Studies of the Adenovirus 5 L1 Gene Aimed at Developing L1 Gene Deficiencies for Use in Gene Therapy Vectors

by

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A thesis submitted for the degree of

PhD

In the University of Warwick

Department of Biological Sciences

September 1999

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Acknowledgements

I wish to thank the past and present members of the Adenovirus and Rotavirus Groups of the Biological Sciences Department for their help and advice during my time at Warwick. I am grateful to Dr. Andy Easton and Prof. Malcolm McCrae for their advice and helpful discussions. I would like to thank to Brian, David, Graham, Jay, Jackie, Jill, Li, Liz, Simone and Ste for their help and suggestions over the years all through my research. Finally I would like to thank my supervisor, Dr. Keith Leppard for giving me the opportunity to do this PhD. I would also like to thank him for his guidance, helpful suggestions and critical reading of this thesis.

Declaration

All the work presented in this thesis was performed by the author in the Biological Sciences Department of the University of Warwick, under the supervision of Dr. K.N. Leppard. None of the material herein has at anytime been presented for examination for any other degree.

Abbreviations

Ω	ohm
μCi	microcurie
μF	microfarad
β-gal	beta-galactosidase
μl	microlitre
AAV	adeno-associated virus
Ad	adenovirus
Ad5	Adenovirus serotype 5
Ads	adenoviruses
amp	Ampicillin
APS	ammonium persulphate
ATP	adenosine triphosphate
bp	base pairs
CAT	chloramphenicol acetyl transferase
CF	cystic fibrosis
Ci	curie
CIAP	calf intestine alkaline phosphatase
cm	centimetre
CMV	cytomegalovirus
cpe	cytopathic effect
DBP	DNA binding protein
dH_2O	distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
ds	double stranded

DTT	dithiothreitol
EBV	Epstein Barr virus
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-*bis*-(β-amino-ethylether)-N,N,N',N'-tetraacetic acid
EIAV	equine infectious-anemia virus
ELISA	enzyme linked immunosorbent assay
ery	erythromycin
exo	exonuclease
FCS	foetal calf serum
g	gravity
gst	glutathione S-transferase
HCAR	Human Coxsackie and Adenovirus Receptor
HEPES	N-2-Hydoxyethylpepirazine-N'-2-ethanesulphonic acid
HIV	human immuno-deficiency virus
hr	hour
HRP	horseradish peroxidase
hyg	hygromycin
IPTG	isopropyl-Thio-β-D-galactopyranoside
ITR	inverted terminal repeat
kan	Kanamycin
kb	kilobase pairs
kD	kilodalton
kV	kilovolts
l	litre
M	molar
mg	milligram
MHC	major histocompatibility complex

min	minute
ml	millilitre
MLP	major late promoter
MLTU	major late transcription unit
MLV	murine leukemia virus
mM	milimolar
mm	millimeter
mmol	millimole
moi	multiplicity of infection
MOPS	3-[N-Morpholino]propanesulphanic acid
mRNA	messenger ribonucleic acid
ms	millisecond
mu	map unit
NCS	new-born calf serum
neo	neomycin
ng	nanogram
nm	nanometer
NP 40	Nonidet P 40
OD	optical density
ONPG	o-nitrophenyl β-D-galactopyranoside
OPD	o-phenylenediamine
orf	open reading frame
ori	origin of replication
P	promoter
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit

pmol	picomole
PMSF	phenylmethanesulphonyl fluoride
psi	pounds per square inch
r	resistance
RCA	replication-competent adenovirus
RCR	replication-competent retroviruses
RNA	ribonucleic acid
rpm	revolutions per minute
RSV	human respiratory syncytial virus
SD	standard deviation
SDS	sodium dodecyl sulphate
sec	second
SIV	simian immuno-deficiency virus
SV40	simian virus 40
TBM	3,3',5',5'-tetramethylbenzidene
TEMED	N,N,N',N'-tetramethyl-ethylenediamide
tet	tetracycline
TK	thymidine kinase
Tris	tris(hydroxymethyl)aminomethane
ts	temperature-sensitive
uv	ultra-violet
wt	wild-type
X-gal	5-Bromo-4-Chloro-3 Indolyl-B-D-galactoside

Summary

Gene therapy is a novel approach to the treatment of human disease that is in its very early stages of development. Its purpose is to add to and/or alter the pattern of gene expression in cells so as to achieve a therapeutic benefit and is being developed for application to such diverse conditions as simple inherited diseases, cancer, AIDS, and cardiovascular disease.

Poor uptake of DNA by cells *in vivo* is a significant technical barrier for gene therapy. Viruses have evolved to carry their nucleic acid contents into cells very efficiently and are thus considered as potential vectors for gene therapy, provided their pathogenicity and other adverse features can be overcome.

Adenovirus is a virus type, which is a prime candidate for development as a vector for efficient transfer of genes into human tissues. In recent years, there have been many studies in order to develop replication deficient adenoviral vectors as gene therapy vectors. Current disadvantages of these vectors include their limited capacity to accommodate exogenous DNA and elucidation of a host immune response against these vectors. Currently they have a capacity for about 9 kilobasepairs of DNA, which is not sufficient for some therapeutic purposes.

The work described in this thesis aimed to provide a way to produce highly deleted adenoviral vectors containing deletions in their L1 52/55-kD, L1 52/55-kD and IV_{a2}, or L1 52/55-kD and IX coding regions. These genes encode proteins, which contribute to virus particles, and their assembly and some of which have been reported to increase expression of the viral late (structural protein) genes. Together with the deletions available in currently used vectors, these new deletions would create vectors with an increased capacity to accommodate exogenous DNA. Furthermore, these deletions were expected to make the vectors more replication-deficient and less immunogenic by decreasing the expression of residual viral genes.

Initially, cell lines expressing adenovirus L1 52/55-kD, L1 52/55-kD and IV_{a2}, or L1 52/55-kD and IX proteins were constructed for use as complementing cell lines. These were designed to supply in *trans* the relevant proteins that would be missing during attempts to construct recombinant viruses with deletion mutations in these gene(s). 293-L1 cells were proven in their ability to complement missing L1 52/55-kD protein function using existing adenovirus L1 52/55-kD point mutants *ts*369 and H5pm8001. However, attempts to isolate an L1 52/55-kD coding region deleted recombinant adenovirus using this cell line were unsuccessful, possibly due to the nature of sequences missing in the deleted L1 52/55-kD coding region which might have currently undefined *cis* acting regulatory functions.

L1 52/55-kD protein is known to form a complex with IV_{a2} and the latter protein has been reported to activate the major late promoter. The effects of L1 52/55-kD protein on the adenovirus major late promoter transactivation were therefore investigated by transient expression experiments carried out by transfection of COS cells with a major late promoter-dependant reporter gene (CAT) and expression vectors for L1 52/55-kD and/or IV_{a2}. These experiments did not reveal any role for the L1 52/55-kD protein in the activation of adenovirus major late promoter.

Chapter 1

Introduction

1.1 Goals of gene therapy

1.1.1 Introduction

Progress in the study of human disorders over the past 25 years has greatly enhanced our ability to describe the molecular basis of many disease states. Molecular genetic techniques have been particularly powerful. They have allowed the isolation of the genes associated with common inherited diseases such as cystic fibrosis (CF), as well as the identification of many other genes that contribute to more complex diseases such as cancers. Because our knowledge of the basic mechanisms underlying many biological processes such as gene expression and protein synthesis has also increased, the challenge today is to use all this information in the development of new treatments for disease.

Gene therapy represents one way in which this knowledge is being applied. Gene therapy consists of the introduction of nucleic acid into cells of a patient in order to use the expression of that nucleic acid for some therapeutic purpose. Though simple, this definition encompasses an extremely wide range of applications such that defining the goals of all gene therapy applications in more precise terms is difficult. While the emergence of gene therapy as an accepted clinical practice has occurred more quickly than many in the field expected, the discoveries that led to the current gene therapy technology derive from the studies in the early 1970s on RNA and DNA tumour viruses (Tooze, 1980; Weiss *et al.*, 1985; Freidman and Roblin, 1972). Investigators probing the mechanisms of transformation by these viruses discovered that viral genetic material was being transferred to the transformed cell, and this led to the concept of using a virus as a gene transfer agent. The advent of recombinant DNA technology in the mid-1970s led to the isolation and manipulation of genes (Danna and Nathans, 1971; Cohen *et al.*, 1973; Maniatis *et al.*, 1978), and made the concept of viral vectors a realistic possibility by the end of the decade. The

development of retroviral-based gene transfer systems in the early 1980s was an essential contribution, demonstrating that genes could be transferred at high efficiency both *in vitro* and *in vivo* (Shimotohno and Temin, 1981; Cone and Mulligan, 1984; Miller *et al.*, 1983).

The original ideas of gene therapy were directed toward treating monogenic (single-gene) disorders, but it has become clear that DNA can be considered a new pharmaceutical agent for treating many types of diseases. Over the last 20 years, the initial thoughts of gene therapy have been transformed into reality with more than 175 clinical trials and 2,000 patients already treated (Ross *et al.*, 1996). Yet with all the trials, there is still no conclusive evidence for efficacy. Although the expectations have exceeded the initial success of this relatively new field, important information has been gained from preclinical and clinical trials. With recent technological advances, gene therapy for treating a wide variety of diseases is likely to become a reality within the early part of the next century. There are clearly a number of obstacles limiting successful gene therapy, but the most difficult to overcome has been the inability to transfer the appropriate gene into a target, non-germ-cell tissue, such that an appropriate amount of gene product (usually a protein) is produced to correct the disease.

1.1.2 Methods of gene transfer

The ideal vector for use in gene therapy is at present a theoretical entity, but one against which current and future vectors can be measured. The ideal vector must achieve regulated and sustained expression of foreign genes in specific cells or tissues. This usually requires the foreign genes to be included in constructs that either integrate within active regions of the genome or that replicate autonomously. And the whole process must be safe, efficient, and selective. Many problems need to be solved in developing any gene-therapy approach: definition of the cells that constitute the target, entry of DNA into those cells, expression of useful levels of

gene product over an appropriate time period, avoidance of the almost inevitable response of the host to the introduced agents, and so on.

The demands made by a gene therapy on its vector are extreme. Vectors have to be able to access the target cell population, introduce the DNA into the cell in a transcriptionally active state, be non-toxic (except where the aim is to kill the cells, such as in cancer therapy) and be able to express the therapeutic gene product at sufficient levels and for an appropriate length of time to correct the disease phenotype. Although existing vectors can achieve some of these aims, none can single handedly provide all the necessary functions (Hodgson, 1995).

There are two approaches that may be utilised for gene therapy - an indirect, *ex vivo* method, in which cells are modified in culture and then transplanted, and a direct, *in vivo* gene transfer method, involving the injection of a vector. In each case, a delivery vector must be used and these can be divided into viral and non-viral systems. In viral methods, a modified virus infects the cells and introduces a viral genome containing the foreign genes. There are four types of virus vectors currently in clinical trials – based on retroviruses, adenoviruses, herpes-simplex viruses and adeno-associated viruses. Non-viral methods, on the other hand, use chemicals mixed with DNA - such as calcium phosphate coprecipitation, liposomes, and molecular conjugates - or physical methods - such as electroporation, microinjection and particle acceleration - to introduce foreign genes into the cell. Although non-viral gene transfer is safer, compared to viral gene transfer, its major drawback is that it can only achieve transient expression because there is no effective mechanism for integration or for autonomous replication, of transfected DNA in human cells.

The main advantage of non-viral methods is that there is no cotransfer of unwanted viral genetic material to the cell. Additionally, non-viral methods lend themselves more readily to the standardised manufacturing conditions that are required if gene

transfer technology is to be applied on a wide scale in medicine (these criteria include scale up and quality control).

An important property of non-viral gene delivery is that DNA only rarely integrates into the chromosomes of host cells. Integration has, in fact, never been reported after non-viral delivery *in vivo*. Instead, the administered gene is intended to persist as an extrachromosomal element (episome) capable of expressing gene products for a discrete period of time before elimination from the host cell by nuclease degradation (Ledley, 1994). This property of non-viral delivery methods will allow gene therapies to be used clinically in a manner similar to conventional medicines, which exhibit a reproducible half-life in the body, leading to a predictable level of the therapeutic product and duration of action. Non-viral gene therapies are usually designed to be applicable to both acute and chronic diseases by repetitive administration, where the dose and schedule of administration can be adjusted to meet the patient's individual needs.

Viruses have evolved to become highly efficient at nucleic acid delivery to specific cell types while avoiding immunosurveillance by an infected host. These properties make viruses attractive gene delivery vehicles, or vectors, for gene therapy. Several types of viruses, including retrovirus, adenovirus, adeno-associated virus (AAV), and herpes simplex virus, have been modified in the laboratory for use in gene therapy applications. Because these vector systems have unique advantages and limitations, each has applications for which it is best suited. Retroviral vectors can permanently integrate into the genome of the infected cell, but require mitotic cell division for transduction. Adenoviral vectors can efficiently deliver genes to a wide variety of dividing and nondividing cell types, but immune elimination of infected cells often limits gene expression *in vivo*. Herpes simplex virus can deliver large amounts of exogenous DNA; however, cytotoxicity and maintenance of transgene expression remain as obstacles. AAV also infects many nondividing and dividing

cell types, but has a limited DNA capacity (Morgan and Anderson, 1993).

1.1.3 Retrovirus-based vector systems

The majority of clinical trials to date have used murine leukaemia virus (MLV) based retroviral vectors for gene transfer (Miller *et al.*, 1992; Miller, 1992; Guild *et al.*, 1988). Wild-type MLV encodes three proteins, from the genes *gag*, *pol* and *env*, which are processed into a number of polypeptides important for replication, encapsidation, infection and reverse transcription. The three proteins can be provided *in trans*, allowing the generation of vectors containing only the *cis*-acting elements that are required for these processes (reviewed by Jolly, 1994). Cell lines that express the three viral proteins stably have been generated, and are termed packaging lines (reviewed by Miller, 1990). These cell lines can be used to produce recombinant, replication-defective virus by either stable or transient transfection. Currently, packaging lines that give titres of greater than 10^7 infectious virus particles per ml have been developed (Cosset *et al.*, 1995). The use of different viral-envelope proteins, such as the G protein from vesicular-stomatitis virus, to produce pseudotype vectors has improved titres following concentration to greater than 10^9 ml^{-1} (Cosset *et al.*, 1995).

The advantage of retroviruses is that they can stably infect dividing cells introducing genes into a single, active region of chromatin in each cell, giving a permanency that facilitates long-term expression by integrating into the host DNA without expressing any immunogenic viral proteins. In theory the integrated retroviral vector will be maintained for the life of the host cell, continuing to express the gene of interest. But a number of problems remain. The most important is the possibility of regenerating replication-competent retroviruses (RCRs) during initial vector production (Muenchau *et al.*, 1990). RCRs can generate fatal neoplasms in primates (Donahue *et al.*, 1992) and, therefore, expensive testing is required for all retroviral vectors

used in human gene therapy. In addition to this major drawback, retroviral vectors accommodate only about 8 kb of foreign DNA sequences, they are rapidly inactivated *in vivo* by serum complement, they carry a risk of oncogenicity due to non-specific integration of the genome, transcription of foreign genes is often inactivated over time, and they can infect only actively dividing cells. However, the recent development of vectors based on lentiviruses [such as human immuno-deficiency virus (HIV), simian immunodeficiency virus (SIV) or equine infectious-anaemia virus (EIAV)], which can infect certain non-dividing cells, should allow the wider *in vivo* use of retroviral vectors for gene-therapeutic applications. Indeed, helper-virus-free stocks of recombinant HIV-based vectors have been generated that can infect a wide range of non-dividing cells, including neurons, pancreatic islets and muscle cells (Blomer *et al.*, 1996; Naldini *et al.*, 1996 (a, b).

1.1.4 Adenovirus-based vector systems.

In recent years, the use of adenoviruses (Ads) as gene therapy vectors has received increasing attention (Bajocchi *et al.*, 1993; Crystal *et al.*, 1994; Davidson *et al.*, 1993; Perricaudet *et al.*, 1992; Wang and Taylor, 1993; Amalfitano *et al.*, 1999; Putzer *et al.*, 1997; Leon *et al.*, 1998) due to a number of properties of the adenovirus system which make it a good candidate for this application, not the least of which is the extensive understanding of the structure and biology of the adenoviruses (Shenk, 1996) that has been gained through their use as model system to study DNA replication, transcription, RNA processing and protein synthesis. Other advantages include the ease with which the genome can be manipulated by simple recombinant DNA techniques, the yields of virus that can be obtained in tissue culture and easily collected and concentrated, and the possibility of high level expression of foreign DNA inserts. The development of adenovirus-based vectors, which is the principal subject of this thesis, is discussed in more detail below.

1.2 Gene therapy using adenoviruses

Adenoviral vectors represent an important class of delivery vehicle for *in vivo* gene therapy of diseases where the tissue to be treated cannot be removed, gene-corrected *ex vivo* and reimplanted. Cystic fibrosis (CF) provides such an example and has stimulated evaluation and development of the adenoviral vector system for clinical use. Several factors account for recent growing interest in adenoviral vectors. First, they can be easily rendered replication deficient by deletion of critical vital regulatory genes. Second, they infect a wide range of cell types. Third, they transduce both dividing and non-dividing cells. Finally, they can be produced easily and at high titers (10^{11}-10^{12} infectious units per ml) in laboratory cell culture. Adenovirus-mediated gene transfer has now been evaluated in various tissues and organs in animals and in several clinical trials in humans. One specialized role of adenoviral vectors for gene transfer involves their use as vaccines. Adenoviruses have also been used in a new type of 'molecular conjugate' gene delivery vector.

1.2.1 General introduction to adenoviruses

The adenoviruses belong to the family *Adenoviridae*, which is divided into two genera, *Mastadenovirus* and *Aviadenovirus* (Norrby *et al.*, 1976). Adenoviruses belonging to genus *Mastadenovirus* infect mammals including humans and the *Aviadenoviruses* infect birds. All members of the adenovirus family have similar chemical and physical properties, but can be distinguished by their individual type-specific antigens. In man, 47 distinct serotypes (Ad1 to Ad47) that form six groups (A to F) have been isolated to date, most of them during the decade following their discovery (Rowe *et al.*, 1953). Human adenovirus type 5 (Ad5), the virus used in this study, belongs to subgroup C and its genome has been completely sequenced (Chroboczek *et al.*, 1992).

Host specificity seems to be very stringent in as much as no spread from one host species to another has ever been reported. An abortive growth cycle usually results

after infection of cells from another species with human adenovirus. Clinical illness associated with adenovirus infection depends on the serotype, but is usually mild and rarely life-threatening. The primary target for adenovirus cytopathology is the epithelial cell. Productive infections of human adenoviruses take place in gastrointestinal, respiratory, or ocular epithelial cells, resulting in pathological alterations mediated by direct tissue damage. For example, some serotypes are associated with "swimming pool conjunctivitis" (Ad 3, 20) or gastroenteritis (Ad40), while Ad5 is responsible for mild upper respiratory tract illnesses. The near-terminally differentiated quiescent cells of the upper respiratory tract lining are the natural host cells in which Ad5 replicates. From the primary sites of infection the viral progeny then enter the bloodstream and spread to the other body tissues. The ultimate outcome of the infection is a chronic and asymptomatic shedding of the virus in tonsilar, adenoid tissues, guts, or kidneys, depending on the infecting serotype. Virus can be excreted for months or years following the initial infection essentially through the oral-fecal and respiratory routes (Evans 1958; Fox *et al.*, 1969). Ad4 and Ad7 cause more severe respiratory infections which can be as bad as pneumonia. This, together with the potential for epidemic spread, has led to the development of a live bivalent vaccine against serotypes 4 and 7 which has been administered as enteric-coated capsules to United States military recruits (Couch *et al.*, 1963; Top *et al.*, 1971).

In 1962, it was shown that the human adenovirus type 12 induced malignant tumors following inoculation into new-born hamsters (Trentin *et al.*, 1962). This was the first time that a human virus had been shown to cause tumors. However, so far, there has been no reported evidence that adenoviruses cause malignant diseases in humans.

1.2.2 Structure and biology of adenoviruses

Adenoviruses have nonenveloped icosahedral (20 facets and 12 vertices) protein

capsids with a "diameter" of 70-100 nm enclosing an inner DNA protein core. The outer capsid is composed of 252 capsomers arranged to form 240 *hexons* (contacting 6 other capsomers, 12 hexons per facet) and 12 *penton bases* (contacting 5 other capsomers); the latter are located at each vertex from which protrude the antenna-like *fibres*. This structure is responsible for attachment of adenoviruses to cells. Adenovirus particles contain 87 % protein, 13 % DNA, no membrane or lipid and only trace amounts of carbohydrate due to the modification of fiber protein by the addition of glucosamine; they have a density of 1.34 g/cm^3 in CsCl (for review, see Shenk, 1996).

All Adenovirus genomes have the same general organization (genes encoding specific functions are located at the same position on the viral chromosome). The double-stranded linear DNA genome of Ad5 is about 36 kb long, conventionally divided into 100 map units (mu). Each extremity of the viral chromosome has a 100-140 bp-long redundancy (length varies with serotype), the *inverted terminal repeat* (ITR) (Garon *et al.*, 1972; Wolfson and Dressler 1972) which is necessary for viral replication. Adjacent to the left ITR are located several specific sequences which direct the packaging of the viral genome into preformed capsids (Hearing *et al.*, 1987). The requirement for these encapsidation signals makes adenovirus virion assembly specific and precludes entry of cellular DNA into adenovirus empty capsids.

By far the most extensively studied adenoviruses are the human serotypes 2, 5, and 12 (for general reviews, see Tooze 1981; Ginsberg 1984). In cultured permissive cells an adenovirus infectious cycle lasts 30-36 hours beginning with an adsorption stage followed by an endocytic entry. Attachment of Ad5 to cells is mediated by the fiber protein binding to its cellular receptor, HCAR (Tomko *et al.*, 1997) an immunoglobulin-like trans-membrane protein. After attachment, Ad5-HCAR complexes are internalised by receptor mediated endocytosis, which is triggered by

the interaction of penton proteins with their cell surface receptors termed integrins (Wickham *et al.*, 1993). However, the mechanism by which the interaction induces endocytosis is not clear. The internalised virus moves from the endosome to the cytosol in a pH-dependent process (Svensson, 1985; Mellman, 1992) by disrupting the endosomal membrane and the penton base is believed to play an essential role in the process. Virus particles are then transported across the cytoplasm to the nucleus, probably by a mechanism involving hexon interaction with microtubules (Dales and Chardonnet, 1973).

After the internalisation, the virion is dismantled by the sequential elimination of its structural proteins (Greber *et al.*, 1993) and only its DNase-sensitive genome and core proteins gain access to the nucleus, allowing the productive cycle per se to commence. Viral DNA replication can begin only after expression of the early genes. Shortly after the onset of replication at 6-8 hours post-infection the late phase of transcription starts. Infection by adenovirus has a profound effect on the host cell since its DNA and protein productions are halted. Synthesis of viral progeny (1000-10,000/cell) leads to cell death through cell lysis (Tollefson *et al.*, 1996).

The genetic organization of the human Ad5 genome is shown in Figure 1.1 where early and late phase transcription blocks can be distinguished by thin and heavy lines respectively. By convention, the map is drawn with the E1 gene at the left end. The viral chromosome carries five early transcription units (E1A, E1B, E2, E3 and E4), two delayed early units (IX and IVa2), and one late unit (the major late transcription unit, MLTU), the transcription from which is processed by alternative splicing to generate five families of late mRNAs (L1 to L5). The two phases of transcription are temporally separated by the onset of viral DNA replication. The early genes are initially expressed from those genome template(s) which have reached the host cell nucleus. Multiple promoters are switched on during the early phase, giving rise to precursor mRNAs which are alternatively processed extensively. During the late

phase, other promoters become activated to ensure expression of structural proteins. However, early genes continue to be expressed at late times after infection and the promoter controlling expression of the major late transcription unit displays a low level of transcription early after infection. The viral genes encoding proteins IV_{a2} and IX begin to be expressed at an intermediate time (Petterson and Roberts, 1986) and thus form a delayed early category. In addition, the early gene E2A encoding the DNA binding protein is transcribed from a late promoter after the beginning of viral replication. Activation of the major late promoter (MLP) at 16 mu gives rise to a giant nuclear RNA spanning close to 80 % of the genome. This precursor RNA is processed in a complex fashion into five families (L1 to L5) of mRNAs, each characterized by the presence of an identical tripartite leader sequence (Figure 1.1). Within each family the mRNAs also possess the same 3' terminus, defined by a common polyadenylation signal. Part of the L1 family is also expressed during early infection. The secondary and/or tertiary structure of the leaders which form the 5'-terminal segment of all mRNA species issued from the MLP clearly facilitates their preferential translation (for review, see Horwitz, 1996). Gene expression is dependent on host cell RNA polymerase II. Adenovirus transcription units tend to encode proteins with related functions, and splicing contributes to a timely expression of proteins with similar functions, and regulates the relative proportions of each.

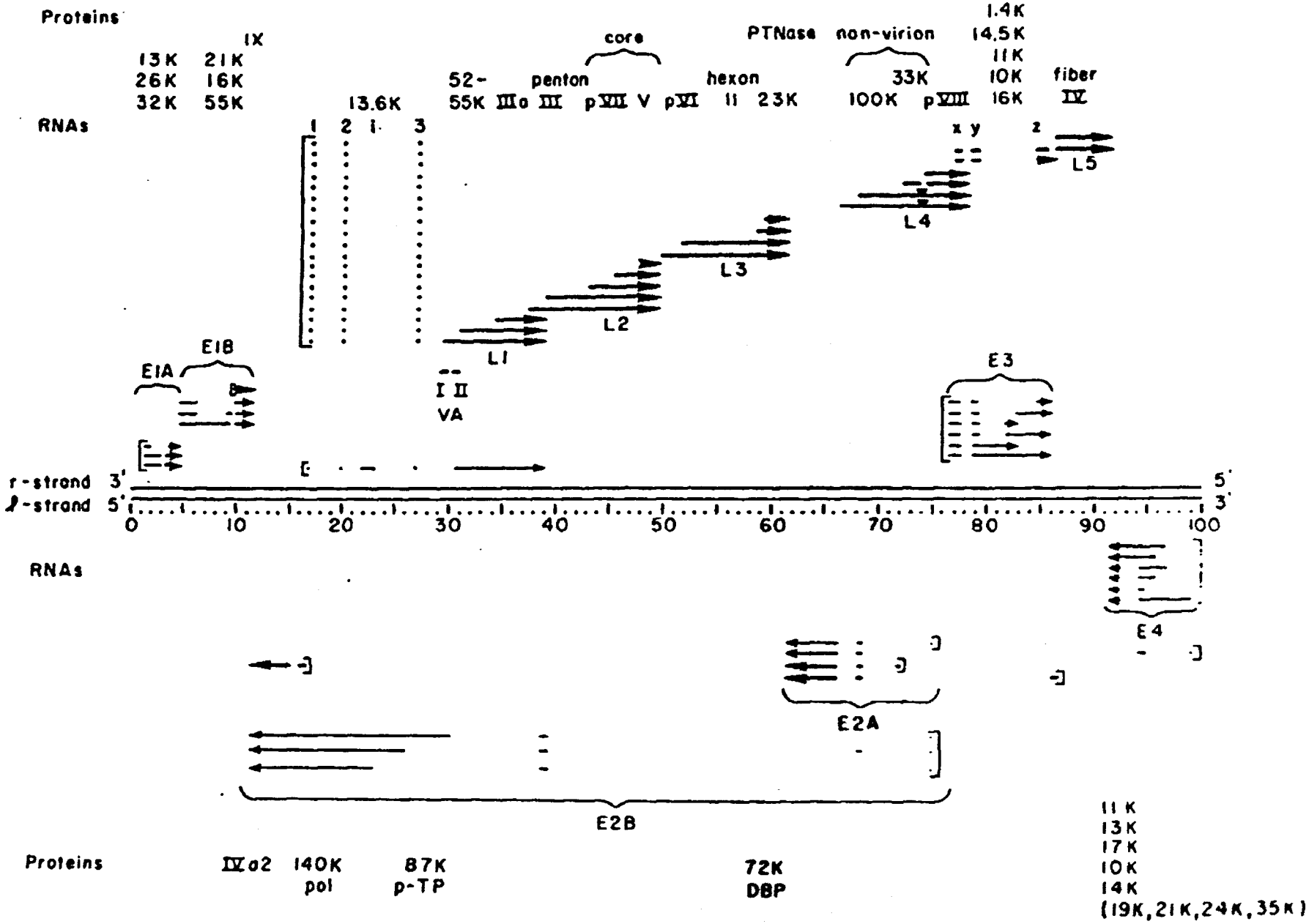

Proteins
IX
13K 21K
26K 16K
32K 55K
13.6K
core
52- penton hexon
55K IIIa III pVIII V pVI II 23K
PTNase non-virion
33K
100K pIX
1.4K
14.5K
11K
10K
16K
fiber
IV
RNAs
1 2 1 3
x y
z
L5
L4
L3
L2
L1
E1B
E1A
I II
VA
E3
r-strand 3' 5'
l-strand 5' 3'
0 10 20 30 40 50 60 70 80 90 100
RNAs
E4
E2A
E2B
Proteins
IVa2 140K 87K
pol p-TP
72K
DBP
11K
13K
17K
10K
14K
(19K,21K,24K,35K)

Figure 1.1 Transcription and translation map of adenovirus.

The early mRNAs are designated E and late mRNAs are designated L. Most late mRNAs originate at 16.3 map units and contain the tripartite leader whose components are labelled 1, 2, and 3. Some of the late mRNAs also contain a fourth leader segment *I*. Polypeptides are identified by the conventional numbering system for the virion structural components and by size for non-structural polypeptides.

The ElA gene is located in the leftmost five percent of the adenovirus genome (Figure 1.1) and is transcribed from a promoter, which is activated by cellular factors immediately after infection. It codes for two predominant, related, multi-functional regulatory proteins. These proteins, by transactivating the transcription of the other early genes, play a key role in the productive infection (Berk *et al.*, 1979; Jones and Shenk, 1979; Nevins, 1981). Three other E1A mRNA species accumulate later during the later stages of the infection, however no definitive unique function has been described for their products (Shenk, 1996). ElA proteins regulate viral genes in a complex manner. The larger polypeptide, 289R (translated from the 13S mRNA), acts either by increasing the DNA-binding activity of transcriptional factors (E2F, E4F, AP1, ATF, TATA factor), or by participating directly in the functional promoter-protein complex through an interaction with a cellular transcription factor. The transactivating function associated with the smaller polypeptide 243R (translated from the 12S mRNA), is due to the release of active E2F cellular transcription factor from pre-existing complexes of E2F with various retinoblastoma protein family members. The free E2F can then interact with a 19K product of the E4 gene to generate a new complex able to bind cooperatively to, and transactivate the viral E2 promoter (for reviews, see Boulanger and Blair, 1991; Nevins, 1991; Nevins, 1992). Moreover, free E2F, by activating cellular genes including dihydrofolate reductase, thymidine kinase and DNA polymerase, also contributes to the establishment of a necessary environment for viral DNA synthesis in otherwise quiescent cells.

Adenovirus infection induces quiescent cells to enter the S phase of the cell cycle to create an environment suitable for viral replication, an effect which is primarily modulated by E1A proteins (Tooze, 1981). E1A proteins interact with a set of cellular proteins, amongst them pRB (retinoblastoma protein) and p300. pRB regulates the ability of the E2F cellular transcription factor to activate transcription and E1A proteins disrupt a series of complexes that contain different pRB family

members, multiple E2F subunits and cyclins with associated kinases. These complexes normally regulate cell cycle progression and their disruption by E1A deregulates normal cell cycle control, allowing quiescent cells to begin DNA synthesis after infection. E1A proteins also interact with a 300 kD cellular protein p300, which is a transcriptional co-activator. Adenovirus E1A mutants lacking pRB binding domains and unable to bind pRB family members can still stimulate cellular DNA synthesis, provided that the p300 binding site remains intact. Thus, the E1A proteins contain two independent domains, both of which can stimulate cells to progress from G_1 to S phase of the cell cycle.

E1A proteins of subgroup C adenoviruses also affect the host immune system by suppressing the anti-viral actions of interferons (IFN) alpha and beta. Another property of E1A-encoded proteins is to render cells susceptible to cytolysis by NK cells and activated macrophages (Cook *et al.*, 1987) and TNF (Duerksen-Hughes *et al.*, 1989). These responses can be detrimental to the survival of adenovirus in an immune-competent environment. However, on the other hand, E1B and E3 products have a role in counteracting the susceptibility to TNF cytolysis (for review, see Wold and Gooding, 1991).

The E1B region found adjacent to E1A (Figure 1.1), codes predominantly for two unrelated proteins, the 55K and 19K. These proteins seem to have an essential role in the progression of a productive infection cycle. Like the E1A proteins, E1B-55K protein also modulates cell cycle progression by targeting the cellular p53 tumor suppressor protein which, like pRB family members, regulates progression from G_1 to S phase. p53 mutations are found in many human tumours since the loss of p53 function by mutation can contribute to tumor progression (Zambetti and Levine, 1993; King and Cidlowski, 1998). p53 is a sequence-specific DNA binding protein that can activate transcription when it binds to p53-response elements, and it can repress a variety of genes that lack a binding site for p53 (King and Cidlowski,

1998). E1B-55K protein antagonises the ability of p53 to influence cell cycle progression by blocking its activation function (Kao *et al.*, 1990; Yew and Berk, 1992).

E1B-55K also forms a complex with the E4 region-encoded 34K and this complex is believed to inhibit translation of cellular mRNAs and to facilitate expression of late viral genes (Sarnow *et al.*, 1984). The other E1B protein, 19K, functions analogously to Bcl-2 to inhibit apoptosis by E1A, p53, and multiple other stimuli, thus delaying cytopathic effects and premature cell destruction. It also prevents degradation of viral and cellular DNAs. Finally, the 19K prevents TNF lysis in human cells (for review, see White, 1998).

The E2 region coded on the left strand is composed of the E2A and E2B units (Figure 1.1). The various proteins encoded in this region are primarily involved in replication of the viral genome. The semi-conservative replication begins 6-8 hours after infection, reaches a maximum rate by 18-20 hours, and it continues until the host cell dies. The inverted terminal repeats of the viral chromosome contain *cis*-acting sequences which serve as replication origins (Challberg and Rawlins, 1984). A complex of two E2B encoded proteins (preterminal protein and the viral DNA polymerase) binds to the highly conserved sequence between base pairs 9 to 18 (Temperley and Hay, 1992). The preterminal protein is synthesised as an 80-kd polypeptide and is found covalently attached to the 5' ends of the viral chromosome. It is subsequently cleaved by proteolysis during assembly of virions to generate a 55-kd fragment (terminal protein) that remains covalently attached to the genome (Challberg and Kelly, 1981). The preterminal protein serves as a primer for DNA replication (Rekosh *et al.*, 1977), preserving the integrity of the viral chromosome's terminal sequence during multiple rounds of DNA replication. The E2B encoded DNA polymerase is a 140-kd protein and contains both 5' to 3' polymerase activity and a 3' to 5' exonuclease activity that serves as a proofreading function during

DNA synthesis (Field *et al.*, 1984). Chain elongation requires E2 encoded DNA polymerase and DNA binding protein, and a cellular protein, nuclear factor II (NFII) which is a topoisomerase.

The virus-coded DNA binding protein (DBP) is a 59 kd phosphoprotein that migrates in SDS-polyacrylamide gels with an apparent molecular weight of 72 kd and it binds tightly in a sequence non-specific fashion to single stranded DNA (Van der Vliet and Levine, 1973). In the presence of DNA binding protein, which coats single stranded replication intermediates, the polymerase is highly processive (Field *et al.*, 1984). As well as its replication role, DBP is involved in the host-specific processing (splicing) of viral fiber mRNAs, and it may also repress viral promoters (Handa *et al.*, 1983). During the early phase of infection, the E2A promoter at 75.1 mu is the same as that used for E2B; at late times, however, alternative promoters give rise to the E2A transcript (Nevins and Winkler, 1980).

The E3 region (76.6-86.2 mu) encodes proteins which allow adenovirus infected cells to evade immunosurveillance. This region is nonessential for viral growth in tissue culture, but has great importance for the virus in the wild since it has been found in many adenoviruses studied (Blair and Hall, 1998). The E3 promoter contains binding sites for the NFkB transcription factor, allowing ElA-independent transcription of this region in lymphoid cells (Williams *et al.*, 1990). Among the nine proteins which are potentially encoded by the pre-mRNA which initiates from the E3 promoter, to date the functions of only four of them can be assigned. The abundant transmembrane gpl9K is localized in the endoplasmic reticulum where it associates with MHC class I molecules and blocks their transport to the cell surface, thus inhibiting cytotoxic T-lymphocyte recognition of adenovirus-infected cells. It is noteworthy that gpl9K has a variable affinity for the different human class I antigens; this implies that the course of adenovirus infections in humans may depend on both the adenovirus serotype and the MHC genotype of the individual. Three

other proteins, the 14.7K and the complex 10.4K/14.5K act to prevent ElA-induced TNF cytolysis of human cells, and this occurs even in the absence of the E1B 19K. The 10.4K/14.5K complex also down-regulates the EGF receptor in adenovirus-infected cells. Thus, it appears that these three E3 proteins, 14.7K, 10.4K and 14.5K, interdict signal transduction for TNF and EGF (for reviews, see Wold and Gooding, 1989; 1991; Blair and Hall, 1998).

The E4 region transcribed from right to left (96.8-91.3 mu) (figure 1.1) is essential for viral growth in cell culture. Its deletion requires that the virus be grown on cells which complement for the E4 defect (Weinberg and Ketner, 1983). However, frame shift mutations in six of the seven open reading frames lying within E4 display only moderate or no effect upon the ability of virus to grow in HeLa cells when studied individually (Halbert *et al.*, 1985). This makes difficult the attribution of specific functions to each of the E4 proteins. However, two E4-encoded products were found to interact with viral and cellular partners: the 19K orf 6/7 protein, which regulates the transcription of E2 after association with E2F (Hardy and Shenk, 1989; Hardy *et al.*, 1989; Huang and Hearing, 1989; Neill *et al.*, 1990; Raychaudhuri *et al.*, 1990; Marton *et al.*, 1990), and a 34K orf 6 protein which has been shown to form a complex with the 55K E1B protein and play an important role in the expression of viral late genes (Sarnow *et al.*, 1984). The E4 proteins are also required for virion assembly and efficient viral DNA replication (Falgout and Ketner, 1987; Halbert *et al.*, 1985; Weinberg and Ketner, 1986; Yoder and Berget, 1986).

Most of the virus-coded proteins synthesised late in infection are virion structural proteins, or their precursors, required for assembly of the nucleoprotein core and the outer capsid. Thus, the monomeric structural polypeptides of the hexon (polypeptide II coded by L3), the penton base (polypeptide III coded by L2), and the fiber (polypeptide IV coded by L5) assemble in the cytoplasm into capsomers which constitute the major structural units of the capsid. The penton base and the fiber

units subsequently combine into the penton. The polypeptides VI and VIII, encoded respectively by blocks L3 and L4, are found associated with the hexons, whereas the polypeptide IIIa (coded by L1) is associated with the peripentonal hexons. Polypeptide IX, encoded by the transcriptional unit embedded in the E1B region, has a particular cementing role. It stabilizes the groups-of-nine hexons which form the central region of each facet of the capsid (Furcinitti *et al.*, 1989). The polypeptides V and VII encoded by the block L2 are linked to the viral DNA in the core structure.

In addition to the structural proteins of the virion, other non-structural proteins involved in various assembly steps are also synthesized as late proteins: A 100K protein (L4) mediates the assembly of the hexon polypeptide into trimeric units and thus functions as a scaffolding protein. It is also required for efficient initiation of translation of viral late mRNA species during the late mRNA species during the late phase of infection (Hayes *et al.*, 1990). Polypeptide IVa2 is also thought to act as a scaffold. A 23K protein (L3) displays a sequence specific proteolytic cleavage activity essential to viral maturation. Several precursor virion polypeptides (pVI, pVII, pVIII, IIIa), as well as the precursor of the terminal protein, are processed by this endopeptidase during maturation of infectious particles.

All adenovirus serotypes may cause cell transformation following infection of primary rodent embryo cells in culture, which are non-permissive. The viral genes responsible for these morphological changes lie within the El region; these genes are systematically found integrated within the host chromosomes of Ad-transformed cells with no specificity with respect to the integration site, and they undergo constitutive expression. The transformation process results, in fact, from the interaction of the El products with host cell cycle regulatory and apoptosis regulatory proteins; the ElA proteins interact with the p105 retinoblastoma susceptibility gene protein (Whyte *et al.*, 1988), while the 55K ElB protein interacts with the p53 (Sarnow *et al.*, 1982). Formation of these two complexes is believed to

release cells from a controlled growth. Finally, the 19K E1B protein (homologous in sequence and function to the Bcl-2 family of apoptosis regulators) blocks apoptosis induced by E1A, p53, TNF-a, Fas, and many other apoptotic stimuli (for review, see White, 1998). E1B 19K protein might also be responsible for the anchorage-independent growth of Ad-transformed cells by disrupting the vimentin-containing intermediate filament network (for review, see Boulanger and Blair, 1991).

The oncogenic potential of adenoviruses in animals clearly varies with the serotype. In addition to their capacity to transform cells in culture, groups A and B adenovirus (Ad12 and Ad7 are prototypes) also display an oncogenic power in newborn hamsters. In contrast, group C viruses (e.g., Ad2 or Ad5) are non-oncogenic *in vivo*. The molecular basis for the oncogenicity is related to the ability of the E1A gene products of these serotypes to down-regulate major histocompatibility (MHC) class I antigen gene expression at the transcriptional level (Bernards *et al.*, 1983; Schrier *et al.*, 1983).

No association of adenovirus infection with tumors in humans has ever been made. In fact, *in vitro* transformation of embryonic cells of human origin is extraordinarily inefficient with adenovirus. However, the transfection of human embryonic kidney cells with Ad5 DNA fragments has led to the establishment of a cell line called 293 where 11 % of the left-end of the viral DNA (0-11 mu) is inserted into the cell genome (Graham *et al.*, 1977; Louis *et al.*, 1997). The availability of these cells is critical to the generation and propagation of El-deleted recombinant adenoviral vectors.

1.2.3 Adenoviruses as cloning vectors

The DNA virus used most widely to date for gene transfer vectors is human adenoviruses (specifically serotypes 2 and 5). Our present understanding of the molecular genetics of adenovirus renders possible its manipulation as a cloning

vector for gene transfer both in cell culture and in animals. The most highly valued advantage of adenoviral-mediated gene therapy is that wild-type adenoviruses are safe. Millions of North American army recruits have been vaccinated by oral administration of enteric-coated capsules containing un-attenuated adenovirus and have rarely displayed adverse side effects (Couch *et al.*, 1963). Furthermore, the adenoviruses that are modified to make the current lines of vectors (Ad5 and Ad2) do not cause tumors in rodents and appear to cause only mild respiratory problems in humans (Shenk, 1996) and they rarely integrate into host genome, thus they have little chance to activate a dormant oncogene or interrupt a tumour suppressor gene. Other suggested advantages are that they are large and can therefore potentially hold large DNA inserts up to 35 kb; they are human viruses and are able to infect a large number of different human cell types at a very high efficiency (nearly 100 % *in vitro*); they can infect non-dividing as well as dividing cells and they can be produced at very high titres in tissue culture (as high as 10^{11} pfu/ml).

