covering the whole mRNA can be generated from a DNA template (Milligan *et al.*, 1987). Then, the cocktail of 21nt-long siRNAs can be generated *in vitro* by cleaving the long dsRNA with recombinant RNase III (Myers *et al.*, 2003). Because the "siRNA cocktail" contains multiple target sequences that cover the entire length of the target mRNA, the chances of gene silencing can be greatly enhanced.

By applying these strategies the silencing of E1A and E1B should be achieved, and in the future the effect of the E1 region on adenovirus integration can be studied using the same system developed in this research.

Even though there was no significant reduction of E1A/E1B mRNA, we still tried to test the HR efficiency. We developed an HR assay system based on bacterial transformation. In this system, the *lacZα* coding region of the plasmid pUC19 was mutated and two plasmids with different *lacZα* deletions (designated

as pUC19-dl1 and pUC19-dl2) were generated. Therefore, these two plasmids can form a functional *lacZα* cassette through intermolecular HR; the two non-recombinant plasmids should form white colonies, while the recombinant plasmid with the functional *lacZα* cassette should form blue colonies.

However, after we isolated the plasmid DNA from mammalian cells and transformed it into bacterial cells we did not find any colonies, even if the transformation efficiency of these competent cells reached as high as 1.8×10^7 colonies per µg of pUC19. It might be that the sensitivity of the bacterial transformation system is too low to detect the plasmid DNA. Therefore, a more sensitive method is required to test the recombination efficiency.

Thus we attempted to evaluate HR efficiency using PCR. As shown in Figure 3.25, in this new method the primers can only anneal to and amplify pUC19, so only the recombinant plasmid formed through intermolecular recombination between pUC19-dl1 and pUC19-dl2 can be amplified. Therefore, the higher the HR efficiency, the more amplification will occur. In this way the HR efficiencies can be compared using PCR.

According to literature, there are various methods available to evaluate the HR efficiency in cultured mammalian cells. In general, these HR studies can be carried out by either analyzing restriction fragment length polymorphism (RFLP) or by testing the expression of functional cassettes formed through the recombination between two dysfunctional cassettes.

The former is more traditional and time-consuming (for example, Young *et al.*, 1984). In this method, the DNA substrates would exchange the genetic

information with each other, so certain pre-made restriction sites will shift from one DNA substrate to the other. Therefore, the recombinant and non-recombinant DNA should show different digestion patterns. Compared to our PCR method, this method is tedious in the performance of Southern blot and the preparation of the restriction sites on the DNA substrates.

The latter is more widely used currently and our design was inspired by this strategy (for example, Vasileva *et al.*, 2006). This strategy is generally carried out by transfecting the cells with two plasmids having different defective reporter cassettes such as GFP (or other fluorescent proteins), and only the cells containing the GFP signal are considered to contain the recombinant functional plasmid. The recombination efficiency can be expressed as the percentage of GFP-expressing cells in all the transfected cells. Other commonly used reporter genes include the antibiotic-resistant genes such as hygromycin phosphor-transferase gene (for example, Yun *et al.*, 2004), so only the cells which survive the hygromycin selection are deemed to contain the recombinant plasmid and the recombination frequency can be calculated from the ratios between the survived cell foci and the total transfected cells.

However, considering that the HR efficiency is generally low, for example, in 10^6 mice cells only 1~5 cells showed the recombinant signal (Wiktor-Brown *et al.*, 2006), the detection of cells expressing this recombinant plasmid would be difficult using techniques such as fluorescence-activated cell sorting or fluorescent microscope. By applying PCR the sensitivity can be enhanced.

Theoretically, as long as one recombinant copy is present in the sample, the amplification would occur.

To our knowledge there was no publication using PCR/ Realtime PCR to perform HR assays. Here we developed a new PCR-based approach, which can be quicker and more sensitive than other HR assay systems.

In addition, as discussed above, by transfecting multiple E1A and E1B siRNAs, the HR efficiency can be evaluated with this system and the effect of the E1 region on HR and adenovirus integration can be studied in the long run.

In gene therapy, targeted integration of the transgene (gene targeting, GT) is desired because the integration of the transgene can provide a persistent expression whereas the specific integration can ensure the safety of the patient. The GT is mediated by HR between the transgene and human chromosomal DNA (Mitani & Kubo, 2002). On the other hand, random integration (RI) might inactivate a tumor suppressor gene or activate an oncogene and should be avoided. Adverse events using retroviral vectors have demonstrated the danger of the RI (Hacein-Bey-Abina *et al.*, 2003; Check, 2005). The RI is mediated by non-homologous end joining (NHEJ) (Pastwa & Blasiak, 2003).

A recent study showed that the RI frequency of adenoviral vectors was much higher than the GT frequency (Ohbayashi *et al.*, 2005). A helper-dependent adenoviral vector (all the virus genes were deleted) carrying an 18.6 kb homology of the hypoxanthine phosphoribosyl transferase (*Hprt*) was used to infect the mouse embryonic stem cells. The frequency of HR reached 0.2% per transduced cell, but the RI revealed extremely high efficiency of 5% per cell. The

results suggested that if the adenoviral vectors integrated then the safety risk would be high. Noticeably, however, the adenoviral vector used in this experiment had the E1 region deleted. Therefore, whether the E1 region has an effect on HR and adenovirus integration still needs to be tested.

If the adenoviral vectors can express the viral protein(s) that can enhance the HR (targeted integration) efficiency and/or decrease the NHEJ (random integration) efficiency, then the safe and persistent gene therapy can be achieved. Some recent papers suggested that adenoviruses indeed encode viral products that can regulate their integration events. It has been shown that the Ad5 E4orf6 protein can inhibit the NHEJ pathway (Mohammadi *et al.*, 2004; Hart *et al.*, 2005), suggesting that this protein can reduce the chance of RI. Considering that the E1 region has multiple functions, if the E1 region can enhance the HR and the targeted integration efficiency then the co-presence of the E1 region and E4 region in an adenoviral vector would provide a safe and persistent approach for human gene therapy.

5. CONCLUSIONS

To sum up, based on the results obtained from this study, the following conclusions can be made:

1) The SubE3 virus cannot be rescued, probably due to the instability of the plasmid pFG173.

2) The E1A and E1B siRNA target sequences used in this study are not potent enough to induce a silenced effect in 293 cells. More siRNA target sequences should be tested in order to silence the E1A and E1B genes.

3) The bacterial transformation system has low sensitivity and cannot be used to test homologous recombination frequencies. However, by using the primers designed inside the deleted parts of the two dysfunctional plasmids, the PCR/ Realtime PCR can be used to evaluate homologous recombination efficiency.

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