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Assessment of the adenovirus E1 proteins for the production of E1-deleted adenoviral vectors in complementing cell lines

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Ce mémoire intitulé:

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Abstract

Gene therapy is the transfer of new genetic material to an individual which results in a therapeutic benefit to that individual. With new genes being discovered almost on a daily basis and the human genome nearly completely sequenced, the focus of research has now turned to improving vectors used in delivering these therapeutic genes. Of the many vectors used to date, adenoviral vectors represent one of the most important delivery systems, especially for *in vivo* gene therapy. Nevertheless, certain problems with these vectors have been raised recently which require special attention before they are to be fully successful. In particular, the development of replication competent adenoviruses (RCAs) during large scale production of adenoviral vectors has been problematic particularly in light of the strict new FDA regulations. For the most part, viral vectors are rendered replication deficient by the deletion of their transcriptional unit. In the case of adenoviruses this represents the early immediate (E1) unit and is usually replaced by the gene of interest. The proteins are provided in transcomplementing cell lines, such as 293, which allow replication of E1-deleted viruses. However, if these replication deficient adenoviral vectors were to reacquire their E1 unit by homologous recombination (hence RCAs) these vectors would then destroy their target cells, not treat them. Moreover, with companies increasing their production scale to 10,000 L the need to improve on existing viral titers is warranted. To address these issues, a new complementing cell line, called BMAdE1, was designed in order to eliminate RCAs and E1-expressing vectors were constructed in order to evaluate the impact that these

proteins have independently on the transcomplementation of replicative defective adenoviral vectors. Experiments carried out with a GFP expressing adenoviral vector showed that BMAdE1 cells were successful in preventing RCAs from developing, however, recombinant virus production was substantially less than what can be obtained in 293 cell. This discrepancy may be due to the varying levels of E1 in the cell at the time of infection and/or due to an intolerance to E1 proteins rendering the cell a poor host for viral replication. Consequently, experiments were done with E1 expressing viruses which augmented levels of each of the E1 proteins at the time of infection. These experiments were, however, not successful since the recombinant viruses which were constructed for this purpose did not express the E1 proteins properly. The only exception was a virus which expressed the E1 protein, E1B19K. The results with this virus showed that increasing levels of E1B19K at the time of infection did not improve viral yields as initially thought and may in fact lower the production capacity of a cell if overexpressed. These results suggest that the overexpression of E1 proteins at the time may not be necessary for the increased viral production and that perhaps other factors which improve the host cell condition should be considered. Furthermore, it was observed during the course of the experiments with the E1B19K virus that the introduction of a multiple cloning site (MCS) upstream of the transgene could potentially lower protein levels. These results were also obtained with an independent protein and thus could serve as an important warning when designing new vectors where the purpose is high level protein production.

Résumé

La thérapie génique, sur laquelle se fondent beaucoup d'espoir médicaux, se veut une méthode thérapeutique consistant à intervenir sur le génome des cellules afin de traiter certaines affections, souvent génétiques. Il s'agit essentiellement d'opérer un transfert de matériel génétique à l'intérieur de cellules spécifiques chez le patient, afin de donner libre cours à l'expression de certains gènes bénéfiques; soit par la méthode *ex vivo* où ces cellules sont d'abord prélevées et recombinées génétiquement *in vitro* avant d'être retransplantées chez le patient, soit par la méthode *in vivo* où certains vecteurs vont larguer le matériel transgénique directement à l'intérieur de l'organisme et atteindre les cellules cibles.

Parmi les vecteurs utilisés pour véhiculer le gène thérapeutique et l'acheminer aux cellules cibles spécifiques en vue de son expression, il y a les adénovirus défectifs pour la réplication. Ils se révèlent relativement faciles à manipuler *in vitro*, croissent en quantité suffisante pour être administrés *in vivo* au patient, et apparaissent appropriés pour un transfert génique dans les cellules musculaires ou nerveuses. Toutefois, l'utilisation des adénovirus comme vecteurs pose deux problèmes: i) l'apparition d'adénovirus compétents pour la réplication lors d'une production à grande échelle avec la lignée de complémentation 293; ii) la nécessité d'augmenter les niveaux de production afin de pouvoir subvenir aux besoins grandissants à l'échelle industrielle. Le contenu de ce mémoire de maîtrise porte donc sur ces problèmes qui sont associés à la biologie des adénovirus.

Au cours de ce projet recherche, une évaluation d'une nouvelle lignée cellulaire de complémentation, BMAdE1, fut faite. Cette lignée se caractérise par sa capacité à contrer l'apparition d'adénovirus compétents pour la réplication. Les résultats de ces expériences ont montré que ces lignées permettent la production de virus délétés de la région E1 sans l'apparition de particules virales aptes à la réplication, mais à des titres inférieurs à ceux souhaités. Une hypothèse fut donc proposée à l'effet que les niveaux de E1 au moment de l'infection ne seraient pas idéaux pour la réplication d'adénovirus recombinants. C'est pour cette raison que furent étudiés les rôles joués par chacune des protéines codées séparément de la région E1. Pour ce faire, des adénovirus recombinants ont été construits de façon à exprimer indépendamment chacune de ces protéines: E1A, E1B19K et E1B55K. Cependant, deux des trois recombinants construits n'ont pu exprimer correctement leur transgène et ne purent donc pas être utilisés pour les expériences. En effet, seul le virus exprimant la protéine E1B19K s'est révélé efficace. Toutefois, contrairement à ce qui pouvait être attendu, l'augmentation des niveaux E1B19K dans les cellules de complémentation au lieu d'être bénéfique à la réplication des vecteurs a engendré plutôt une baisse de la production de virus recombinants. Il se peut que les cellules de la lignée de complémentation soient sensibles à l'effet toxique induit par la protéine et que des niveaux élevés de la protéine peuvent être toujours utiles mais à condition d'avoisiner ceux produits normalement par un virus à l'état sauvage. Enfin, durant le cours de ces expériences, une baisse importante de la production de la protéine recombinante fut remarquée lorsqu'un site de clonage multiple fut introduit