Adenoviral vectors are made up of at least two components, the modified viral genome and the virion structure surrounding it. Most present generation vectors comprise virion particles based largely on the wild type virus structure although some new approaches have been taken to redirect adenovirus tropism by genetically or non-covalently incorporating non-native receptor ligands onto the virus coat proteins (Gall *et al.*, 1996; Krasnykh *et al.*, 1996; Wickham *et al.*, 1995; Michael *et al.*, 1995; Wickham *et al.*, 1996). This structure packages and protects the viral nucleic acid and provides the means to bind and enter target cells. In contrast to the virion structure, the viral nucleic acid in a vector designed for gene therapy is changed in many ways. The goals of these changes are to disable growth of the virus in target cells while maintaining its ability to grow in vector form in available helper cells, to provide space within the viral genome for insertion of exogenous DNA sequences, and to incorporate new sequences that encode and enable appropriate expression of the gene of interest. With durable gene transfer as the objective,

infection of the targeted cells should not lead to cell death (except where the aim is to kill the cells, such as in cancer gene therapy). Thus, the deletion of key viral genes constitutes a prerequisite to block the genetic program of the virus.

Adenovirus vector development has focused on crippling the virus by deleting essential genetic regions and complementing the missing function(s) in *trans*. Two general ways for the complementation of the missing function(s) have been designed. One entails the propagation of replication-defective adenovirus vectors by the use of a complementing cell line. Complementing cell lines are cells engineered to express, either inducibly or constituvely, one or more viral gene products. The second entails the propagation of the recombinant in the presence of a helper adenovirus for complementation of viral functions lacking in the recombinant.

Recombinant adenoviruses with insertions in place of some viral sequences are traditionally produced by overlap recombination after cotransfection of appropriate complementing cells (and/or cells infected with the complementing helper virus) with a plasmid shuttle vector containing part of the viral genome in which a cassette for transgene expression has been placed and one or more overlapping viral genomic DNA fragments, which together comprise a full length genome. The recombinant viruses generated following this procedure contain the transgene cassette, usually in place of essential genes, and thus cannot replicate efficiently after the infection of non-complementing cell lines.

The so-called "first generation" of adenoviral vectors were made by the deletion of the E1 region from the viral genome. This deletion rendered the virus replication defective, so they had to be propagated in cells which had been manipulated to complement the missing E1 function in *trans*. The standard cell line used for this complementation was the human embryonic kidney cell line 293, (which carries a

chromosomally integrated segment of Ad5 DNA from the left end of the genome, encompassing the E1 region (Graham *et al.*, 1977) as mentioned above). These vectors retained the immediate 5' end of the viral genome, including the terminal repeats, sequences required for packing, and the overlapping E1 enhancer region. All coding sequences for E1A were deleted, but often some of the 3' ends of the E1B gene and all of the protein IX coding sequences were retained since pIX is indispensable for encapsidation of full-length genome recombinants (Ghosh-Choudhury *et al.*, 1987). All of the remainder of the viral genome could be left intact, but usually the sequences encoding the E3 region were also removed in order to create more space for the insertion of transgenes. The E3 region, as mentioned earlier, functions to suppress the host immune response during virus infection *in vivo*, but is not required for replication or packaging in tissue culture cells. The icosahedral viral capsid can package viral genomes up to 105 % the length of wild type viral DNA (Bett *et al.*, 1993); in "first generation" adenoviral vectors with E1 and E3 deletions this corresponds to an insertion capacity of about 8 kb of foreign DNA.

The "first generation" vectors have been shown to be capable of *in vivo* delivery of transgenes to a wide variety of both dividing and non-dividing cells, as well as mediating high levels of transgene expression (Kozarsdy and Wilson, 1993). However, these vectors elicited strong inflammatory and specific viral immune responses (Ali *et al.*, 1994) which lead to the elimination of transduced cells by cytotoxic T cells. Since first generation vectors are deleted in the E1 region, which *trans*-activates the other viral transcription units, it was envisioned that such viruses would not express the remaining adenovirus genes. However, Engelhardt *et al.* (1993) found that low levels of the adenovirus E2A DNA binding protein (DBP) were expressed *in vivo*, with high levels detected in a subset of cells showing marker gene expression. This result indicated that the E1 deletion was not enough to preclude expression of other viral genes, an effect observed previously *in vitro* with

a high moi of E1 deleted virus. This is probably a reflection of the fact that, at high multiplicities of infection, the E1 region becomes dispensable for replication (Jones and Shenk, 1979 (b)). This may be related to the expression of specific host gene products with E1A-like activity (Reichel *et al.*, 1987). This hypothesis is supported by the fact that it had previously been shown that cellular transcription factors, such as NF-IL6, could compensate for a lack of E1A (Spergel *et al.*, 1992). Leaky viral DNA replication is followed by low level viral late protein production. Accumulation of adenoviral cytotoxic late gene products following gene transfer causes direct cytopathic effects on the transduced cells (Zhang and Schneider, 1994) and triggers host cellular immune responses. These adverse effects can be aggravated by the emergence of E1-containing replication-competent adenovirus (RCA) during the production of E1-deleted virus in 293 cells (Lochmuller *et al.*, 1994). Homologous recombination between the genome of the E1-deleted virus and the adenovirus sequences integrated in the 293 cells has been suggested as the mechanism for the appearance of the E1+ RCA in passaged stocks of previously purified recombinant E1-deleted virus. However, a new cell line for complementation of E1 was developed by Imler *et al.* (1996) based on the A549 cell line (human lung carcinoma cell line). E1 expression in this cell line was achieved from a plasmid expression casette. E1 sequences did not contain the same extended portion of the viral genome since they lacked the E1B and pIX polyadenylation signal. Thus the probability of producing RCA through homologous recombination between the E1-deleted virus genome and the existing E1 sequences in the cells was eliminated.

Cytotoxicity of viral late proteins and the host cellular immune response against the adenoviral antigens results in local inflammation and destruction of transduced cells (Yang *et al.*, 1994 (a,b)). The resulting limited persistence of transgene expression *in vivo* observed with "first generation" adenovirus vectors rules out their use in applications that require long-term expression of transgene products.

It was hoped that a weaker immune response would result if additional viral genes were deleted from the vector. Thus, second generation adenoviral vectors have been designed by the introduction of further mutations into additional adenovirus early gene regions whose products are required to support and regulate viral DNA replication which is in turn required for the expression of late genes. Vectors with a deletion of E1 coupled with a deletion or inactivating mutation of other essential early genes, E2b, E2a and/or E4 (Engelhardt *et al.*, 1994; Gao *et al.*, 1996; Gorziglia *et al.*, 1996; Moorhead *et al.*, 1999), or vectors with all of the viral genes deleted (so called 'gutless' vectors) (Haecher *et al.*, 1996; Chen *et al.*, 1997) have been constructed and tested in animals. However, there have been conflicting reports regarding the immunogenicity of these vectors, and the stability and persistence of gene expression *in vivo* from them (Lusky *et al.*, 1998; Goeziglia *et al.*, 1996; Kaplan *et al.*, 1997; Gao *et al.*, 1996).

In the development of adenoviral vectors, deleting more and more viral genes may not always be advantageous because some of these genes may have beneficial features, for example suppressing an immune response against the vector. Their removal could increase the rate at which the vector is eliminated. As an example, the E3 region encodes a protein of relative molecular mass 19K that protects the virus from host immune surveillance. In addition, *cis* acting sequences may exist that help maintain the stability of the adenoviral genome in the cell.

There are two other disadvantages associated with the use of adenoviral vectors in addition to host immune response: first, there is no mechanism whereby these vectors can persist in dividing cells; second, they have insufficient insert carrying capacity for some gene therapy applications which require the insertion of relatively large DNA molecules beyond the capacity of current adenoviral vectors, such as the full length dystrophin cDNA (14 kb; Koenig *et al.*, 1987). Several attempts have

been made to increase the capacity of adenovirus vectors for heterologous DNA by the introduction of further deletions of viral sequences.

A cell line for the complementation of Ad5 protein IX deficiency was developed by Caravokyri and Leppard (1995), based on the 293 cell line. Since pIX is within the E1B region and is necessary for packaging full-length genomes (Ghosh-Choudhury, 1987), its deletion from vectors previously placed a restriction on the size of transgene which could be accomodated. Use of this cell line allowed the construction of a virus with an increased capacity for insertion of foreign DNA, up to 9.2 kb in length. Krougliak and Graham (1995) also used 293 cells to express pIX and coupled this with expression of E4, potentially allowing the generation of vectors able to accommodate inserts up to 11 kb in length, with deletions in E1, E3 and E4.

By engineering the correct combination of viral genes (incorporating immunosuppressive genes, perhaps from other sources, while deleting immune stimulating gene products and reducing, if possible, the immunogenicity of viral capsid proteins), it is likely that adenoviral vectors can be generated that have low toxicity, that do not generate an immune response, and that, therefore can be given repeatedly. This would also decrease the requirement for the persistance of the vector in the host due to the possibility of repeated administration of the vector. Alternatively, chimeric viral-vector systems that combine advantageous properties of two or more viral systems could be explored in order to make it possible to achieve persistance by the incorporation of features in the adenoviral vectors exploited by other viruses, such as integration into the host genome or autonomous episomal replication in the host cell nucleus. The insert capacity of the adenoviral vectors can be increased considerably (up to 35 kb) by the use of so called 'gutless' adenoviral vectors which contain only the essential adenovirus packing signals and the origins of replication present at the both end of the genome. Currently these

vectors are produced in the presence of helper virus but the difficulty of separating helper virus from the recombinant gutless vector makes this method unsuitable for producing vectors for use in humans. However, this type of vector might be generated without the need for a helper virus if a packaging cell line was available that possessed all the complementing functions required. Though difficult to create, once such a packaging cell line becomes available, all the viral sequences except the ones required *in cis* for replication and packaging may be removed from the vector genome.

1.3 Adenovirus type5 L1-52/55kD protein and the adenovirus major late transcription unit (MLTU)

1.3.1 Ad5 MLTU

Adenovirus late genes begin to be expressed efficiently at the onset of viral DNA replication. The adenovirus late coding regions are organised into a single large transcription unit whose primary transcript is about 29000 nucleotides in length (Evans *et al.*, 1979). This transcript is processed by differential poly(A) site utilisation and splicing to generate at least 18 distinct mRNAs. These mRNAs have been grouped into five families, termed L1 to L5, based on the utilisation of common poly(A) addition sites (figure 1.1) (Chow *et al.*, 1977; Nevins and Darnell, 1978). Expression of this large family of late mRNAs is controlled by the major late promoter (MLP). This promoter exhibits a low level of activity early after infection. However, following the onset of viral DNA replication, the rate of transcription from MLP increases to levels at least 20 times higher than those achieved by the other viral promoters (Shaw and Ziff, 1980).

Although the promoter for the major late transcription unit (MLP) is active at early times after the infection, only mRNAs corresponding to the L1 encoding region are produced (Shaw and Ziff, 1980; Nevins and Wilson, 1981). Full expression of the

late genes requires viral DNA replication. The major late promoter from adenovirus types 2 and 5 has been most extensively studied as a model for eukaryotic transcription (reviewed by Berk, 1986; Logan and Shenk, 1986). Basal transcription from this promoter depends on a set of well defined promoter elements: a properly positioned TATA box, an upstream element (UE) located between positions -67 and -49 relative to the MLP transcriptional start site, a CAAT box between -80 and -76, an initiator element spanning the start site, and additional elements located beyond position +140 (Concino *et al.*, 1984; Hen *et al.*, 1982; LeBowitz *et al.*, 1989; Mason *et al.*, 1990; Moncollin *et al.*, 1986; Reach *et al.*, 1990; Reach *et al.*, 1991; Zock and Doerfler, 1990)

Sequence elements located downstream of the MLP start site have been identified and shown to be essential *in vivo* for promoter activation (Alonso-Caplen *et al.*, 1988; Leong *et al.*, 1990; Mansour *et al.*, 1986; Mason *et al.*, 1990). DNase I protection and dimethyl sulphate interference mapping experiments have delineated two main downstream sequence elements, DE1 and DE2, located between +85 and +98 (DE1) and +100 and +120 (DE2). These elements specifically bind proteins that are detected only in adenovirus infected cells in the late phase of infection. The binding of these proteins correlates with the transcriptional activation of the MLP, as measured by cell-free transcription (Jansen-Durr *et al.*, 1988; Jansen-Durr *et al.*, 1989) or during infection with recombinant viruses (Leong *et al.*, 1990; Mason *et al.*, 1990). Footprinting experiments combined with *in vitro* transcriptional analyses of selected promoter mutations suggested that the late-phase specific stimulation of the MLP results from a co-operative action of the UE and DE elements (Mondesert and Kedinger, 1991).

The presence of two virally induced factors, DEF-A and DEF-B, correlates with late phase MLP stimulation. DEF-A binds specifically to DE1 and also, with lower affinity, to the 3' portion of the DE2 (DE2a). DEF-B interacts with the 5' part of the

DE2 element (DE2b). When added together, DEF-A and DEF-B were found to co-operatively assemble onto the DE2 element as a complex that was more stable than that formed by each protein alone (Jansen-Durr *et al.*, 1989; Mondesert *et al.*, 1992).

Purification of DEF-B allowed identification of the protein as the product of the adenovirus IV_{a2} gene product (pIV_{a2}) and both DNA binding and transcriptional experiments have confirmed that the IV_{a2} protein contributes to the late phase-specific activation of major late promoter (Tribouley *et al.*, 1994; Lutz and Kedinger, 1996).

1.3.2 L1-52/55kD protein

As mentioned earlier, although the major late promoter is active early after infection, only the 5' proximal L1 family of mRNAs accumulates in the cytoplasm at this time. This finding results from transcriptional termination midway through the transcription unit and preferential utilisation of the L1 poly(A) addition site (Iwamoto *et al.*, 1986). The termination is partially relieved as the infection enters the late phase, allowing expression of full set of mRNAs encoded by the major late unit.

The L1 family of mRNAs encode three known proteins: the 52- and 55-kilodalton (52/55-kD) proteins and the IIIa protein. Protein IIIa is a structural component of the virion (Boudin *et al.*, 1980). The 52/55-kD proteins are phosphoproteins that are produced as a result of the differential phosphorylation of a 48 kD precursor protein (Hasson *et al.*, 1992) but they have not been detected in virions (Hasson *et al.*, 1989; Lucher *et al.*, 1986). Both phosphoforms were shown to be present within all suspected virus assembly intermediates but not within mature virions (Hasson *et al.*, 1992). Only L1 52/55-kD mRNA is produced at early times post-infection.

At late times during adenovirus infection, two abundant particle types are formed

that can be separated by CsCl equilibrium centrifugation (Maizel *et al.*, 1968). The heavier of these particles is the mature virus, while the lighter particles are empty capsids. Proteins that are not found in the mature virions but are found in empty capsids may function as scaffolding proteins during the assembly process (Rosenwith *et al.*, 1974). Pulse-chase experiments combined with analyses of defective particles formed during infection of cells with temperature-sensitive mutants revealed a third, less-abundant class of particles known as assembly intermediates (D'Halluin *et al.*, 1978; Edvardsson *et al.*, 1976). Further characterisation of these particles by reversible cross-linking revealed that they could be separated into two components, termed heavy and light intermediates. Light intermediates have the same protein composition as empty capsids but are associated with a small fragment of the viral genome. The heavy intermediates contain the full-length viral genome and lack all scaffolding proteins. A precursor/product relationship between assembly intermediates and mature virions was suggested by kinetic analyses showing that radiolabel incorporated into assembly intermediates could be chased into mature virions (D'Halluin *et al.*, 1978; Edvardsson *et al.*, 1976). A fourth type of particle known as the young virion was also identified (Ishibashi and Maizel, 1974). Young virions are similar to mature virions except that several viral proteins are present in a precursor form and some proteins are absent. Overall these findings suggest that the first step in viral morphogenesis is association of viral proteins (some in precursor form) with scaffolding proteins to form the empty capsid. The association of the viral DNA is the next detectable step and results in the formation of light intermediates. The DNA is then encapsidated and the scaffolding proteins are degraded or released to produce heavy intermediates. Young virions are formed by the incorporation of viral core proteins, and the final step is the cleavage of precursor proteins by the viral protease to produce the mature virion.

Characterisation of an adenovirus harboring a temperature-sensitive mutation in the

L1 52/55-kD protein (*ts369*) revealed that this protein is required for viral assembly (Hasson *et al.*, 1989). When HeLa cells were infected with *ts369* at the non-permissive temperature, light intermediates accumulated. Analyses of these intermediates indicated that they were associated with the left end of the viral genome, suggesting that the 52/55-kD protein has a role in DNA encapsidation. Later findings indicated that early assembly intermediates have many copies of the 52/55-kD protein and that these structures gradually lose the 52/55-kD protein as they mature into virions (Hasson *et al.*, 1992). This led to the suggestion that the 52/55-kD protein may act as a scaffolding protein (Hasson *et al.*, 1992). However, the analyses of *H5ts369* could not differentiate between a role for the 52/55-kD protein as a scaffolding protein or in DNA encapsidation (Hasson *et al.*, 1992). More recently it has been shown that the 52/55-kD protein mediates encapsidation of the viral genome rather than functioning as a scaffolding protein (Gustin and Imperiale, 1998). This indication came by the characterisation of assembly intermediates that form in the absence of the 52/55-kD protein in cells infected with an adenovirus mutant incapable of expressing the 52/55-kD protein (Gustin and Imperiale, 1998).

Previously, it was reported that the 52/55-kD protein interacts with the IV_{a2} protein during infection (Gustin *et al.*, 1996). Since the IV_{a2} protein is a late stage specific transcriptional activator of the major late promoter (Tribouley *et al.*, 1994), this suggested that a possible additional role for the 52/55-kD protein might be to regulate proper temporal activation of late gene expression by interacting with the IV_{a2} protein. The apparently specific production of the 52/55-kD protein at earlier times post-infection than other MLTU products agrees with this idea. A potential role for the IV_{a2}-52/55-kD protein interaction in the regulation of transcription from the major late promoter is investigated in chapter 7.

1.4 Aims of the project

The main purpose of the work described in this thesis was to increase the capacity of

the current adenovirus type 5 vectors for the insertion of exogenous DNA to be used in gene therapy applications. This involved the construction of complementing cell lines expressing adenovirus type 5 L1 52/55-kD proteins and/or various other late proteins to support the production of deleted adenoviral vectors and the deletion of L1 52/55-kD coding region from the adenovirus 5 genome. The other purpose of this work was to investigate the effects of adenovirus L1 52/55-kD protein on the activation of adenovirus major late promoter, which is described in a separate chapter (chapter 7).

Chapter 2

Material and Methods

2.1 Materials

A list of commonly used buffers and solutions, and suppliers are presented in Appendices B and C respectively.

2.2 Bacteriological techniques

2.2.1 Bacterial strains and plasmids and media

A list of bacterial strains and plasmids used in this study and their sources are shown in Table 2.1 and Table 2.2 respectively. *Escherichia coli* strains were grown in LB broth (1 % (w/v) bacto-tryptone, 1 % (w/v) NaCl, 0.5 % yeast extract) at 37 °C with continuous agitation. M17 (Difco Laboratories) medium supplemented with 0.5 % (w/v) glucose (GM 17) (Terzhagi and Sandine, 1975) was used for static growth of *Lactococcus lactis* strains at 30°C. Where antibiotic selection of transformed bacteria was required, transformation mix was plated on LB media containing 1.5 % (w/v) agar and appropriate antibiotic.

2.2.2 Maintenance of bacterial strains

E. coli strains were streaked on LB agar and *L. lactis* strains were streaked on GM17 agar containing appropriate antibiotics when needed. All bacterial strains were stored at 4 ^{0}C and sub-cultured monthly. For long term storage 1 ml of a freshly saturated culture was added to a freezing vial containing 1 ml DMSO (dimethylsulfoxide) solution (7 % (v/v)) and stored at –70 ^{0}C.

2.2.3 Rapid preparation of plasmid DNA from *E. coli*

Rapid plasmid DNA preparation was performed either by using Qiagen's QIAprep plasmid kit, according to the manufacturer's instructions, or by the following method (Birnboim, 1989). 1.5 ml of each overnight culture was transferred to a 1.5 ml Eppendorf tube for extraction. Tubes were centrifuged for 15 sec and the

supernatant was removed. Each pellet was resuspended in 0.1 ml of lysozyme solution (50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl, pH 8. Lysozyme was added at a concentration of 1 mg/ml shortly before use) and held at 0 °C for 5 min. 0.2 ml alkaline SDS (0.2 N NaOH, 1 % (w/v) SDS) at room temperature was

Strain	Relevant genotype or phenotype	Reference
E. coli		
XL1Blue	*lacZ*, *recA*$^-$, *tet*^{r} plasmid-free strain	Stratagene
XL1Blue MR	*lacZ*, *recA*$^-$ plasmid-free strain	Stratagene
MC 1061	*recA*$^+$, *str*^{r} plasmid free-strain	Wertman, K.F. *et al*, 1986
L. lactis		
MG 1363	Plasmid-free strain	Gasson, 1983

Table 2.1 List of bacterial strains

Plasmid	Relevant genotype	Reference
pAd5Cla1-Nhe1	Ad5 Cla1, Nhe1 fragment (917-10809 bp) cloned in pBR322	This work
pAd5LE-ΔL1	Ad5 left end genome (ΔL1) (1-16746 bp), amp^r in pBR322	This work
pAd5LE-ΔL1-tet	tet^r gene cloned in PAd5LE-ΔL1	This work
pAd5LE-10809	Ad5 Nhe1 fragment (1-10809 bp) cloned in pBR322	This work
pAd5RE-ΔL1	Ad5 right end genome (ΔE3) (10294-35938 bp), kan^r, amp^r in pBR322	This work
pBR322	ColE1 ori, ROP, amp^r, tet^r	Watson, N., 1988
pcDNA3.1/His B/lacZ	P_{CMV}, LacZ, amp^r, ColE1 ori, SV40 ori, neo^r	Invitrogen
pCMV55K	P_{CMV}	White, E. and Cipriani, R., 1989
pCMV-CAT	CAT gene cloned downstream of P_{CMV}	
pCMV-IX	Ad5 pIX cDNA	Caravokyri, C. and Leppard, K., 1995
pCR II	amp^r,kan^r,lacZ, F1 ori, ColE1 ori	Invitrogen
pCR II-L1	Ad5 L1-52/55 kD orf cloned in pCR II	This work
pFG	Ad5 SalI fragment (ΔE3) (16746- 35938 bp)	Caravokyri, C. (unpublished)
pGEX-2T	amp^r, P_{lac}, gst, colE1 ori	Pharmacia
pGEX-2T-L1	Ad5 L1-52/55 kD orf cloned in pGEX-2T	This work
pIL253	Ery^r, *L. lactis* ori	Simon and Chopin, 1988
pIL253-LE	Ad5 left end genome (1-10809 bp) cloned	This work

	in pIL253, ery^r	
pIV_{a2}	Ad5 IV_{a2} cDNA	Brey, S., 1999
pMEP4	amp^r, Ori P, ColE1 ori, $P_{hmetIIa}$, SV40 pA, EBNA-1, hyg^r	Invitrogen
pMEP-CMV	$P_{hmetIIa}$ in pMEP4 replaced by P_{CMV} from pCMV55K	This work
pMEP-CMV-IV_{a2}	Ad5 IV_{a2} cDNA cloned in pMEP-CMV, downstream of P_{CMV}	Brey, S. 1999
pMEP-CMV-L1	Ad5 L1-52/55-kD orf cloned in pMEP-CMV, downstream of P_{CMV}	This work
pMLP-CAT	CAT gene cloned downstream of Ad5 MLP	Lutz, et al., 1997
pREP9	Amp^r, Ori P, ColE1 ori, P_{RSV}, SV40 pA, EBNA-1, neo^r	Invitrogen
pREP9-IV_{a2}	Ad5 IV_{a2} cDNA cloned in pREP9	This work
pREP9-IX	Ad5 pIX cDNA cloned in pREP9	This work
pSal1C-ΔL1	Ad5 L1-52/55-kD orf (11050-12158 bp) deleted from pSal1C	This work
pSal1C-ΔL1-Kan	kan^r gene cloned in pSal1C	This work
pSalIC	Ad5 SalI fragment (9841-16746 bp) cloned in pBR322	Leppard, K.N. (unpublished)
pXhoIC	Ad5 XhoI fragment (1-5788 bp) cloned in pBR322	Leppard, K.N. (unpublished)

Table 2.2 List of plasmids

added and the sample was mixed gently by inversion several times followed by incubation at 0 °C for 5 min. 0.15 ml High-salt solution (3 M potassium acetate, 1.8 M formic acid) was added and held at 0 °C for 15 min after mixing gently, until a curd-like precipitate was formed. Centrifugation was performed for 2 min and the supernatant was transferred into another tube. Cold ethanol (0.9 ml) was added to the sample, which was held at –20 °C for 15 min, then centrifuged for 1 min. The pellet was dissolved in 0.1 ml of acetate-MOPS (0.1 M sodium acetate, 0.05 M MOPS, adjusted to pH 8 with NaOH) and reprecipitated with 0.2 ml ethanol. The final pellet was suspended in 0.04 ml of dH_2O.

2.2.4 Rapid preparation of plasmid DNA from *L. lactis*

The method described by O'Sullivan and Klaenhammer (1993) was used for the small-scale preparation of lactococcal plasmid DNA. The procedure was devised by modifying steps from *E. coli* mini-prep procedures to meet the specific needs of lactococcal plasmid isolation. 5-10 ml of culture grown overnight in GM17 medium was harvested by centrifugation at 5000 rpm for 10 min, followed by resuspension in 25 % sucrose containing 30 mg/ml lysozyme, to a final volume of 200 µl. After being transferred to Eppendorf tubes, and incubated at 37 °C for 15 min, 400 µl of alkaline SDS (3 % SDS-0.2 N NaOH) solution was added and incubated for 7 min at room temperature after mixing. After incubation, 300 µl of ice cold 3 M sodium acetate (pH 4.8) was added, mixed, and centrifuged for 15 min at 4 °C in a bench centrifuge. The supernatant was transferred to a new tube and 650 µl of isopropanol at room temperature was added, which was then mixed and centrifuged for 15 min at 4 °C. The pellet was resuspended in 320 µl dH_2O and 200 µl of 7.5 M ammonium acetate containing 0.5 mg/ml ethidium bromide, together with 350 µl of phenol/chloroform was added to the suspension. The solution was mixed, centrifuged for 5 min at room temperature, and the resulting upper phase was transferred to a new tube. 1 ml of –20 °C ethanol was added and centrifuged for 15 min at 4 °C after mixing thoroughly. Then the pellet was resuspended in 40 µl dH_2O

or in TE containing 0.1 mg/ml RNase to remove the RNA where needed. The method is effectively applicable for the isolation of plasmid DNA that has a size up to 80 kb.

2.2.5 Large scale preparation of plasmid DNA from *E. coli*

Large scale preparation of plasmid DNA was routinely performed either by Qiagen's plasmid Maxi kit, according to the manufacturer's instructions or by the following method (Sambrook *et al*, 1989). *E. coli* cells grown overnight in 1000 ml LB harvested by centrifugation and were resuspended in 25 % sucrose in 50 mM Tris-HCl and 1 ml lysozyme mix with 2 ml 0.25 M EDTA, pH 8 was added to the resuspended solution (50 mg/ml lysozyme, 0.25 M Tris-HCl, pH 8). The mixture was then left for 5 min at room temperature and 8 ml of Triton solution (2 % (v/) Triton X-100, 50 mM Tris-HCl pH 8, 62.5 mM EDTA) was added. The tubes containing the mixture were inverted until the samples became sticky and then centrifuged at 17000 rpm at 4 °C for 25 min. The supernatant was taken and 1 g of CsC1 was added for each ml of supernatant together with 1/8 volume of 5 mg/ml ethidium bromide. The samples were then centrifuged at 70000 rpm at 10 °C for 4 hours in a Beckman ultracentrifuge. The tubes were observed under ultra-violet light and the lower band corresponding to plasmid DNA was removed using a syringe and a needle. Ethidium bromide was removed by extraction 3 times with CsCl-saturated isoamylalcohol and dialysed overnight against 2 l of TE buffer. The sample was then transferred to Eppendorf tubes and was stored at –20 °C.

2.2.6 Preparation of competent *E. coli* and transformation with plasmid DNA

A single colony of cells was picked and transferred to 2 ml LB broth and grown overnight at 37 ^{0}C. The next day, the 2 ml culture was diluted to 100 ml with fresh LB and incubation was continued for another 2 hours at 37 ^{0}C. Then the bacteria

were harvested by centrifugation at 5000 rpm for 5 min using Sorvall JA20 rotor and the supernatant was discarded. The bacterial pellet was resuspended very gently in 50 ml of ice-cold 0.1 M $CaCl_2$, kept on ice for 30 min and harvested as before. After resuspension of the bacterial pellet in 1 ml of ice-cold $CaCl_2$, cells were transferred into Eppendorf tubes in 100 µl aliquots, which were kept on ice. Following the preparation of competent bacteria, DNA (0.1 µg-3 µg) diluted to 100 µl with sterile dH_2O was added to 100 µl of competent bacteria, mixed gently and kept on ice for 40 min. The mixture was then heat-shocked in a waterbath at 42 °C for 1 min followed by further incubation on ice for 2 min. To allow the cells to recover from transformation, 0.8 ml of pre-warmed LB medium was added to the transformation mixture, incubated at 37 °C for 1-2 hours and aliquots (1 %, 5 %, 10 %, 20 % of the 1 ml bacterial suspension) were plated out on LB plates containing the appropriate antibiotic(s). To allow the growth of antibiotic-resistant single colonies, plates were incubated 15-48 hours at 37 °C.

2.2.7 Electroporation of *L. lactis* with plasmid DNA

The protocol of Holo and Nes (1989) was used to prepare electrocompetent cells. To obtain competent cells, cultures were grown to an optical density at 600 nm of 0.5 to 0.8 and then diluted 100-fold in SGM17 (GM17 containing 0.5 M sucrose) supplemented with 2.5 % glycine to weaken the bacterial cell wall. After growth at 30 °C to an optical density at 600 nm of 0.4 to 0.7, the cells were harvested by centrifugation at 4 °C at 5000 g. Following two washes in ice-cold 0.5 M sucrose containing 10 % glycerol, the cells were suspended in 1/100 culture volume of this solution and then stored in 40 µl aliquots at –80 °C until use. Before electroporation, the frozen cell suspensions were thawed on ice. Portions were mixed with 1 µl of DNA dissolved in dH_2O and then transferred to an ice-cold electroporation cuvette (2 mm-electrode gap) and exposed to a single electrical pulse. A Gene Pulser (Bio-Rad Laboratories, Richmond, California) delivered the pulse set at 25 µF and at 2.5 kV. The cuvette was connected parallel to 200-Ω resistor (pulse controller; Bio-

Rad), resulting in time constants of 4.5 to 5 ms. Immediately following the discharge, the suspension was mixed with 0.96 ml of ice-cold SGM17 containing 20 mM $MgCl_2$ and 2 mM $CaCl_2$ (SGM17MC) and left on ice for about 5 min. Appropriate dilutions were then made in SGM17MC, and the cells were incubated at 30 °C for 2 hours before 100 µl portions were spread on selective GM17 plates containing appropriate antibiotic. Transformants were enumerated after 2 days of incubation at 30 °C.

2.3 Tissue culture and virus techniques

2.3.1 Mammalian cells and virus strains

A list of mammalian cells and virus strains used in this study and their sources are shown in Table 2.3.

2.3.2 Maintenance and passage of mammalian cell lines

293, HeLa and COS1 cell lines were maintained in 90 mm tissue culture dishes containing 10 ml of medium (other culture vessels used in experiments contained volumes of medium in proportion to their surface areas). 293 and HeLa cells were grown in DMEM-10 % NCS, and COS1 cells were grown in DMEM-5 % FCS. All cells were grown at 37 °C in a 5 % CO_2 humidified atmosphere.

293 cells were passaged at an area ratio of 1:4 or 1:6 and COS1, and HeLa cells were passaged at a ratio of 1:8 or 1:10 every 3-4 days. All cell monolayers were washed with versene and passaged with 5:1 versene/trypsin (0.25 % w/v) solution. Trypsin was neutralised by the addition of serum equal to the volume of versene/trypsin used.

Cell or virus strain	Source and/or genotype	Reference
293	Human kidney cells expressing Ad5 E1 genes.	Graham *et al.*, 1977
293-L1	293 cells expressing Ad5 L1 52/55-kD protein.	This work
293-L1-IV_{a2}	293 cells expressing Ad5 L1 52/55-kD and IV_{a2} proteins.	This work
293-L1-pIX	293 cells expressing Ad5 L1 52/55-kD and pIX proteins.	This work
COS1	African green monkey kidney cells expressing SV40 T antigen	Gluzman, 1981
HeLa	Human cervical epitheloid carcinoma	Scherer *et al.*, 1953
Ad5 *wt*300	Wild-type Ad5	Jones & Shenk, 1979 (b)
Ad5 *ts*369	An adenovirus mutant containing a temperature sensitive L1 gene	Hasson *et al.*, 1989
H5pm8001	An adenovirus mutant incapable of expressing the L1 52/55-kDa protein	Gustin & Imperiale, 1998

Table 2.3 A list of mammalian cells and virus strains

2.3.3 Freezing and recovery of cell stocks

For long term storage, mammalian cells were frozen in 2 ml freezing vials after resuspension of the trypsinized monolayer cells from a 90 mm tissue culture dish in 1ml of freezing mixture (92 % serum, 8 % DMSO). Freezing was done slowly at –70 °C for 24 hours by wrapping the freezing vials with a thick layer of tissue paper. The vials were then transferred to liquid nitrogen storage freezer.

When frozen cells were needed for study, the vials were thawed rapidly in a 37 °C water bath and the cells were plated at high density onto a 90 mm dish containing 9 ml of prewarmed growth medium. After an incubation of 24 hours the medium was replaced by 10 ml of fresh growth medium.

2.3.4 Virus infection of cell monolayers

Appropriate dilution of virus stock in 0.5 ml DMEM per plate were added onto 75 % confluent cells in 90 mm dish after removing the growth medium, and incubated at 37 °C for 1-1.5 hours with regular (every 15 min) rocking. Then 10 ml of appropriate growth medium preheated to 37 °C was added onto each dish and incubated at the desired temperature for a desired amount of time. Other culture vessels used in experiments contained volumes of medium or virus dilution in proportion to their surface areas.

2.3.5 Harvesting and storage of virus particles from infected cells

Infected cells were harvested when cytopathic effect was visible and lysed by three freeze-thaw cycles. Cell debris was removed by centrifuging (Beckman GPR centrifuge, GH 3.7 rotor, 1500 rpm, 3 min) and the supernatant (virus stock) stored in 2-3 ml aliquots at –70 °C.

2.3.6 Transfection of cell monolayers by the calcium phosphate precipitation method

The 293 cells from one 90 mm dish were passaged and divided between three 60 mm dishes 24 hours before transfection. Four hours before transfection the medium was changed to DMEM/10 % FCS. DNA/calcium phosphate precipitates were prepared by mixing 50 µl of 2x HEPES buffer (16 g/l NaCl, 0.74 g/l KCl, 2 g/l glucose, 10 g/l HEPES), 3 µl of 100x phosphate solution (9.9 g/l Na_2HPO_4), and DNA solutions + water to 90 µl, then adding 10 µl of 1.25 M calcium chloride and blowing air through the solution. The precipitates were allowed to form for 30 min at room temperature, then the medium was removed from the dishes and the cells were overlaid with the precipitate suspensions diluted to 5 ml with DMEM-10 % FCS. The cells were then incubated for 4 hours at 37 °C (5 % CO_2), with occasional rocking. The medium was removed from each dish, and 1 ml of 20 % (v/v) glycerol in TS buffer was carefully added to each monolayer. This was removed after exactly 1min, and the cells were washed twice with TS, then overlaid with DMEM-10 % NCS. The cells were incubated at 37 °C for 12 hours, and the medium was replaced by fresh medium.

2.3.7 Transfection of cell monolayers by lipofection

When higher efficiency of transfection was required in experiments, a lipofection method using LIPOFECTACE reagent (GibcoBRL) was performed according to the instructions supplied by the manufacturer (5 µl LIPOFECTACE per 1 µg of DNA).

2.3.8 Small scale preparation of adenovirus DNA

Infected 293 cell monolayers in 90 mm dishes were harvested when full cytopathic became visible (usually in 3-4 days) and cells were pelleted by centrifuging 5 min at 1500 rpm in a Beckman GPR centrifuge (GH 3.7 rotor). The supernatant was removed and the pellet was resuspended in 0.4 ml of TE (pH 9)/10 mM spermine tetrahydrochloride (Sigma). The suspension was mixed with 0.4 ml DOC lysis

buffer (20 % (v/v) ethanol, 100 mM Tris-HCl pH 9, 0.4 % (w/v) sodium deoxycholate) and cell debris was pelleted by centrifugation (15 min, 12000 rpm, microcentrifuge). The supernatant was collected and 60 µl 10 % (w/v) SDS, 40 µl 0.25 M EDTA, and 20 µl of 20 mg/ml proteinase K (Boehringer) were added. The solution was mixed several times by inversion and incubated at 37 °C for 1 hr. Residual protein was then extracted with phenol/chloroform and the DNA precipitated by adding 30 µl 5 M NaCl and 0.6 ml of propan-2-ol at room temperature. The solution was mixed several times by inversion and the DNA was pelleted by centrifuging for 15 min at 12000 rpm in a microcentrifuge (room temperature). The DNA pellet was dried and resuspended in 50 µl distilled water.

2.3.9 Large scale preparation of adenovirus DNA

Infected Hela or 293 cells from ten 90 mm dishes were harvested as described above resuspended in 10 ml of 0.1 M Tris-HCl (pH 8). The infected cell suspension was disrupted by sonication on ice (two sets of 10 x 1s pulses separated by 30 sec using a Jencons GE375 ultrasonic processor and 3 mm tapered microtip) and transferred to a 30 ml Corex tube to be clarified by centrifugation (6000 rpm for 10 min, 4 °C, in a Beckman J2-21M centrifuge with JS-13.1 rotor). The supernatant was collected and 5 ml fractions were layered over CsCl step gradients consisting of 2 ml of CsCl in TD (density 1.40 g/ml) with an overlay of 3 ml of CsCl in TD (density 1.25 g/ml). The gradients were centrifuged at 35000 rpm for 1 hour (15 °C) in a Beckman L8-70M ultracentrifuge (SW 41 rotor). The virus formed a clearly visible band below cellular debris, which was recovered by puncturing the bottom of the tube and collecting drip fractions. Collected virus from all the tubes was then pooled and centrifuged to equilibrium (at least 15 hr) in CsCl/TD of density 1.35 g/ml at 40000 rpm (15 °C, Beckman L8-70M ultracentrifuge, SW 50.1 rotor). The virus band was collected (as above), diluted with two volumes of water and precipitated with six band volumes of ethanol at –70 °C for 15 min. Centrifuging for 20 min at 7000 rpm (4 °C) in a Beckman J2-21M centrifuge (JS-13.1 rotor) pelleted the virus. The pellet

was dried and gently resuspended in 2 ml of TNE, then 120 µl 10 % (w/v) SDS, 40 µl 0.25 M EDTA and 20 µl 10 mg/ml proteinase K were added. The solution was incubated at 37 °C for 1-2 hours then extracted with 2 ml of phenol/TNE at room temperature. The tube was gently swirled for 1 min, 1 ml chloroform/amyl alcohol was added, and the solution was again gently swirled to mix. Phases were separated by centrifuging for 10 min at 3000 rpm (4 °C) in a Beckman GPR centrifuge, and the organic extraction and centrifugation were repeated with the aqueous phase. DNA was precipitated from the final aqueous phase by adding sodium acetate solution to 0.3 M and two volumes of ethanol. The DNA was allowed to precipitate at –70 °C for 30 min, and then pelleted at 7000 rpm for 30 min (4 °C) in a Beckman J2-21M centrifuge (JS-13.1 rotor). The DNA pellet was washed with 70 % ethanol, dried, and allowed to redissolve in 0.5 ml TE at 4 °C overnight.

2.3.10 Determination of virus stock titers by plaque assay

Serial dilutions of virus stocks were prepared in DMEM in the range of 10^{-1} - 10^{-9} and aliquots of the dilutions were used to infect duplicate cultures of cells in 6 well tissue culture dishes. Immediately after infection, cells were overlaid with semi-solid medium (4 % (v/v) NCS, 0.375 % (w/v) $NaHCO_3$, 1 % (w/v) noble agar, in DMEM). After an incubation period of 3-4 days, a second overlay (1 % (v/v) NCS, 0.188 % (w/v) $NaHCO_3$, 1 % (w/v) noble agar, in DMEM) was added. When the plaques became visible (usually within 4-6 days), they were stained by the addition of an overlay containing neutral red (1 % (v/v) NCS, 0.188 % (w/v) $NaHCO_3$, 1 % (w/v) noble agar, 0.01 % (w/v) neutral red, in DMEM) and counted the next day.

2.4 DNA manipulation techniques

2.4.1 Digestion of DNA with restriction enzymes

Restriction enzymes were purchased either from Gibco BRL or New England Biolabs and the digestions were carried according to the manufacturer's instructions,

using concentrated buffers supplied with the enzyme. Multiple enzyme digests were carried in the same reaction tube provided that their buffers were compatible. Otherwise the reactions were carried out sequentially, DNA being purified between each reaction.

2.4.2 End-fill and overhang removal reactions

To produce blunt end termini of DNA fragments for cloning by blunt end ligation, T4 DNA polymerase (Gibco BRL) was used with all four dNTPs according to the manufacturer's instructions. The DNA was then purified by phenol/chloroform extraction as explained in section 2.4.3.

T4 DNA polymerase produces blunt ends by removing 3' protruding ends because of its strong 3' to 5' exonuclease activity. Similarly if the termini have 5' protruding region, the enzyme will simply extend the recessed 3' termini due to its polymerase activity.

2.4.3 Dephosphorylation of DNA

In order to prevent self-ligation of vector DNA termini in cloning experiments, 5' termini of vector DNA were dephosphorylated by using calf intestinal phosphatase (CIP) (Boehringer). 5 µl of phosphatase buffer (supplied by the manufacturer), 14 µl dH_2O and 7 units of CIAP was added to 30 µl of reaction mixture containing 1.5 µg plasmid DNA digested with restriction endonuclease(s). The reaction was carried out at 37 ^{0}C for 15 min and the CIAP was inactivated by further incubation at 75 ^{0}C for 15 min. DNA was then purified by phenol/chloroform extraction.

2.4.4 DNA ligation reactions

In order to join duplex DNA restriction fragments having either blunt ends or compatible cohesive ends for cloning purposes, T4 DNA ligase (Gibco BRL) was used according to the manufacturer's instructions. For blunt end ligation, reactions

were carried out at room temperature and cohesive end ligation reactions were carried out at 15 ^{0}C. Both kinds of reactions were incubated overnight.

2.5 Separation and purification of DNA fragments

2.5.1 Agarose gel electrophoresis

Agarose gels were cast by melting the agarose in 50 ml of TBE buffer in a ratio of 0.5-1.5 % (w/v) until a clear transparent solution was achieved. 2 µl of ethidium bromide (10 mg/ml) was added to the melted solution and then poured into a horizontal gel apparatus and allowed to harden. The gel was submerged in TBE buffer and samples, which were mixed with the gel loading buffer, were applied in the wells formed by the removal of the comb after the gel was hardened. An electric field of 8 volts/cm was applied until the bromophenol blue dye, present in the gel loading buffer, reached the 2/3 length of the gel. The gel was removed and the DNA fragments were visualised under ultraviolet light.

2.5.2 Purification of DNA fragments from agarose gels

After visualisation of the DNA fragments following separation by agarose gel electrophoresis, the required fragments were cut out of the gel by using a scalpel and placed in dialysis tubing filled with TBE buffer. The tubing was sealed at both ends by clippers and the DNA was electro-eluted into the TBE buffer present in the dialysis tube in a gel tank containing TBE buffer. The eluted DNA was then transferred into a microfuge tube and extracted with phenol/chloroform.

Alternatively, for the purification of DNA fragments in the size range of 500-10000 bp, the DNA extraction kit from Qiagen was used according to the instructions supplied by the manufacturer.

2.5.3 Phenol/chloroform extraction and ethanol precipitation of DNA

An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the DNA solution to be purified in a 1.5 or 2 ml microcentrifuge tube and mixed by vortexing, followed by centrifugation at 15000 rpm in a microcentrifuge for 1 min. The resulting aqueous layer was transferred to a fresh tube (extraction was sometimes repeated several times to achieve improved purification) and the DNA was recovered by the following ethanol precipitation method.

In order to recover DNA from solutions, precipitation of DNA by ethanol was carried out by adding 0.1 volumes of 3 M sodium acetate, pH 5.2, and 2 volumes of cold ethanol (at -20^{0}C) followed by incubation at –70 ^{0}C for 30 min. The mixture was then centrifuged for 10 min at 1500 rpm in a microcentrifuge and the pellet was dried and resuspended in desired volume of dH_2O.

2.5.4 Spectrophotometric quantification of DNA

DNA in a solution was quantified by taking absorbance readings at 260 nm in a spectrophotometer. An absorbance of 1 corresponds to 50 µg/ml of double stranded DNA and 20 µg/ml of single stranded DNA oligonucleotide. Absorbance readings of the samples were also taken at 280 nm and the ratio A_{260}-A_{280} was used to assess the purity of the solutions. Ratios lower than 1.6-1.8 indicate contamination of the solution with other molecules and prevents accurate quantification by this method.

2.6 Polymerase Chain Reaction (PCR)

2.6.1 Primers

Designed primers were ordered from Gibco BRL. The sequences of the primers used are given in Appendix A.

2.6.2 PCR amplification reactions

Appropriate primers were used to amplify predetermined regions of a template DNA in a 50 µl volume of PCR mixture (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.01 % gelatine, 0.2 mM of each dNTP, 0.1 % Triton-X100 and 0.25-3 mM $MgCl_2$ (concentrations differ for specific PCR)) containing 1 µM of each primer and 0.4 units of Taq DNA polymerase, overlaid with 80 µl of liquid paraffin. Standard reaction conditions were 25-30 cycles of 1 min denaturation at 92 °C, 1 min annealing at an appropriate temperature for the choosen oligonucleotides (42 °C-60 °C) and 3 min elongation at 72 °C. Actual conditions were optimised for each primer-template combination. 15 µl of the resulting PCR reaction mixture was analysed by agarose gel electrophoresis. As required, the PCR products were purified by using Qiagen's DNA purification from PCR kit, as recommended by the manufacturer.

2.6.3 Detection of recombinant plasmids in bacterial colonies by PCR

Cells from a colony were inoculated (as DNA source) using a sterile toothpick directly into a 50 µl PCR mixture and PCR was performed as described in section 2.5.2.

2.6.4 Detection of Adenovirus recombinants by PCR

a) from cultured cells overlaid with liquid medium

Cells in a 60 mm dish were collected after trypsinisation and pelleted (Beckman GPR centrifuge, GH 3.7 rotor, at 1500 rpm for 3 min). After resuspension in 400 µl of NP40 lysis buffer, cells were incubated on ice for 10 min and pelleted as before. The resulting supernatant containing any viral particles were transferred to a clean microfuge tube and incubated at 37 ^{0}C for 4 hours with 0.5 mg/ml proteinase K and 0.5 % SDS (for 500 µl of supernatant 27 µl of 10 mg/ml proteinase K and 27 µl of 10 % SDS) to degrade viral coat proteins. Viral DNA was then recovered by three

times phenol/chloroform extraction, ethanol precipitation and resuspension in 50 µl of dH_2O. 5 µl of this suspension was used in PCR amplification reactions for detection as described in section 2.5.2.

b) from cultured cells overlaid with agar medium

Identified virus plaques were taken off the tissue culture plate using a Pasteur pipette and resuspended in 1 ml of DMEM-10 %FCS. 370 µl of the suspension was taken and added to 40 µl of 10x DNase I buffer (400 mM Tris-HCl pH 7.5, 60 mM $MgCl_2$) and 10 units of DNase I (Boehringer) for 1 hour at 37 ^{0}C. Then, DNase I and viral capsid proteins were denatured by the addition of 32 µl of 0.25 M EDTA, 0.25 M EGTA, 10 µl of 20 % SDS, 5 µl of 16 mg/ml proteinase K and incubation for 2 hours at 56 ^{0}C. DNA was recovered by phenol/chloroform extraction, ethanol precipitation and resuspension in 20 µl of dH_2O. 5 µl of this suspension was used for PCR amplification.

2.7 DNA sequencing

2.7.1 Construction of nested deletions of cloned DNA for use in DNA sequencing

10 µg of plasmid containing the cloned DNA to be sequenced was completely digested with restriction enzymes that left a 3' overhang adjacent to the primer binding site and a 5' overhang or blunt end adjacent to the insert DNA (see Figure 7.2.1). The 5' or blunt restriction site was positioned between the 3' restriction site and the insert and complete digestion was verified running a small aliquot on an agarose minigel. DNA was recovered by phenol/chloroform extraction, ethanol precipitation and dissolved in 1x exo III buffer (15 mM Tris-HCl, pH 8.0 and 0.66 mM $MgCl_2$) for a final concentration of 0.1 µg/µl. 25 µl of the solution was taken to a new tube and 150 units of exo III per pmol susceptible 3' ends was added. At 1

min intervals, 3 µl aliquots were removed to individual tubes and placed immediately on dry ice for 5 min. 3 µl of dH_2O was added to each sample and incubated for 10 min at 70 ^{0}C to inactivate exo III.

To create blunt ends on the exo III-treated DNA by removing single stranded portions, 4 µl (4 units) of S1 nuclease together with 15 µl S1 nuclease buffer (16 mM sodium acetate, pH 4.6, 400 mM NaCl, 1.6 mM $ZnSO_4$, 8 % glycerol) was added to each aliquot and incubated 20 min at room temperature. S1 nuclease reaction was stopped (via pH shift) by adding 5 µl S1 nuclease stop buffer (0.8 M Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, 80 mM $MgCl_2$) to each sample. The extent of deletion in each sample was examined by running 8 µl of each sample on agarose gel electrophoresis. Then, 1 µl (2 units) DNA polymerase Klenow fragment with 0.25 mM dNTP mix was added to the remaining 22 µl of each sample and incubated at 37 ^{0}C for 10 min to fill in any recessed 3' ends.

DNA in each sample was purified by phenol/chloroform extraction, ethanol precipitation and resuspension in 29 µl dH_2O, and then recircularizied by the addition of 6 µl (6 units) T4 DNA ligase, 0.5 µl 0.1 M ATP, 4 µl 10x ligase buffer (500 mM Tris-HCl, pH 7.5, 70 mM $MgCl_2$, 10 mM DTT) and incubation overnight at room temperature.

Competent *E.coli* cells were transformed with 10 µl ligation reaction from each sample. One clone from each transformation was analysed by restriction analysis after plasmid isolation and linearization by a suitable restriction enzyme and plasmids showing the desired extents of deletions were saved for DNA sequencing.

2.7.2 DNA sequencing by dideoxy method

Sequencing reactions were carried out by using the Sequenase (Version 2.0) kit from

United States Biochemical Corporation according to the instructions provided with the kit and ^{35}S-dATP (Amersham).

2.7.3 Denaturing polyacrylamide gel electrophoresis for DNA sequencing reactions

Denaturing polyacrylamide gels were cast by the polymerisation of acrylamide:bisacrylamide in the presence of urea. For this, 3.42 g acrylamide, 0.18 g bisacrylamide, 26.4 g urea were dissolved in freshly prepared 25 ml TBE buffer and the volume was made up to 60 ml by using TBE. This acrylamide solution was filtered to remove any impurities and to this filtrate 240 µl 10 % ammonium persulfate (APS) and 24 µl TEMED was added to start the polymerisation process. Immediately after mixing, the solution was poured into vertical sequencing gel apparatus (BioRAD) and the top of the apparatus was sealed by the placement of a shark's tooth comb. After the samples were loaded into the wells, the electrophoresis was run at 2000 volts until the bromophenol dye reached the end of the gel. The gel was then soaked for at least 20 min in 5 % acetic acid, 15 % methanol to remove the urea and dried at 80 ^{0}C for 2 hours under vacuum. The bands were visualised by autoradiography (section 2.9.4).

2.8 Analysis of Proteins

2.8.1 Spectrophotometric determination of protein concentration

The protein concentration of sample solutions was determined using the BioRAD protein assay kit (according to the manufacturer's microassay procedure) and a spectrophotometer.

2.8.2 Separation of proteins by SDS polyacrylamide gel electrophoresis

Discontinuous SDS-polyacrylamide gels (Laemmli, 1970) were prepared and run in a Gibco BRL vertical gel electrophoresis apparatus according to description of

Sambrook *et al.* (1989). Protein samples were heated to 95 ^{0}C for 3 min in loading buffer (100 mM DTT, 25 mM Tris-HCl pH 6.8, 10 % glycerol, 2 % SDS and 0.005 % bromophenol blue) just before applying to the gel. After performing electrophoresis until the blue dye reached the bottom of the gel, it was either fixed by soaking in 25 % methanol, 7 % glacial acetic acid for at least 30 min and dried at 70 ^{0}C for 2 hours for the detection of radiolabelled samples by autoradiography (section 2.9.4) or stained by soaking the gel in at least 5 volumes of staining solution (0.25 g of Coomassie Brilliant Blue R250 in 90 ml of methanol:H_2O (1:1 v/v) and 10 ml of glacial acetic acid) for 4 hours at room temperature. The gel was destained by soaking it in methanol/acetic acid solution (as before) without the dye on a slowly rocking platform for 4-8 hours changing the solution 3 or 4 times and stored in water in a sealed plastic bag.

2.8.3 Transferring proteins from acrylamide gels to nitro-cellulose membrane

When the SDS polyacrylamide gel was approaching the end of its run, the gel was removed and transferred to a tray of water and then placed in contact with a piece of prewetted nitro-cellulose filter (Hybond-C, Amersham) which was cut at the same size as the gel. The gel and filter were then sandwiched between Whatman 3MM paper, two porous pads and two plastic supports, all prewetted in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037 % SDS, 20 % methanol, pH 8.3). The entire construction was then immersed in an electrophoresis tank equipped with standard platinum electrodes, which contained transfer buffer. The nitro-cellulose filter was placed toward the anode (Figure 2.2) and an electric current of 70 volts was applied for 2 hours to transfer the proteins on to the nitro-cellulose filter.

2.8.4 Detection of proteins by Western Blotting

After the transfer of proteins to the nitro-cellulose filter, any remaining protein binding sites on the were blocked by incubation in 5 % (w/v) non-fat dried milk in PBS for 1 hour at 37 ^{0}C, rocking occasionally. The filter was washed with PBS.

Primary antibodies reactive against the target protein(s) (diluted 1:100-1:1000 in 5 % non-fat dried milk in PBS) were added to the filter and incubated at room temperature for 1.5 hours with constant rocking. The filter was washed again by two 10 min incubations in PBS with constant rocking. Then biotinylated secondary antibodies (e.g. biotinylated anti-mouse IgG from goat) (diluted 1:500 in 1 % non-fat dried milk in PBS) against primary antibodies were added on the filter and incubated at room temperature for 1 hour with constant rocking. The filter was washed as before and incubated once more at room temperature for 45 min after the addition of HRP-strepdavidin complex diluted 1:500 in 1 % non-fat dried milk in PBS. The filter was washed by three 10 min incubations in 1 % Tween-20 in PBS and one 5 min incubation in PBS. For the detection of target protein bands TMB stabilised substrate for HRP (Promega) was added on the filter and waited for 10-20 min until the development of a purple colour. The filter was washed for the last time in H_2O and the result was recorded by digitally scanning the filter (Hewlett Packard) using Deskscan software.

2.8.5 Detection of proteins by ELISA

Detection of antibodies against a target protein in a serum sample was carried by using an ELISA (enzyme linked immunosorbent assay) method. Target antigen was diluted to a concentration of 1 μg/ml in 100 mM sodium bicarbonate and 50 μl was applied to each well on a 96 well ELISA plate and dried at 37 ^{0}C overnight. Wells were blocked by the addition of 200 μl 5 % w/v non-fat dried milk in wash buffer (0.244 % (w/v) Tris base, 0.8 % (w/v) NaCl, 0.1 % (v/v) Tween-20, adjusted to pH 7.4 with HCl) for 45 min and then washed 3 times for 5 min each wash with wash buffer. The serum samples were titrated from 1:30 to 1:7801250 in a 5-fold series in wash buffer and 50 μl were added to the wells (all the wells in row A receiving the lowest dilution and so on, Figure 4.1). Serum samples were incubated for 2 hours with the bound antigen in the wells and then the wells were washed with wash buffer as before to remove unbound antibodies in the serum. Then, 100 μl of biotinylated

anti-rabbit IgG antibodies diluted 1:5000 in wash buffer were added on to each well and incubated 1.5 hours at room temperature. Wells were washed as before, 100 µl of HRP strepdavidin complex diluted 1:5000 in wash buffer was added on to each well and incubated 1 hour at room temperature. Once again the wells were washed as before and 100 µl of OPD substrate (10 mg OPD, 9 ml 0.11 M disodium hydrogen phosphate, 1 ml 0.5 M citric acid and 10 µl hydrogen peroxide) per well was added. After yellow colour development, reactions were stopped by the addition of 50 µl of 2.5 M H_2SO_4 to each well and the absorbance of each well at 492 nm was determined spectrophotometrically by using a 96-well plate reader.

CAT ELISA kit (Boehringer Mannheim) which is based on the sandwich ELISA principle was routinely used according to the manifacturers instructions in experiments for the detection of CAT expression in transfected mammalian cell lysates.

2.8.6 Screening of cell lysates for β-galactosidase activity

10 µl of cell lysate was placed in a 1.5 ml microcentrifuge tube and Z buffer (16.1 g/ml $Na_2HPO_4.7H_2O$; 5.5 g/ml $NaH_2PO_4.H_2O$; 0.75 g/ml KCl; 0.246 g/ml $MgSO_4.7H_2O$, pH 7.0) was added to a final volume of 400 µl followed by 5 min incubation at room temperature. Then, 100 µl of freshly prepared 4 mg/ml ONPG (o-nitrophenyl β-D-galactopyranoside) in Z buffer was added, mixed by vortexing, and incubated at room temperature until the samples became yellow. Finally the reaction was stopped by adding 500 µl of 1 M sodium carbonate, mixed by vortexing, and the absorbance of the samples were measured by spectrophotometry at 420 nm.

2.9 Purification of GST-fusion Proteins in E.coli

2.9.1 Preparation of small scale crude cell extracts of GST-fusion proteins

Proteins were expressed as glutathione S-transferase fusion proteins in E. coli strain XL1-Blue. Freshly transformed bacteria were grown in 20 ml LB/ ampicillin (50 μg/ml) at 37 ^{0}C until an OD_{600} of 0.5 was reached. Fusion protein expression was induced in the culture by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 1 mM final concentration. The culture was then moved to 30 ^{0}C and 2 ml sample was collected at a desired time point post-induction and cells were pelleted by centrifugation for 0.5 min at 15000 rpm in a microfuge. Cells were resuspended in 1 ml ice-cold PBS and lysed by sonication on ice for 0.5 min (using Jencons GE375 ultrasonic processor with microtip set at 4 (power output), 50 % duty cycle using 10 sec pulses). The solution was then centrifuged for 5 min at 15000 rpm in a microfuge to separate the soluble extract from the insoluble pellet. The sample was then analysed by SDS-PAGE.

2.9.2 Large scale preparation of GST-fusion proteins

1 L cultures of freshly transformed bacteria were grown to an OD_{600} of 0.5 and protein expression induced by addition of IPTG to a final concentration of 1 mM. The culture was moved to 30 ^{0}C and fusion proteins harvested at the desired time post-induction. Cells were pelleted by centrifugation at 4 ^{0}C for 15 min at 1600 g, washed with 40 ml PBS containing protease inhibitors (500 μM DTT, 500 μM PMSF, 10 μM leupeptin, 2 μM pepstatin A, 10 mM EDTA) then resuspended in 10 ml PBS containing protease inhibitors and frozen at –70 ^{0}C. Cells were lysed using a French pressure press at 1010 psi and cellular debris removed by centrifugation at 32000 g for 60 min at 4 ^{0}C. The lysate was diluted to 100 ml in PBS/ 1 % Triton X-100 and incubated with 2 ml of a 50 % suspension of glutathione-Sepharose 4B beads (Pharmacia) for 1 hr at room temperature. The beads were washed four times in 50 ml PBS, collected in a column and protein eluted with 50 mM Tris-HCl pH 8,

10 mM reduced glutathione. 0.5 ml fractions were collected and protein concentrations determined using the BioRad protein assay (section 2.7.1). Finally, their protein content was assessed by SDS-PAGE and Western blotting.

2.10 Analysis of viral and cellular protein expression

2.10.1 ^{35}S-methionine labelling of cellular proteins

Virus-infected or mock-infected cell monolayers on 60 mm dishes were incubated with 1 ml of methionine free DMEM (ICN-Flow) for 30 min. Then, 1 ml of methionine free DMEM supplemented with 100 μCi of ^{35}S-methionine [1000 Ci/mmol, Amersham] was added. The dish was incubated for a further 2 hours and the labelling medium was removed. The cells were washed with ice-cold PBS and lysed by the addition of 1 ml RIPA buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.6, 1 % (v/v) Triton X-100, 1 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS). The dish was incubated for 5 min on ice, the lysate was transferred into a microfuge tube, vortexed and then centrifuged at top speed in a micro-centrifuge. The supernatant was transferred into a fresh tube and stored at –70 ^{0}C.

For the labelling of proteins expressed in smaller quantities, cell monolayers were labelled for 24 hours. This was done by using a slightly modified labelling medium which consisted of 4 ml of 2 % dialysed FCS, 5 % normal DMEM and 100 μCi ^{35}S-methionine in methionine-free DMEM.

2.10.2 Immunoprecipitation of ^{35}S-methionine labelled proteins

Antibodies (1-2 μl of polyclonal antisera) against the target protein(s) were added to the radiolabelled, infected or mock-infected cell lysates and incubated for 1 hour at room temperature on a rotator. Then, 50 μl of protein A sepharose (Sigma) slurry (slurry was prepared according to the manufacturer's instructions) was added to each lysate and incubated for a further 30 min at room temperature. The lysate was

centrifuged in a microcentrifuge at 400 rpm for 2 min and the supernatant was discarded. The pellet was washed 2-3 times in RIPA buffer and resuspended in gel loading buffer (see section 2.7.2). The samples were either stored at –70 ^{0}C or immediately boiled for 3 min and subjected to SDS polyacrylamide gel electrophoresis.

2.10.3 Autoradiography

Autoradiography was done by exposing an X-ray film (Fuji RX) to dried radioactive gels at –70 ^{0}C in complete darkness for at least 12 hours. The film was then developed and fixed by using chemicals (Kodak) according to the supplier's instructions.

Chapter 3

Cloning and sequencing of the adenovirus 5 L1-52/55 kD gene

3.1 Introduction

This chapter describes the starting point of the experimental work presented in this thesis: cloning of the adenovirus type 5 (Ad5) L1-52/55 kD gene.

As stated in the general introduction, the aim of this study was to construct human cell lines that could complement deletions of Ad5 late genes without a need for a helper virus. To do this, the missing function(s) had to be supplied by cell lines that expressed the genes deleted from the virus genome.

To facilitate the expression of the desired genes in cell lines, they had to be first cloned into suitable vectors (see chapter 5) which would carry out the expression of the gene(s) within the cells upon transfection.

Although adenovirus DNA can be manipulated directly with restriction enzymes, we decided to amplify the desired Ad5 gene by PCR and directly clone it into a bacterial plasmid vector designed for the easy cloning of PCR fragments. This made the sequencing and the further cloning of the gene into other plasmids possible due to the presence of universal primer binding sites and a multiple cloning site.

3.2 PCR amplification and cloning of the Ad5 L1 52/55-kD gene

In order to facilitate its cloning, the Ad5 L1 52/55-kD coding region was amplified by PCR. Primers AP1 and AP2 (Appendix A), containing BamH1 and EcoR1 recognition sequences respectively at their 5' ends, were designed according to published sequence data of Ad5 (Chroboczek *et al.*, 1992) to amplify the L1 52/55-kD open reading frame (orf). These primers were homologous to the Ad5 genome at positions 11050 to 11067 and 12300 to 12281 (on the opposite strand) respectively (Figure 3.1).

To be used as a template for the PCR, Ad5 *dl*309 DNA was obtained from particles purified by caesium chloride centrifugation (section 2.2.9) and 100 ng was used for each PCR. Test PCRs were performed to determine the optimum reaction conditions for the amplification of L1 52/55-kD orf by using primers AP1 and AP2. Optimum PCR conditions for this specific amplification were 94 ^{0}C for 1 min, 55 ^{0}C for 1 min, and 72 ^{0}C for 3 min for 30 cycles. The $MgCl_2$ concentration used in the reaction buffer for this particular pair of primers was 0.75 mM (Figure 3.2).

The 1.3 kb amplification product, containing the L1 52/55-kD orf, was then gel purified and ligated directly into TA cloning vector pCR II (Invitrogen) (Figure 3.3). This vector is designed for direct cloning of PCR fragments into a multiple cloning site (MCS). This MCS is present in the orf of the β-galactosidase gene α-peptide and bacterial colonies, of suitable strains, harbouring this gene can be distinguished by a blue colour when grown on an agar medium containing X-gal (40 mg/ml (w/v) in dimethyl formamide) (a substrate for β-galactosidase). If a PCR fragment is cloned at this position, the orf is disrupted and the colonies harbouring this plasmid can be distinguished by their white colour. After transformation of *E. coli XL-1 Blue* with the ligation mixture, positive (white) colonies harbouring pCR II-L1 were checked for the presence of an insert by the colony PCR method, using the primers AP1 and AP2. One of these colonies was picked for further analysis, which was done by restriction digestion on purified plasmid DNA, using BamH1 and EcoR1 restriction enzymes (Figure 3.4).

3.3 Construction of nested deletions of the cloned L1 gene for DNA sequencing

Due to the absence of a proofreading activity, Taq polymerase enzyme used in PCRs has a low fidelity with an error rate of about $2x10^{-4}$ (Dunning *et el.*, 1988), which is about 10^4 times higher than a bacterial DNA polymerase (around $8x10^{-8}$). Considering the possibility of an error being introduced into the amplified sequence

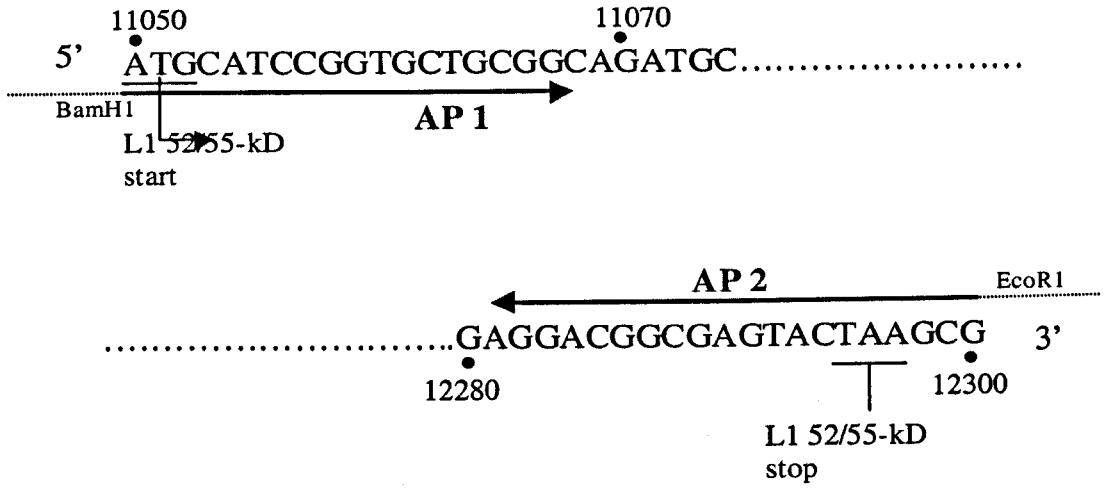


Figure 3.1 Part of the Ad5 genome coding for L1 52/55-kD orf
Primers and their direction of polymerization are shown by arrows.

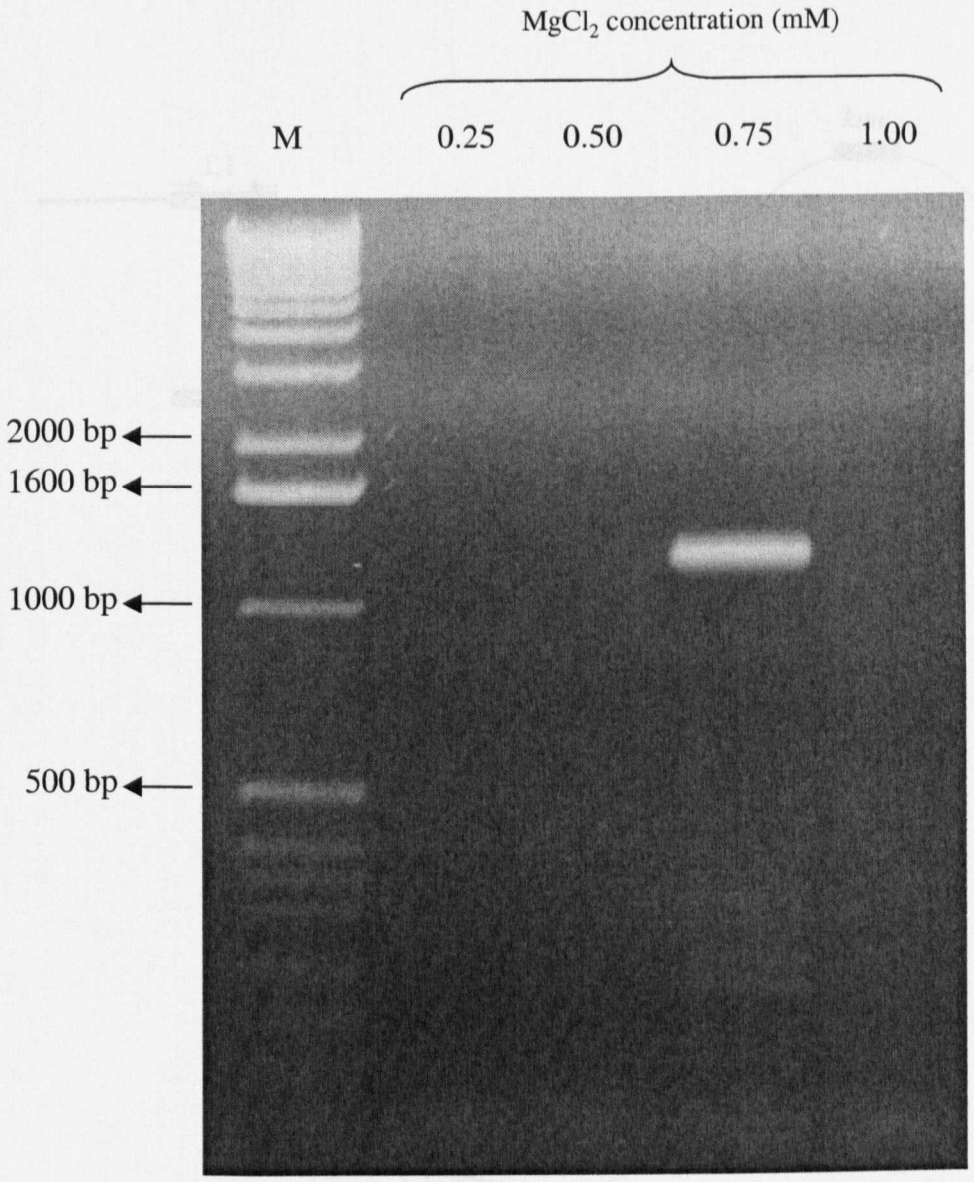


Figure 3.2 Determination of optimum $MgCl_2$ concentration for the amplification of L1 52/55-kD orf (1.3 kb) by PCR using primers AP1 and AP2.

Different concentrations of $MgCl_2$ were used in individual PCRs as indicated above the lanes. The expected size of the AP1/AP2 amplification product was about 1200 bp. M-dsDNA size marker as indicated.

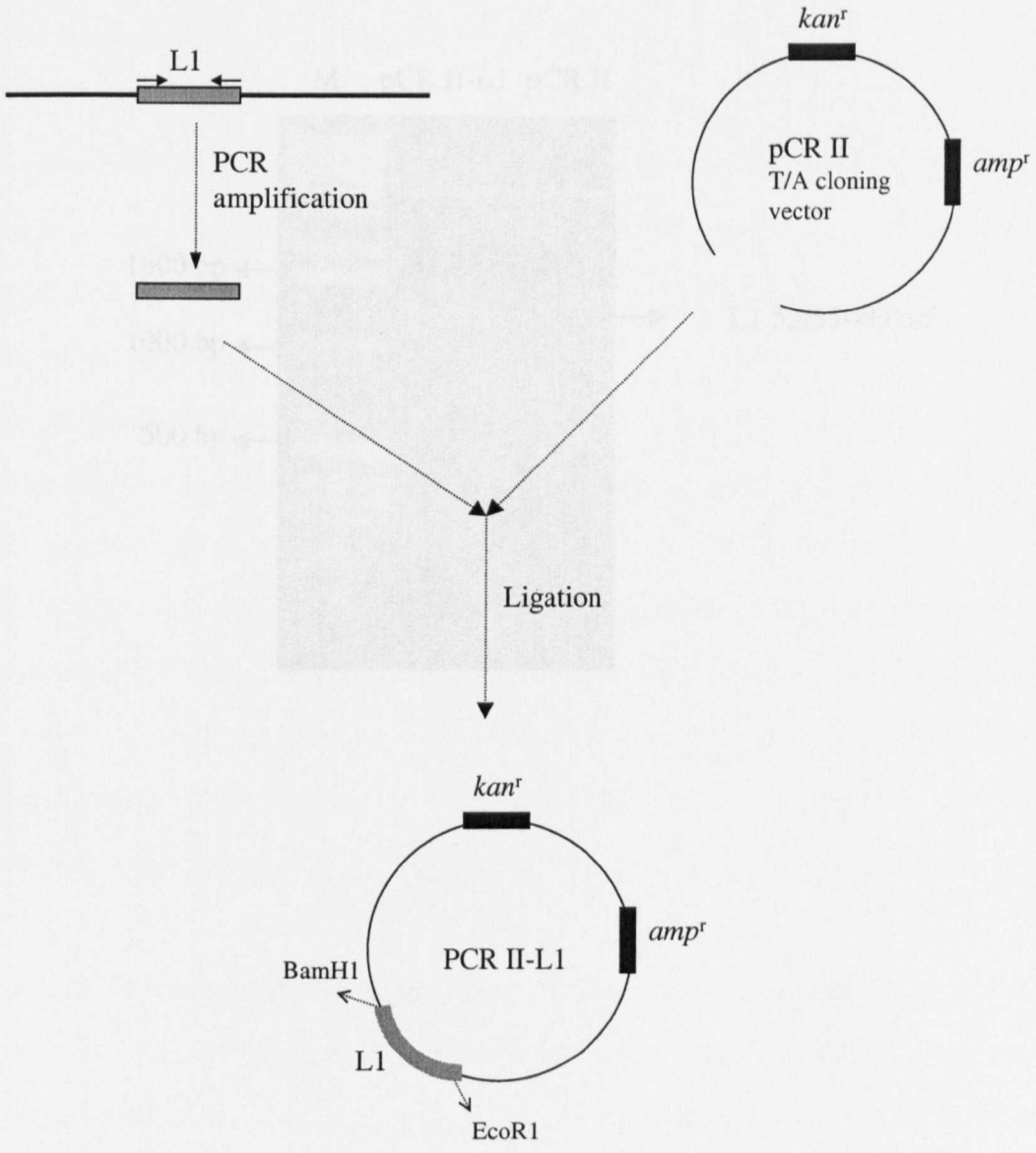


Figure 3.3 PCR amplification and cloning of the L1 52/55-kD. open reading frame
The 1.3 kb amplification product was cloned into vector pCR II using the A overhangs created by Taq polymerase.

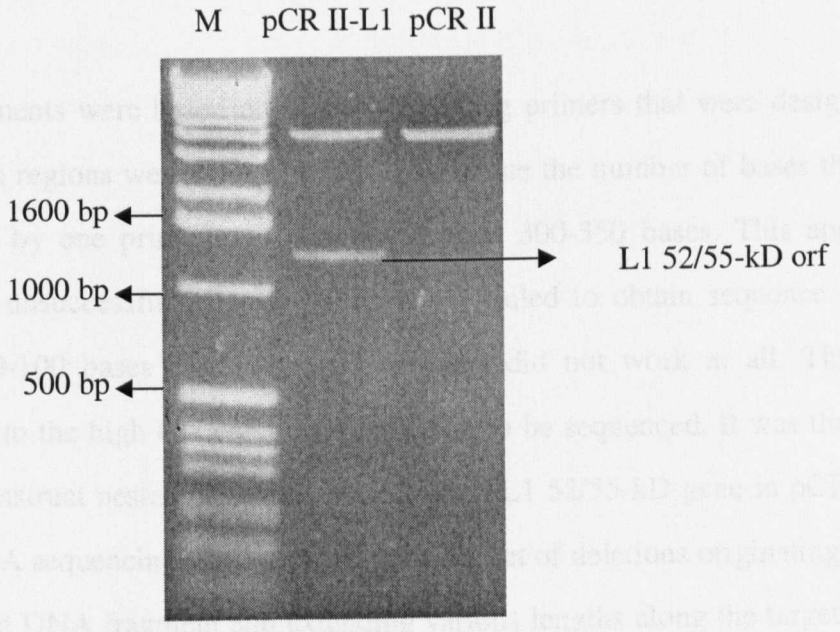


Figure 3.4 Restriction enzyme analysis of plasmid pCR II-L1
Plasmids pCR II-L1 was digested with enzymes BamH1 and EcoR1 to release the expected 1.3 kb insert. Plasmid pCR II was similarly digested as a control. M-dsDNA size markers as indicated.

of L1 52/55-kD, we decided to subject the cloned 1.3 kb PCR fragment to DNA sequencing analysis because any error introduced at this stage could prevent the expression of a normal protein in future experiments.

Initial experiments were based on sequencing using primers that were designed to hybridise with regions were about 300 bp apart, since the number of bases that can be sequenced by one primer was typically around 300-350 bases. This approach proved to be unsuccessful because we regularly failed to obtain sequence results more than 80-100 bases long and some primers did not work at all. This was probably due to the high GC content of the DNA to be sequenced. It was therefore decided to construct nested deletions of the cloned L1 52/55-kD gene in pCR II-L1 for use in DNA sequencing. Nested deletions are a set of deletions originating at one end of a target DNA fragment and extending various lengths along the target DNA. Each successive longer deletion brings new regions of the target DNA into sequencing range (about 300 bp for normal sequencing gels) of the primer binding site (figure 3.5).

The method for constructing nested deletions is based on the enzymatic properties of exo III, a 3' exonuclease specific for double-stranded DNA. Exonuclease III can initiate digestion at blunt ends or ends with a 5' overhang, but cannot efficiently initiate digestion at a 3' overhanging end. The protocol contained the following steps (figure 3.5): (1) cloning of target DNA into a polylinker in a suitable sequencing vector. Between the target DNA and the sequencing primer binding site there must be a recognition site for a restriction enzyme that generates a 5' overhanging end or a blunt end. Also between that restriction site and primer binding site there must be a recognition site for a restriction enzyme that generates a four base 3' overhanging end that will thus be protected from exo III digestion. The plasmid vector pCR II used for the cloning of L1 52/55-kD gene complies with these requirements. (2) double digestion of pCR II-L1 at a site adjacent to the cloned target DNA with an

enzyme that left a 5' overhanging or blunt end (SpeI) and an enzyme that left a 3' overhanging end of four bases (KpnI). (3) exo III digestion for varying lengths of time to create unidirectional digestion of the target sequence. (4) treatment with S1 nuclease (a single-stranded nuclease) to remove the 5' single strand, and repair of ends with Klenow fragment of *E. coli* DNA polymerase I; and (5) circularization by ligation with T4 DNA ligase and transformation of competent *E. coli* cells.

During exo III digestion 10 samples were taken at 1 min intervals and aliquots from each sample were analysed by agarose gel electrophoresis to check the extent of digestion (Figure 3.6). After the construction of plasmids containing nested deletions of the L1 52/55-kD gene (see section 2.6.1), and transformation of competent bacteria, one colony from each transformation was picked, plasmid DNA prepared and analysed by restriction digestion to determine the typical extent of deletion in the first 7 samples (Figure 3.7 (b) and 3.7 (c)). The restriction map of the undeleted plasmid is represented in figure 3.7(a). Figure 3.7 (b) shows that the first deletion sample contains two of the expected NsiI fragments (573 and 3747 bp) and lacks the third fragment (751 bp fragment). Instead, there is a deletion fragment of about 400 bp in length. After digestion with EcoR1 and HindIII this same DNA gives two rather than the expected three bands, one corresponding to a small fragment of 535 bp in length (between the HindIII site in the L1 52/55-kD gene and the EcoR1 site outside it), another corresponding to a large fragment of 3883 bp in length (between EcoR1 and HindIII sites in the vector); the third fragment, corresponding to the exo III deleted portion between the two HindIII sites is absent. From these results, it was concluded that the exo III deletion had also extended towards the Kpn1 protected direction (although to a much smaller extent than in the other direction) stopping just before Nsi1 site but abolishing the HindIII site (Figure 3.7 (a)). For the deletion samples 2 and 3, the exo III deletion towards the protected Kpn1 site goes beyond the Nsi1 site thus producing two bands after Nsi1 digestion (Figure 3.7 (b)).

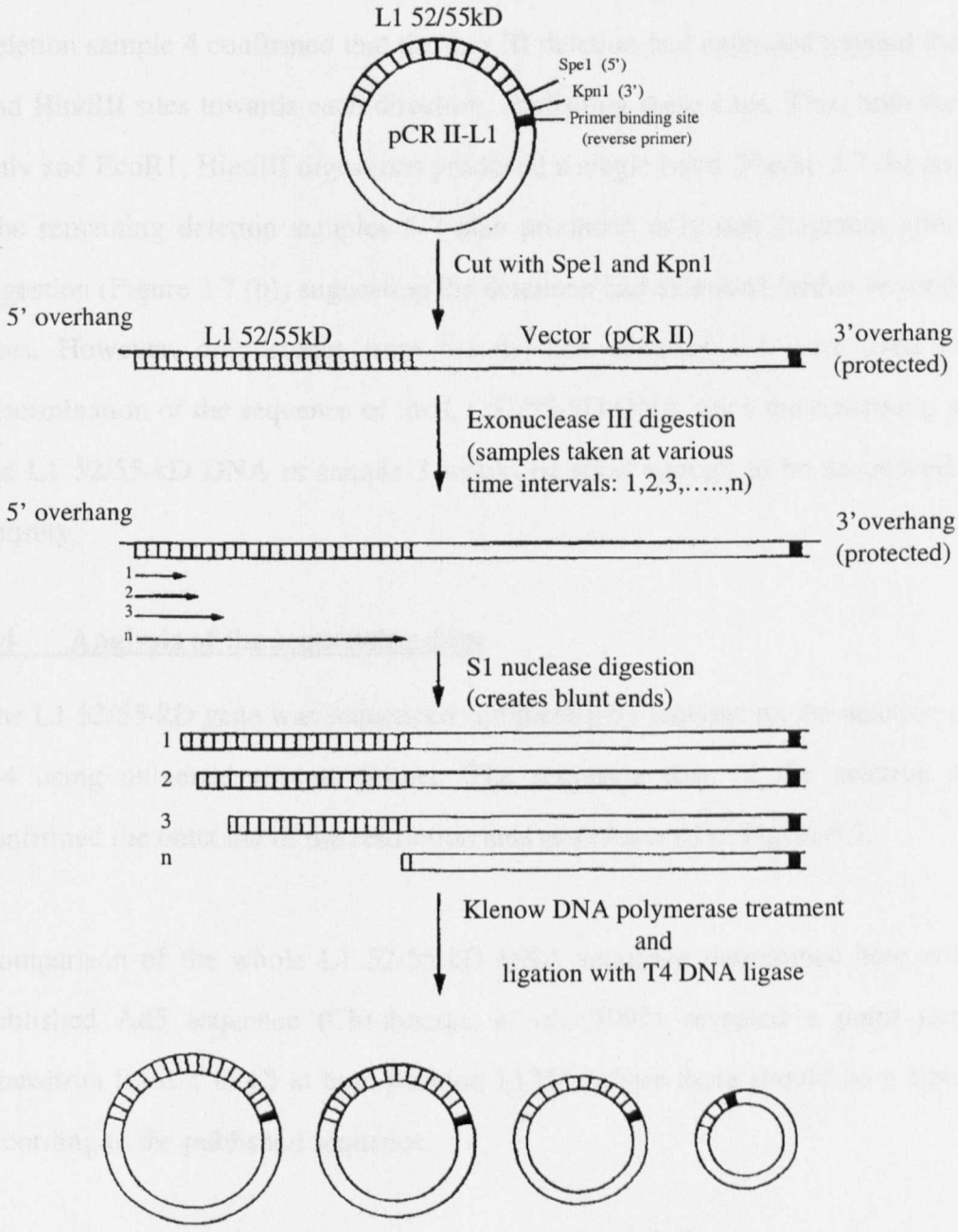


Figure 3.5 Construction of nested deletions of pCR2-L1 for sequencing
The pCR II-L1 plasmid was digested with Spe1 and Kpn1 which generated a 3' overhang next to the sequencing primer site and a 5' overhang next to the L1 52/55-kD gene. Digestion with exo III generated unidirectional deletions from the end with the 5' overhang. Treatment with S1 nuclease and repair with Klenow fragment created blunt ends from both the exo III treated ends and the 3' overhang, which had been protected from the digestion. The deleted plasmids were circularised with DNA ligase and used to transform *E. coli*.

However, although we cannot say towards which direction, it is evident that the deletion increases progressively at each sample (Figure 3.7 (c)). The results for deletion sample 4 confirmed that the exo III deletion had extended beyond the Nsi1 and HindIII sites towards each direction, abolishing these sites. Thus both the Nsi1 only and EcoR1, HindIII digestions produced a single band (Figure 3.7 (b) and (c)). The remaining deletion samples 5-7 also produced only one fragment after Nsi1 digestion (Figure 3.7 (b)) suggesting the deletions had extended further beyond these sites. However, only clones from the deletion samples 1-4 were used for the determination of the sequence of the L1 52/55-kD DNA since the remaining part of the L1 52/55-kD DNA in sample 3 would be short enough to be sequenced in its entirety.