en amont du gène E1B19K afin de faciliter le clonage de nouveaux gènes. Fait intéressant à noter, cette observation fut aussi faite avec la protéine GFP, qui est une protéine non reliée à E1B19K. Les résultats obtenus semblent indiquer l'introduction d'un site de clonage multiple en amont d'un transgène pourrait avoir un impact sur les niveaux d'expression de protéines et devrait en conséquence être prise en considération lorsqu'on envisage un nouveau vecteur dont le but est d'optimaliser les niveaux de protéines.

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List of commonly used abbreviations

Ad-	adenovirus
AdVs-	adenoviral vectors
AES-	adenovirus expression system
cDNA-	complement DNA
CPE-	complete cytopathic effect
DNA-	deoxyribonucleic acid
E1-	early-immediate transcriptional unit
FDA-	Food and Drug Administration
GFP-	green fluorescent protein
GTU-	gene transfer unit
ITRs-	inverted terminal repeats
K7-	cassette
Kb-	kilobase
kDa-	kilodalton
MCS-	multiple cloning site
ml-	milliliter
MLP-	major late promoter
MOI-	multiplicity of infection
mRNA-	messenger RNA
PCR-	polymerase chain reaction
PFU-	plaque forming unit
RCA-	replication competent adenoviruses
RNA-	ribonucleic acid
rtTA-	reverse tetracycline-controlled transactivator
TR5-	tetracycline responsive promoter
tTA-	tetracycline-controlled transactivator
UTR-	untranslated region
µg-	microgram
wt-	wild type

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Chapter 1
Introduction

1.1 The expectation of gene therapy

The future of medicine is expected to rely heavily on recombinant DNA technology for improvements in patient care. This includes the development of new molecular techniques for the diagnosis of diseases and the development of new therapeutic drugs. Dramatic advances in molecular genetics have also heightened expectations for gene therapy.

Gene therapy is essentially the transfer of genetic material into specific cells of a patient to treat human diseases. Two separate methods of delivering the therapeutic gene are possible. Gene transfer can be carried out *in vivo*, where the vector containing the expression cassette is delivered directly to target cells within the individual. Alternatively, due to the inability of certain vectors to infect or reach the target tissue, cells from the latter are removed, grown *in vitro*, and infected/transfected with the recombinant vector. The altered cells producing the foreign protein are then transplanted back into the patient. This procedure is known as '*ex vivo* gene therapy'.

1.2 Vectors used in gene therapy

There are essentially two types of gene delivery vehicles, more commonly known as vectors. In both cases, however, the gene of interest is first placed in the vector which in turn is capable of delivering the gene into specific target cells for its expression. Ideally, the vector used should be safe and deliver genes efficiently. From the start, viruses have attracted the attention of scientists for their innate ability to specifically enter cells and express their genes (Friedmann and Roblin, 1972). As such, if the viruses' genes responsible for replication and virulence were to be replaced with therapeutic genes then, in theory, the recombinant virus should transfer helpful genes to cells but should not be able to replicate or produce disease. However, due to the ever present ability of viruses to induce disease, scientists have also developed nonviral methods for the therapeutic transfer of genes. The obvious advantage of these vectors is that they are safe and provide an alternative to those in the medical field who have difficulty accepting viruses as therapeutic vectors.

1.2.1 Non-viral vectors

Many scientists feel that gene therapy protocols will have to gain the acceptance of physicians as well as patients if they are to become alternatives to conventional pharmaceutical, biological and surgical therapies. As viral vectors continue to show great promise as therapeutic vectors there is always the fear that the residual viral elements within the viral vector may become cytopathic and cause harm

to the patients. Even though clinical trials to date have limited evidence of disease caused by viral vectors, there is continued apprehension in using viruses as gene delivery vehicles. As such, many methods used to deliver conventional drugs and biological products are now being used to deliver genes. Liposomes, cationic lipids, polymers, and endosomal lysis peptides are examples of non viral methods used today to deliver genes for therapeutic purposes (reviewed in: Ledley., 1994; Ledley 1995). The major advantage of nonviral vectors is that they are safe. They have not been associated with any immunological response and eliminate the possibility of generating a pathogenic vector as a result of rare recombination events. They also have a very large insert capacity. Their drawbacks, however, have severely limited their application *in vivo*. Non viral vectors have a very low gene transfer efficiency, about 10,000 times less efficient when compared to viral vectors. For example, there have been problems with the release of the therapeutic gene from the endosome following uptake by the cell of the nonviral vector. These and other problems associated with nonviral vectors are currently being addressed (reviewed in: Cooper., 1997) and the hope is that nonviral vectors will be used in gene therapy protocols aimed at achieving short-term expression of therapeutic genes in selected tissues with a high degree of safety.