3.4 Analysis of the sequencing data

The L1 52/55-kD gene was sequenced completely by sequencing the deletion clones 1-4 using universal reverse primer. The sequence data of the deletion clones confirmed the outcome of the restriction analysis observed in Figure 3.7.

Comparison of the whole L1 52/55-kD DNA sequence determined here with the published Ad5 sequence (Chroboczek *et al.*, 1992) revealed a point mutation (transition from T to C) at base position 11284, where there should be a Kpn1 site according to the published sequence.

In order to check whether a mutation had been caused by Taq DNA polymerase during PCR amplification or if the virus stock carried a mutation at this position, the Ad5 genome was subjected to restriction analysis by Kpn1 (Figure 3.8). Digestion of the Ad5 *wt*300 stock DNA revealed the absence of a Kpn1 site, which should be present in the L1 52/55-kD coding region according to the published sequence.

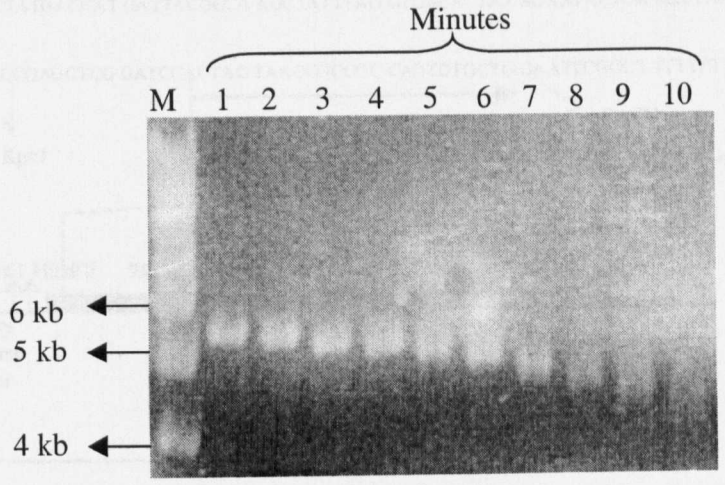


Figure 3.6 Ethidium bromide stained gel depicting exo III digestion of pCR II-L1. PCR II-L1 was cut with Spe1 and Kpn1. The linearised plasmid was then subjected to exo III digestion for 10 different time intervals, followed by S1 nuclease digestion. Approximately 1 μg of each DNA sample was loaded on the gel. The DNA fragments in the lane labelled "M" are dsDNA size markers, as indicated.

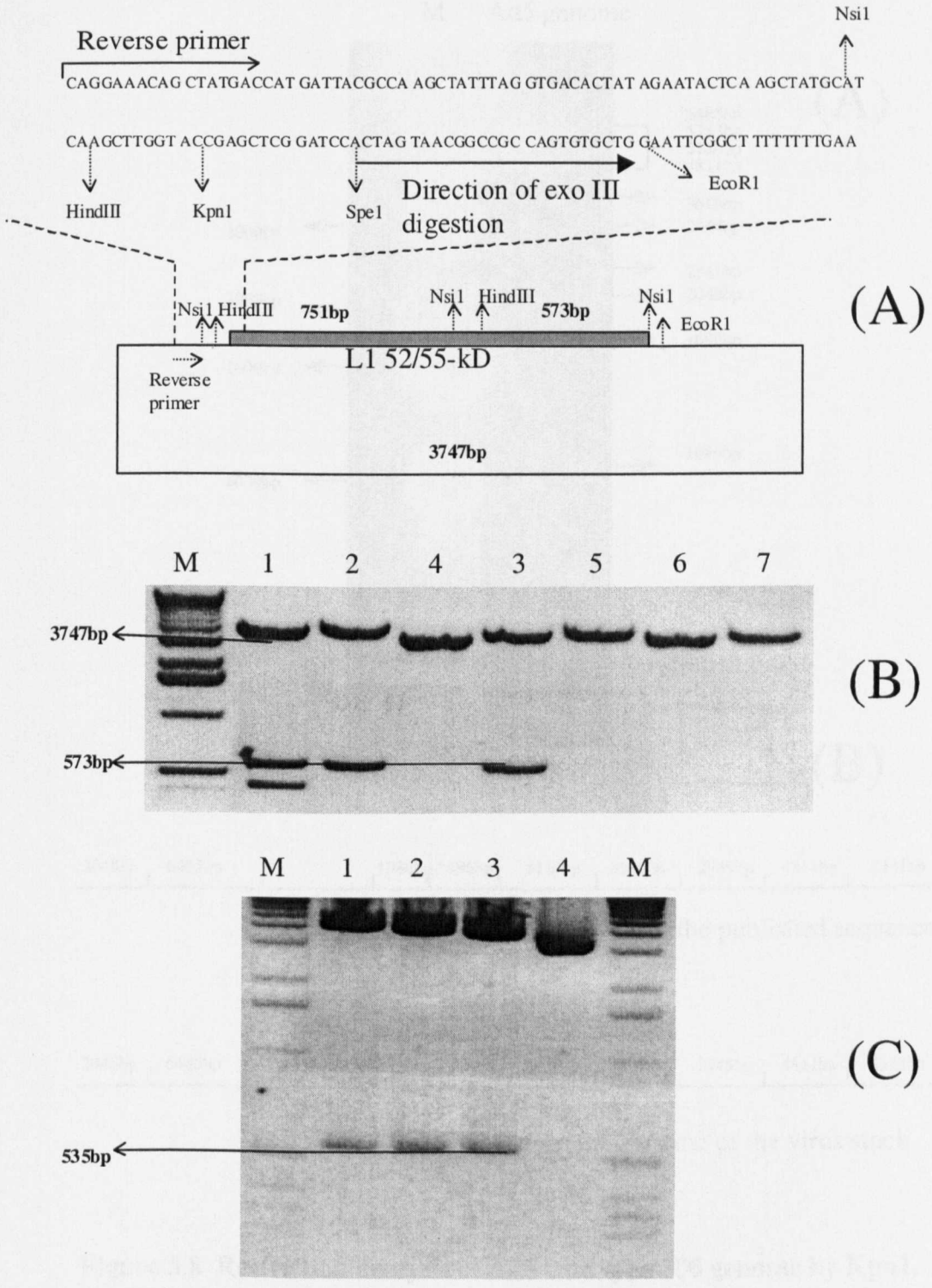


Figure 3.7. Determination of the extent of deletion in each deletion sample. (A) Schematic representation of pCR II-L1 (digested with Kpn1 and Spe1 before exo III treatment). (B) Single clones from the first seven of the deletion samples, analysed by Nsi1 digestion (see text). Lane M:Marker DNA, Lanes 1-7: deletion samples. (C) Single clones from the first four of the deletion samples analysed by EcoR1 and HindIII digestion (see text). Lanes M: Marker DNA, Lanes 1-4: deletion samples.

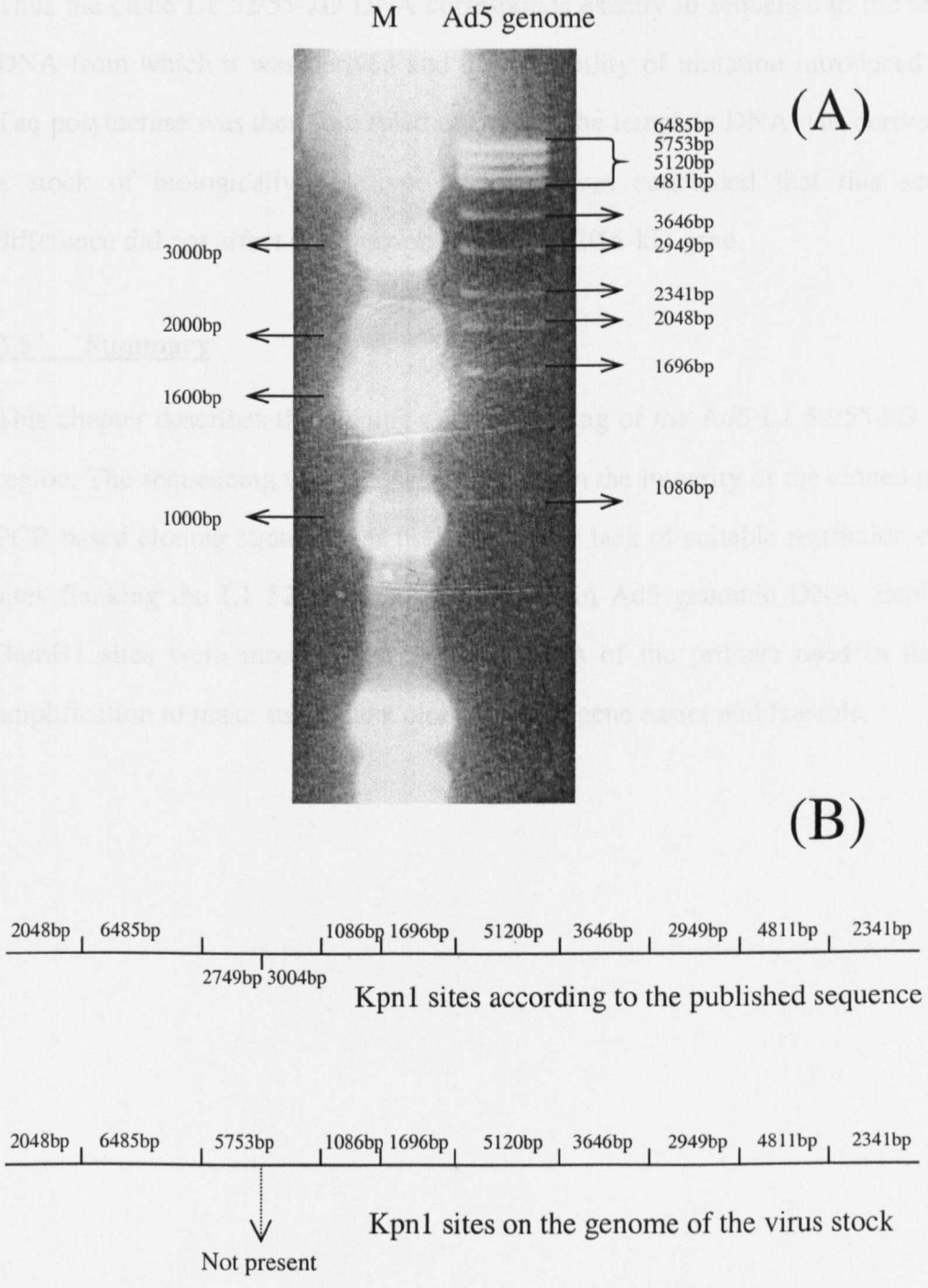


Figure 3.8 Restriction analysis of Ad5 wild type 300 genome by Kpn1.
(A) Ethidium bromide staining of Kpn1 digested Ad5 genome DNA analysed by agarose gel electrophoresis. M: molecular markers, Ad5: Ad5 genome digested with Kpn1.
(B) Schematic representation of Kpn1 sites on Ad5 genome and sizes of the predicted digested fragments (not scaled). "Not present" shows the site that should be present according to the published Ad5 sequence (Chroboczek *et al.*, 1992) but which is not present in the wt 300 DNA according to this analysis.

Thus the clone L1 52/55-kD DNA corresponds exactly in sequence to the template DNA from which it was derived and the possibility of mutation introduced by the Taq polymerase was therefore ruled out. Since the templete DNA was derived from a stock of biologically wild-type virus, it was concluded that this sequence difference did not affect the function of the L1 52/55-kD gene.

3.5 Summary

This chapter describes the cloning and sequencing of the Ad5 L1 52/55-kD coding region. The sequencing was necessary to confirm the integrity of the cloned gene. A PCR based cloning strategy was used due to the lack of suitable restriction enzyme sites flanking the L1 52/55-kD coding region in Ad5 genomic DNA. EcoR1 and BamH1 sites were incorporated at the 5' ends of the primers used in the PCR amplification to make subsequent cloning of the gene easier and feasible.

Chapter 4

Production of antibodies against adenovirus 5 L1-52/55-kD protein

4.1 Introduction

In this chapter I will explain the production of polyclonal antibodies against L1 52/55-kD protein. The aim of producing antibodies against the L1 52/55-kD in this project was to facilitate the detection of the protein by immunological methods (e.g. Western blot, immunoprecipitation, ELISA, etc.). For this purpose, I decided to express the L1 52/55 kD in *E. coli* as a GST fusion protein to be used as immunogen for the production of antibodies in rabbit.

E. coli has two characteristics that make it ideally suited as an expression system for many kinds of proteins to be used as antigens in antibody production: it is easy to manipulate and it grows quickly in inexpensive media.

In general, GST fusion proteins are soluble and are easily purified from lysed cells under nondenaturing conditions by adsorption with glutathione-agarose beads, followed by elution in the presence of free gluthathione (Smith and Johnson, 1988). If the DNA sequence of the gene coding for the protein of interest is known, this method is ideal for the expression of proteins to be used as immunogens and allows researchers to avoid expensive and time-consuming purification of the protein from cell lysates.

The basic approach used to express foreign genes as GST fusions *in E. coli* begins with insertion of the gene into an expression vector (e.g. pGEX-2T). This vector contains several elements: (1) sequences encoding ampicillin resistance (β-lactamase) that assure maintenance of the vector in the cell; (2) a controllable transcriptional promoter (P_{tac}) which, upon induction by IPTG, can produce large amounts of mRNA from the cloned fusion gene; (3) a polylinker to simplify the insertion of the gene in the correct orientation within the vector. Once constructed,

the expression vector containing the gene to be expressed is introduced into an appropriate *E. coli* strain by transformation.

Foreign polypeptides are expressed as fusions to the C terminus of glutathione-S-transferase (GST), a common 26 kD cytoplasmic protein of eukaryotes. The GST gene used to generate the pGEX vectors was originally cloned from the parasitic helminth, *Schistosoma japonicum* (Smith *et al.*, 1986). The fusion proteins typically remain soluble within the bacteria and can be purified from lysed cells because of the affinity of the GST moiety for glutathione immobilized on agarose beads. Recovery of the fusion proteins is by elution with free reduced glutathione at neutral pH.

The main advantage of this system for expressing and recovering foreign proteins from *E. coli* is that most fusion proteins remain soluble; native proteins over expressed in *E. coli* often denature and precipitate. Furthermore, denaturing conditions are not required at any stage during purification, and consequently, foreign polypeptides may retain their functional activities and antigenicity. Additional features are the efficiency and rapidity of purification, the high level of inducible expression achieved with the strong tac promoter, and the broad range of suitable bacterial hosts (because the pGEX vectors carry the *lacI* repressor allele).

The main determinant for successful purification of foreign polypeptides using the pGEX system is solubility of the fusion protein. To some extent, this can only be discovered empirically. More difficulties are encountered as the size of the desired fusion protein increases (particularly when >50 kD), or when the protein contains regions that are strongly hydrophobic or highly charged. Insoluble fusion proteins can sometimes be coaxed into solution or can otherwise be purified after solubilization in denaturing reagents (Smith, 1993).

After purification, the GST moiety can if necessary be removed from fusion proteins by cleavage with site-specific proteases at a recognition site encoded within the polylinker region of the vector. However, often the GST carrier does not compromise the antigenicity or functional activity of the foreign polypeptide. Modified versions of the original pGEX vectors have been produced that simplify cloning, cleavage or detection of fusion proteins (for review, see Smith, 1993).

4.2 Expression of the L1 protein as a GST-fusion protein

4.2.1 Cloning of L1 gene into a GST-fusion vector

In order to achieve the expression of L1 52/55-kD gene in *E. coli*, the L1 52/55-kD coding sequence was cloned into the GST fusion protein expression vector pGEX-2T (Figure 4.2). This vector expresses the cloned L1 52/55-kD as a GST fusion protein in frame with GST (see Figure 4.1 and Primer AP1 in Appendix A). The PCR amplified L1 52/55-kD gene contains a BamH1 site at its 5' end. Vector pGEX-2T also has a BamH1 site in its polylinker. The PCR-incorporated BamH1 site was designed so that cloning of L1 52/55-kD gene by the use of the BamH1 site placed it in frame with the GST coding sequence (Note that there are other pGEX vectors in which the cloning sites are present in different reading frames, so the right vector can be chosen (Pharmacia).).

The cloning was done by digestion of the plasmids pCR II-L1 and pGEX-2T with BamH1 and EcoR1 enzymes (figure 4.2). The relevant fragments from both reactions were purified from agarose gels after subjecting the reaction mixtures to agarose gel electrophoresis. The L1 52/55-kD fragment and the pGEX-2T vector backbone were ligated and the ligation mixture was used to transform *E. coli, XL1-Blue* strain. The resultant ampicillin (*amp*) resistant colonies were replica plated to kanamycin (*kan*) agar plates and growth compared with the original ampicillin plates. Colonies which were *kan*-sensitive (the ones which did not contain pCR II

backbone) were subjected to colony PCR analysis, using primers AP1 and AP2, in order to check the presence of L1 52/55-kD DNA. One colony which showed a band corresponding to the right size of 1250 bp after the PCR analysis (Figure 4.3 (a)) was picked for further analysis. Plasmid DNA from this colony was prepared in small scale and analysed by digestion with BamH1 and EcoR1 restriction enzymes (Figure 4.3 (b)). This analysis confirmed the presence of L1 52/55-kD DNA within the pGEX-2T vector.

4.2.2 Analysis of the expression and purification of the GST-L1 fusion protein

Although GST fusion proteins are usually stable in *E. coli*, if the protein coded by the insert DNA is bigger than 50 kD the fusion product can be degraded extensively due to the instability of the large protein. After the confirming that the L1 52/55-kD cDNA had been cloned into the GST fusion vector pGEX-2T, the next step was to ask whether the GST-L1 52/55-kD protein was being expressed in detectable levels in *E. coli*. To do this, an overnight culture of the strain harbouring plasmid pGEX-2T-L1 was diluted 1/100 in fresh medium and incubated until a culture density of OD_{600} 0.5 was reached. At this level the culture was divided into two and in one of the cultures, the expression of GST-L1 fusion protein was induced by the addition of 0.1mM IPTG to the medium. Samples were taken from both induced and uninduced cultures at 0 hr, 2 hr, 4 hr and 6 hr intervals and the cells in each sample were lysed by sonication. Insoluble material was separated by centrifugation and the supernatants were used for analysis on SDS-polyacrylamide gel electrophoresis (Figure 4.4). Analysis of the gel after staining with Coomassie blue showed increasing amounts of a novel, induced protein as the duration of induction increased (lanes 3 to 5). In the sample collected at 0 hr there was not any detectable level of this protein (lane 2). When compared with the marker protein molecular weights (lane 1), it can be seen that this protein had a molecular weight of around 70 to 75 kD. This was roughly equal to the size of the expected GST-L1 (26 kD + 48 kD) fusion protein. The same protein was seen in the uninduced sample

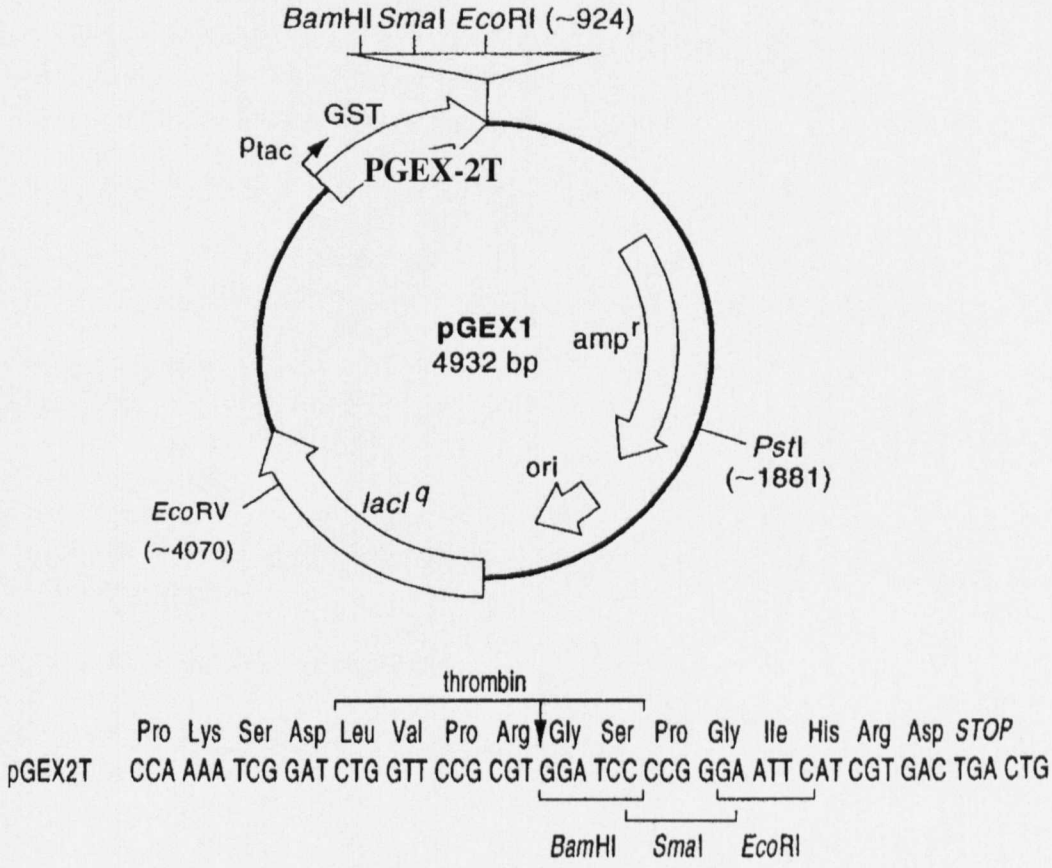


Figure 4.1 GST fusion protein expression vector.

PGEX-2T is a fusion protein expression vector that expresses a cloned gene as a fusion protein to GST. The *lac* repressor (product of the *lacI* gene) binds to the P_{tac} promoter, repressing the expression of GST fusion protein. Upon induction with IPTG, derepression occurs and GST fusion protein is expressed. The gene of interest can be inserted into the polylinker located at the end of the GST gene. The polylinker sequence is shown below the map of pGEX-2T, where the restriction endonuclease and protease cleavage sites are bracketed.

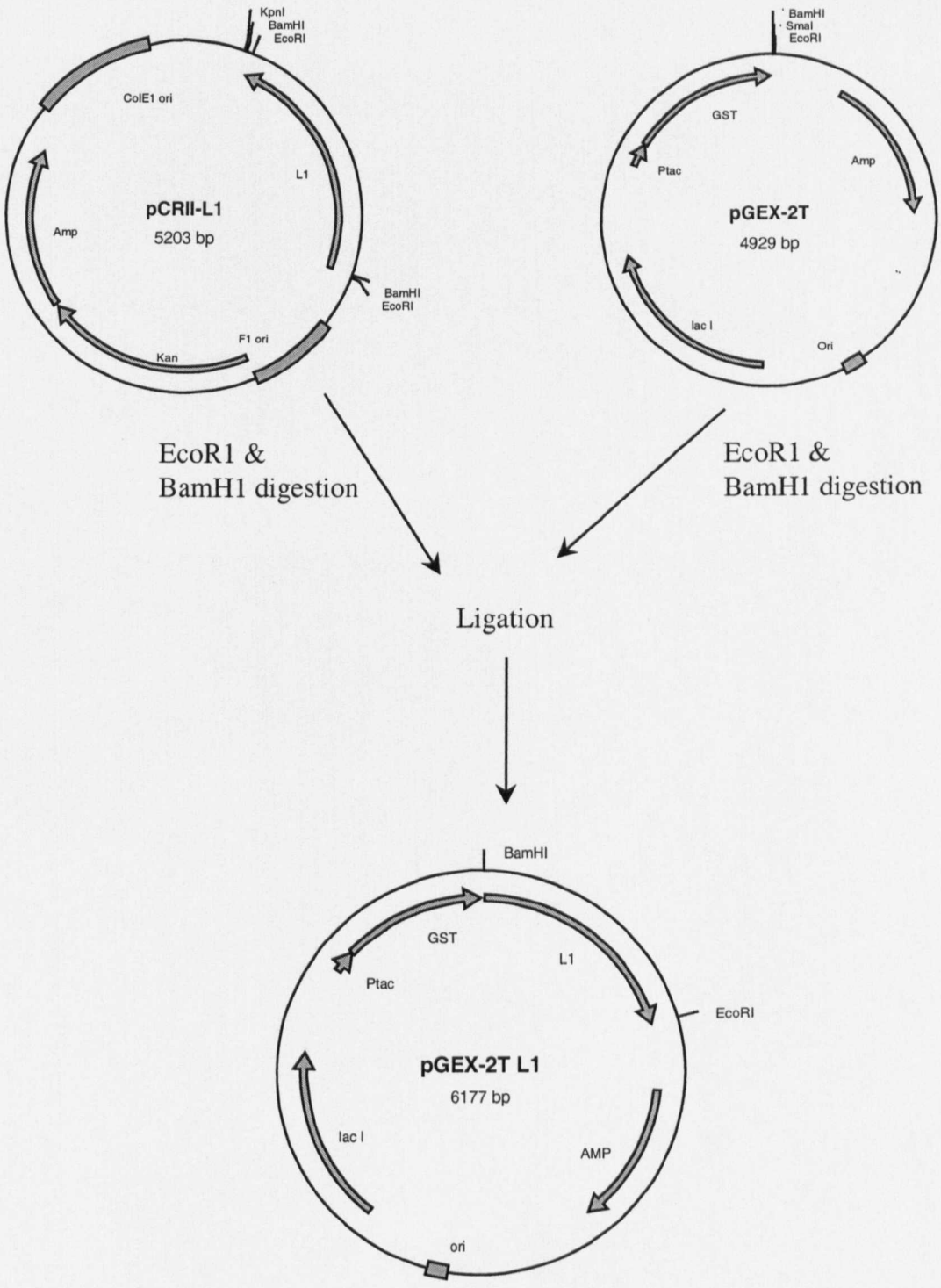


Figure 4.2 Cloning of L1 52/55-kD into GST fusion protein expression vector pGEX-2T.

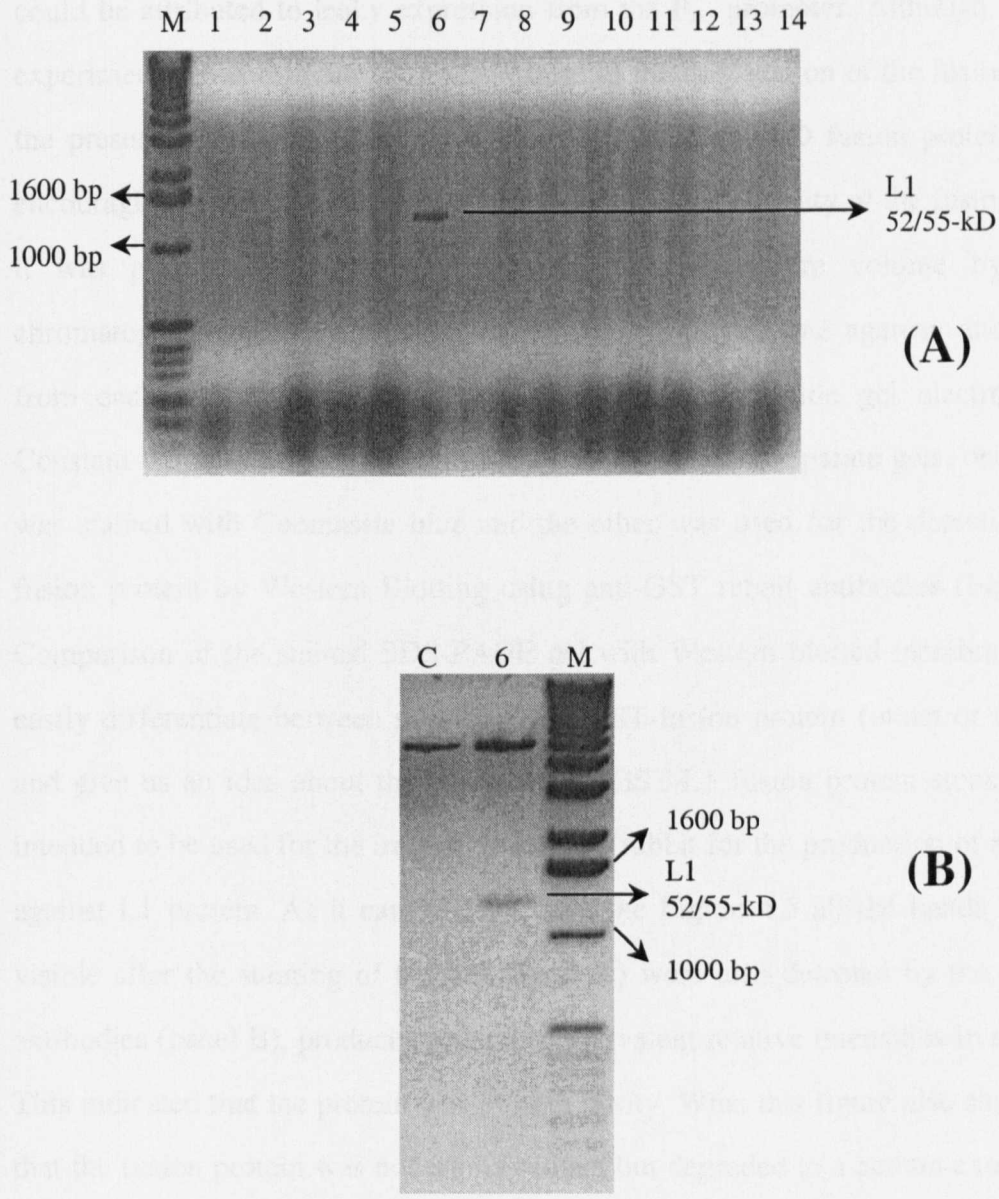


Figure 4.3 PCR and restriction analysis of transformants for pGEX-2T-L1.

(A) PCR analysis of *kan*-sensitive colonies by primers Ap1 and Ap2. M: molecular markers, 1-14: transformants analysed.

(B) Restriction analysis of plasmid DNA from transformant 6 by enzymes EcoR1 and HindIII. M: molecular markers, 6: transformant 6, c: control DNA pGEX-2T.

collected at 6 hr post-induction (lane 6), however, at a much lower amount. This could be attributed to leaky expression from the P_{tac} promoter. Although from this experiment it was difficult to say the extent of the degradation of the fusion protein, the presence of detectable levels of the GST-L1 52/55-kD fusion protein was an encouraging result. In order to investigate in detail the stability of the fusion protein, it was purified in large scale from a 500 ml culture volume by affinity chromatography (see materials and methods) on glutathione agarose and aliquots from each fraction were analysed by SDS-polyacrylamide gel electrophoresis. Constant volumes of each fraction were analysed on two separate gels; one of these was stained with Coomassie blue and the other was used for the detection of the fusion protein by Western Blotting using anti-GST rabbit antibodies (Figure 4.5). Comparison of the stained SDS-PAGE gel with Western blotted membrane would easily differentiate between non-GST and GST-fusion protein (intact or degraded) and give us an idea about the purity of the GST-L1 fusion protein stock that was intended to be used for the immunization of a rabbit for the production of antibodies against L1 protein. As it can be seen from the Figure 4.5 all the bands that were visible after the staining of the gel (panel A) were also detected by the anti-GST antibodies (panel B), producing bands of equivalent relative intensities in each case. This indicated that the protein was of high purity. What this figure also showed was that the fusion protein was not entirely intact but degraded to a certain extent. Since the bands smaller than the expected fusion protein were also detected by the anti-GST antibodies, they were concluded to be degradation products of the GST-L1 52/55-kD fusion protein.

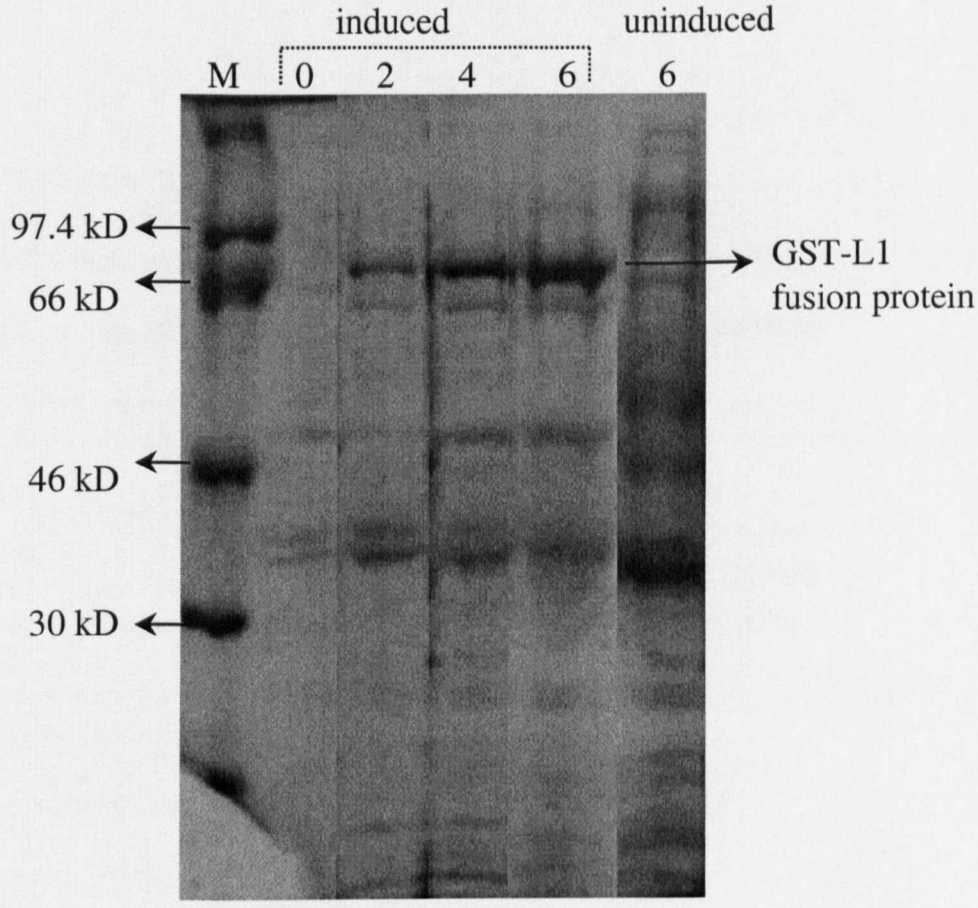


Figure 4.4 Analysis of the expression of GST-L1 fusion protein
M: molecular size markers, induced: samples taken after the induction of the P_{tac} promoter by IPTG, Uninduced: sample taken before the induction. numbers indicate the time (in hours) intervals the samples were taken post induction

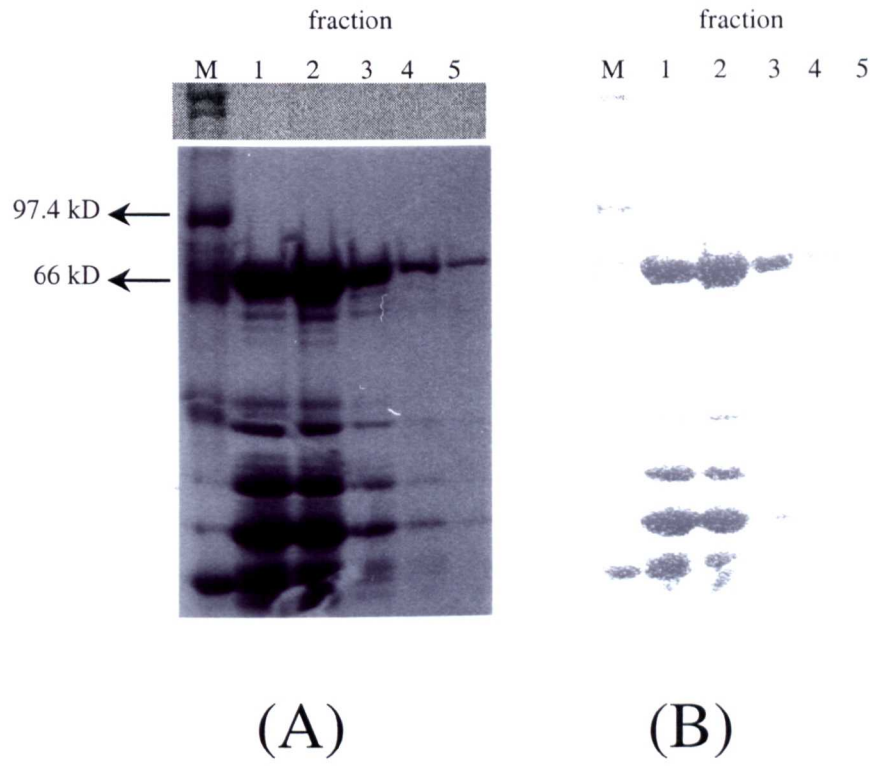


Figure 4.5 Large-scale purification of GST-L1 52/55-kD fusion protein by affinity chromotography.
Aliquots from each fraction collected during elution from glutathione-agarose were analysed by SDS-PAGE and proteins detected by Coomassie blue staining(A) and by Western Blotting using anti-GST antibodies (B).
M: Molecular size markers, 1-5: eluted fractions from affinity chromotography

4.3 Antibody production

The typical kinetics of the development of a specific antibody response upon immunization with antigen is illustrated in Figure 4.6. After the primary immunization, naive B cells are stimulated to differentiate into antibody-secreting plasma cells. For most soluble protein antigens, specific antibody begins to appear in the serum 5 to 7 days after the animal is injected. The antibody concentration (titer) continues to rise and peaks around day 12, after which it decreases (Klinman and Press, 1975).

In addition to differentiating into antibody-forming cells, the antigen-stimulated B cells proliferate to form a large population of "memory" B cells, which quickly become activated after further antigen is administered. Thus, the lag period before the appearance of the specific antibody is much shorter after a booster injection than that observed for the initial immunization. In addition, a significantly higher titer of specific antibody is achieved and is sustained for a longer period of time. The peak of antibody production occurs 10 to 14 days after boosting. As a consequence of the existence of the memory B cells, less antigen is required to stimulate a strong secondary response. Memory B cells are long-lived, therefore a specific antibody response can be elicited 6 months to a year after the last booster. Finally, the average affinity and degree of specificity of the antibody population for the antigen increase with repeated immunizations (Klinman and Press, 1975).

Adjuvants administered with an antigen dose greatly enhance the specific antibody titer, as they allow the antigen to be released slowly, ensuring the continual presence of antigen to stimulate the immune system. Freund's adjuvant is most commonly

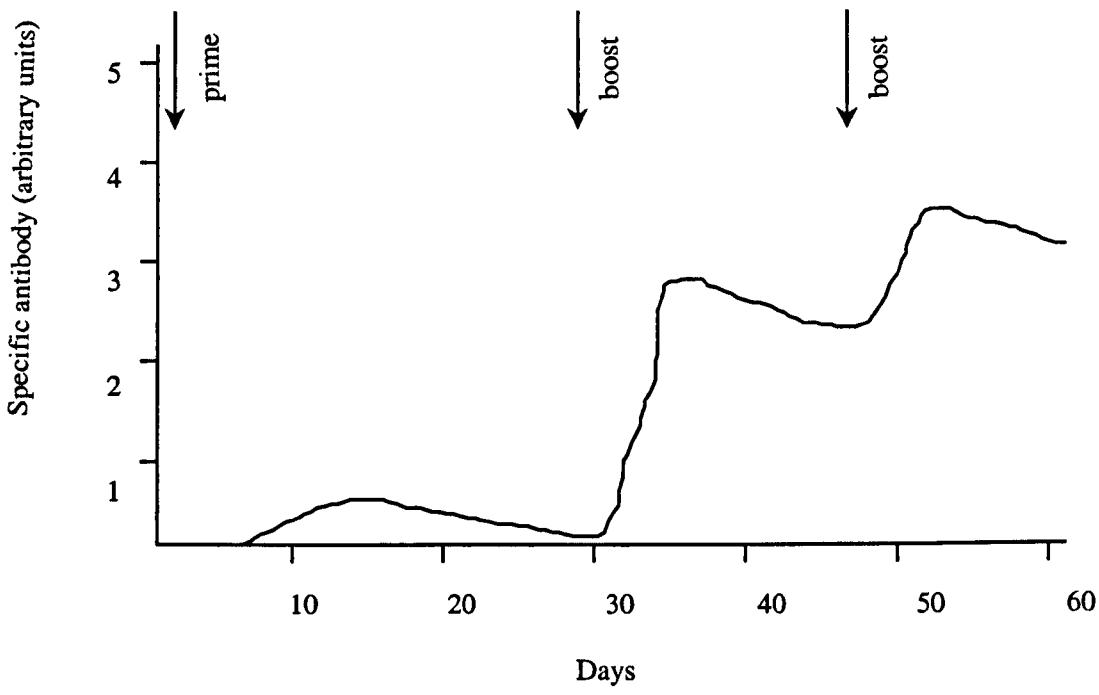


Figure 4.6 Typical kinetics of development of a specific antibody response.
Arrows indicate the timing of priming and boosting immunization. Actual amounts of specific antibody produced will vary considerably depending on immunogenicity of the protein.

used in the preparation of antisera because it induces a high, long-lasting antibody titer that is often still measurable 25 weeks or more after boosting. The presence of mycobacteria in complete Freund's adjuvant non-specifically activates the T-cell population, ensuring that lymphokine production is maximal for B-cell stimulation. Complete Freund's adjuvant may cause granulomas, however, and can therefore be used only for primary immunization. Incomplete Freund's adjuvant, lacking the mycobacteria is adequate for booster injections.