1.2.2 Viral vectors

The viral vectors used as gene delivery vehicles include retroviruses, adenoviruses, adeno-associated virus, herpes simplex virus, vaccinia virus, and more

recently lentiviruses. (reviewed in: Ali et al., 1994; Joly, 1994; Methali, 1996; and Verma and Somia, 1997). The first human trial used recombinant retrovirus genomes as vectors and showed promising although not completely successful results (Rosenberg et al., 1990). Today, the two most widely used viral vectors for gene therapy are the retroviruses (RVs) and adenoviruses (AdVs). Both types of viruses provide a good illustration of how the specific advantages and limitations of each predispose them to specialized field of applications. For example, RVs are capable of integrating the host's genome and giving a long-lasting, perhaps life-long, expression of the recombinant gene. However, they are difficult to grow to high titres and purify for direct administration, which requires that the patient's cells be cultured and transduced *in vitro*. Furthermore, they cannot infect non-dividing cells such as somatic cells, the only exception being lentiviruses, which are presently being investigated as potential vectors. Finally, there is always the risk, although remote, of insertional mutagenesis and/or activation of cellular oncogenes. AdVs, on the other hand, are relatively easy to manipulate *in vitro*. They grow to high titres and can be purified for *in vivo* administration to the patient. Moreover, AdVs can transduce both proliferating and non-proliferating cells making them better suited for gene transfer in post-mitotic cells such as muscle and nerve cells. The major drawbacks associated with AdVs are i) the generation of replication competent adenoviruses (RCAs) during large scale production (see below section 1.5 for more details). The presence of RCAs or partly attenuated vectors can lead to misinterpreted results or pathogenesis, especially when administered to the lungs. ii) since AdVs remain episomal multiple doses are required to maintain the expression of the transgene. However, a strong

immune response has been associated with AdVs and renders the results of readministration ineffective.

As can be seen by the brief description of RVs and AdVs, much work remains before these vectors are routinely used as vehicles for therapeutic genes. In fact, much of the focus today is now concentrated on the development of new vectors that can circumvent the problems mentioned.

1.3 Adenovirus Biology

1.3.1 Historical perspective

Adenoviruses were first isolated in the 1950's by Rowe et al. (1953), in an attempt to identify the etiologic agent of the common cold and by Hillerman and Werner (1954) while studying an epidemic of respiratory illness among army recruits. However, it was Hubener that demonstrated that both had in fact isolated the same virus (Hubener et al., 1954). The two original groups had observed the spontaneous degeneration of cell cultures made from primary human adenoid and tracheal cells isolated from individuals with acute respiratory illness. In 1956, the term adenovirus was designated for this viral agent, due to the source of tissue (adenoid) from which the virus was isolated (Enders et al., 1956). By the late sixties, however, it was apparent that adenoviruses were not the causative agent of the common cold and in

fact cause limited respiratory illnesses in children and even less in the general population (Brandt et al., 1969; Maller et al., 1966).

Adenoviruses came to the forefront of research with the discovery that a human adenovirus, serotype 12 (Ad12), could cause the induction of tumors when injected into newborn hamsters (Trentin et al., 1962). This finding led to widespread suspicion within the scientific community that a human cancer might be associated with adenovirus infection. This phenomenon, however, was found to be associated solely with rodents and no human cancers have been linked with any of the adenovirus DNA sequences (Green et al., 1980; Mackey et al., 1976). Adenoviruses have proven over the years to be an invaluable model for the study of many basic aspects of cell biology and gene expression in mammalian cells.

1.3.2 Classification

Adenoviruses belong to the family of Adenoviridae. This family is subdivided into two genuses, *Mastadenovirus* and *Aviadenovirus*. Human adenoviruses are members of the *Mastadenovirus* genus which also includes simian, bovine, equine, porcine, ovine, canine, and opossum viruses. The Aviadenoviruses only infect birds and are sufficiently distinct to merit a separate genus.

Table 1.1

CLASSIFICATION OF HUMAN ADENOVIRUSES

Subgroup	Serotypes	Oncogenic Potential	
		Tumours in animal	Transformation in tissue culture
A	12,18,31	High	+
B	3,7,11,14,16,21,34,35	Moderate	+
C	1,2,5,6	Low or none	+
D	8,9,10,19,20,22-30,32,33,36-39,42-47	Low or none	+
E	4	Low or none	+
F	40,41	Not determined	+

Adapted from (Shenk, 1996)

To date, forty-seven human adenovirus serotypes have been isolated, based on type specific neutralizing antigenic determinants (Toogood et al., 1992). Each serotype (Rosen, 1960; Norrby, 1969) was then classified into one of six subgroups (see Table 1), A-F, based on the serotype's ability to agglutinate red blood cells (Hierholzer, 1973). Of particular note is subgroup C, which is non-tumorigenic and contains the two serotypes, 2 and 5, which have been historically the most extensively studied.

1.3.3 Adenoviral lytic cycle

Structurally, the adenovirus is a non-enveloped icosahedral shaped virus. It is a medium sized virus, 70 to 100 nm in diameter (Horne et al., 1959), with 20 triangular surfaces containing 12 vertices at each corner. The virion is made up of a protein shell, called the "capsid", surrounding the "core" of the virus where the DNA is located. The icosahedral shape is the evolutionary choice of the majority of animal viruses, probably because it is the most efficient geometric shape in which to form a closed shell. Moreover, due to the complex nature of capsid assembly, very few variations are permitted. All in all, the adenoviral virion is composed of more than 2700 polypeptides and approximately 36 kilobase DNA molecule.

1.3.4 The viral capsid

The adenovirus proteins are located in both the outer shell of the virion and in the core. The outer shell or capsid is comprised of seven distinct polypeptides. The capsid is composed of 240 hexons and 12 penton subunits (Ginsberg et al., 1966; Valentine and Pereira, 1965). As suggested by their Greek root, pentons and hexons are comprised of five and six subunits, respectively. The hexon is a tight association of three molecules of polypeptide II located on the surfaces of the icosahedral structure, also referred to as the *hexon capsomere,* and explains why it is the most abundant virion constituent (Horowitz et al., 1970). Pentons, on the other hand, are located at each of the twelve vertices. They act as a base for the fiber protein and are composed of an association of five copies of polypeptide III (van Oostrum et al., 1985). The fiber proteins are long protrusions which originate, as mentioned before, from the penton base. They are composed of a trimeric complex of polypeptide IV (van Oostrum et al., 1985) and possess at their tips, globular knob domains which recognize its cellular receptor, the coxsackievirus and adenovirus receptor (CAR) (Bergelson et al., 1997). The other four polypeptides of the capsid, VI, VIII, IX and IIIa, all play associated structural roles in the outer shell. Polypeptides VI, VIII and IX, for example, are all involved in stabilizing the hexon capsomeres whereas polypeptide IIIa seems to function as a link between the viral capsid and the core (Everitt et al., 1975). Interestingly, adenovirus mutants which fail to synthesize polypeptide IIIa are unable to assemble into virions (Devaux et al., 1982).

1.3.5 The viral core

The viral core is defined as the region of the adenoviral particle that is left when the outer icosahedral particle is stripped away. It consists of five identified proteins as well as the viral genome. The predominant protein of the core, with about 1000 copies present per virion, is polypeptide VII (Corden et al., 1976; Sung et al., 1977). This 18 kDa protein is positively charged which explains why it is so extensively associated with the viral DNA (Chatterjee et al., 1986). In fact, polypeptide VII may play the role of a histone-like molecule since the packaging within the viral DNA resembles that of the nucleosome structures found in cellular chromatin (Corden et al., 1976). Polypeptides V, X and μ are all minor components of the viral core and are associated with the viral DNA (Chatterjee et al., 1986). The fifth protein of the core, the terminal binding protein (TP), was initially not detected by SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis) analysis since there are only two copies of this protein per virion. However, its ability to mediate the circularization of the viral genome by a protease sensitive, non-covalent mechanism indirectly led to its discovery (Rekosh et al., 1977). As its name implies, this 55 kDa protein is bound to the ends of both strands via a phosphodiester bond with a serine residue (Desiderio and Kelley, 1981; Smart and Stillman, 1982) and serves several critical functions during viral DNA replication (discussed in section 1.4.4).

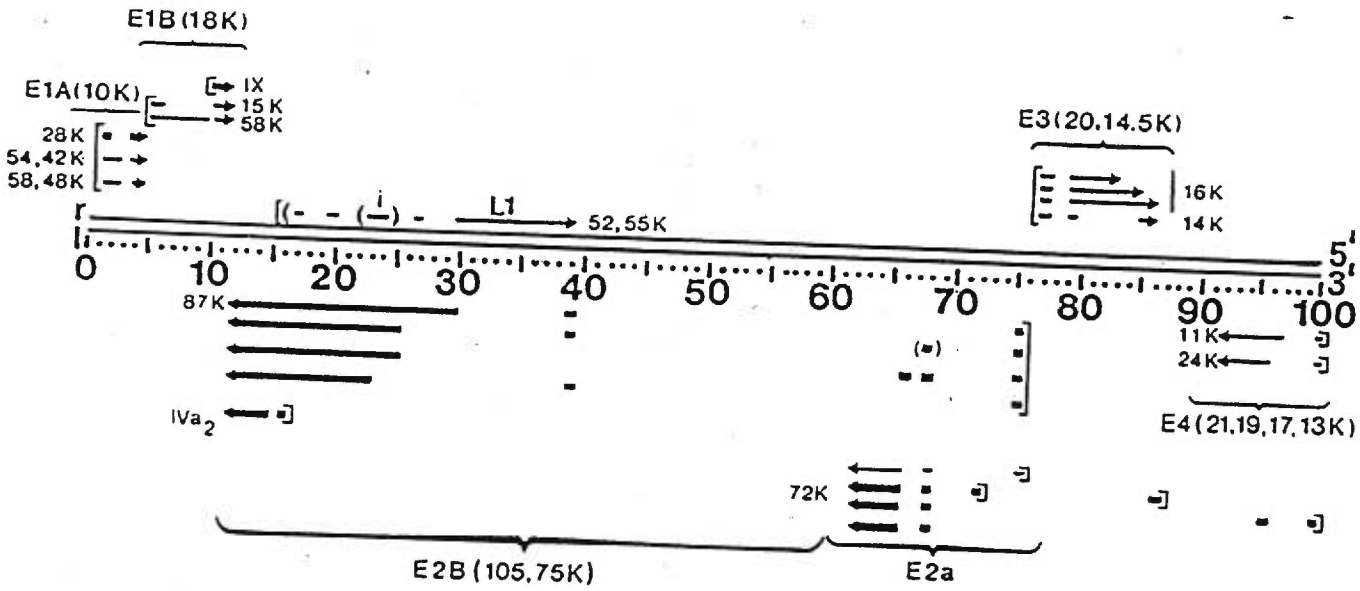
1.3.6 Structure of the adenovirus genome

The final element to be found in the viral core is the double-stranded DNA molecule (Green and Pina, 1963). The genome is roughly 36,000 base pairs long and is divided into 100 map units (see Fig. 1.1). Both DNA strands are used for transcription with the rightward strand coding for E1A, E1B, IX, major late, VA RNA, and E3 units and the leftward strand coding the E4, E2, and IVa2 units. By convention, the rightward strand which codes for about 70% of the genetic information is designated as "r", whereas the leftward strand is designated as "l".