4.3.1 Immunization of rabbit against GST-L1 fusion protein

Polyclonal antiserum was raised against GST-L1 52/55-kD fusion protein by subcutaneous injection into an outbred half-lop rabbit. Approximately 300 μg of purified fusion protein, in Freund's complete adjuvant, was used for the initial immunization, which was followed by four successive booster immunizations with 300 μg of protein in Freund's incomplete adjuvant in each case. Serum was obtained before each immunization and tested for GST-L1 52/55-kD by indirect ELISA (Figure 4.7). Specific antibodies were detected by coating the wells of a microtiter plate with antigen (GST-L1 52/55-kD fusion protein), incubating the coated plates with dilutions of serum samples obtained before each immunization, and washing away unbound antibodies. A solution containing biotinylated anti-rabbit IgG antibodies was then added onto each well. After incubation, unbound anti-rabbit antibodies were washed away and detection was performed by the addition of the substrate for HRP which produces a colour upon hydrolysis of the substrate. The hydrolyzed substrate amount, which was assessed with a spectrophotometer, is proportional to the amount of specific antibody in the test solutions. The results presented in Table 4.1 clearly showed a reactivity between the collected antisera and the GST-L1 52/55-kD fusion protein. The reactivity increased further in each collected sample until week 12 of the immunisation schedule. For the sample collected at week 16, the reactivity was comparable to the levels seen for the week

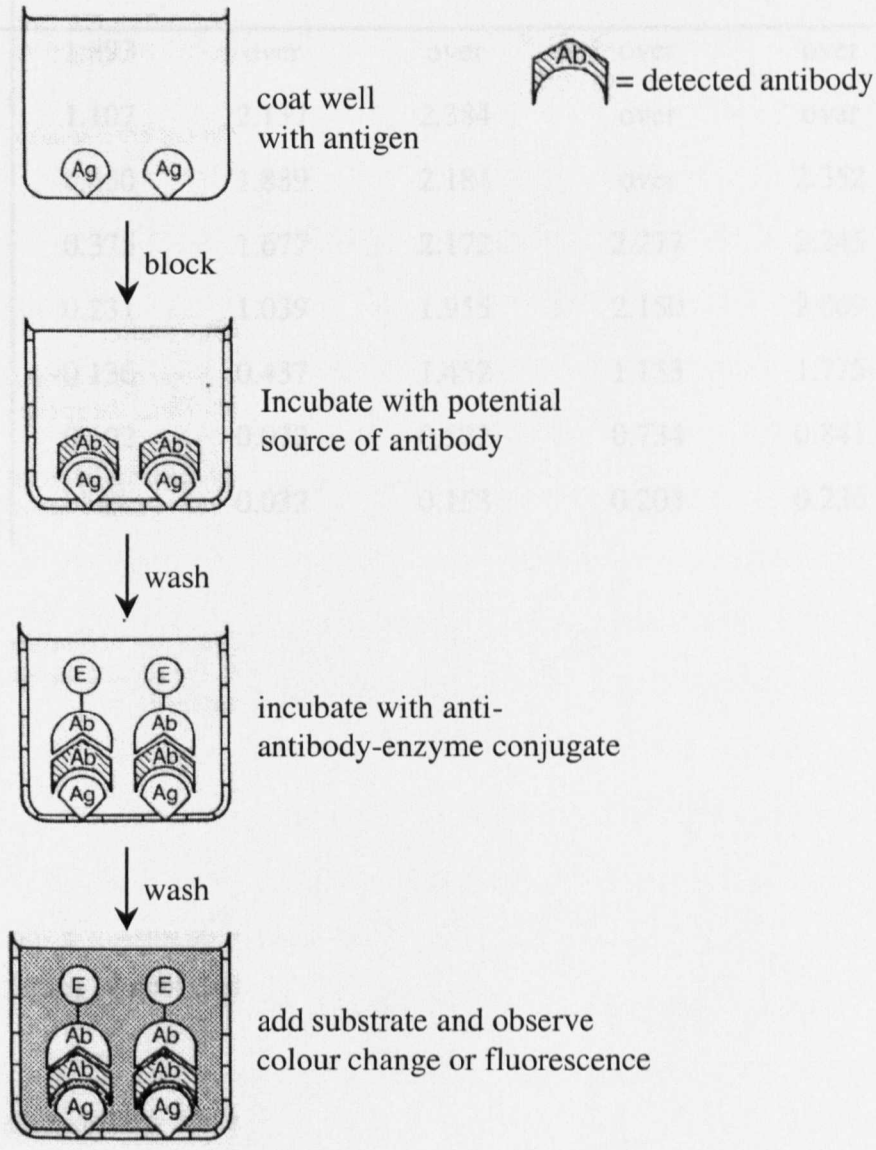


Figure 4.7 Indirect ELISA to detect specific antibodies.
Ag=antigen; Ab=antibody; E=enzyme

Dilution factor	Pre bleed	post 4 weeks	post 8 weeks	post 12 weeks	post 16 weeks
1:10	1:893	over	over	over	over
1:50	1.107	2.137	2.384	over	over
1:250	0.650	1.889	2.184	over	2.352
1:1250	0.375	1.677	2.172	2.277	2.245
1:6250	0.231	1.039	1.955	2.150	2.069
1:31250	0.136	0.437	1.452	1.133	1.775
1:156250	0.102	0.072	0.522	0.734	0.841
1:7801250	blank	0.032	0.158	0.203	0.236

Table 4.1 Indirect ELISA test on sera collected after immunisation with GST-L1 52/55-kD protein.

Specific antibodies bound to GST-L1 52/55-kD fusion protein were detected with biotinylated anti-rabbit antibodies and streptavidin-HRP complex. After addition of a colourimetric substrate for HRP, absorbance was read at 490nm.

12 sample, suggesting that the maximum specific antibody levels had been reached.

4.3.2 Immunoprecipitation of L1 protein from Ad5 infected 293 cells

Although the antisera collected from the immunized rabbit were shown to react with the GST-L1 52/55-kD fusion protein by ELISA, this result could be due to the presence of anti-GST antibodies reacting with the GST part of the fusion protein. In order to confirm the presence of antibodies reactive against the L1 52/55-kD protein, adenovirus infected and mock-infected 293 cells were radiolabelled with ^{35}S-methionine and all the cell extracts were immunoprecipitated with either pre-immune or 8 weeks post-immunization serum (figure 4.8). As a positive control for the determination of Ad5 infection, ^{35}S-methionine labelled Ad5 or mock infected 293 cells were immunoprecipitated by monoclonal antibodies against Ad5 DBP (Reich *et al.*, 1983). The result of this immunoprecipitation experiment indicated the presence of a band corresponding to L1 52/55-kD protein, only in the Ad5-infected cell extracts when immunoprecipitated with the immune serum. Two separate bands corresponding to differentially phosphorylated forms of the L1 protein as previously reported (Lucher *et al.*, 1986) (52 kD and 55 kD respectively) were not distinguishable in this autoradiograph. This was most probably due to the high amount of each protein making the two bands appear as a large single band. Alternatively, the labelling protocol used may have differentially labelled the newly synthesised, under-phosphorylated form.

4.4 Summary and Discussion

In this chapter I have described the production of polyclonal antiserum against the Ad5 L1 52/55-kD protein. I chose to express the L1 52/55-kD protein as a GST fusion protein in *E. coli* by using a suitable GST fusion protein expression vector (pGEX-2T). After its purification, the GST-L1 52/55-kD fusion protein was used for the immunisation of a rabbit and serum collected prior to each booster immunizations, with four weeks time intervals. This approach yielded a high titre

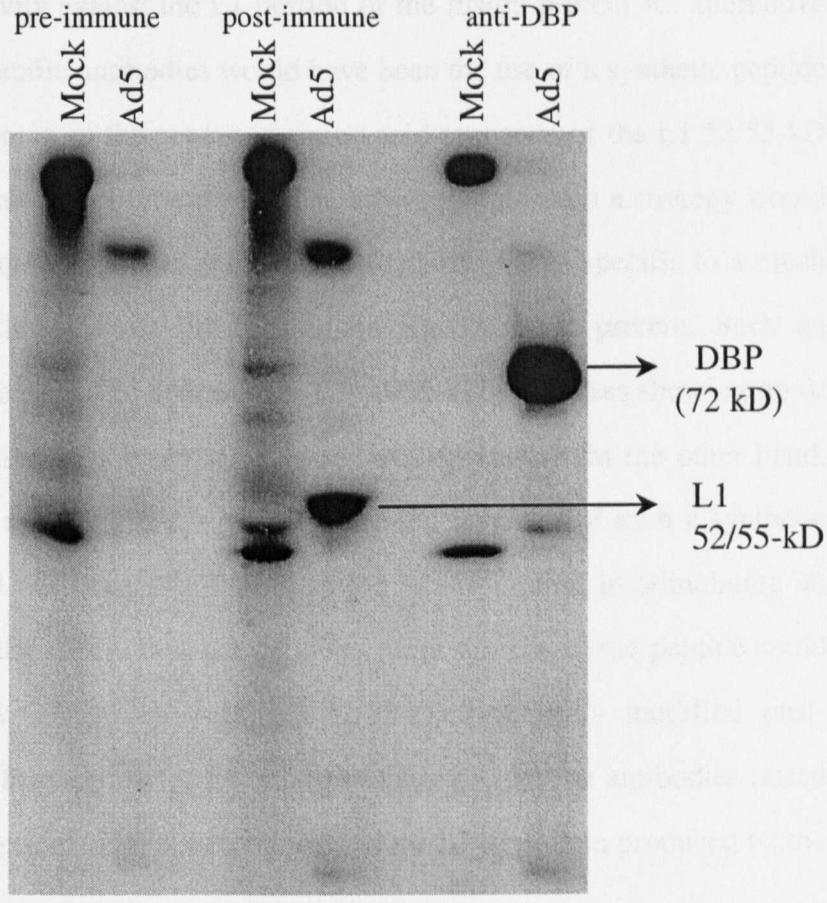


Figure 4.8 Immunoprecipitation of L1 52/55-kD protein from Ad5-infected cells.

Ad5 or mock-infected 293 cells were radiolabelled with ^{35}S-methionine and total cell extracts immunoprecipitated by using either anti-GST-L1 52/55-kD pre-immunisation serum or anti-GST-L1 52/55-kD 8 weeks post-immunisation serum or anti-DBP monoclonal antibodies. Immunoprecipitation by anti-DBP monoclonal antibodies was done as a positive control to determine Ad5 infection in the experiment .

antiserum with reactivity against the L1 portion of the fusion protein An alternative method for raising specific antibodies would have been the use of a synthetic peptide corresponding to a region of the predicted amino acid sequence of the L1 52/55-kD protein for the immunisation of the rabbit. The advantages of such a strategy would be its shorter time requirement and the production of antibodies specific to a much smaller number of the epitopes present on the L1 52/55-kD protein. Such an antiserum would be expected to contain anti-L1 52/55-kD antibodies showing no or little specific interaction with other viral or non-viral proteins. On the other hand, due to the small and relatively much less complicated structure of such a synthetic peptide, it might had not been as efficient as the whole protein in stimulating an immune response in the rabbit, thus the antibody titers specific to the peptide could have been very low. Since the Ad5 L1 52/55-kD protein is modified post-translationally in the host cell by differential phosphorylation, the antibodies raised against a synthetic peptide might not recognize the modified protein produced by the infected host cell.

Chapter 5

Construction of cell lines expressing adenovirus 5 late genes

5.1 Introduction

Construction of adenoviruses deficient in viral genes responsible for the replication and growth of virus requires the use of either an helper virus or complementing cell lines expressing the missing viral genes. The use of a helper virus is generally not desired due to the high risks of contamination of the recombinant virus stocks with the helper virus. In this chapter, I will describe the construction of complementing cell lines with the aim of producing replication-deficient Ad5 recombinants deficient in one or more late genes.

In previously published work, an Ad5 pIX-deficient mutant was constructed and complemented by a 293 cell line expressing the Ad5 pIX protein (Caravokyri and Leppard, 1995). Since the aim was to produce highly deleted Ad5 recombinant virus deficient in L1 52/55-kD and/or other late genes, it was decided to make complementing cells lines expressing Ad5 L1 52/55-kD, L1 52/55-kD plus pIX and L1 52/55-kD plus IV_{a2} genes in order to be used to construct only L1 52/55-kD, both L1 52/55-kD and pIX and both L1 52/55-kD and IV_{a2} deleted viruses respectively. The planned strategy was first to construct the L1 52/55-kD deleted virus. Upon construction of such a virus the next step would be to construct viruses containing the deletions of L1 52/55-kD with pIX and L1 52/55-kD with IV_{a2} genes.

There are two widely used methods for the constitutive expression of proteins in eukaryotic cell lines. The first one of these methods involves the integration of DNA containing an expression cassette of the desired protein into the host cell genome. By this way, the integrated copy of the gene expressing the desired protein is maintained in a stable form and passed onto to next generation of cells without being lost. However, the amount of protein produced from the integrated expression cassette is variable and was anticipated to be too low for effective complementation of adenoviruses deficient in functions normally expressed to high levels late in

infection, due to the presence of a single copy of the expressed gene in each cell. The second method involves the episomal expression of the desired gene in the nucleus of the transfected cells. In this method, the gene to be expressed is cloned into a Epstein Barr Virus (EBV)-based plasmid vector which is maintained as multiple copies/cell by episomal replication in the host cell nucleus, hence expressing high levels of recombinant proteins.

EBV based vectors are maintained extrachromosomally in primate and canine cell lines (Chittenden, *et al.*, 1989) and offer the following features (Figure 5.1): ColE1 origin of replication and ampicillin-resistance gene for growth selection in *E.coli*; the EBV origin of replication (*ori*P) and nuclear antigen gene (EBNA-1) to allow high copy episomal maintenance; a selectable marker gene (e.g. hygromycin-resistance) for stable maintenance of the vector; a multiple cloning site flanked by a strong promoter and a polyadenylation signal for the cloning and expression of the recombinant protein. *Ori*P and EBNA-1 are necessary for the extrachromosomal maintenance of the plasmid in mammalian cells. EBNA-1 protein acts as both an enhancer and an activator of the EBV *ori*P. Cells transfected with such a plasmid (e.g. pMEP 4) can be selected by growing them in a medium containing the appropriate drug (e.g. hygromycin). Generally, as the concentration of the drug used for the selection of the plasmid increases, the copy number of the plasmid increases and higher levels of recombinant protein expression are achieved. The highest possible drug concentrations that do not affect the cell growth have to be determined experimentally for different types of cell lines.

5.2 Constitutive episomal expression of the Ad5 L1 gene in 293 cells

To express L1 52/55-kD from a strong promoter, the L1 52/55-kD open reading frame from plasmid pCR II-L1 was cloned downstream of the human cytomegalovirus immediate early (CMV-IE) promoter in an episomal expression vector, pMEP-CMV (figure 5.5). This is a very strong constitutive promoter and

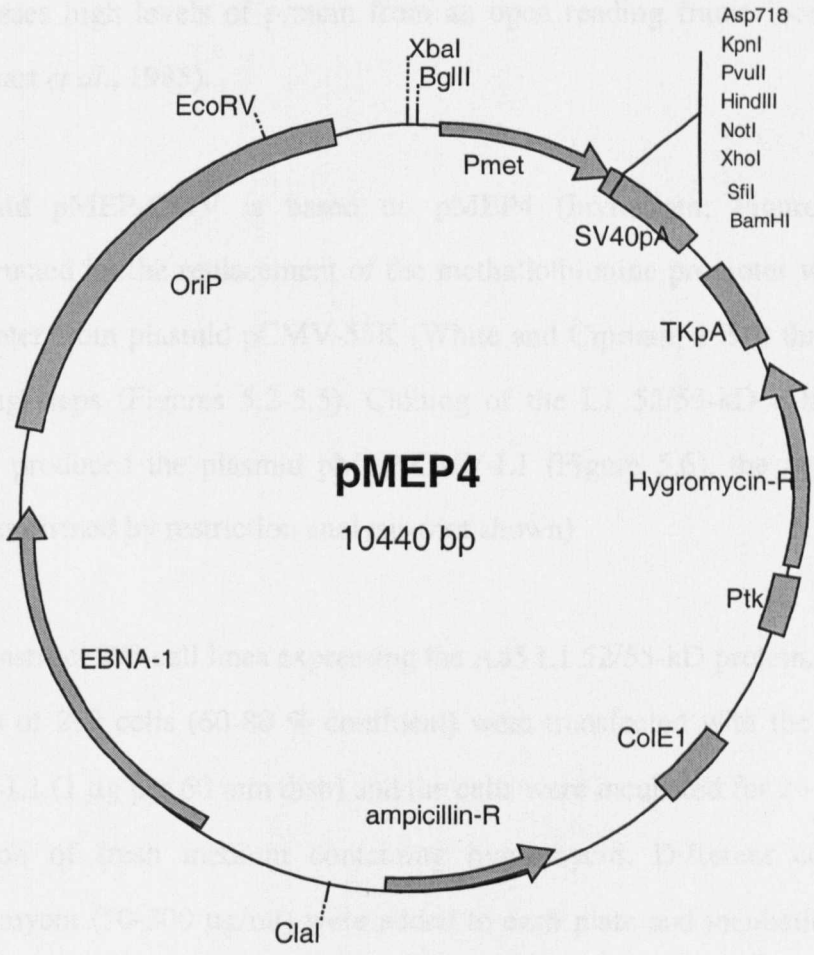


Figure 5.1 pMEP4: An EBV-based episomal mammalian expression vector (Invitrogen)

pMEP9 is maintained extrachromosomally in primate and canine cell lines (Chittenden *et al.*, 1989) and offers the following features: ColE1 origin of replication and ampicillin resistance gene for growth selection in E.coli; the EBV origin of replication (*ori*P) and nuclear antigen (EBNA-1) to allow high copy episomal expression; a selectable marker (hygromycin-resistance gene) flanked by a promoter and a polyadenylation signal sequence for stable maintenance of the vector ; a MCS flanked by methallothionine promoter and another polyadenylation signal for the cloning and expression of the recombinant protein.

expresses high levels of protein from an open reading frame located downstream (Boshart *et al.*, 1985).

Plasmid pMEP-CMV is based on pMEP4 (Invitrogen; Figure 5.1) and was constructed by the replacement of the methallothionine promoter with the CMV-IE promoter from plasmid pCMV-55K (White and Cipriani, 1990) through a series of cloning steps (Figures 5.2-5.5). Cloning of the L1 52/55-kD cDNA into pMEP-CMV produced the plasmid pMEP-CMV-L1 (Figure 5.6), the structure of which was confirmed by restriction analysis (not shown).

To construct 293 cell lines expressing the Ad5 L1 52/55-kD protein, multiple 60 mm dishes of 293 cells (60-80 % confluent) were transfected with the plasmid pMEP-CMV-L1 (1 μg per 60 mm dish) and the cells were incubated for 24 hours before the addition of fresh medium containing hygromycin. Different concentrations of hygromycin (50-300 μg/ml) were added to each plate and incubation continued for several weeks (replacing the medium in each dish regularly with fresh medium containing hygromycin) until stable cell lines growing at a rate comparable to wild type 293 cells (i.e. could be passaged at a 1 in 4 area split every 3-4 days) were established.

To check the level of expression of the L1 52/55-kD protein in each cell line grown in the presence of different concentrations of hygromycin, cell lysates from 60 mm tissue culture dishes were analysed by Western blotting using polyclonal antibodies raised against L1 52/55-kD. Cells lines grown in the presence of 200 μg/ml hygromycin showed the highest amount of L1 52/55-kD protein expression (data not shown). Propagation of this cell line was continued, and upon confirmation of the expression by another Western blot (Figure 5.7), the cell line was named 293-L1 cell line. As it can be seen in the Figure 5.7, the L1 52/55-kD protein expression in 293-L1 cells is at comparable levels to the expression in Ad5 infected 293 cells lysed at

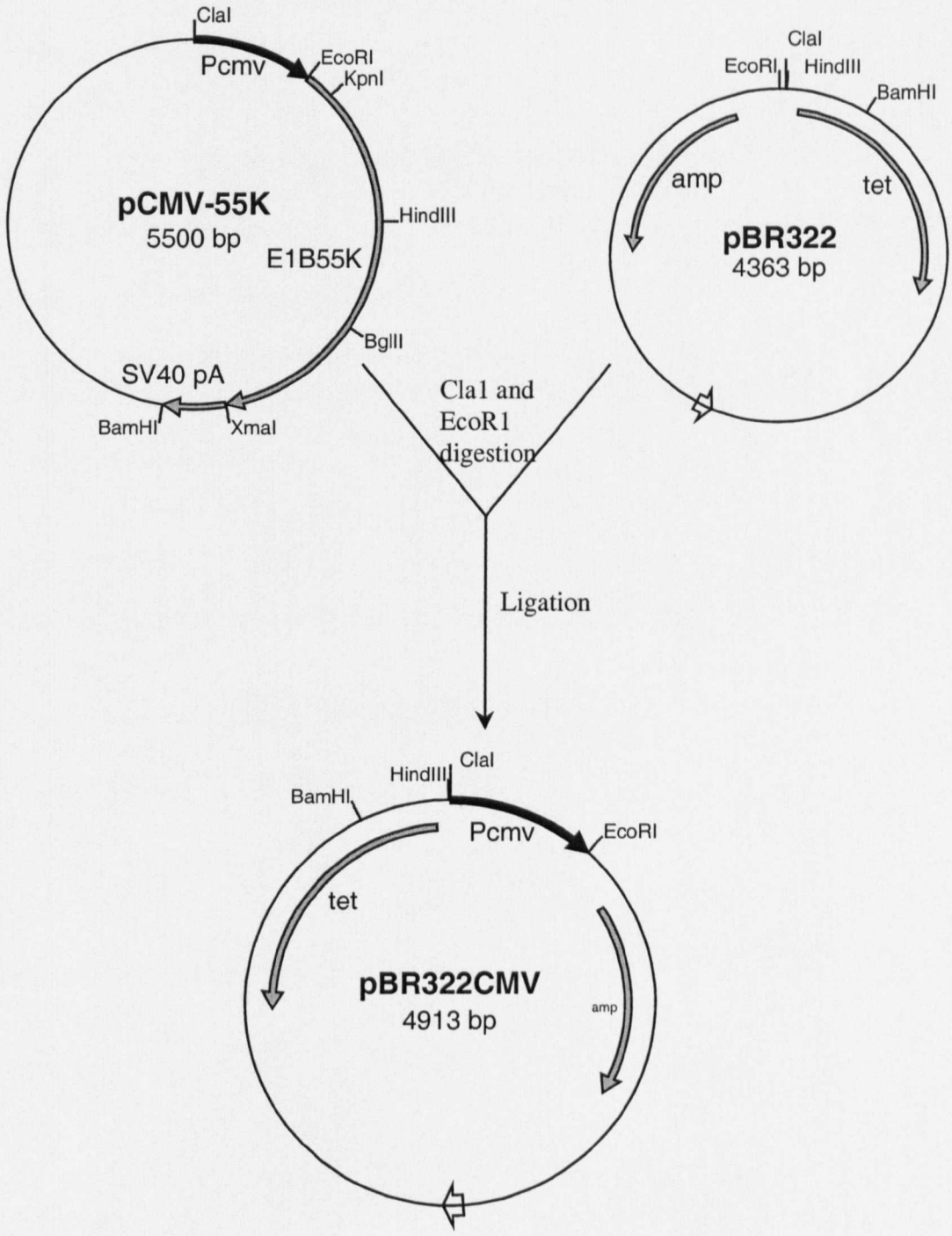


Figure 5.2 Construction of pMEP-CMV (step 1)
The CMV promoter from pCMV-55K was cloned as a Cla1-EcoR1 fragment into pBR322.

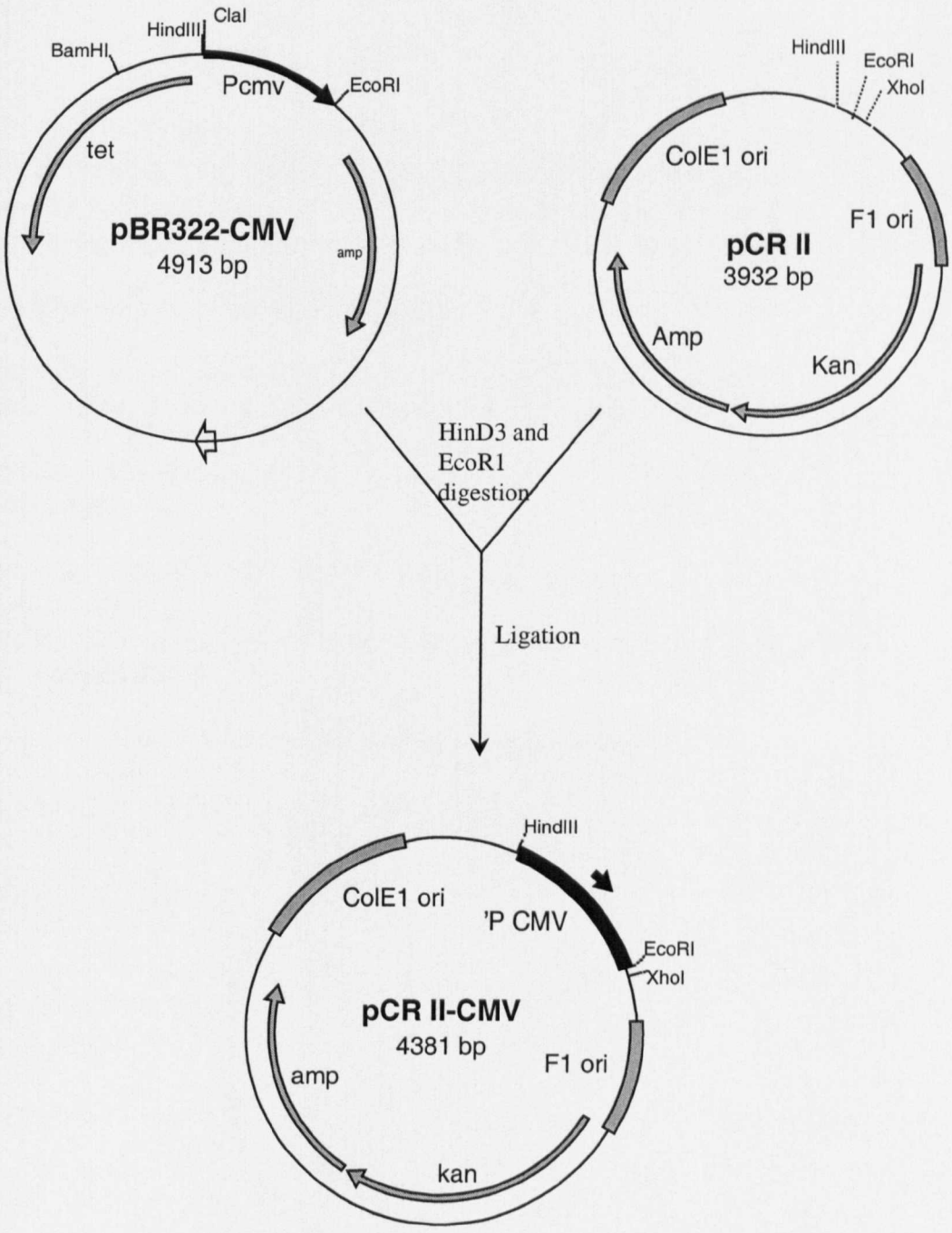


Figure 5.3 Construction of pMEP-CMV (step 2)
The CMV promoter from pBR322-CMV was cloned as a HindIII-EcoRI fragment into pCR II.

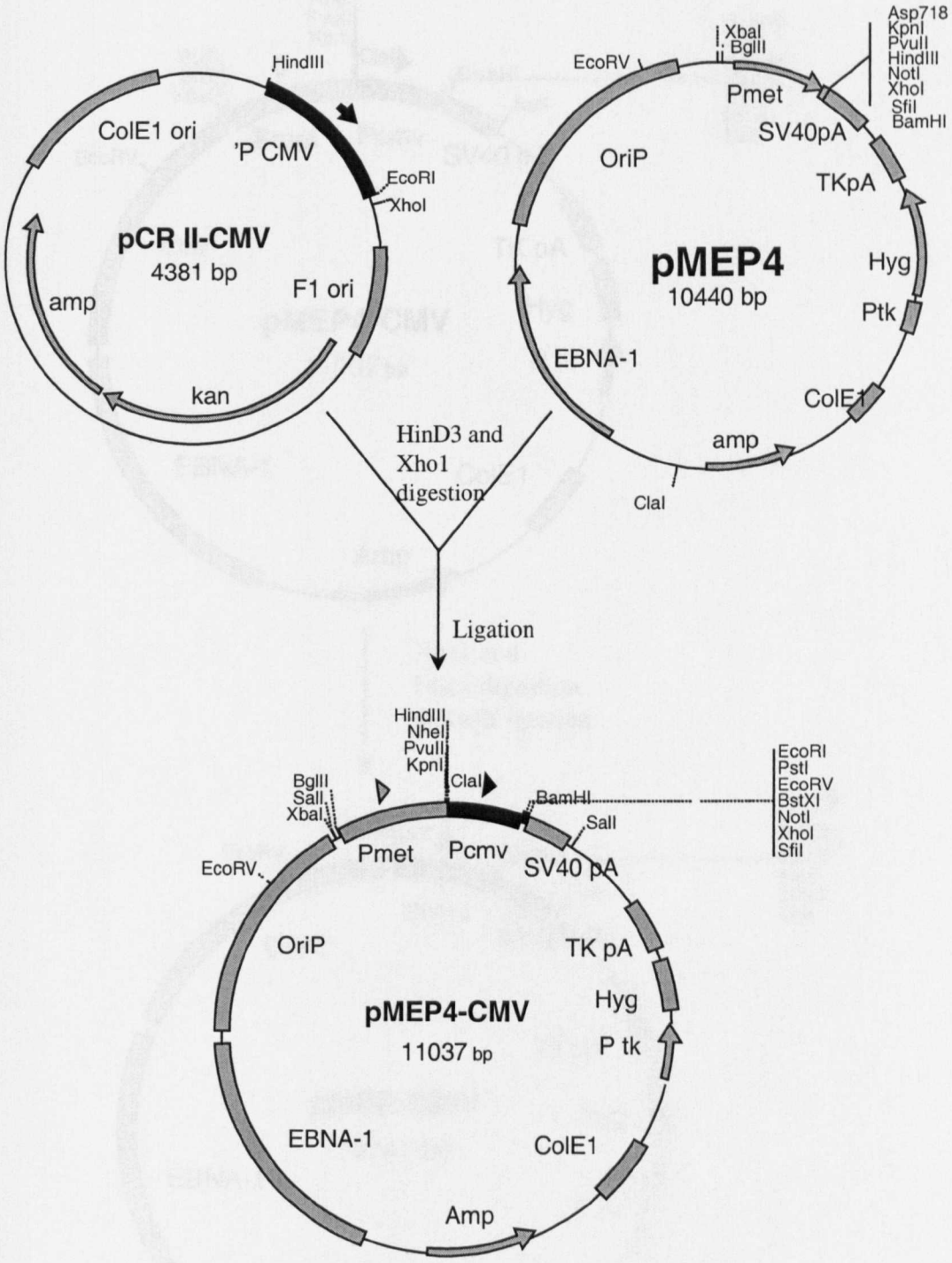


Figure 5.4 Construction of pMEP-CMV (step 3)
The CMV promoter from pCR II-CMV was cloned as a HindIII-Xho1 fragment into pMEP4.

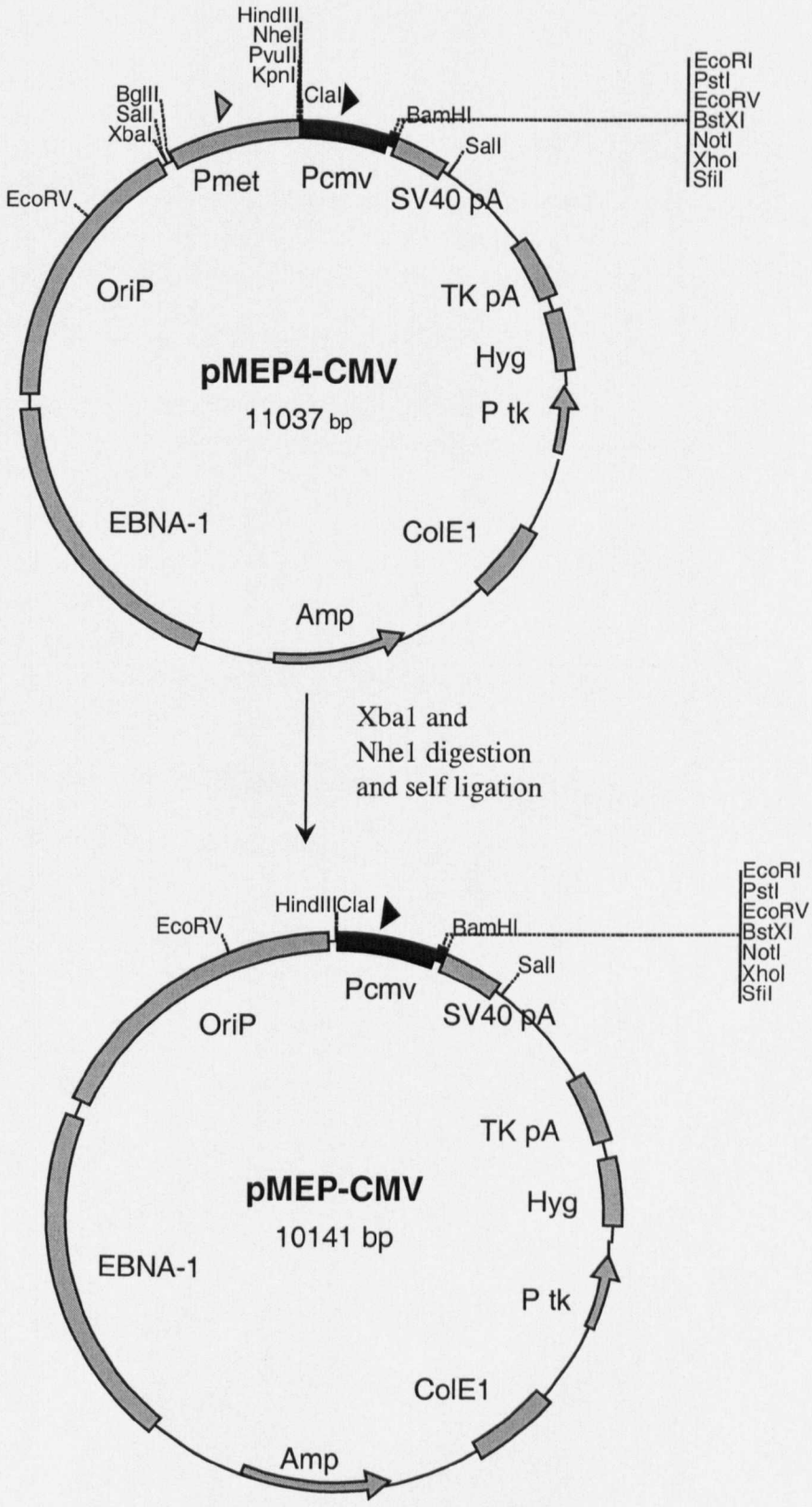


Figure 5.5 Construction of pMEP-CMV (step 4)
Deletion of the P_{met} promoter from pMEP4-CMV by Xba1 and Nhe1 digestion and religation.

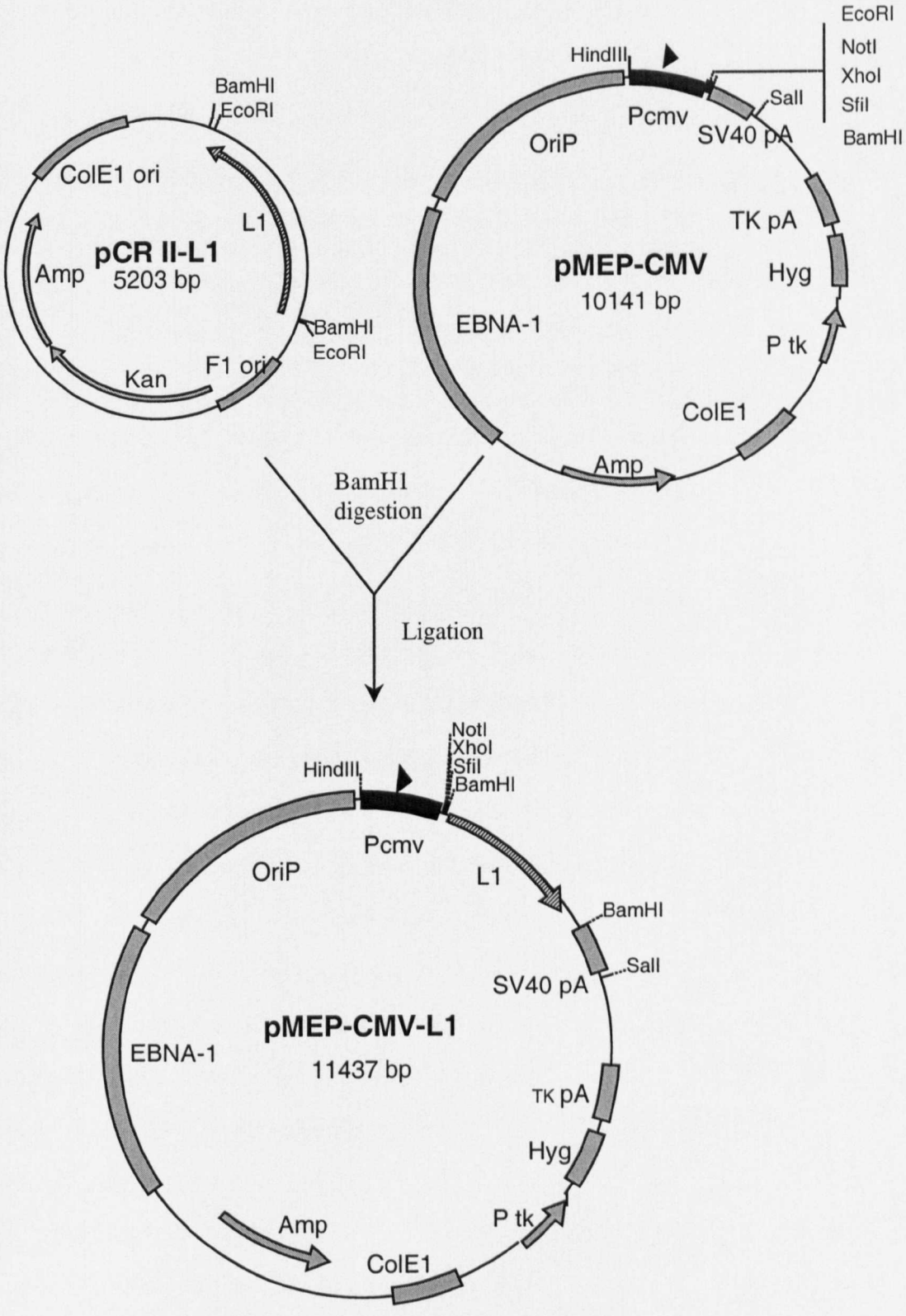


Figure 5.6 Construction of pMEP-CMV-L1
Insertion of the L1 52/55-kD cDNA into pMEP-CMV as a BamH1 fragment into pMEP-CMV.

18-20 hours post-infection. As expected, there is no expression at all in 293 cells.

5.3 Complementation of Ad5 L1 mutants by 293-L1 cell lines

To assess the degree of complementation afforded by 293-L1 cells, Ad5 mutants *ts*369 (Hasson *et al.*, 1989) and H5pm8001 (Gustin and Imperiale, 1998) were used. Ad5 *ts*369 produces L1 52/55-kD proteins with a two amino acid substitution and its ability to form plaques is temperature-sensitive. The yield of *ts*369 is reduced by a factor of 10^3 when it is propagated at 39.5 ^{0}C (non-permissive temperature) as compared with 32 ^{0}C (permissive temperature). On the other hand H5pm8001 is an L1 null mutant incapable of expressing the 52/55-kDa protein at all.

A complementation assay for Ad5 *ts*369 was devised in order to investigate the level of complementation, if any, provided by the 293-L1 cells (Figure 5.8). Two 60 mm dishes of both 293 and 293-L1 cells were infected with *ts*369 with a multiplicity of infection (moi) of 10 pfu for each dish. One dish from each cell type was incubated at permissive temperature (32 ^{0}C) and the remaining dishes were incubated at non-permissive temperature (39.5 ^{0}C). Also two 60 mm dishes of 293-L1 cells were infected with wild type Ad5 (moi=10 pfu) as controls, and while one of them was incubated at permissive temperature, the other one was incubated at non-permissive temperature. After an incubation period of 3 days, virus was harvested from each infected dish by three freeze & thaw cycles and dilutions from each were assayed for plaque formation on 293 cells at the permissive temperature (Figure 5.9).

Ad5 mutant *ts*369 grown at non-permissive temperature in 293 cells lines produced a titre of $2x10^6$ pfu/ml, 1000 fold lower than the titre when grown at the permissive temperature which was $2x10^9$ pfu/ml. This result is consistent with the one published by Hasson *et al.*, 1989. On the other hand, growth of the *ts* mutant in 293-L1 cells at the non-permissive temperature produced a titre of $6x10^8$ pfu/ml while growth at the permissive temperature produced a titre of $4x10^9$. Compared to the *ts*

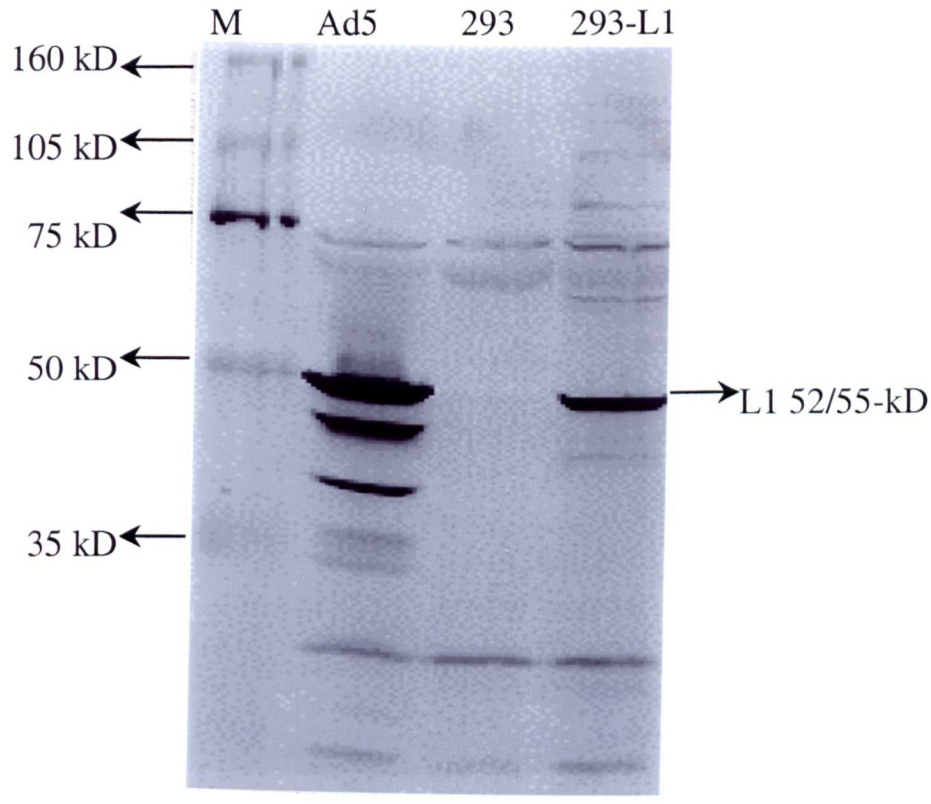


Figure 5.7 Analysis of L1 52/55-kD protein expression in hygromycin resistant 293 cells transfected with plasmid pMEP-CMV-L1 by western blotting

Cell lysates from mock-infected (293), Ad5-infected (Ad5) 293 cells and 293-L1 cells were immunoblotted by using anti GST-L1 polyclonal antibodies. M- protein molecular mass markers.

mutant virus titre when grown at permissive temperature in 293 cells, there was a two-fold increase in the titre when the *ts* mutant virus was grown at the same temperature in 293-L1 cells. Although not a big difference, this two-fold increase in the virus titre was most probably due to the higher levels of L1 52/55-kD protein present in the 293-L1 cells.

However, when a comparison was made between the virus titre of the *ts* mutant grown in 293 cells at the non-permissive temperature and in 293-L1 cells at the same non-permissive temperature, there was a 300-fold increase in the titre of the *ts369* virus grown in 293-L1 cells. This 300-fold increase in the virus titre showed the level of complementation of the *ts* mutant virus by the 293-L1 cell line and thus proved that this cell line was expressing the L1 52/55-kD protein in a biologically functional form. In the control experiment, where wild-type adenovirus was grown in 293-L1 cells at both permissive and non-permissive temperatures, there was no marked difference in the virus yield. This observation ruled out the possible effects of temperature shift on the wild-type virus titres.

In another experiment, the adenovirus L1 null mutant H5pm8001 was used for a similar complementation assay. This time, both wild-type and L1 null mutant (H5pm8001) viruses were grown in two type of cell lines, 293 and 293-L1 (figure 5.10). Virus was harvested from the cells three days after infection and the virus titre was determined by plaque assay on 293-L1 cells (figure 5.11).