The distinguishing feature of the adenovirus genome is the presence of inverted terminal repeats (ITRs). ITRs are short, highly conserved DNA sequences with a core region of 10 nucleotides (Tolun et al., 1979). Each terminal repeat has one origin of replication as well as a *cis*-acting packaging sequence (Hammarskjold and Winberg, 1980). The presence of a packaging sequence which interacts directly with encapsidating proteins assures specific recognition of the adenoviral genome (Grable and Hearing, 1992).

The adenovirus genome is divided according to the type of protein encoded and the time it is transcribed during a productive infection (reviewed in: Petterson and Roberts, 1986). Essentially, genes transcribed before viral replication occurs are designated early (E) genes and those after are called late (L) genes. The five early transcription units (E1A, E1B, E2, E3, and E4) encode proteins that function to

<u>Figure 1.1:</u> Genomic map of adenovirus coded early proteins and their mRNAs.



Adapted from (Shenk, 1996)

prepare the virus and the host cell for viral replication, as well as encoding proteins that impair immune responses. The single late gene (L1) driven by the powerful major late promoter (MLP) encodes proteins necessary for the structural components of the virus.

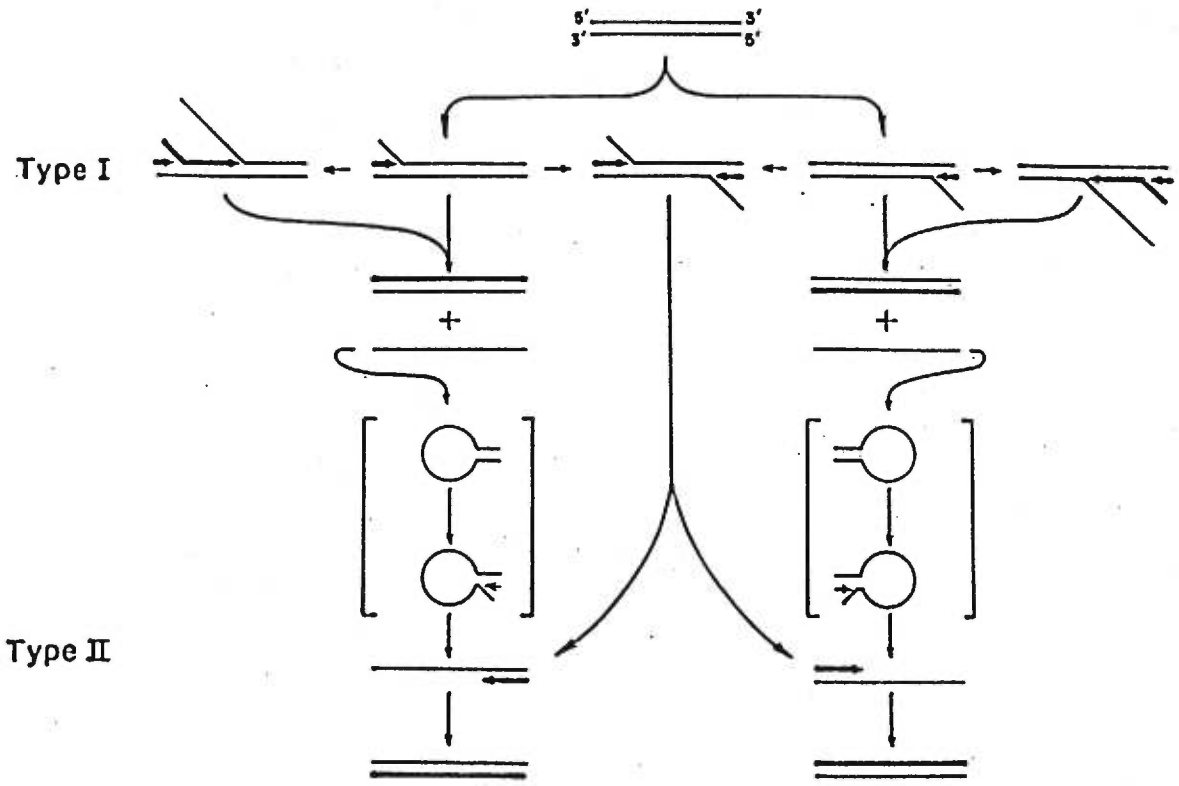
1.3.7 Adenovirus DNA replication

As with all viral infections, adsorption of the adenovirus to the cell surface initiates all subsequent steps in viral replication. The initial binding of the virus is mediated through the carboxy terminus of the fiber protein (Philipson et al., 1968) to its cellular component, the coxsackievirus and adenovirus receptor (Bergelson et al., 1997). In addition, a second protein-protein interaction is essential for proper internalization of the virus particle. This involves the penton base protein which interacts with the integrin family of cell surface proteins via an Arg-Gly-Asp motif present on the penton (Bai et al., 1993; Wickham et al., 1993). Once endocytosis of the viral particle has occurred, the virus exits the endosomes into the cytosol by a process that is dependent on the low pH of the endosome and the penton base protein of the virus (Svensson, 1985). Once liberated in the cytosol, the virus capsid is disassembled in a stepwise manner in order to expose the viral core during its transport to the nucleus (Dales and Chardonnet, 1973; Greber at al., 1993). The transport of viral DNA to the nucleus is completed with the binding of the terminal protein to the nuclear matrix and entry of the genome into the nucleus (Fredman and Engler, 1993; Shaack et al., 1990).