H5pm8001 produced a titre of 1.5×10^6 pfu/ml in 293 cells, around 1000-fold lower than the titre obtained from wild-type virus grown in the same 293 cell lines (1×10^9 pfu). This difference was consistent with the result published by Gustin and Imperiale (1998). Growth of the wild type virus in 293-L1 cell lines led to a minor increase in the titre (around 4-5 fold) when compared with the titre obtained from growth in 293 cells. However, the L1 null mutant virus H5pm8001 titre was

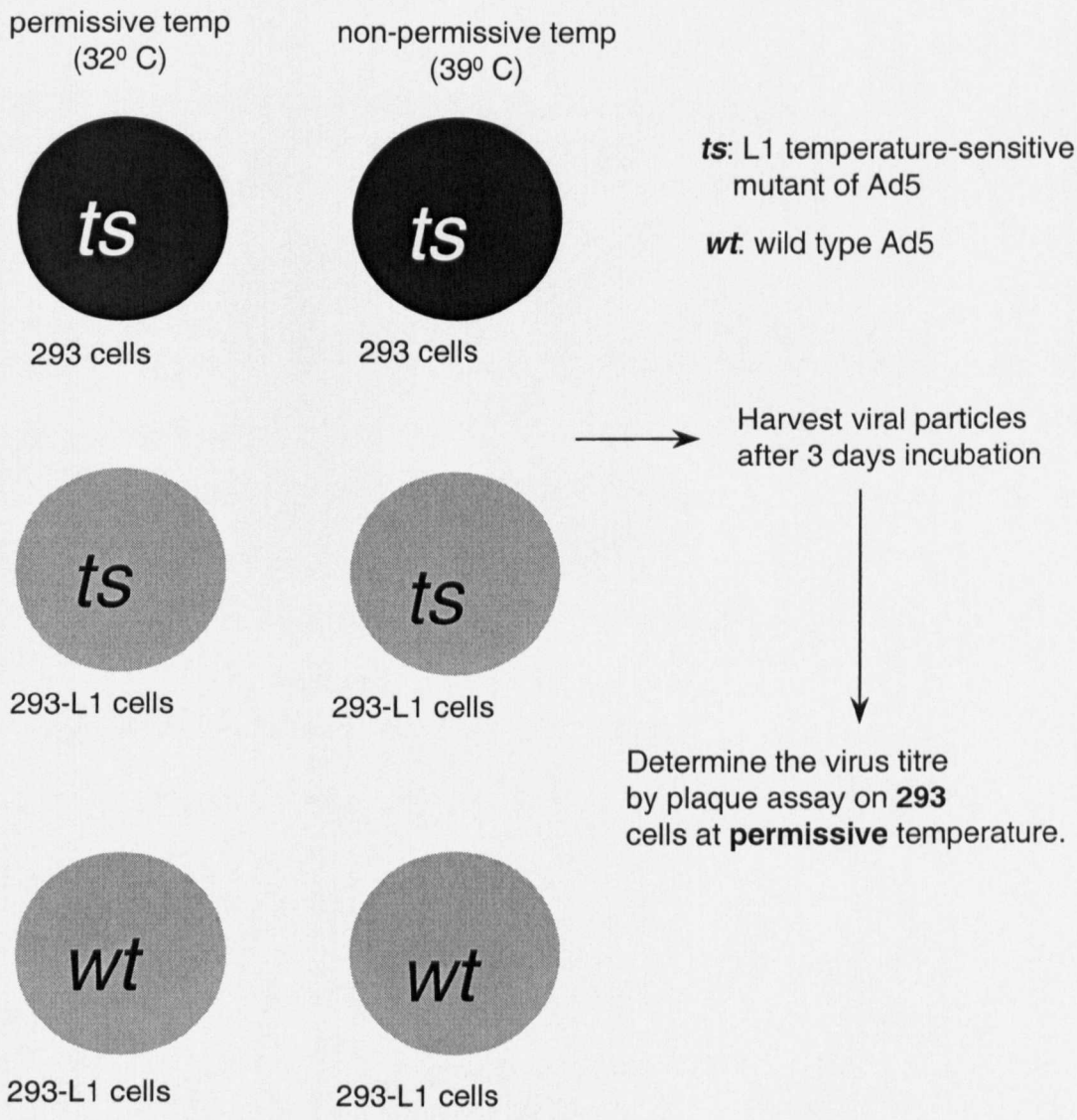


Figure 5.8 Experimental setup for the complementation assay using Ad5 mutant *ts*369

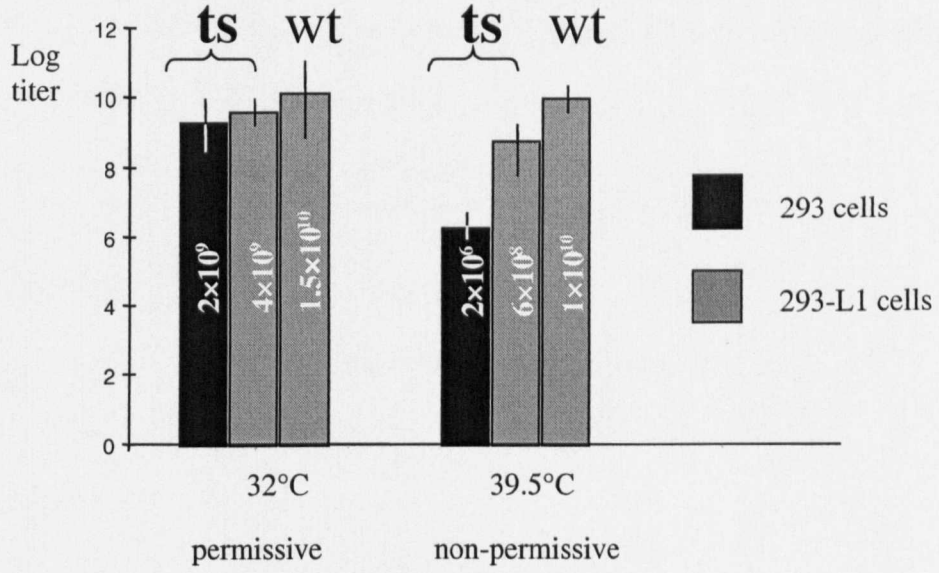


Figure 5.9 Complementation assay of the adenovirus mutant *ts*369 by the 293-L1 cell line.

Wild-type virus was grown in 293 L1 cells and L1 temperature mutant *ts*369 virus was grown in two cell lines, 293 and 293-L1 (figure 5.8). The cells were infected with the relevant virus at an moi of 10 pfu. Virus was harvested from the infected cells three days after the infection and the virus titre was determined by plaque assay on 293-L1 cells. Error bars show the standard deviation of results obtained in three independent complementation experiments calculated according to the equation shown in Table 7.1.

drastically increased (around 200-fold) when it was grown in 293-L1 cells. This increase was comparable to the 300-fold increase obtained in the complementation assay for the *ts369* and once again showed that the 293-L1 cells were capable of complementing the L1 52/55-kD protein deficiency during an infection of this cell line by a mutant adenovirus incapable of expressing the L1 52/55-kD gene.

These results obtained from both complementation assays were encouraging and indicated that the 293-L1 cell line could successfully complement a missing L1 52/55-kD protein function during my attempts to construct an L1 52/55-kD gene-deleted adenovirus in the future experiments.

5.4 Constitutive episomal expression of the Ad5 IV_{a2} and pIX genes in 293-L1 cells

Confirmation of the capability of the 293-L1 cells to complement the Ad5 L1 52/55-kD protein deficiency in Ad5 mutants *ts*369 and H5pm8001 let me proceed to construct cell lines expressing other Ad5 late genes, IV_{a2} and pIX respectively in 293-L1 cells. The main aim of construction of such cell lines was to use them as complementing cell lines for the construction of recombinant Ad5 lacking IV_{a2} or pIX coding regions together with L1 52/55-kD coding region. These cell lines were also required for experiments to investigate the effects of Ad5 late proteins in the activation of Ad5 major late promoter (see chapter 7).

Construction of 293 cell lines expressing Ad5 L1 52/55-kD and IV_{a2} proteins or L1 52/55-kD and pIX proteins involved the coexpression of the relevant proteins in the same cell. Potentially, the cDNAs encoding the relevant proteins could be cloned and expressed from the same expression vector provided that each cDNA was flanked by a different promoter and polyadenylation sequences. However, since a cell line expressing the Ad5 L1 52/55-kD protein was already constructed, it was

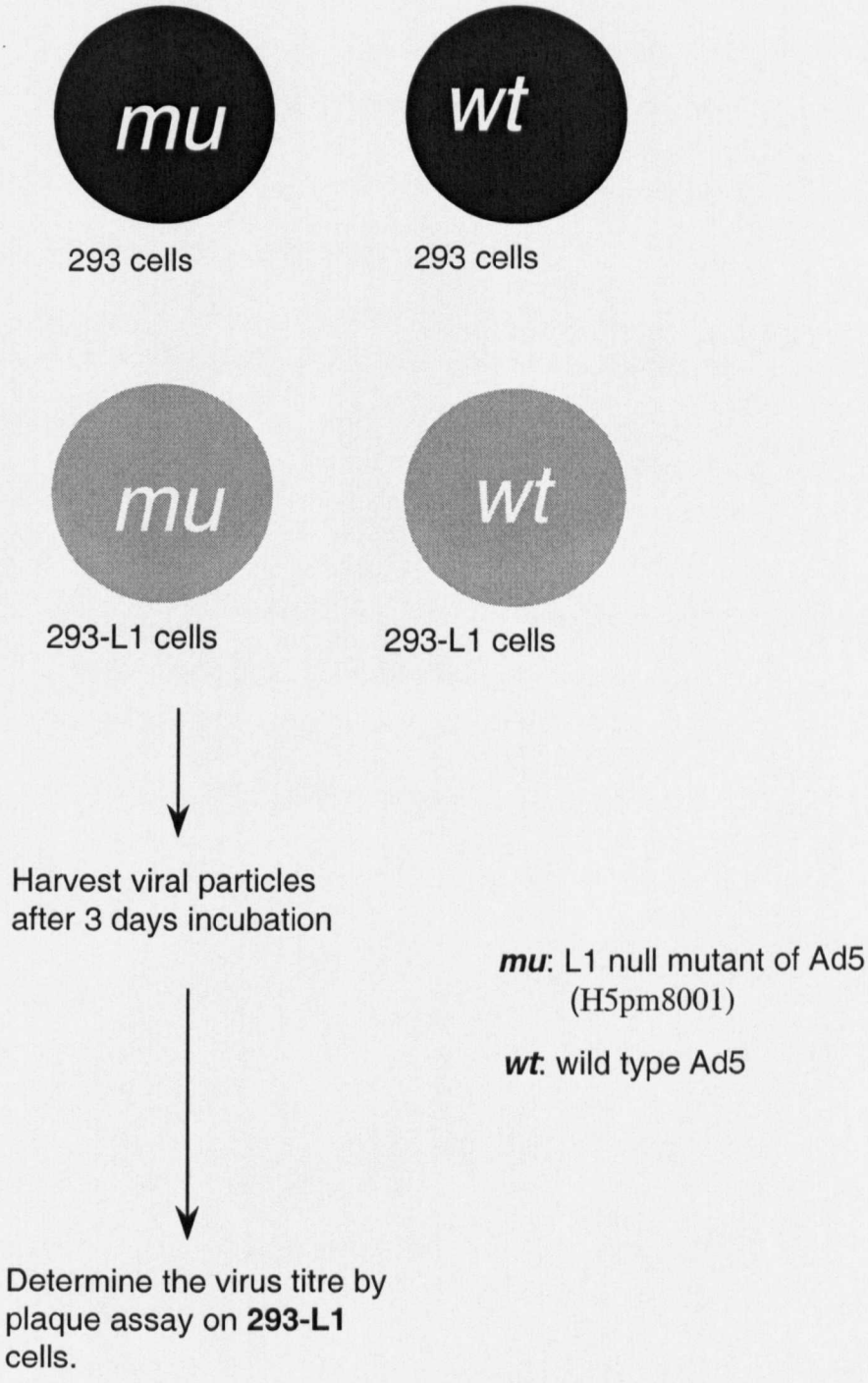


Figure 5.10 Experimental setup for the complementation assay on Ad5 mutant H5pm8001

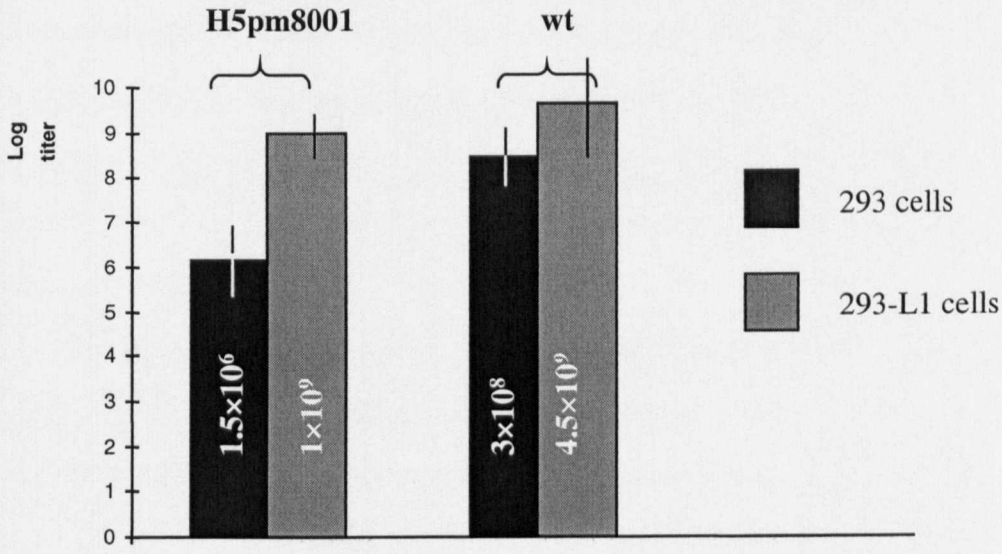


Figure 5.11 Complementation assay of the Ad5 L1 null mutant H5pm8001 by the 293-L1 cell line.

Wild-type and L1 null mutant (H5pm8001) viruses were grown in two type of cell lines, 293 and 293-L1 (figure 5.10). The cells were infected with the relevant virus type with a moi of 10 pfu. Virus was harvested from the infected cells three days after the infection and the virus titre was determined by plaque assay on 293-L1 cells. Error bars show the standard deviation of results obtained in three independent complementation experiments calculated according to the equation shown in Table 7.1.

decided to use a separate episomal expression vector for the expression of the second Ad5 protein (IV_{a2} or pIX). Such a coexpression strategy required the use of different selectable markers for each expression vector in order to amplify and select them independently from each other. This ensures the selection of cells harbouring both vectors driving the expression of the different cloned cDNA molecules when grown in the presence of the relevant antibiotics for which the genes present on the vectors provide resistance.

For this purpose, cDNAs of the Ad5 IV_{a2} and Ad5 pIX genes were cloned into another EBV-based mammalian episomal expression vector, pREP9 (figure 5.12). This plasmid has similar features to pMEP4 (figure 5.1) except that it contains the RSV promoter instead of the metallothionine and also a G418 (neomycin) resistance gene instead of the hygromycinB resistance gene present on pMEP4.

To be used in the construction of 293 cell lines expressing Ad5 L1 52/55-kD and Ad5 IV_{a2} genes (293-L1-IV_{a2}), IV_{a2} cDNA from plasmid pIV_{a2} was cloned into the multiple cloning site of pREP9 downstream the RSV promoter (figure 5.13 (a)). Similarly, for the construction 293 cell lines expressing Ad5 L1 52/55-kD and Ad5 pIX (293-L1-pIX), pIX cDNA from plasmid pCMV-IX was cloned at the same position of pREP9 (figure 5.13 (b)). Constructed plasmids were named as pREP9-IV_{a2} and pREP9-IX respectively.

Construction of the 293-L1-IV_{a2} and 293-L1-pIX cell lines was done using the same methodology as described for the construction of the 293-L1 cell line (section 5.2) except that varying concentrations of G418 were used instead of hygromycinB. To maintain expression of L1 52/55-kD, a fixed concentration of hygromycinB (200 μg/ml) was also included in the growth medium.

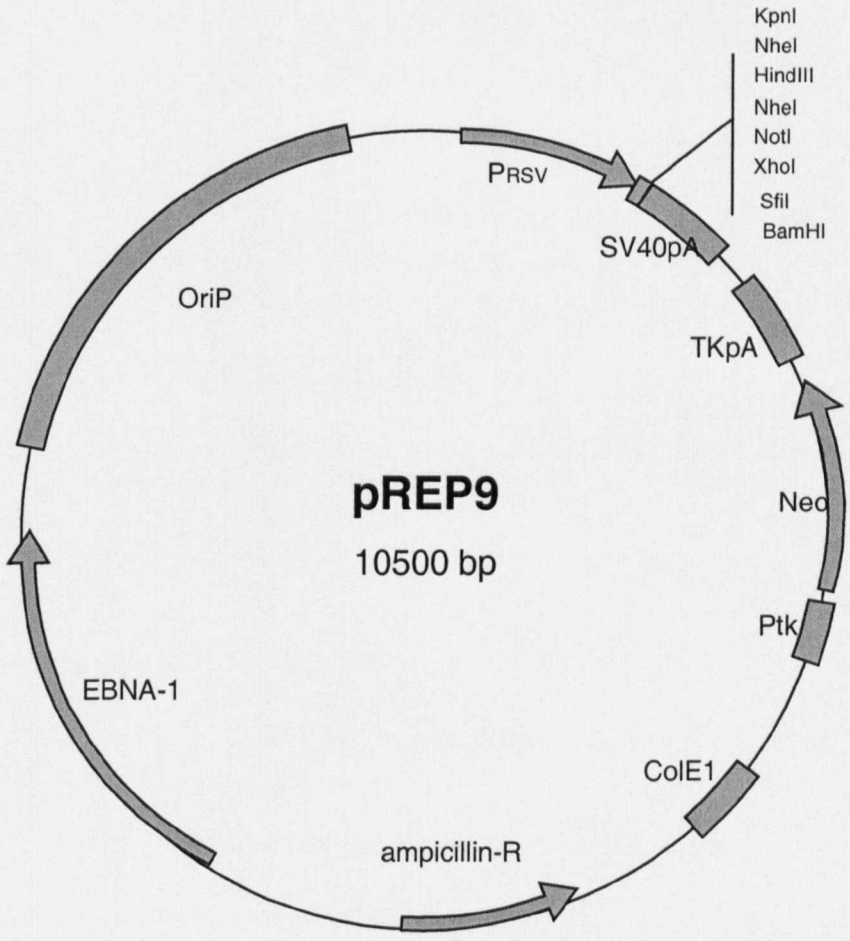


Figure 5.12 pREP 9: An EBV-based episomal mammalian expression vector (Invitrogen)

pREP 9 is maintained extrachromosomally in primate and canine cell lines (Chittenden *et al.*, 1989) and offers the following features: ColE1 origin of replication and ampicillin resistance gene for growth selection in E.coli; the EBV origin of replication (*ori*P) and nuclear antigen (EBNA-1) to allow high copy episomal expression; a selectable marker (neomycin-resistance gene) flanked by a promoter and a polyadenylation signal sequence for stable maintenance of the vector ; a MCS flanked by RSV promoter and another polyadenylation signal for the cloning and expression of the recombinant protein.

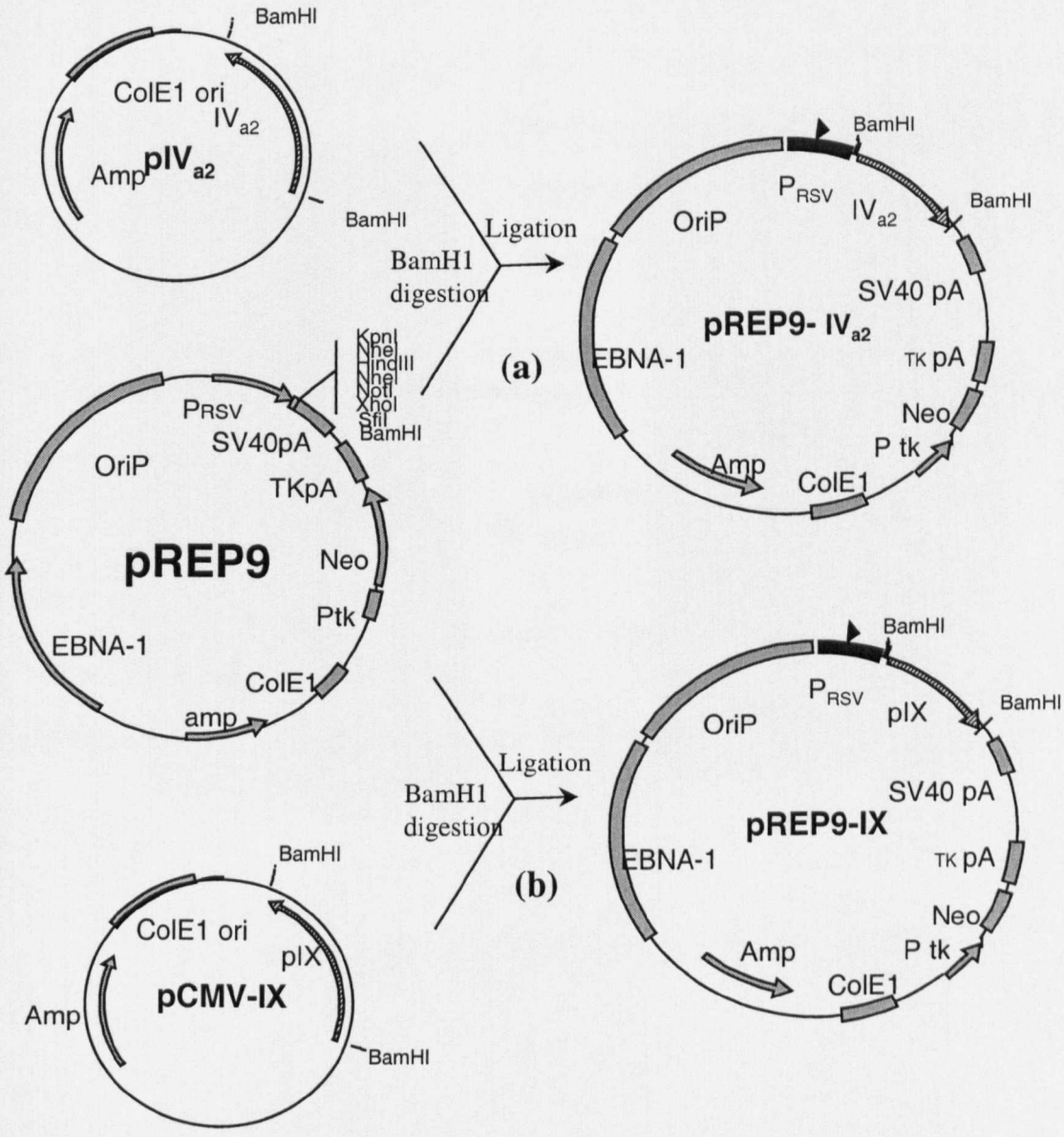


Figure 5.13 Construction of pREP9-IV_{a2} (a) and pREP9-IX (b).
For the construction pREP9-IV_{a2}, Ad5 IV_{a2} cDNA from plasmid pIV_{a2} was cloned in to the MCS of the plasmid pREP9 by using BamH1 restriction enzyme (a) and similarly for the construction of pREP9-IX, Ad5 pIX cDNA was cloned into the MCS of pREP9 again by using BamH1 restriction enzyme (b). The orientation of cloned inserts was confirmed by restriction analysis (data not shown).

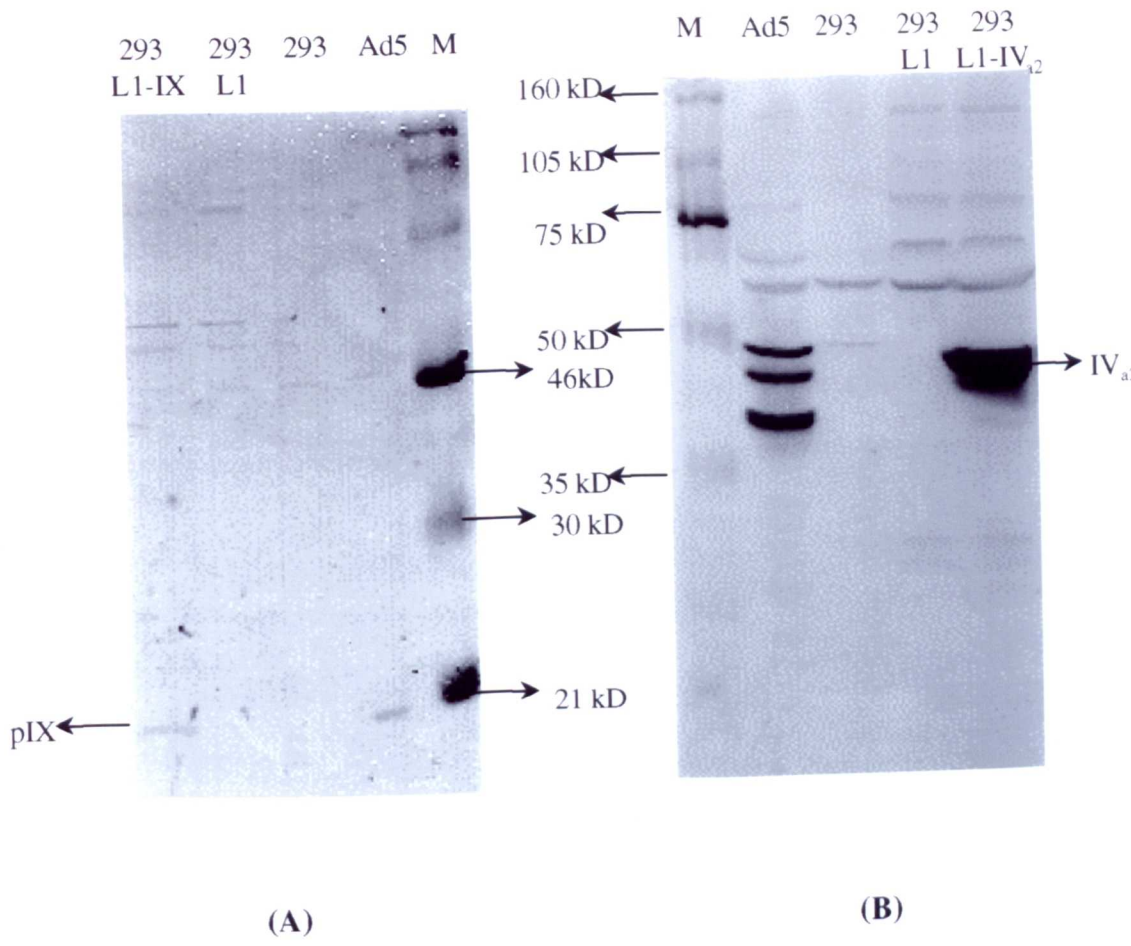


Figure 5.14 Analysis by Western blotting of pIX (a) and IV_{a2} (b) protein expression in G418 resistant 293 cells transfected with plasmid pREP 9-IX and pREP 9- IV_{a2} respectively.

(a) Cell lysates from mock-infected (293) or Ad5-infected (Ad5) 293 cells, 293-L1 cells and 293-L1-IX cells were immunoblotted by using anti GST-pIX polyclonal antibodies.

(b) Cell lysates from mock-infected (293) or Ad5-infected (Ad5) 293 cells, 293-L1 cells and 293-L1-IV_{a2} cells were immunoblotted by using anti GST- IV_{a2} polyclonal antibodies.

To check the level of expression of the L1 52/55-kD and IV_{a2} proteins or L1 52/55-kD and pIX proteins in each cell line grown in the presence of different concentrations of G418, cell lysates were analysed by Western blotting using polyclonal antibodies raised against L1 52/55-kD (Chapter 4), IV_{a2} (Brey, 1999) and pIX (Caravokyri and Leppard 1995). Cells lines grown in the presence of 300 μg/ml G418 showed the highest amount of IV_{a2} and pIX expression in 293-L1-IV_{a2} cells and 293-L1-pIX cells respectively (data not shown). Propagation of these cell lines was continued, and upon confirmation of the expression by another Western blot (figure 5.14), several aliquots from each cell line were stored by freezing in liquid nitrogen to be used in future experiments.

5.5 Summary and Discussion

In this chapter I have described the construction of 293 based cell lines 293-L1, 293-L1-IV_{a2}, and 293-L1-IX expressing Ad5 proteins L1 52/55-kD, L1 52/55-kD and IV_{a2}, and L1 52/55-kD and IX respectively in order to be used as complementing cell lines. These were designed to supply in *trans* the relevant proteins that would be missing during attempts to construct recombinant viruses with deletion mutations in these gene(s). Although the 293-L1 cells were shown to be expressing the L1 52/55-kD protein by Western blotting experiments, the functionality of the expressed protein could not be demonstrated by this method. Another concern about the expressed L1 52/55-kD protein was that the constituve presence of this protein in the cells prior to infection could affect the normal replication cycle of an L1 52/55-kD deleted virus. This could have unpredictable effects and even cause decrease in the virus titers rather than increase by complementation. Thus, in order to test both the functionality and the complementing ability of the 293-L1 cells, a complementation assay was devised to compare the titers of L1 52/55-kD mutant viruses grown in 293 and 293-L1 cells. The Ad5 L1 52/55-kD mutants used for this purpose were *ts*369 and H5pm8001. These viruses contained a *ts* or null mutation in the L1 52/55-kD orf respectively and were both shown to be complemented by the 293-L1 cells.

Chapter 6

Attempts to construct L1-52/55-kD deleted adenovirus 5 recombinant

6.1 Construction by *in vivo* homologous recombination in 293-L1 cells

6.1.1 Introduction

As noted in the general introduction, one of the major aims of this study was to construct an Ad5 mutant containing a deletion of the L1 52/55-kD coding sequences in order to open space in the viral genome for heterologous genes. In this section I will describe my attempts to create such a virus by *in vivo* overlap recombination methods. The general strategy employed for this purpose is shown in figure 6.1.

Although adenovirus DNA can be manipulated directly, it is generally more convenient to work on smaller fragments of the genome subcloned into plasmid vectors. Complete viral DNA can be reconstructed either by ligation of appropriate cloned and viral genomic fragments, or by overlap recombination *in vivo* between such fragments (Chinnadurai *et al.*, 1979).

In this study, deletion of the L1 52/55-kD coding region was not possible by direct manipulation of the whole viral genome due to the lack of suitable unique restriction sites. Thus sub-clones of the viral fragments were created in order to enable the use of restriction sites unique to each sub-clone which are not unique in the whole viral genome.

6.1.2 Subcloning of the viral genome right half into a plasmid vector

In order to introduce an L1 52/55-kD deletion into the right hand Ad5 genome, plasmid pSal1C was used in the first step of the extensive cloning experiments. pSal1C contains the Ad5 Sal1 fragment from 9841 bp to 16746 bp cloned into the Sal1 site of pBR322 (figure 6.2). Within this Ad5 Sal1 fragment, the L1 52/55-kD coding region (Ad5 11050 bp to 12297 bp) is also present and a fragment of 1108 bp

(from 11050 bp to 12158 bp) corresponding to the majority of the L1 52/55-kD coding region could easily be deleted from the plasmid by digestion with enzymes Nsi1 and Apa1 (figure 6.2). Hence plasmid pSal1C-ΔL1 was created by the deletion of an 1108 bp fragment (referred to as L1 deletion or ΔL1 in the rest of the thesis) from plasmid pSal1C (figure 6.2). Later kanamycin-resistance gene from plasmid pCR II was cloned into pSal1C-ΔL1 (figure 6.3) in order to make the screening of recombinants in subsequent cloning steps easier. This new plasmid was called pSal1C-ΔL1-kan.

In the following step, the large Sal1 fragment from the plasmid pSal1C-ΔL1-kan containing the L1 deletion within Ad5 sequences 10294 to 16746 bp was cloned into plasmid pFG that contains the Ad5 right end genome from the Sal1 site present at 16746 bp on the Ad5 sequence (figure 6.4) to the right end and was called pAd5RE-ΔL1.

6.1.3 Subcloning of the viral genome left half into a plasmid vector

A plasmid harbouring the left half of the Ad5 genome up to the Sal1 site located at 16746 bp on the viral genome and containing the L1 52/55-kD coding region deletion was constructed in three sequential steps. In the first step, the 9892 bp fragment of Cla1 (917 bp) and Nhe1 (10809 bp) digested Ad5 genome was cloned into Cla1 and Nhe1 digested pBR322 (figure 6.5) to create plasmid pAd5Cla1-Nhe1.

Plasmid pAd5Cla1-Nhe1 was then used in order to extend the Ad5 sequences present in the plasmid pXho1C which contains Ad5 sequences from the left hand end to the Xho1 site located at 5788 bp on the viral genome (figure 6.6). For this purpose, both pXho1C and pBRAd5Cla1-Nhe1 were digested with EcoR1 and AflII enzymes and the small fragment of pXho1C was inserted into the large fragment of pBRAd5Cla1-Nhe1 (figure 6.6). The new recombinant plasmid was called pAd5LE-10809.

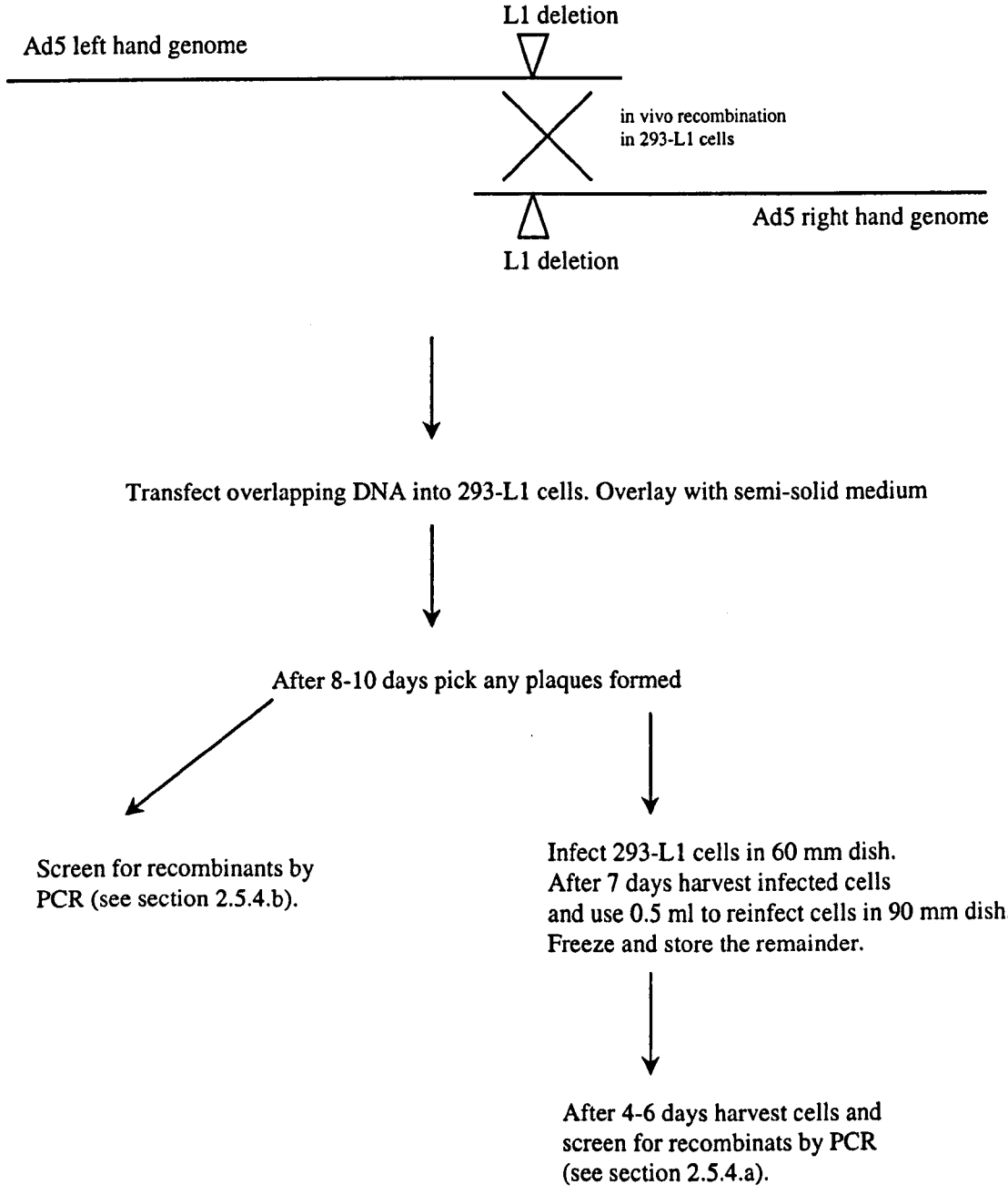
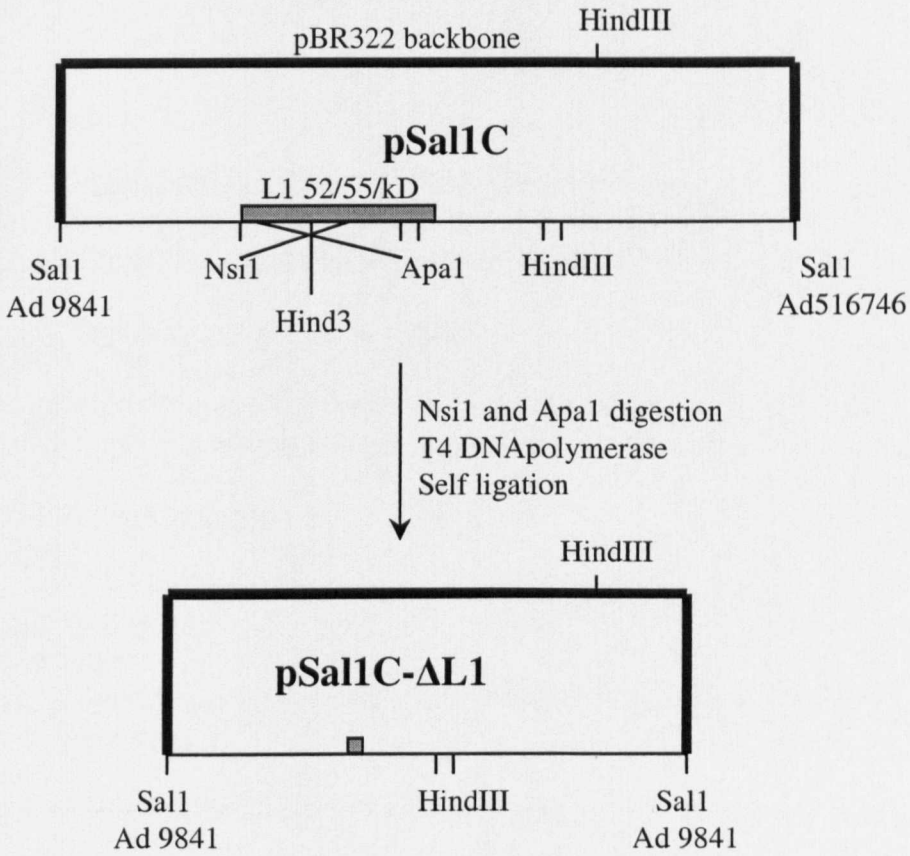
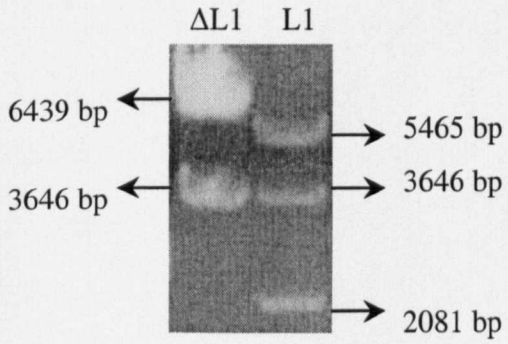


Figure 6.1 General Strategy used for the construction and isolation of L1 52/55-kD deleted Ad5 recombinant.
The diagram lists the stages involved in isolating recombinant adenoviruses by the methods detailed in chapter 2.



(A)



Expected sizes of the fragments upon HindIII digestion (bp)

ΔL1	L1
6439	5465
3646	3646
75	2081
	75

(B)

Figure 6.2 Construction of the pSal1C-ΔL1

(A): Schematic representation; Most of the L1 52/55-kD coding region in pSal1C was removed by the digestion of the plasmid with Nsi1 and Apa1 followed by treatment with T4 DNA polymerase to create blunt ends and self ligation of the plasmid.

(B): Restriction digestion analysis of the plasmids pSal1C-ΔL1 and pSal1C by HindIII that confirms the deletion of the L1 52/55-kD coding region present in the plasmid pSal1C-ΔL1.

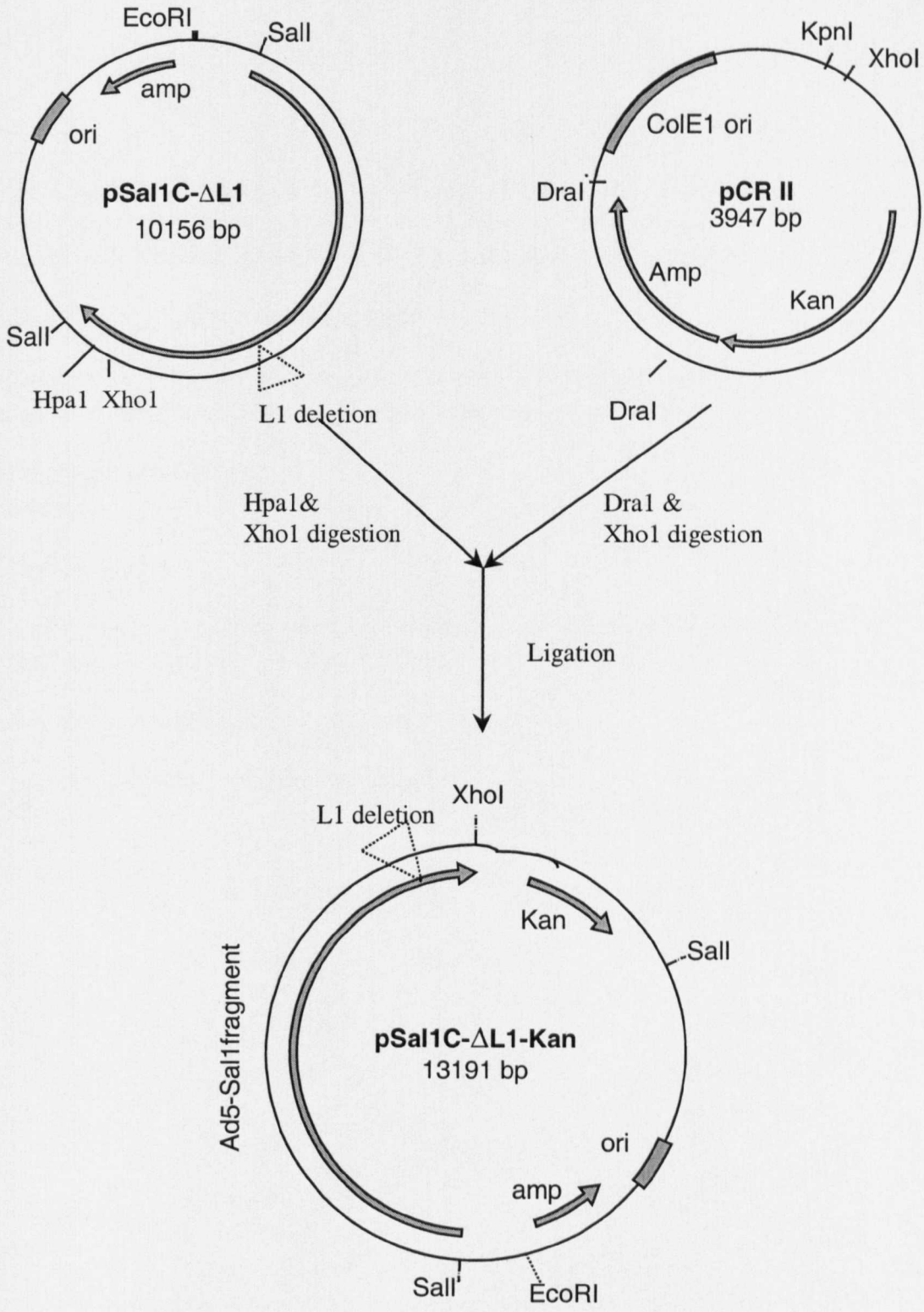


Figure 6.3 Construction of pSal1C-ΔL1-Kan
The kanamaycin-resistance gene was cloned into pSal1C-ΔL1 by taking it out of pCR II using enymes Dra1 and Xho1 and ligating into the Hpa1 and Xho1 digested pSal1C-ΔL1. Note that Hpa1 and Dra1 produce blunt ends and can be ligated without prior manipulation of the DNA.