As early as one hour post-infection, viral mRNAs from the E1A gene can be observed (Nevins et al., 1979). The E1A gene product prepares the cell for viral DNA replication by enhancing early transcription by 50-100 fold (Berk et al., 1979; Jones and Shenk, 1979) as well as inducing cell cycle progression to the S-phase (Chellappan et al., 1992). The viral replication occurs in two steps which are shown schematically in Fig 1.2 (Lechner and Kelly, 1977). Initially, only one of the two strands is replicated resulting in a fully replicated double stranded genome and a displaced complementary single strand as a byproduct. In the second step, annealing of the complementary sequences in the ITRs of the displaced genome leads to a circularization of the single stranded viral genome. The "panhandle" structures formed by the annealed ITRs are then used by the cell's replicative proteins to complete the viral DNA replication process.

Many different viral and cellular proteins are essential for viral replication to proceed efficiently. The E2 transcription unit codes for three proteins, all of which are essential for adenovirus DNA replication. In addition to the E2 transcription unit, two other *cis* acting viral proteins are required for initiation of DNA replication, the 55 kDa terminal protein and the 105 kDa viral DNA polymerase (Temperley and Hay, 1992). Two cellular factors have also been shown to play essential roles in initiation of DNA replication, namely, nuclear factor I and III (Chen et al., 1990; Mul et al., 1991).

Figure 1.2: Model for adenovirus DNA replication



The TP effectively localizes the adenovirus polymerase to the site of initiation (Enomoto et al., 1981; Lichy et al., 1982; Stillman et al., 1982; Temperley and Hay, 1992). In fact, the TP plays two important roles in adenoviral replication. i) acts as a primer for DNA replication and ii) it maintains the integrity of the viral genome's extremities (Rekosh et al., 1977). The third E2 encoded protein, the 72 kDa DNA-binding protein (van der Vliet et al., 1975), allows for efficient elongation of the nascent DNA strand.

1.3.8 Expression of late viral proteins

Expression of late viral proteins begins as soon as viral DNA replication is initiated. The major late promoter (Berget and Sharp, 1979; Nevins and Wilson, 1981; Shaw and Ziff, 1980) is responsible for driving the single transcription unit on which all late viral proteins are encoded. Five families of late transcripts designated L1-L5 encompass the twenty cytoplasmic mRNA species generated from the large primary transcript (Chow et al., 1977; Nevins and Darnell, 1978; Ziff and Fraser, 1978). All late mRNAs contain a common 5' untranslated region of approximately 200 bp in length called the tripartite leader (Akusjarvi and Petteron, 1979; Berget et al., 1977; Chow et al., 1977; Zain et al., 1979; Ziff and Evans, 1978). Splicing of the tripartite leader occurs in three regions which map to positions 16.5, 19.6 and 26.5 map units. Of note, the tripartite leader has been shown to enhance translation of mRNAs late although not early in infection (Berkner and Sharp, 1985; Logan and Shenk, 1984).

1.3.9 Virus assembly

Virus assembly represents the culmination of the viral cycle. Structural proteins produced from the late transcripts and viral DNA are assembled into an infective viral particle ready to be liberated from the cell and start a new round of replication. The virus produces in excess all of the components necessary for its assembly. In fact, only about 1-5% of the fibers and pentons and 20-30% of the hexons are actually incorporated into virus particles (Everitt et al., 1971; White et al., 1969). Furthermore, about 20% of the virus genomic DNA molecules synthesized during replication are incorporated into capsids.

Assembly of the viral particles begins with the formation of the capsid followed by the entry of the viral genome (Sundquist et al., 1973). Encapsidation of the viral genome is orientation dependent with the left end always being packaged first (Tibbets, 1977). In fact, a packaging sequence located 260 bp from the left end of the genome has been shown to be essential for genome encapsidation (Grable and Hearing, 1992; Hammarkjold and Winberg, 1980; Hearing et al., 1987; Tibbets, 1977).

Several viral proteins are implicated in proper assembly of the viral particles. The viral protease, which is encoded by the L3 message, is responsible for proteolytic cleavage of several structural proteins. Mutations in this protein result in failure to assemble virus particles (Weber, 1976). Also, the 100 kDa protein encoded in the L4

region acts as a chaperon-like protein and facilitates the formation of hexon (Cepko and Sharp, 1982).

1.4 Adenoviral E1 proteins and Transformation

All adenoviruses serotypes are capable of causing morphological transformation of cells in culture (Freeman et al., 1967; Freeman et al., 1967; Gilden et al., 1968). However, only group A adenoviruses are capable of inducing tumors into newborn hamsters (Trentin et al., 1962). The viral genes responsible for cellular transformation have been shown to be located within the E1 transcription unit (Graham et al., 1974). The proteins coded by both E1 subunits, E1A and E1B, play important roles in transformation. Mutant viruses where one of the two E1 subunits is disrupted have among other things been shown to be defective for transformation.

1.4.1 The E1A proteins

Early region 1A (E1A) gives rise to two major mRNAs, 13S and 12S, by alternative splicing (Perricaudet et al., 1979) very early following adenoviral infection (Nevins et al., 1979). The difference between the two E1A encoded proteins is a 46 amino acid region which is unique to the 13S product. Analysis of E1A amino acid sequences among many serotypes has revealed the presence of three conserved regions, designated CR1, CR2 and CR3 for conserved region 1, 2, and 3

(van Ormondt et al., 1980). The unique 46 amino acids are found in the CR3 region and are exclusive to the 13S mRNA which is also known as the 289R protein. The CR3 is now known to be a very effective transcriptional transactivator of early adenoviral promoters (Berk et al., 1979; Jones and Shenk, 1979). However, the E1A protein made from the 13S transcript does not have sequence-specific DNA binding properties. Rather it acts as a trans-acting transcriptional regulator whose precise mode of action is not known (Chatterjee et al., 1986; Ferguson et al., 1985).

1.4.1.1 Induction of DNA synthesis by E1A

Aside from activating early viral genes, the E1A proteins have a profound effect on the cell in order to optimize conditions for viral productions. The first condition to many viral infection is the induction of a replicative state within the cell. Since adenoviruses usually infect terminally differentiated epithelial cells, passage into the S-phase of the cell cycle is of paramount importance. Several pathways to induce DNA synthesis are possible and E1A targets them by associating with a set of cellular proteins involved in cell cycle control.

1.4.1.2 Retinoblastoma class proteins

The protein p105, which is a product of the retinoblatoma (Rb) susceptibility gene, was the first cellular protein to be associated with E1A (Whyte et al., 1988).

The Rb gene plays an important role in cell cycle control by arresting cells in the G_1 phase and preventing cells from entering into the S-phase (Goodrich et al., 1991; Qin et al., 1992). Rb blocks entry into the S-phase by binding to the transcriptional transactivator E2F (Flemington et al., 1993; Helin et al., 1993). E2F, as its name implies, binds and activates the adenovirus E2 promoter (Kovesdi, 1986). Moreover, E2F stimulates the transcription of many growth factors as well as the activation of genes required for DNA synthesis (reviewed by Nevins, 1992). Therefore, the observation that E1A binds Rb led to the model where E1A mediates the release of E2F and indirectly induces DNA synthesis through E2F. In addition, other Rb related proteins, namely p107 and p130, respectively, are capable of binding E2F and have been shown to bind with E1A proteins (Ewen et al., 1991; Hannon et al., 1993; Li et al., 1993). This reinforces the E1A-mediated E2F release model for the purpose of disrupting cell cycle regulation.

1.4.1.3 p300 class proteins

In addition to the Rb binding model, E1A proteins induce DNA synthesis by an alternative pathway. In this pathway, E1A binds to the E1A-associated cellular protein p300 protein (Eckner et al., 1994) and to the 265 kDa nuclear protein, CBP (Lundbland et al., 1995). Elevated levels of second messenger cAMP leads to the activation of protein kinase A (PKA) which phosphorylates a DNA binding protein called, cAMP responsive element binding protein (CREB). p300 and CBP both bind

to the phosphorylated forms of CREB (Gonzalez and Montminy, 1989) and this complex is capable of transcriptional activation (Arany et al., 1995; Lundbland et al., 1995). As such, transcriptional activation of the p300/CBP complex with phophorylated CREB is repressed by E1A. The significance of this repression is that the p300/CBP-CREB complex functions to stimulate differentiation of the cell as well as maintaining terminally differentiated cells in quiescence (G_0) (Kato et al., 1994). Therefore, binding of these proteins by E1A proteins allows for progression through G_0/G_1, which is essential for adenoviruses since they target cells that tend to be terminally differentiated. E1A's role in inducing cellular transformation is related to its ability to induce DNA synthesis. Mutants of E1A which are unable to bind to p300/CBP or to Rb are severely retarded in their ability to cause transformation (Egan et al., 1989; Whyte et al., 1989).

1.4.2 The E1B proteins

E1A-mediated apoptosis, the cell's ability to commit suicide, has negative consequences on virus production. Viral inhibition of apoptosis, therefore, represents a strategic mechanism in order to maintain cell viability long enough to allow for maximal viral production. That is why many viruses possess genes which are able to delay the onset of apoptosis long enough for viral production to take place. In the case of adenoviruses, these functions are found within the two proteins expressed by the E1B transcription unit, E1B19K and E1B55K. These two genes are

independently capable of transforming primary rodent cells in coordination with E1A while both act with greater efficiency when expressed together (Barker and Berk, 1987; Bernards et al., 1986; Mclorie al., 1991; White and Cipriani, 1990).

1.4.2.1 Functional homology between E1B19K and Bcl-2

The first protein to show a clear ability to inhibit the cytopathic effect induced by E1A was the E1B19K protein (Pilder et al., 1984; Subramanian et al., 1984; White et al., 1991). E1B19K belongs to a growing family of cellular and viral proteins known to be involved in the inhibition and acceleration of apoptosis (reviewed by White, 1996). The prototype protein of this family is Bcl-2 which acts as an anti-apoptotic protein, protecting cells from apoptosis when overexpressed. E1B19K has been shown to be functionally equivalent to Bcl-2 in its ability to inhibit apoptosis (Huang et al., 1997). Both proteins can associate with two proteins known to accelerate the process of apoptosis, Bax (Chen et al., 1996; Han et al., 1996; Huang et al., 1997) and Bak (Farrow et al., 1995; Huang et al., 1997). Interaction with these proteins by E1B19k and Bcl-2 prevents homodimerization, thus preventing their pro-apoptotic function.