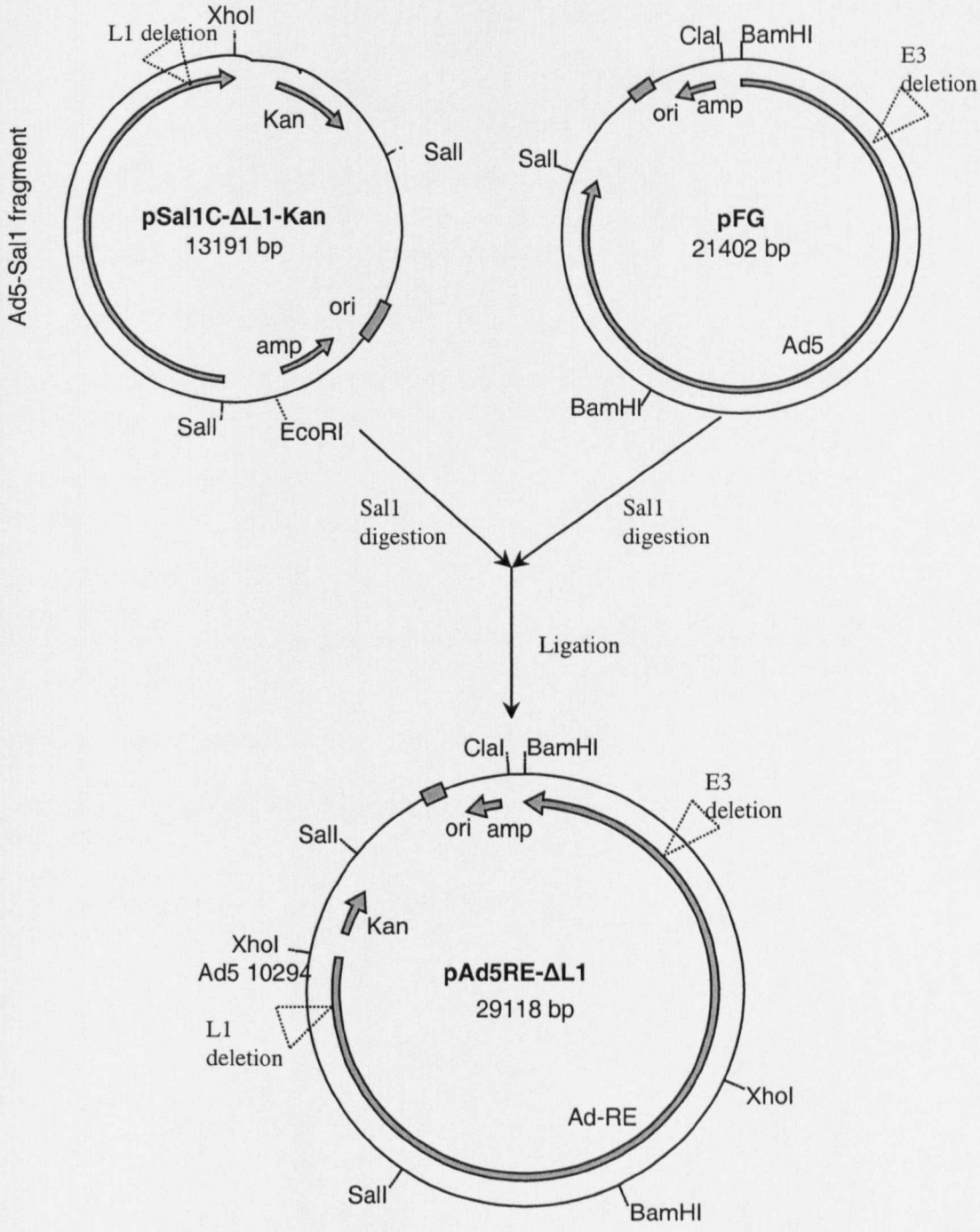


Figure 6.4 Construction of pAd5RE-ΔL1
Ad5 right hand genomic sequences in pFG were extended until the Xho1 site located at 10294 bp on the Ad5 genome by cloning the large Sal1 fragment of pSal1C -ΔL1 into the Sal1 site of pFG. Recombinant plasmid was screened by kanamycin and the orientation of the cloned insert was determined by restriction analysis (data not shown).

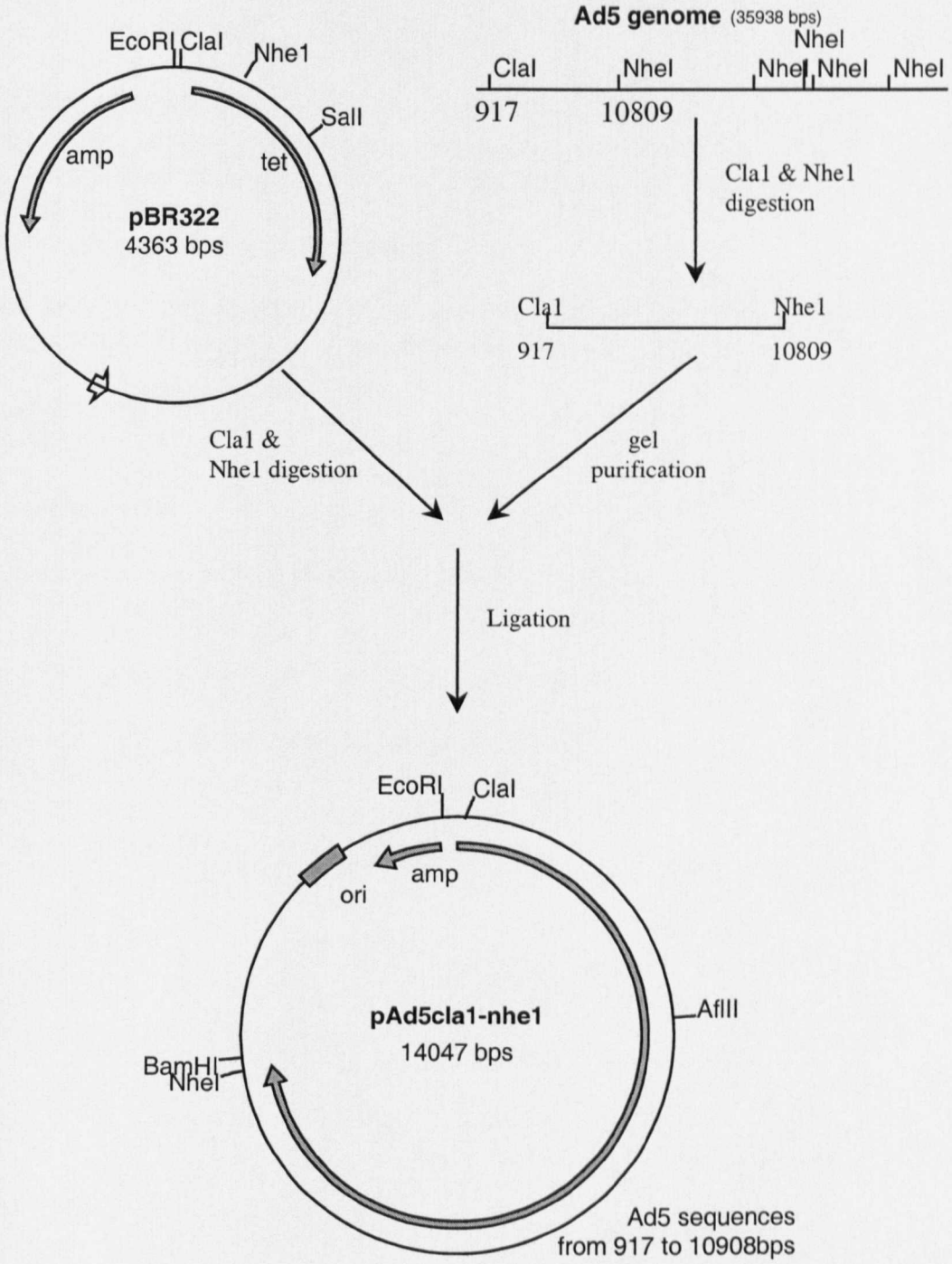


Figure 6.5 Construction of pAd5cla1-nhe1
9892 bps fragment of Cla1 and Nhe1 digested Ad5 genome was gel purified and then cloned into Cla1 and Nhe1 digested pBR322 to create plasmid pAd5cla1-nhe1.

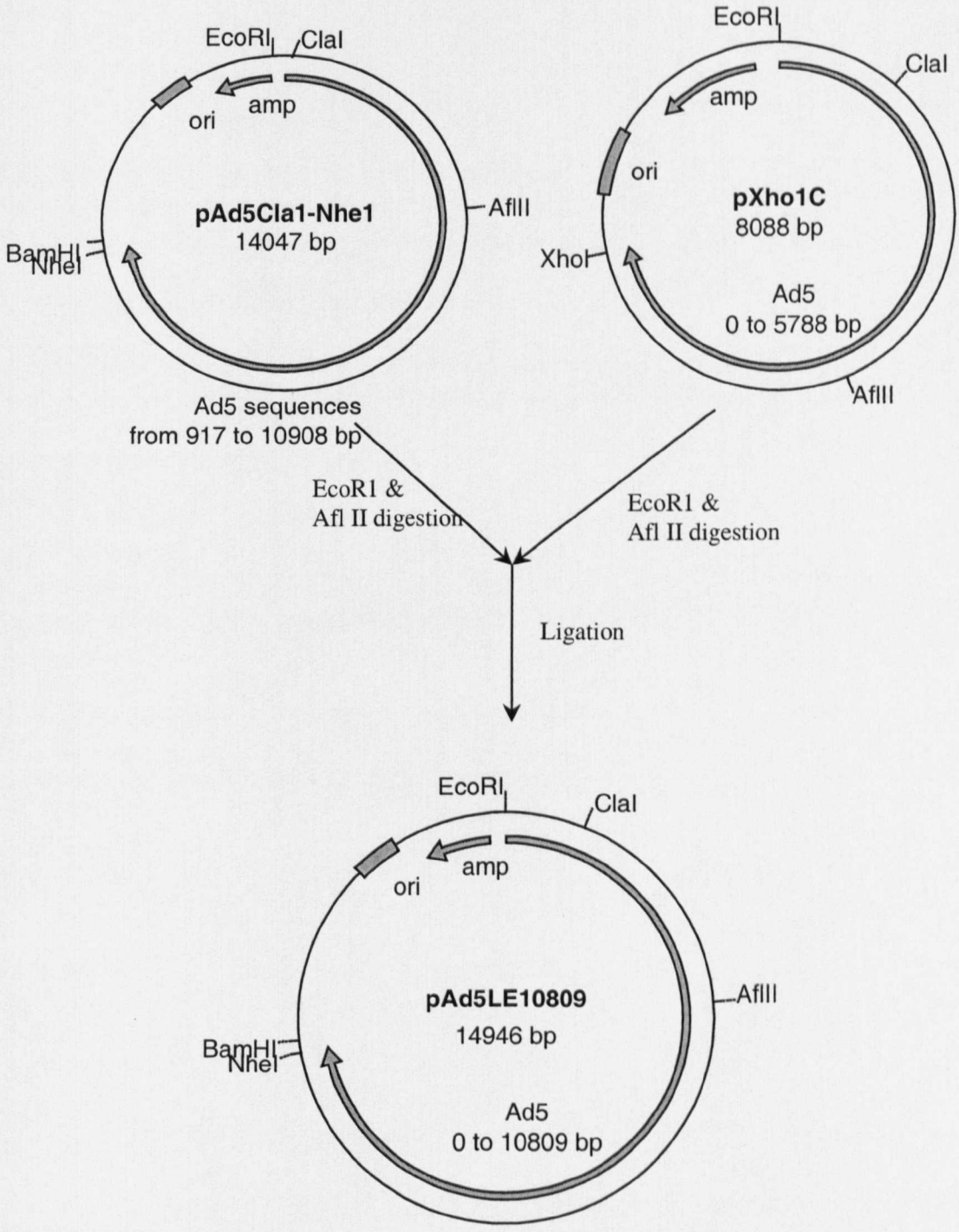


Figure 6.6 Construction of pAd5LE-10809
Both pXho1C and pBRAd5Cla1-Nhe1 were digested with EcoR1 and AflII enzymes and the small fragment of pXho1C was inserted into the large fragment of pBRAd5Cla1-Nhe1 in order to extend the Ad5 sequences to the Nhe1 site located at 10809 bp on the Ad5 genome.

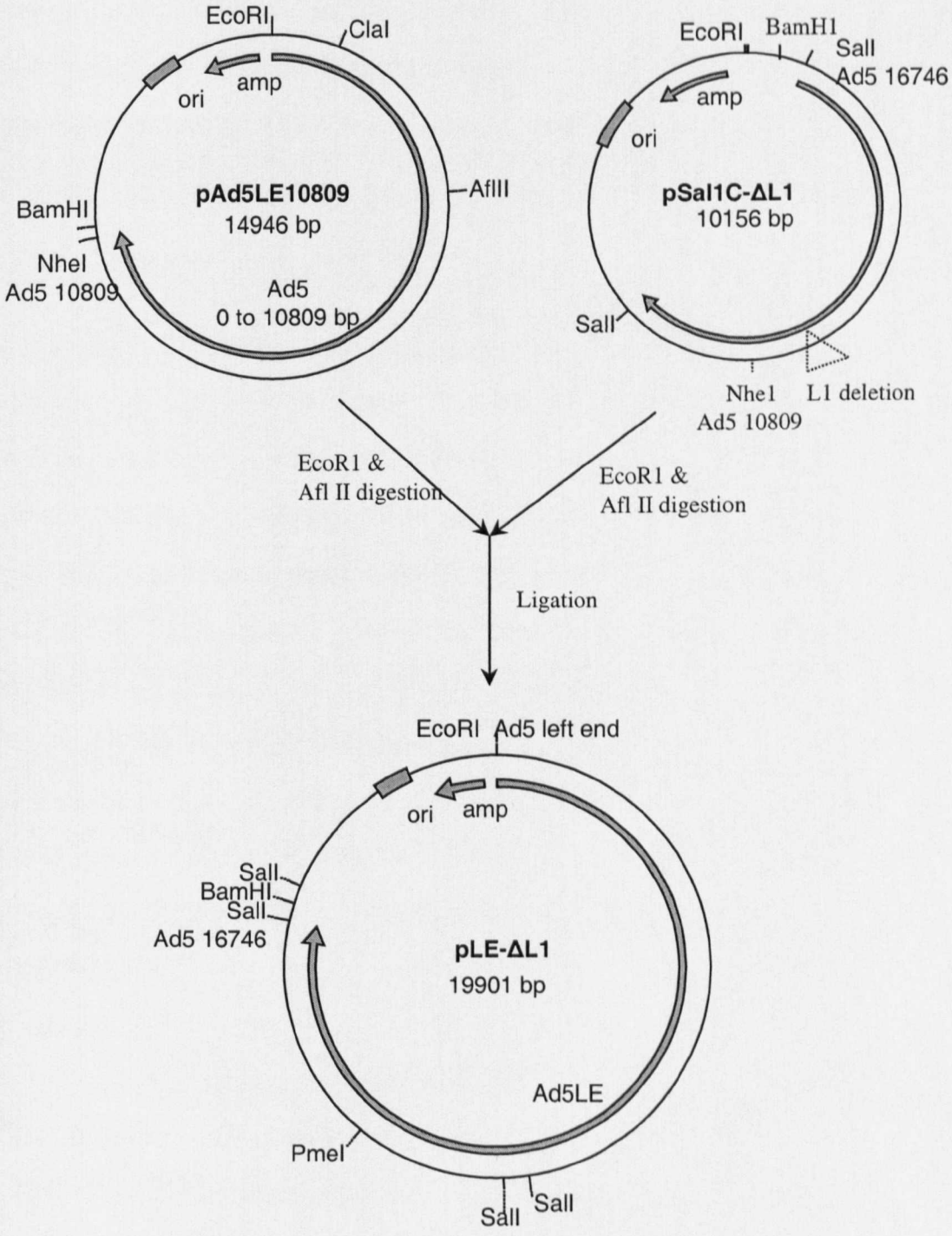


Figure 6.7 Construction of pLE-ΔL1
Large fragment of Nhe1 and BamH1 digested pSal1C-L1 (contains an L1 52/55-kD deletion in the Ad5 sequences) was cloned into pAd5LE-10809 digested by the same enzymes extending the left end Ad5 sequences up to the Sal1 site located at 16746 bp on the viral genome.

In the last step, the large fragment of Nhe1 and BamH1 digested pSal1C-ΔL1 (contains an L1 52/55-kD deletion in the Ad5 sequences) was cloned into pAd5LE-10809 digested by the same enzymes (Figure 6.7) extending the left hand Ad5 sequences up to the Sal1 site located at 16746 bp on the viral genome. This plasmid was then called pAd5LE-ΔL1.

6.1.4 *in vivo* overlap recombination experiments

Having constructed the two plasmids, one harbouring the left end part of the Ad5 genomic DNA and the other the right end part of the Ad5 genomic DNA (pAd5LE-ΔL1 and pAd5RE-ΔL1 respectively), the next step was to try to reconstitute a virus genome from them *in vivo* by overlap recombination after transfecting 293-L1 cells with linearized forms of both of the plasmids. Since both the pAd5LE-ΔL1 and pAd5RE-ΔL1 carry the L1 52/55-kD coding region deletion at the appropriate location in region where the Ad5 genomic sequences in the plasmids overlap each other and since the naked adenovirus genomic DNA is infectious, any viral DNA reconstituted due to an overlap recombination event inside the transfected 293-L1 cell nucleus would be expected to be infectious, leading to the replication of the reconstituted L1 52/55-kD deleted viral DNA and thus production of new mutant viral particles.

Overlap recombination experiments involved the transfection of 293-L1 cells with three different combinations of molecules (Figure 6.8). The first combination contained linearized plasmids pAd5LE-ΔL1 with pAd5RE-ΔL1 (digested with EcoR I and Cla I respectively), the second combination contained linearized pAd5LE-ΔL1 with the 24315 bp fragment (corresponding to right end of the viral genome) of NsiI-digested *wt* Ad5 DNA and the third combination contained linearized pAd5RE-ΔL1 with the 11311 bp fragment (corresponding to left end of the viral genome) of MluI-digested *wt* Ad5 DNA. The sizes of the overlapping regions in each

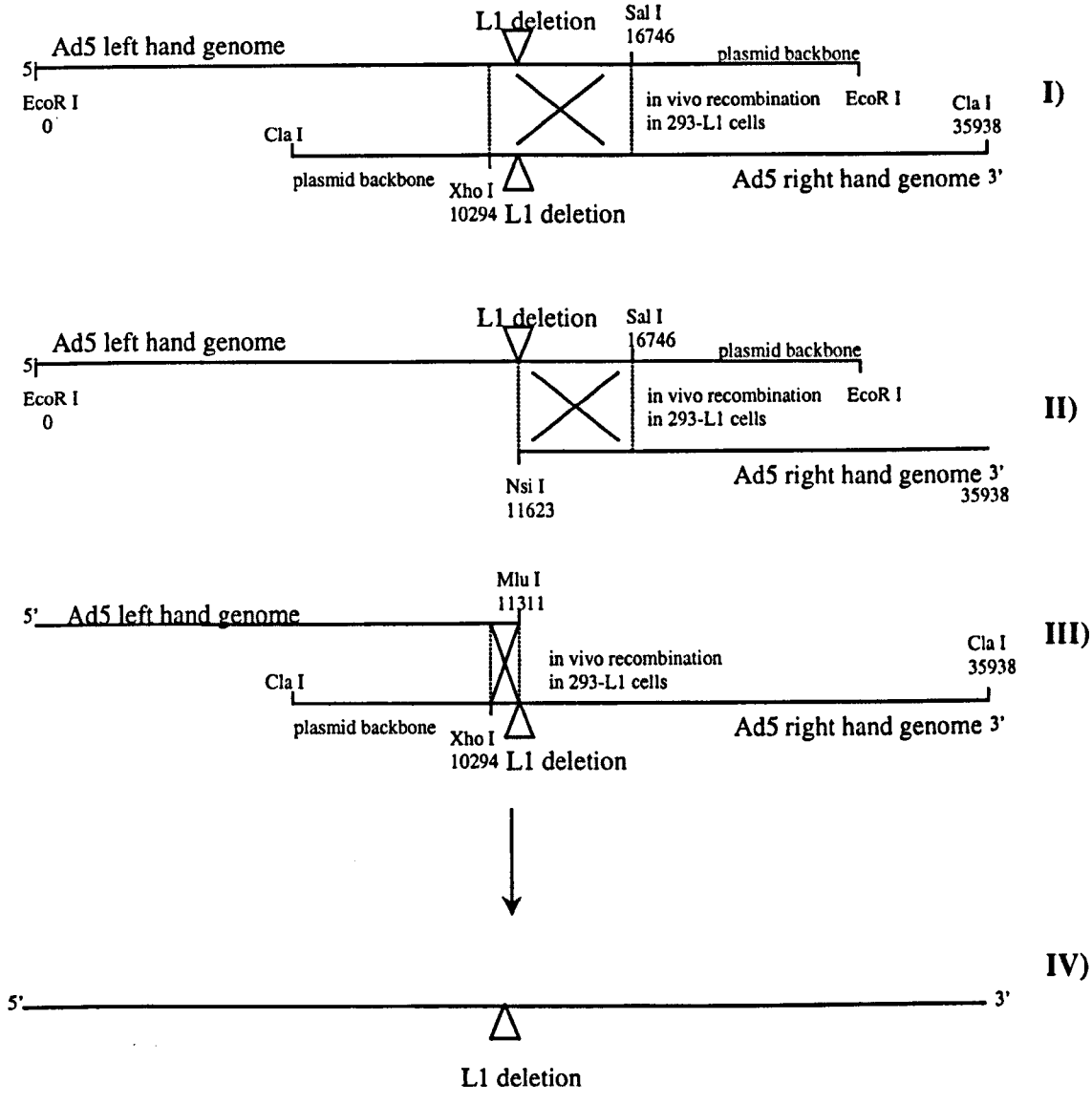


Figure 6.8 Three different combinations of molecules used in overlap recombination experiments.
I) linearized plasmids pAd5LE-L1 with pAd5RE-L1 (digested with EcoRI and ClaI respectively); II) linearized pAd5LE-L1 with the 24315 bp fragment (corresponding to right end of the viral genome) of NsiI-digested *wt* Ad5 DNA and ;III) linearized pAd5RE-L1 with the 11311 bp fragment (corresponding to left end of the viral genome) of MluI-digested *wt* Ad5 DNA. IV) Any recombination event occurring between the molecules in each combination gives rise to infectious viral DNA lacking the L1 52/55-kD coding region.

of molecules were 5344 bp, 4588 bp and 1017 bp respectively and any recombination event occurring between the molecules in each combination giving rise to infectious viral DNA would be expected to carry a deletion of the L1 52/55-kD coding region (Figure 6.8). The experiments were carried out as follows: 5 μg of each DNA molecule were used for transfection of 293-L1 cells in 60 mm dishes by lipofection (see Chapter 2) and the dishes were overlaid with semi-solid medium (as it was done in plaque assay experiments, see chapter 2). After 8-10 days of incubation any plaques formed were picked and the agar plugs were resuspended in 1 ml of DMEM-10 % FCS; 370 μl of each suspension were used for the detection of recombinants by PCR (see Section 2.5.4.b). The remaining 630 μl of each plaque suspension were used to infect 293-L1 cells in 60 mm dishes and the infected cells were harvested after four days and lysed by three freeze and thaw cycles. 0.5 ml from each cell harvest was then used for another round of infection of 293-L1 cells in 90 mm dishes and the remaining lysates were stored at –70 ^{0}C. After 4-6 days of incubation, the infected cells were harvested and screened by PCR for the recombinants. To detect recombinants by PCR, primers L5 and L6 were designed: These primers hybridise to sequences flanking the L1 52/55-kD coding region (Figure 6.9) and amplify a fragment of 194 bp from Ad5 sequences containing a deletion of the L1 52/55-kD region and a fragment of 1302 bp from Ad5 sequences containing an intact L1 52/55-kD coding region.

In all the experiments carried out on cells transfected with the combination of linearized plasmids pAd5LE-ΔL1 with pAd5RE-ΔL1 no clearly identifiable plaques were observed. Any morphological formations which resembled that of plaque formation were treated as if they were plaques and subjected to at least 3 rounds of plaque amplification and PCR analysis, however there was no amplification of a diagnostic fragment in any of them. On the other hand, in control experiments where purified 24315 bp fragment (corresponding to the right end of the viral genome) of NsiI-digested *wt* Ad5 DNA and 11311 bp fragment (corresponding to the left end of

the viral genome) of MluI-digested *wt* Ad5 DNA were used for the transfection of 293-L1 or 293 cells, a 1302 bp PCR fragment corresponding to amplification from *wt* viral DNA was consistently identified from material in any of the formed plaques.

In contrast to the result with fragment combination I (figure 6.8), plaques were regularly observed and amplified from cells transfected with fragment combinations II and III (figure 6.8). However, in all of the plaques amplified and analysed by PCR, a fragment of around 1300 bp corresponding to an intact L1 52/55-kD region was detected. This meant that the plaques were formed by a wild type virus in each case and this was most probably due to the presence of incompletely digested wild type Ad5 DNA in the transfection mixture.

The same results were obtained after several repetitions of these experiments suggesting two possibilities; 1. the expected overlap recombination could be occuring at a very low frequency such that it would require much higher numbers of repetitions of the transfection experiments to be detected. Although in the control experiments, where purified 24315 bp fragment (corresponding to right end of the viral genome) of NsiI-digested *wt* Ad5 DNA and 11311 bp fragment (corresponding to left end of the viral genome) of MluI-digested *wt* Ad5 DNA were used for the transfection, all the plaques screened contained wild type Ad5, it is possible that it was due to incomplete digested viral DNA during the preparation of the relevant virus genome fragments rather than reconstitution of the viral genome due to overlap recombination. 2. Another possibility is that an Ad5 virus deficient in L1 52/55-kD coding sequences can be replication incompetent due to the reason mentioned above.

Although 293-L1 cells were shown to complement the missing L1 52/55-kD protein function in Ad5 L1 mutants *ts*369 and H5pm8001 (see section 5.3), this does not necessarily mean that these cells would also be able to complement the same

missing protein function of a reconstituted Ad5 genomic DNA containing a deletion of the L1 52/55-kD coding sequences. Those sequences may well be carrying one or more yet undiscovered *cis*-acting elements having functions that are important directly or indirectly for the replication of the virus, rendering construction of such a virus impossible.

6.2 Construction in *E. coli*

6.2.1 Attempts to clone the entire Ad5 genome containing a deletion of the L1 52/55-kD into a bacterial plasmid.

Since the failure to construct an L1 52/55-kD-deleted Ad5 by overlap recombination in 293-L1 cells could be due to very low frequencies of the expected recombination event, one way to test such a theory would be to attempt to construct the virus by transfecting 293-L1 cells with a plasmid containing the entire Ad5 sequences except the L1 52/55-kD coding sequences. Such an experiment would also prove whether or not the deleted L1 52/55-kD coding sequences harbours any *cis*-acting sequences required directly or indirectly for the replication of the virus.

One way to construct such a plasmid containing the entire Ad5 sequences except the L1 52/55-kD coding sequences was to take out the Ad5 sequences present in the plasmid pAd5LE-ΔL1 and insert them into an appropriate site in pAd5RE-ΔL1, as shown in figure 6.11. In this way, a plasmid containing the entire Ad5 genome could be amplified in bacteria and then transfected into 293-L1 cells after being purified and linearised. Since there is no need for a recombination event in order to reconstitute a viral genome, the probability of isolating an L1 52/55-kD deleted mutant Ad5 would be much higher. In order to achieve this, the tetracycline-resistance gene from pBR322 was first cloned into pAd5LE-ΔL1 to ease the selection of recombinant plasmids in subsequent cloning steps (Figure 6.10). The next step consisted of taking out the tetracycline resistance gene and Ad5 left end

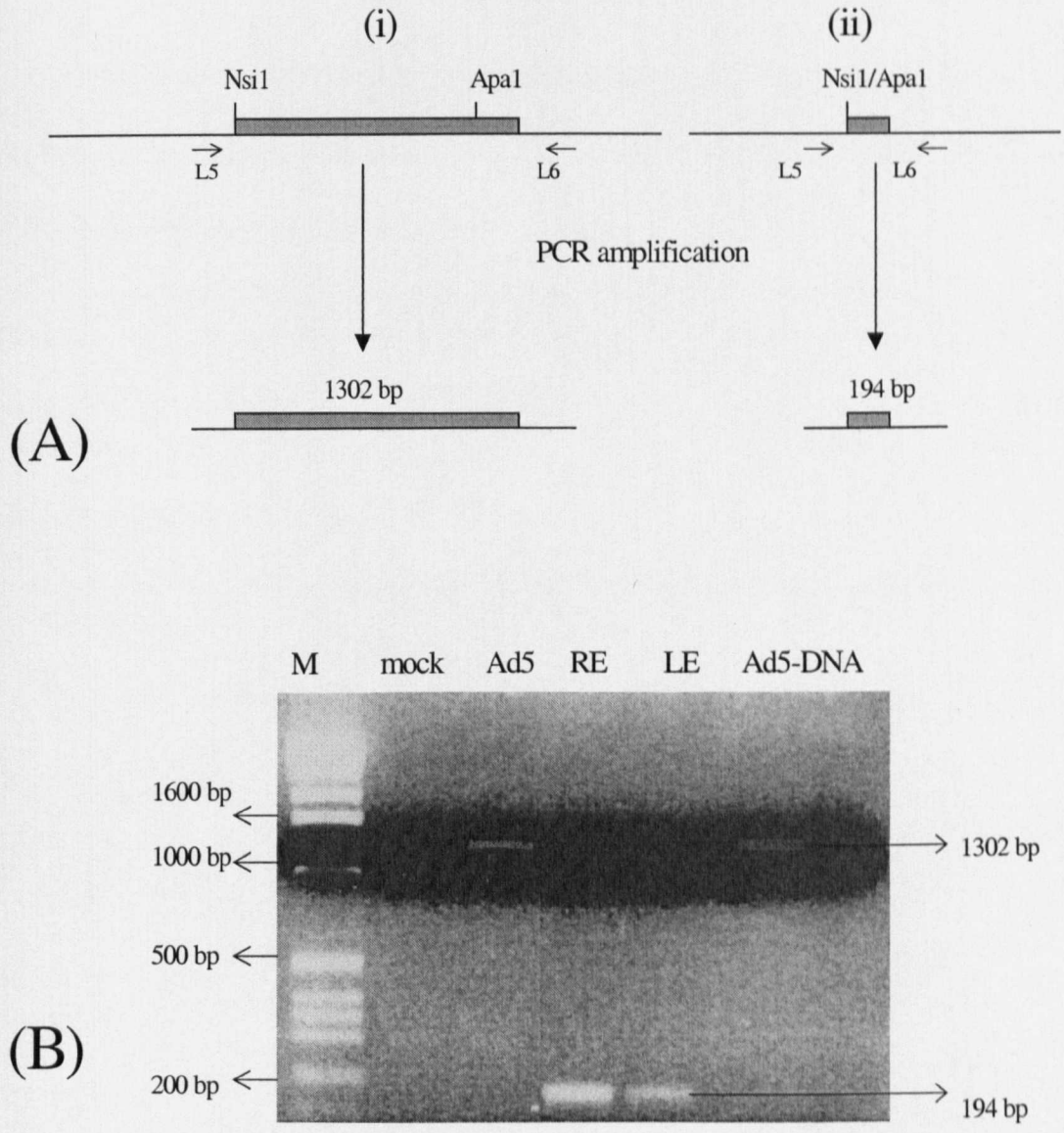


Figure 6.9 Primers used for the detection of L1 52/55-kD deleted recombinants
(A) Primers and their direction of hybridization are shown by arrows. (i) and (ii) show the amplification of fragments from wild type and L1 52/55-kD deleted Ad5 sequences respectively.
(B) PCR amplification by primers L5 and L6 from different templates to test the reliability of the detection method. M: molecular size markers; mock: DNA extract from mock-infected 293-L1 cells; Ad5: DNA extract from Ad5-infected 293-L1 cells; RE: plasmid pAd5RE-ΔL1; LE: plasmid pAd5LE- ΔL1; Ad5-DNA: purified Ad5 genomic DNA.

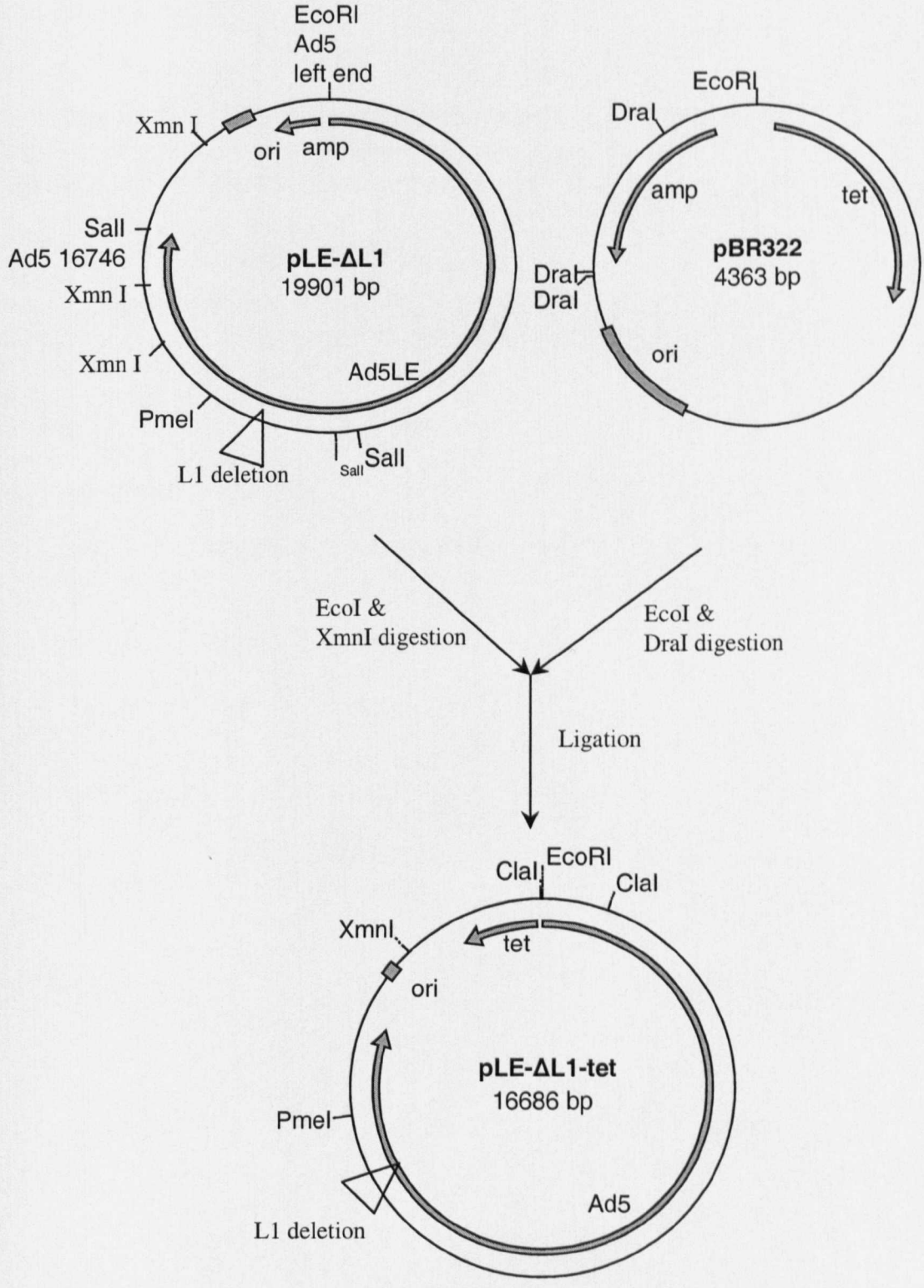


Figure 6.10 Construction of pLE-ΔL1-tet
The large fragment of XmnI and EcoRI digested pLE-ΔL1 (contains an L1 52/55-kD deletion in the Ad5 sequences) was ligated to the large fragment of DraI and EcoRI digested pBR322.

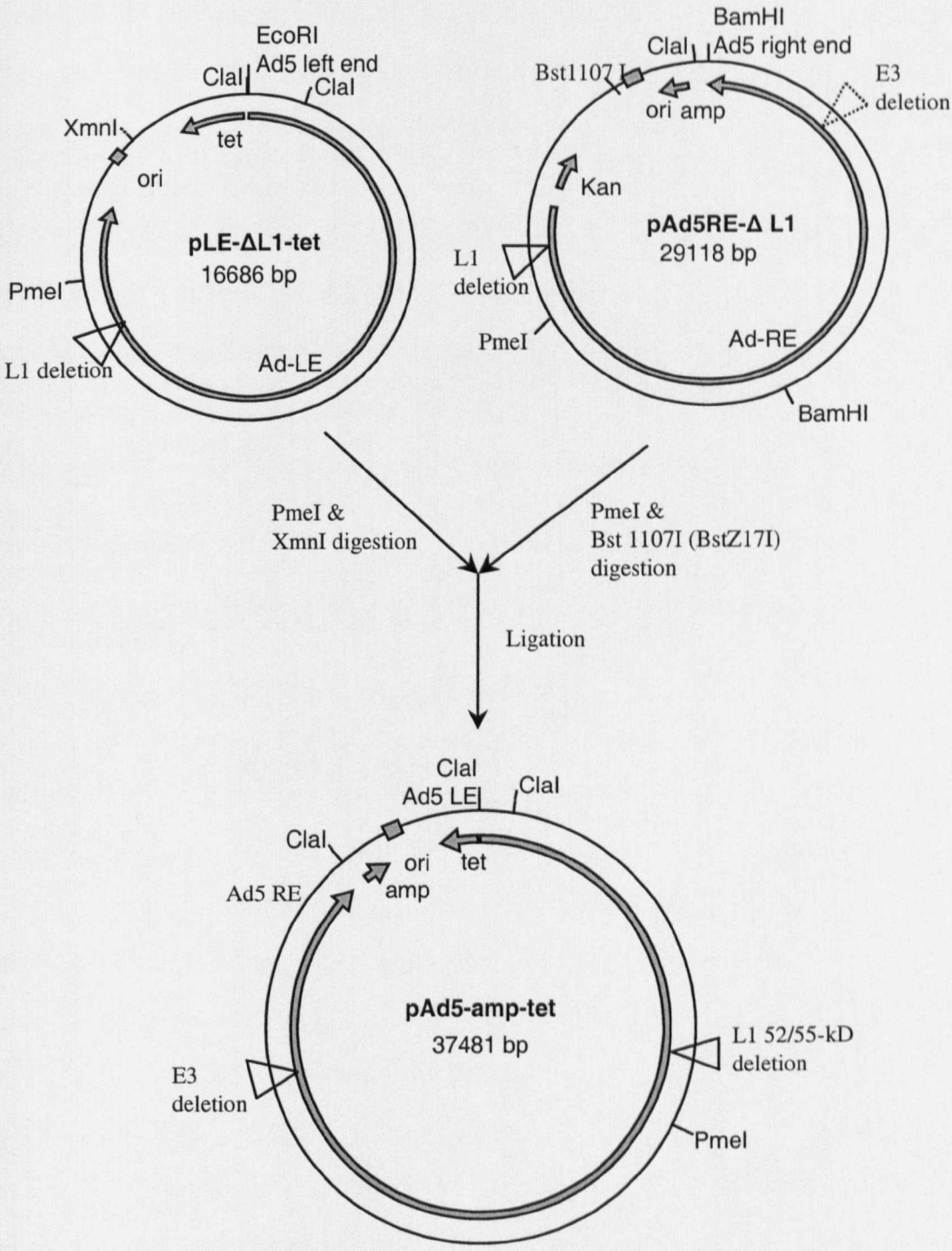


Figure 6.11 Attempted construction of pAd5-amp-tet
Large fragment of Xmn I and pMe I digested pLE-ΔL1-tet was tried to be inserted into the large fragment of Pme I and Bst1107 I digested pAd5RE-ΔL1. Plasmid pAd5-amp-tet could then be isolated by ampicillin and tetracyclin selection and the orentation could be determined by restriction digestion analysis.

genome from plasmid pLE-ΔL1-tet by the enzymes XmnI and PmeI (both produce blunt ends) and cloning the fragment into pAd5RE-ΔL1 digested by Bst1107I and PmeI (both produce blunt ends), reconstituting the entire Ad5 genome with a deletion of the L1 52/55-kD coding region (Figure 6.11). Once created, the entire Ad5 sequences then could be taken out from this plasmid by Cla I digestion (note that the Cla I site present towards the left end of the viral genome is protected by *dam* methylase in *E. coli*) and used for the transfection of 293-L1 cells. However all the attempts to construct pAd5-amp-tet failed, possibly due to instability of such a large plasmid or to the poor efficiency of ligation of the two large blunt ended fragments.

6.2.2 Attempts to construct by homologous recombination in *E. coli*

Having failed in both *in vivo* overlap recombination in 293-L1 cells and construction of a plasmid harbouring the entire manipulated Ad5 sequences, a different strategy was planned. This strategy was based again on overlap recombination for the reconstitution of an infectious viral genome but this time in *E. coli* rather than 293-L1 cells. For this purpose a *Lactococcus lactis* plasmid vector, pIL253, which does not replicate in gram-negative bacteria including *E. coli*, was used. This plasmid contains a lactococcal origin of replication, a multiple cloning site and an erythromycin-resistance gene which can be used for selection in both *L. lactis* and *E. coli* strains. Ad5 left end genome from the plasmid pAd5LE-10809 was cloned into pIL253 in *L. lactis* (Figure 6.12). The new plasmid was called pIL-LE and used for the transformation of the *E. coli* strain *MC 1061* harbouring the plasmid pAd5RE-ΔL1. The purpose of this experiment was to reconstitute the Ad5 viral genome containing a deletion of the L1 52/55-kD coding region in *E. coli* by an overlap recombination event between the plasmids pIL-LE and pAd5RE-ΔL1 (Figure 6.13). Such an overlap recombination would lead to the formation of single large plasmid termed pIL-Ad5. Since pIL-LE, which contains an erythromycin-resistance gene, is incapable of replicating in *E. coli*, any colonies grown on a selective agar medium

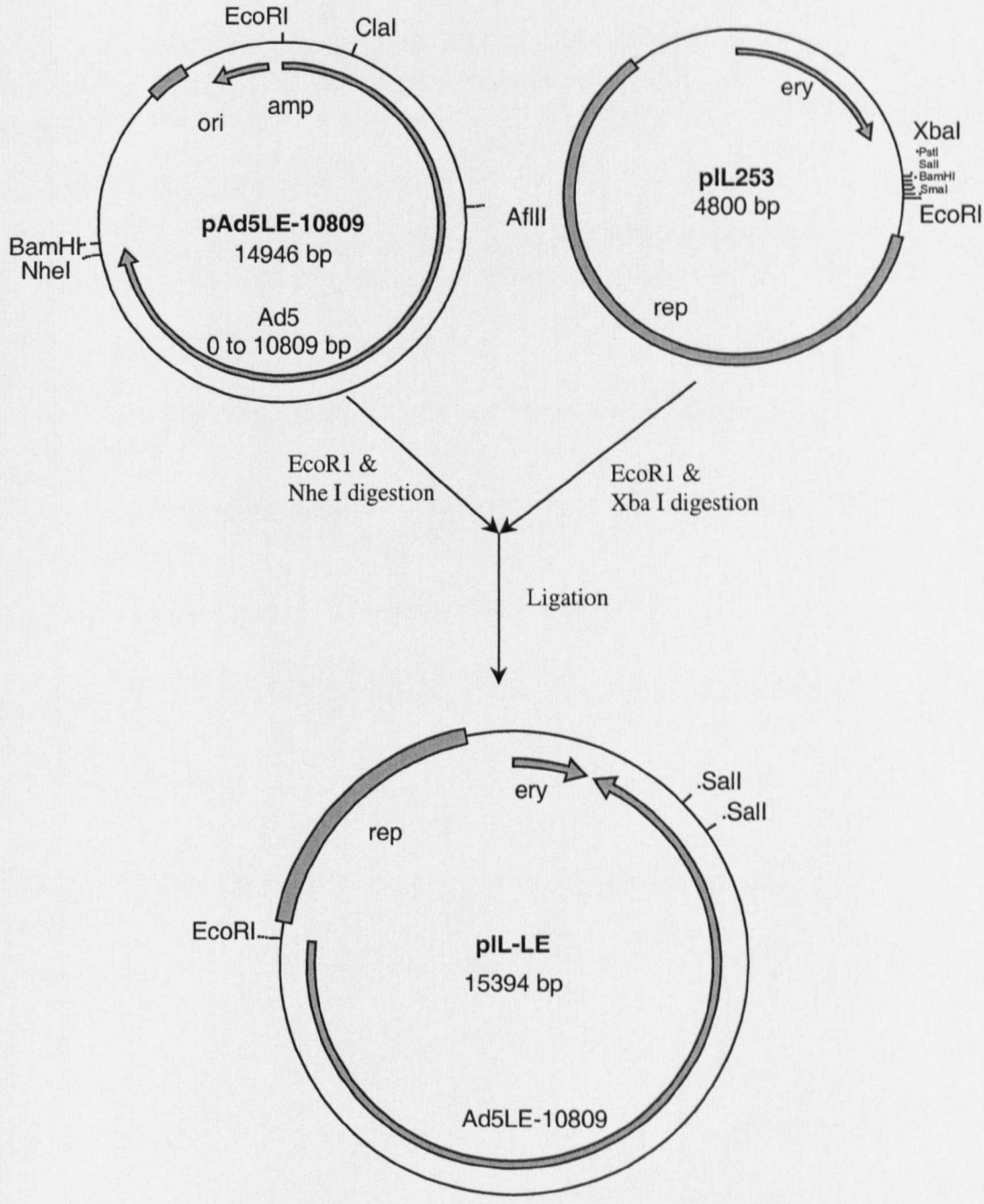


Figure 6.12 Construction of pIL-LE
A fragment containing the Ad5 sequences from pAd5LE-10809 was taken out by enzymes EcoR I and Nhe I and cloned into the lactococcal plasmid pIL253 which was digested with Xba I and EcoR I. Note that Nhe I and Xba I produce compatible cohesive end that can be ligated to each other without a need for further manipulation of the ends.

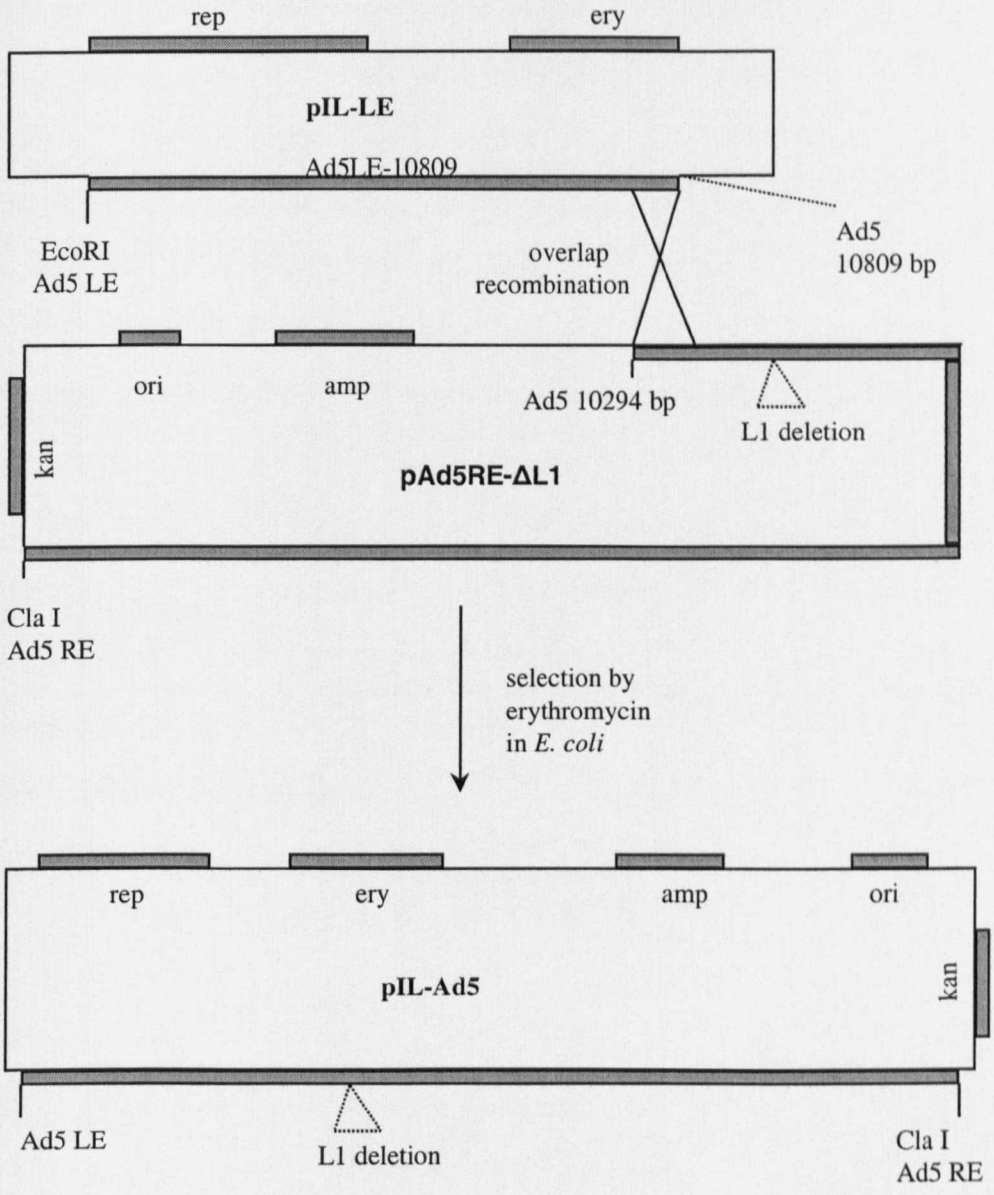


Figure 6.13 Attempts to reconstitute Ad5 genomic DNA by overlap recombination in *E. coli*.
E. coli strain *MC 1061* harbouring the plasmid pAd5RE-ΔL1 was transformed with the plasmid pIL-LE which acts as a suicide vector in *E. coli* due to its inability to replicate in gram-negative bacteria. Upon selection of the transformed bacteria with erythromycin, colonies containing the plasmid pIL-Ad5, which would be formed by an overlap recombination between the plasmids pIL-LE and pAd5RE-ΔL1, could be picked.

containing erythromycin would be expected to harbour recombinant plasmid pIL-Ad5 in which the entire Ad5 genomic sequence containing a deletion of the L1 52/55-kD coding region would reside. This plasmid could then be purified, linearized by using ClaI, and used for the transfection of 293-L1 cells in an attempt to make an L1 52/55-kD deleted Ad5 virus.

However, although several erythromycin-resistant colonies of *E. coli* were isolated, restriction analysis of the plasmid preparations from these colonies revealed that they all contained the plasmid pAd5RE-ΔL1 rather than the expected pIL-Ad5. The experiment was repeated several times but same results were obtained consistently, suggesting that the erythromycin-resistant colonies were possibly either due to the integration of the plasmid pIL-LE into the bacterial genome or to the selection of erythromycin escape mutants of *E. coli*. The underlying reason for not being able to isolate a colony containing the expected plasmid pIL-Ad5 could the plasmid's instability because of its large size (around 44000 bp).

6.3 Summary

This chapter describes attempts to make an adenovirus type 5 recombinant lacking 1108 bp of the 1247 bp L1 52/55-kD coding region. Failure to make such a virus described in section 6.1 could be due to the sequences missing in the deletion of the L1 52/55-kD coding region having other functions such as to destroy the viability of the intended deleted virus, even in the presence of effective L1 52/55-kD complementation. Since it is not possible to distinguish between this hypothesis and the alternative of failure to create the required recombinant genome by the traditional overlap recombination experiments in mammalian cell lines, a better approach would be to reconstitute the entire viral genome in a single plasmid prior to the transfection of the cell lines. By this way the construction of the desired viral genomic sequences would not depend on a low frequency recombination event. In section 6.2, attempts to construct the viral genome in a single bacterial plasmid prior

to the transfection of the cell lines by using two different strategies were described and possible reasons for the failure of those attempts were explained.

Chapter 7

Investigation of the effects of adenovirus 5 L1-52/55 kD protein on major late promoter (MLP) activity

7.1 Introduction

The adenovirus L1 52/55-kD protein is expressed both in the early and late stages of infection, raising the possibility that it has multiple roles in the viral life cycle. Two temporal classes of mRNAs, early and late, are produced during adenovirus infection. The late transcripts are further divided into five families of 3' coterminal transcripts, L1 to L5 (Fraser and Ziff, 1978). The L1 transcripts encode the IIIa and 52/55-kD proteins. Unlike the other families of late transcripts, the L1 mRNAs are detectable very early after infection has commenced (Akusjarvi and Persson, 1981; Nevins and Wilson, 1981) and the 52/55-kD protein is detectable 6 or 7 hours postinfection (Lucher *et al.*, 1986). Analysis of the 52/55-kD protein indicates that it is required for the encapsidation of the viral genome during packaging (Gustin and Imperiale, 1998) and also it has been shown that the L1 52/55-kD protein interacts specifically with adenovirus IV_{a2} protein (Gustin *et al.*, 1996). The early appearance of the 52/55-kD protein makes it unique among the gene products expressed from the adenovirus major late promoter (MLP) and its interaction with the IV_{a2} protein suggests that the 52/55-kD protein may have functions in addition to those involved in encapsidation of viral DNA (Gustin and Imperiale, 1998).

The IV_{a2} protein has been shown to be a late-stage-specific transcriptional activator of the MLP (Tribouley *et al.*, 1994). It is a component of two complexes, DEF-A and DEF-B, that bind to and activate the MLP (Tribouley *et al.*, 1994; Lutz and Kedinger, 1996) (Figure 7.1 a). DEF-B consists of a homodimer of the IV_{a2} protein. The DEF-A complex has been partially purified and shown to be composed of IV_{a2} associated with a viral or virally induced factor (Lutz and Kedinger, 1996). It is conceivable therefore that the L1 52/55-kD protein associates with the IV_{a2} protein to form the DEF-A complex. A 40 kD peptide present in infected cells has been shown to co-purify with the DEF-A complex (Lutz and Kedinger, 1996). Previous immunoblot analysis of the 52/55-kD protein has indicated that there is major band

at 40 kD that presumably arises from the action of the viral protease at a consensus cleveage site (Hasson *et al.*, 1992). It is possible that this cleavage product is the functional partner of IV_{a2} in the DEF-A complex.

7.2 Results

With the precedent of IVa2 studies in mind, which revealed the contribution of this intermediate gene product in MLP activation (Lutz and Kedinger, 1996), I examined the effect of L1 52/55-kD expression on MLP activity.

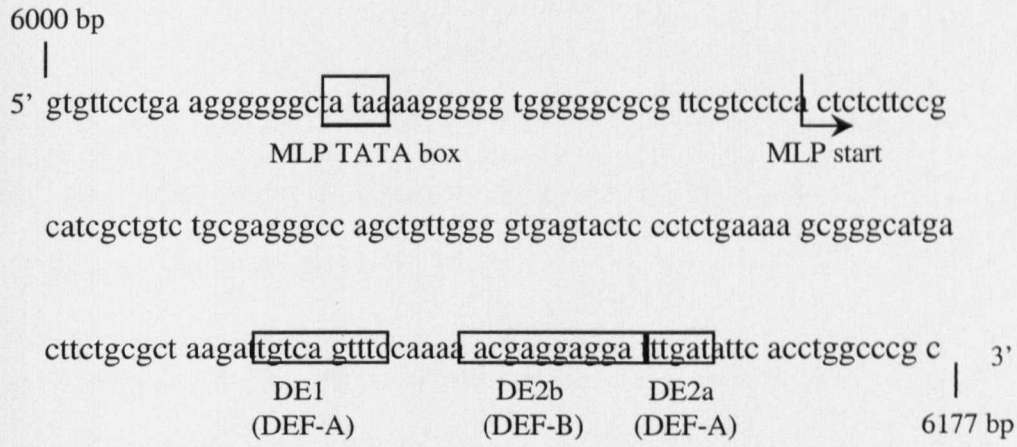
In order to confirm the interaction of the L1 52/55-kD protein and the IV_{a2} protein *in vivo*, radiolabelled extracts from cell lines 293-L1 and 293-L1-IV_{a2} were immunoprecipitated by using anti-L1, anti-L1 pre-immune, anti-IV_{a2}, and anti-IV_{a2} pre-immune polyclonal antisera. Figure 7.2 represents the co-immunoprecipitation of L1 52/55-kD and IV_{a2} proteins by both anti-L1 and anti IV_{a2} polyclonal antibodies and supports the finding by Gustin *et al.*, 1996. In addition, this result also shows that L1 52/55-kD and IV_{a2} proteins interact in the absence of infection, confirming that no other viral proteins are required for their interaction.

The experimental strategy employed for the investigation of the effects of L1 52/55-kD protein on MLP involved the transfection of COS cells with plasmids pMLP-CAT (Lutz *et al.*, 1997), a reporter plasmid which contains chloramphenicol acetyltransferase (CAT) as the reporter gene inserted down-stream of the Ad5 wild-type MLP sequence (-246 to +131, with respect to the MLP start site) (Figure 7.1 b), and one or more of the plasmids pCDNA3.1/HisB/lacZ, pMEP-CMV-L1 and pREP9-IV_{a2}. Plasmids pMEP-CMV-L1 and pMEP-CMV-IV_{a2} express Ad5 genes L1 52/55-kD and IV_{a2} genes respectively (see Chapter 5), and pCDNA3.1/HisB/lacZ expresses the *LacZ* gene from the CMV promoter. Initial attempts to study the effects of L1 52/55-kD protein on MLP activity involved the transfection of Ad5 or

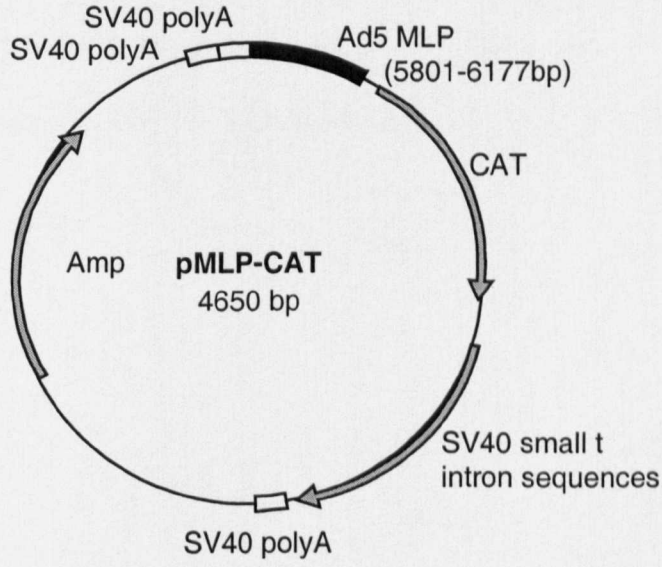
mock-infected 293-L1, 293-IV_{a2} (Brey, 1999), and 293-L1-IV_{a2} cells with the reporter plasmid pMLP-CAT but it was failed to get detectable levels of CAT expression from the reporter plasmid in any of the transfected samples (data not shown). Therefore it was decided to switch to the system employed by Lutz *et al* (1997) in which the COS1 cells are transfected with the reporter plasmid and other constructs which express the relevant proteins. Thirty-six hours after transfection, the cells were harvested, extracts were prepared and aliquots, normalised by protein concentration and beta-galactosidase activity, were assayed by CAT-ELISA (see Chapter 2) to determine the level of CAT expression. The relative MLP activities of the samples were calculated as shown and described in Table 7.1.

Initially, control experiments were performed, employing pIX expression vector pREP9-IX together with the pMLP-CAT and pCDNA3.1/HisB/lacZ plasmids for the transfection of COS1 cells in order to control the reliability of the method by comparing the results with those obtained by Lutz *et al.* (1997). According to their finding, the expression of the product of the adenovirus gene IX stimulated adenovirus MLP in a dose-dependent fashion, independent of other viral proteins.

5 μg of pMLP-CAT, 1 μg of pCDNA3.1/HisB/lacZ and 500 ng of pREP9-IX or control pREP9 plasmids were used for the transfection of 60 mm dish COS1 cell cultures. In each transfection, the final amount of pREP9 vector sequences was adjusted to 500 ng by addition of empty pREP9. Figure 7.3 shows the mean values (with the standard deviation, SD) of four independent MLP activity experiments in COS1 cells transfected with either pREP9-IX or empty vector pREP9. The experiment showed 2.4-fold increased MLP activity in the cells that received pIX expressing vector pREP9-IX, in agreement with the results published by Lutz *et al.* (1997). Also, in another experiment, the effects of different concentrations of pIX on MLP activation were investigated. The result of this experiment is shown in Figure 7.4. A dose-dependent increase MLP activation up to an input of 100 ng pREP9-IX



(A)



(B)

Figure 7.1 The Ad5 MLP and the reporter plasmid pMLP-CAT (Lutz *et al.*, 1997)

(A) The sequence of the MLP region and minimal targets (DE1, DE2a and DE2b) for the DEF-A and DEF-B binding activities, which activate MLP at late times. (B) Map of plasmid pMLP-CAT. Plasmid contains SV40 polyadenylation signals, the *cat* gene, the SV40 small t intron sequences, the ampicillin-resistance gene, and the Ad5 MLP.

A	B	C	D	E	F	G	H
Transfected sample	**relative protein []**	**b-gal activity**	**b-gal activity relative to protein []**	**CAT activity**	**CAT activity relative to protein []**	**CAT activity relative to protein [] and b-gal activity**	**relative MLP activity**
	Absorbance at 595nm of fixed volumes of the samples in BioRad protein assay	Absorbance at 420nm of fixed volumes of the samples in β-gal assay	maximum relative protein con. among the whole samples multiplied by individual sample's b-gal activity and divided by the individual sample's relative protein conc. B_{max}* (C/B)	Absorbance at 405nm of fixed volumes of the samples in CAT-ELISA	maximum relative protein con. among the whole samples multiplied by individual sample's CAT activity and divided by the individual sample's relative protein conc. B_{max}* (E/B)	maximum b-gal activity relative protein con. among the whole samples multiplied by individual sample's CAT activity relative to protein conc. and divided by the individual sample's b-gal activity relative to protein conc. D_{max}* (F/D)	Cat activity relative to protein conc. and b-gal activity divided by minimum cat activity relative to protein conc and b-gal activity among the samples G/G_{min}

Standard deviation of the relative MLP activities of samples from each independent experiments were calculated and plotted in the graphs according to the following formula:

standard deviation, $s = \sqrt{s^2}$,

where $s^2 = \Sigma (x_i)^2 / (n-1)$,

$\Sigma x_i = x_1 + x_2 + x_3 + ... + x_i$

x = sample value and

n = sample size

Table 7.1 Method for the calculation of the relative MLP activies and standard deviations of the samples observed in the transfected cells.

For the determination of protein concentration, b-gal activity and CAT activities see chapter 2.

Max : the maximum value observed in the indicated column letter.

Min : the minimum value observed in the indicated column letter.

[] : concentration.

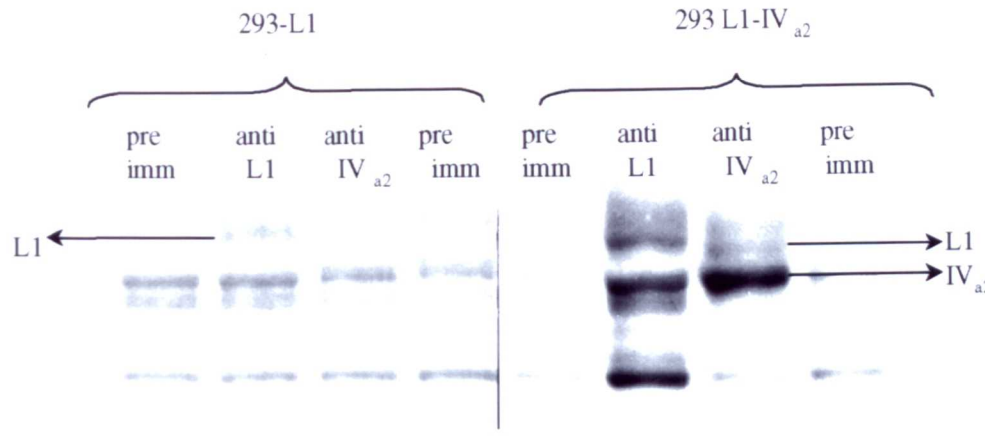


Figure 7.2 Co-immunoprecipitation of L1 52/55-kD and IV_{a2} proteins from 293-L1- IV_{a2} cells

Proteins from extracts of radiolabelled 293-L1 and 293-L1- IV_{a2} cells were immunoprecipitated by using polyclonal anti-sera, or matched control pre-immune sera, against L1 52/55-kD or IV_{a2} proteins as indicated. Samples were analysed on a 15 % SDS polyacrylamide gel and detected by autoradiography.

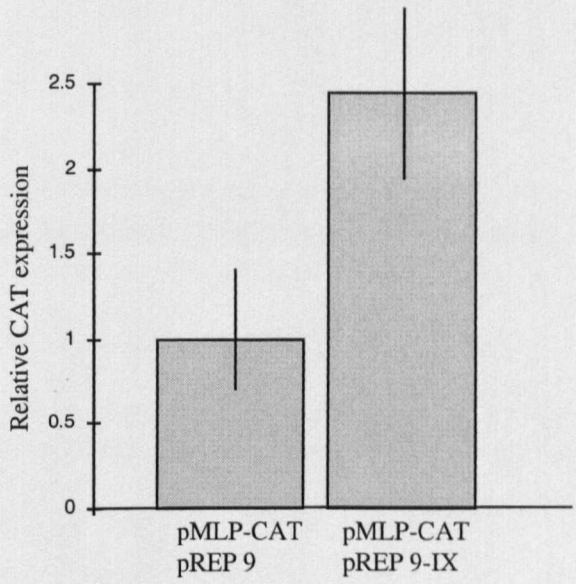


Figure 7.3 Effects of pIX on Ad5 MLP activity
5 μg of pMLP-CAT, 1 μg of pCDNA3.1/HisB/lacZ and 250 ng of pREP9-IX or pREP9 plasmids were used for the transfection of 60 mm dish COS1 cell cultures. CAT protein in cell extracts prepared 36 hours post-transfection was quantified by a CAT-ELISA. Each transfection was performed in triplicate and each extract assayed in duplicate. Error bars show the standard deviation of results obtained in four independent experiments.

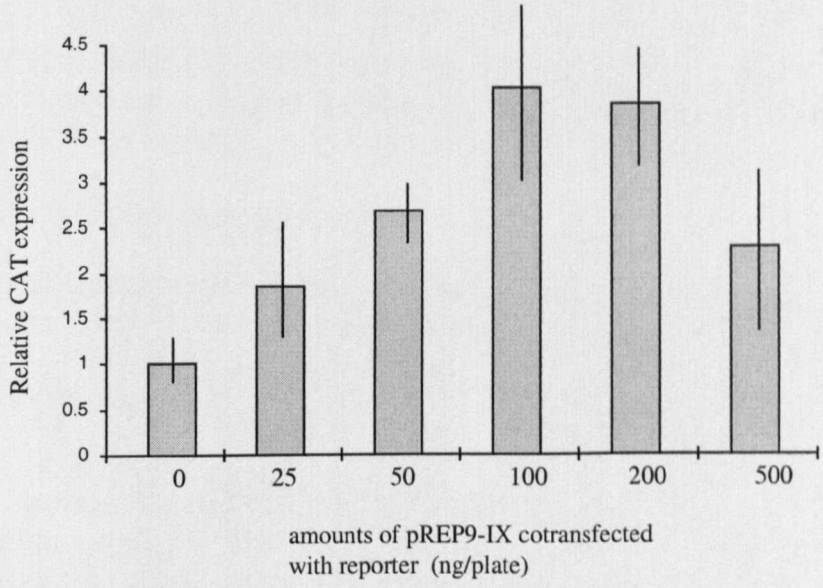
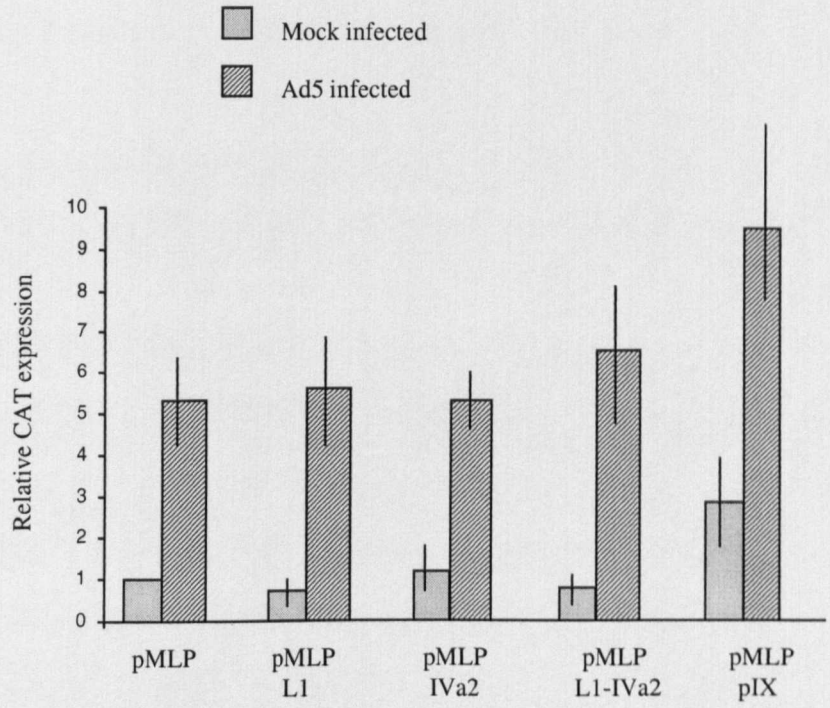


Figure 7.4 Effects of different concentration of pIX on Ad5 MLP activity
5 μg of pMLP-CAT, 1 μg of pCDNA3.1/HisB/lacZ and different amounts of pREP9-IX (the amount of pREP9 vector sequences was adjusted to 500 ng in each case by the addition of empty pREP9) were used for the transfection of 60mm dish COS1 cell cultures. CAT protein in cell extracts prepared 36 hours post-transfection was quantified by a CAT-ELISA. Each transfection was performed in triplicate and each extract assayed in duplicate. Error bars show the standard deviation of results obtained in four independent experiments.



L1 : pMEP-CMV-L1
IVa2 : pMEP-CMV-IVa2
pIX : pREP9-IX

Figure 7.5 Effects of different Ad5 late proteins on Ad5 MLP activity
5 μg of pMLP-CAT, 1 μg of pCDNA3.1/HisB/lacZ and 100 ng of pMEP-CMV-L1 and / or pMEP-CMV-IVa2, or pREP9-IX (in each transfection the amount of pMEP-CMV and pREP9 vector sequences were adjusted to 200 ng by the addition of empty pMEP-CMV or pREP9) were used for the transfection of 60 mm dish COS1 cell cultures and the cells were either mock or Ad5 (moi of 10) infected 12 hours after transfection. CAT protein in cell extracts prepared 36 hours post-transfection was quantified by a CAT-ELISA. Each transfection was performed in triplicate and each extract assayed in duplicate. Error bars show the standard deviation of results obtained in four independent experiments.

per plate was observed, which again correlates with the published data.

Reproduction of the previously published data published by Lutz *et al.* (1997) gave a good indication of the reliability of the method employed for the investigation of the effects of other Ad5 late proteins on the adenovirus MLP activity. The next step was to investigate the effects of Ad5 IV_{a2}, L1 52/55-kD and both IV_{a2} and L1 52/55-kD proteins on the adenovirus MLP activity. For this, COS1 cells in 60 mm dishes were transfected with 5 μg of pMLP-CAT, 1 μg of pCDNA3.1/HisB/lacZ and 100 ng of pMEP-CMV-L1 or pMEP-CMV-IV_{a2} or both. In each transfection the the amount of pMEP-CMV vector sequences was adjusted to 500 ng by the addition of empty pMEP-CMV (in case of control transfections involving Ad5 infection, the cells were infected with wild type Ad5 12 hour after transfection). Thirty-six hours after transfection, the cells were harvested, extracts were prepared and aliquots, normalised by protein concentration and beta-galactosidase activity, were assayed by CAT-ELISA for the determination of CAT protein concentration. The mean values from the results from four independent experiments, and their standard deviations are shown in a graphical form in figure 7.5. As it can be seen in the figure, neither the L1 52/55-kD nor the IV_{a2} protein alone had any effect on the reporter activity, as would be expected if both proteins are needed to form the DEF A complex which binds to MLP to cause its activation together with the DEF B complex. However, the expression of both proteins at the same time in the same cells also had no effect on the MLP, disproving the idea that the L1 52/55-kD protein is involved with IV_{a2}, in the activation of MLP. In the control experiments where the transfected cells were infected with wild type Ad5, there was marked increase in the CAT reporter activity as expected due to the presence of viral or virally induced factors in the cells. Also in cells transfected with the pIX expression plasmid as a positive control there was increased reporter activity in agreement with earlier observations.

7.3 Summary and Discussion.

In this chapter I have described an investigation of the effects of the Ad5 L1 52/55-kD protein on Ad5 MLP activity by transfection of COS1 cells using a MLP driven CAT reporter construct.

The early appearance of the 52/55-kD protein has been taken as an indication that it might have additional functions at early times during infection (Hasson *et al.*, 1989). Also, previous publications had led to the hypothesis that this L1 protein might activate MLP in a complex with IV_{a2} protein (Gustin *et al.*, 1996). However, according to a recently published article by Gustin and Imperiale, (1998) their analysis of infection by an adenovirus harbouring a mutation that blocks expression of the L1 52/55-kD protein, H5pm8001, did not reveal a requirement for the 52/55-kD at early times. As they determined by examining the onset of DNA replication and gene expression, H5pm8001-infected 293 cells made the transition from the early to late stage infection at the same time as did wild type Ad5 infected cells and the 52/55-kD protein was not required for the activation of the MLP during infection. The results of the transient expression experiments in COS cells presented in this chapter also support the idea that L1 52/55-kD protein does not have any effects on the activation of the MLP. The infected cell-specific partner protein with IV_{a2} in the MLP-activating factor DEF-A therefore remains unidentified.

Thus it would be appropriate to speculate that, the interaction between the IV_{a2} and 52/55-kD proteins is involved in another function, possibly in the packaging of the viral genome since the 52/55-kD protein mediates encapsidation of the viral genome (Gustin and Imperiale, 1998).

Chapter 8

General discussion

The enabling technology of gene therapy is based on strategies for delivering genes. To do this, gene delivery vehicles called vectors have been developed, which encapsulate therapeutic genes for delivery to cells. Many of the vectors currently in use are based on attenuated or modified versions of viruses. Over billions of years of evolution, viruses have developed extraordinarily efficient ways of targeting cells and delivering their genomes into the cells, which unfortunately leads to disease. The challenge in developing such viruses as vectors is to remove the disease-causing components of the virus and insert recombinant genes whose expression will be therapeutic to the patient. The modified viruses cannot be allowed to replicate in the patient, but must retain the ability to efficiently deliver genetic material.

Of the numerous viral vectors investigated, those based on adenoviruses have proved efficient for gene transfer both in vivo and in vitro. They are relatively safe and the extensive knowledge of their biology has made them strong candidates for use in gene therapy applications. Despite their advantages, the lack of long-term persistence of gene expression from cells transduced with these vectors, the generation of host immune responses against them, and the limited insert-carrying capacity of these vectors has greatly limited their application.

The aim of the work presented here was to achieve improvements over currently existing adenovirus vectors for gene therapy through increasing their insert carrying capacity by the deletion of some of the viral late gene(s). Attention was focused primarily on the L1 52/55-kD protein-coding region. The effect of this gene product (L1 52/55-kD protein) on the expression of other late genes was also investigated.

In the preceding chapters I have described experiments designed to construct complementing cell lines that would support the growth of adenoviral vectors containing deletions in their L1 52/55-kD, L1 52/55-kD and IV_{a2}, and L1 52/55-kD and IX coding regions. Both IX and IV_{a2} proteins have functions that are involved in

the transactivation of the adenovirus MLP, hence the production of other viral late proteins. Initially, L1 52/55-kD protein was also believed to be involved in the transactivation of the viral MLP. Thus the construction of recombinant adenoviral vectors containing deletions in their L1 52/55-kD, L1 52/55-kD and IV_{a2}, and L1 52/55-kD and IX coding regions together with the deletions available in currently used vectors was expected to result not only in increased insert capacity but also to decreased immunogenicity due to decreased levels of viral late gene expression.

Initial experiments described in Chapters 3-5 involved the cloning and sequencing of the Ad5 L1 52/55-kD cDNA and production of antibodies against L1 52/55-kD protein. The antibodies were later used for the detection of the L1 52/55-kD protein from 293-L1 cells. 293-L1 cells were constructed by transfection of a stably maintained episomal mammalian expression vector harboring the L1 52/55-kD expression cassette. Similarly, cell lines 293-L1-IV_{a2} and 293-L1-IX expressing the L1 52/55-kD and IV_{a2} and L1 52/55-kD and IX proteins respectively were constructed using appropriate episomal expression vectors.

In Chapter 6, attempts to construct an adenovirus recombinant deficient in L1 52/55-kD coding region were described. Although various different strategies were employed for the construction, isolation of such a recombinant proved more difficult than anticipated, and ultimately no such virus was isolated. One possible explanation for the failure to isolate such a virus could be due to the nature of sequences missing in the deleted L1 52/55-kD coding region. Adenovirus gene expression is complex (see Chapter 1) and the sequences may well be carrying one or more yet undiscovered *cis*-acting elements which have functions that are important directly or indirectly for the replication of the virus, rendering construction of such a virus impossible. It is also possible that, during the cloning steps described in chapter 6, a mutation or mutations might have been introduced into the viral sequences elsewhere in the genome destroying the infectivity of the recombinant viral genome.

These reasons are largely the subject of speculation, and require experimental evaluation. One way of doing this would be the construction of both the wild type and recombinant viral sequences in separate single plasmids before transfection of the cell lines in an attempt to make the virus. This way it would be possible to test the presence of any mutations in the cloned viral sequences that would affect the infectivity of the viral genome. The advantages this system would provide are firstly there is no need for a recombination event in order to reconstitute a viral genome *in vivo* and secondly, ease of production of large amounts of the recombinant viral genome DNA. However, several attempts to carry out this experiment failed and consequently, the work had to be abandoned due to the time limitations.

Had the work described in Chapter 6 resulted in the isolation of an L1 52/55-kD deleted virus, insertion of IV_{a2} and/or IX coding region deletions into the L1 52/55-kD deleted viral genome would have been attempted. The ultimate target would be to create a recombinant adenovirus with deletions of E1, E4, L1 52/55-kD, IV_{a2}, and IX coding regions, and containing a reporter (e.g. β-gal) expression cassette. The duration of reporter gene expression and the nature and extent of the immune response to the vector in mice would be compared to those produced by a recombinant adenovirus containing the same reporter cassette and the deletions of E1 and E4 region only and the possible advantages or disadvantages of using such a vector would therefore be assessed.

Experiments described in Chapter 7 were done in order to investigate the effects of L1 52/55-kD on the adenovirus MLP. Early reports suggested that a possible role for the 52/55-kD protein might be to regulate proper temporal activation of late gene expression by interacting with the IV_{a2} protein, since IV_{a2} was known to be involved in this process by interacting with another yet unknown viral or infection-induced protein. However transient expression experiments carried out by transfection of COS cells with a MLP dependent reporter gene (CAT) and expression vectors for L1

52/55-kD and/or IV_{a2} did not reveal any role for the L1 52/55-kD protein in the activation of MLP. Therefore, since the L1 52/55-kD protein is known to be involved in the process of viral genome encapsidation, it can be speculated that the interaction between IV_{a2} and the L1 52/55-kD proteins is involved in packaging the viral genome. Experiments designed to examine if these proteins, alone or in combination, have any affinity for the adenovirus packaging signal would be an appropriate investigation of this speculation.

The basic challenge in gene therapy is to develop approaches for delivering genetic material to the appropriate cells of the patient in a way that is specific, efficient and safe. This problem of "drug delivery," where the gene is a drug, is particularly challenging for genes which are large and complex and require targeting to the nuclei of cells. However, if genes are appropriately delivered they can persist for the life of the cell and potentially lead to a cure. For gene therapy to become a widely used medical therapy, several technical hurdles need to be overcome. First, gene transfer vectors are needed that can be injected directly into the patient. In addition the injected vector would need to target specifically to and enter a particular tissue or organ. Second, once the gene vector has reached the cell of interest, its genetic content needs to integrate safely into a non-critical site on the chromosome, homologously recombine with the defective gene it is trying to replace or otherwise achieve long-term persistence. Finally, ways must be found to control adverse immune response to vector and transgene products.

Further developments of adenovirus vectors, of the type described and attempted in this thesis represent a likely avenue through which some or all of these obstacles may be overcome.

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Appendix A : Primers

AP1:

5' TTT TTG GAT CCA TGC ATC CGG TGC TGC GG 3'

AP2:

5' TTT TTG AAT TCC GCT TAG TAC TCG CCG TCC T 3'

L5:

5' GGA AAC AGG GAC GAG CC 3'

L6:

5' TGA TCA GAA ACA TCA CCG GC 3'

Reverse primer:

5' CAGGAA ACAGCTATGA C 3'

Appendix B: Buffers and Solutions

LB	1 % (w/v) bactotryptone, 1 % (w/v) NaCl, 0.5 % yeast extract
LB agar	1 % (w/v) bactotryptone, 1 % (w/v) NaCl, 0.5 % yeast extract, 1.5 % (w/v) bacto-agar
PBS	137 mM NaCl, 2.7 mM Na_2HPO_4, 1.4 mM KH_2PO_4
TBE	89 mM Tris-HCl, 89 mM boric acid, 1 mM EDTA pH 8.0
TD	25 mM Tris-HCl, pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4
TE	10 mM Tris-HCl, pH 8.0, 1 mM EDTA
TNE	10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0
TS	25 mM Tris base, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 1 mM $MgCl_2$
10X DNA gel loading buffer	0.21 % Bromophenol Blue, 0.21 % Xylene Cyanol FF, 0.2 M EDTA, pH 8.0 and 50 % Glycerol

Antibiotic and X-gal stock solutions

Antibiotic	Stock Solutions Conc	Working Concentration
Ampicillin	50 mg/ml in H_2O	100 μg/ml
Chloramphenicol	34 mg/ml in ethanol	170 μg/ml
Erythromycin	50 mg/ml in athanol	500 μg/ml for *E. coli* 5 μg/ml for *L. lactis*
Kanamycin	10 mg/ml in H_2O	50 μg/ml
Streptomycin	10 mg/ml in H_2O	50 μg/ml
Tetracycline HCl	5 mg/ml in ethanol	50 μg/ml
X-gal	40 mg/ml in dimethyl formamide	500 μg/ml

Appendix C: Suppliers

All chemicals, of analytical or molecular biology grade, were supplied by BDH (Poole, Dorset, UK), Sigma (Poole, Dorset, UK) or Fisons Scientific (Loughborough, UK) unless otherwise stated below.

Amersham (Amersham, Bucks, UK)

Biotinylated goat anti-rabbit polyclonal antibody, streptavidin-biotinylated horseradish peroxidase, Hybond C membrane, SDS-PAGE rainbow markers, T7 Sequenase (version 2.0) DNA polymerase

Becton Dickinson (Cockeysville, USA)

Yeast extract

BioRad (Hemel Hempsted, Herts, UK)

Acrylamide, N,N'-methylene-bis-acrylamide, TEMED, ammonium persulphate

BOC (Surrey, UK)

N_2, CO_2, dry ice

Boehringer Mannheim (Lewes, East Sussex, UK)

Hygromycin B, CIAP

Difco Laboratories (Basingstoke, UK)

Bacto-agar, bactotryptone, M17, noble agar

Fisher Scientific (Loughborough, UK)

Chloroform, ether, caesium chloride, SDS

Gibco BRL (Renfrewshire, UK)

DMEM, dsDNA markers, FCS, G418, Klenow DNA polymerase, Lipofectace, NCS, Restriction enzymes and buffers, T4 DNA ligase, T4 DNA polymerase, Taq polymerase, trypsin, versene

Invitrogen (Carlsbad, USA)

Mammalian expression vectors

Kodak (London, UK)

X-ray developing and fixation solutions, X-ray film

New England Biolabs (Herts, UK)

Restriction enzymes

Pharmacia Biotech (Herts, UK)

dNTPs, glutathione-Sepharose 4B beads, pGEX-2T

Qiagen (Hilden, Germany)

Qiaprep plasmid miniprep and maxiprep kits

Stratagene (Cambridge, UK)

E. coli XL1 Blue, E. coli XL1 Blue MR

University of Warwick media preparation service

PBS, dH_2O, trypsin/versene, neutral red