1.4.2.2 The p53 tumor suppressor

E1A proteins induce apoptosis by the activation of the tumor suppressor gene p53. Increased levels of p53 results from E1A disruption of Rb normal function in

the cell. p53 then represents a defense mechanism by the cell in response to the abnormal conditions within the cell, due to the expression of cell cycle disrupters by the virus. Apoptosis by the cell would then protect neighboring cells from viral infection by eliminating the virus in its uncapsidated form. However, p53 mediated induction of apoptosis is countered by the binding to the activation domain of p53 by E1B55K protein. By binding to p53, E1B55K blocks the activation of p53-responsive genes (Yew et al., 1994), thus preventing p53-mediated apoptosis.

1.5 Large-Scale production of recombinant adenoviruses

Preclinical gene transfer studies with adenovirus recombinants progress in a logical manner from small animals such as mice to larger animals such as rats, dogs, primates and ultimately to phase 1 clinical studies with human volunteers. However, as the size of the animal increases so does the amount of virus required for efficient gene transfer. For example, the anterior tibialis of a mouse requires 2×10^9 viral particles (20µl of a 10^{11} pfu/ml) in order to transduce more than 80% of the muscle fibers, but the same muscle is 2500 times larger in humans. Therefore, to obtain the same efficiency of transduction with humans, one would need 5×10^{12} viral particles per muscle. This means that at the existing production scale, a 20L culture would provide sufficient material for only four subjects. When one considers the cost of production and purification, this level of production does not represent an

economically viable process in the long run. Yet, if viral yields could be augmented by two or three fold, a 100L culture would allow 56 more patients to receive treatment. In order to achieve this goal, it was the objective of this thesis to examine potential schemes for the improvement of viral yields by studying the role that adenovirus E1 proteins play in viral production in complementing cell lines.

1.6 The need for a new complementing cell line

Replication-defective human adenovirus recombinants have been shown to be efficient tools for the transfer of various genes *in vitro* and *in vivo*. Virtually all of the E1-deleted adenoviral vectors (AdV) used in gene therapy protocols are produced in 293 cells (Graham, 1977), a human embryonic kidney cell line expressing E1 functions from an integrated segment of the left end of the Ad5 genome (see Fig. 1.3). These cells, however, have been shown to allow the generation of replication competent particles as a result of homologous recombination between the AdV's DNA and the integrated adenovirus sequences present in the 293 cell line (Lochmüller et al., 1994). With the growing number of clinical trials planned using AdVs for the treatment of human diseases, the presence of replication competent adenoviruses (RCAs) in viral production raises serious health and safety issues that could compromise the use of AdVs in human gene therapy trials. Therefore, the search for a new complementing cell line capable of eliminating RCAs is of paramount importance if AdVs are to be used as vehicles for therapeutic drugs.

In order to circumvent the problems mentioned above, a new complementing cell line was engineered that expresses the E1A and E1B adenovirus proteins (Massie, 1998b). The human lung carcinoma A549 cell line was stably transfected with the expression vector pHβE1AE1B. This plasmid contains the Ad5 E1-coding region (532-3525) where the expression of E1A is controlled by the strong constitutive β-actin promoter and the expression of E1B is controlled by its own E1A-responsive promoter. More importantly, this new construct limits the overlapping sequence to only 200bp (3334-3525) and does not contain the packaging and ori sequences (0-350) that are present in the 293 cell line. These modifications greatly reduce the possibility of generating RCAs since even in the rare event of recombination between the AdV and host cell genome no RCAs can be generated without the ori and packaging sequences. This new E1-complementing cell line (BMAdE1) is capable of supporting the growth of E1-deleted adenoviruses, but to levels inferior to those obtained with the 293 cell line. The first objective of this study was to quantitatively compare the production capabilities of the BMAdE1 clones relative to that of the standard 293 cell line.

The reduced capacity of the BMAdE1 cells to support the production of E1-deleted adenoviruses could be due to potentially toxic effects of E1 protein expression or alternatively there is the possibility that the levels of E1 proteins were suboptimial to allow for maximal viral production. This second possibility was suggested by the results of a comparison of E1 levels produced in cells infected by a

wild-type adenovirus and those present in the 293 cell line. These results showed that an Ad5dl309 virus produced 5 to 10-fold more E1B19K than the 293 cell line, whereas the other E1 proteins were made at similar levels. It was conceivable that this increased abundance of E1B19K could explain the higher production capacity of the wild type virus compared to that of an E1-deleted adenovirus in 293 cells. An increase in the production capacity of E1-deleted adenoviruses to that of the wild type virus could significantly reduce production costs since an E1-deleted adenovirus produces, at best, 30% the level of virus particles per cell as the wild type virus does. Therefore, the major goal of this thesis was to investigate the role that each of the AdE1 proteins plays in supporting AdV production.

The approach that was taken was to construct three E1-deleted adenoviruses expressing each of the E1 proteins separately. These E1A, E1B55K and E1B19K expressing adenoviruses could then be used to infect 293 cells and the effect of their expression on viral production could be assessed. During the course of constructing the transfer plasmids that were used to generate the recombinant AdV it was found that the inclusion of a multiple cloning site, to facilitate the insertion of various inserts, had a dramatic effect on the levels of expressed protein. Since this result is of significant importance when using the adenoviral expression system (AES) to maximize protein expression, an investigation was carried out to verify this phenomenon and to determine the mechanism of inhibition.

Chapter 2
Materials and Methods

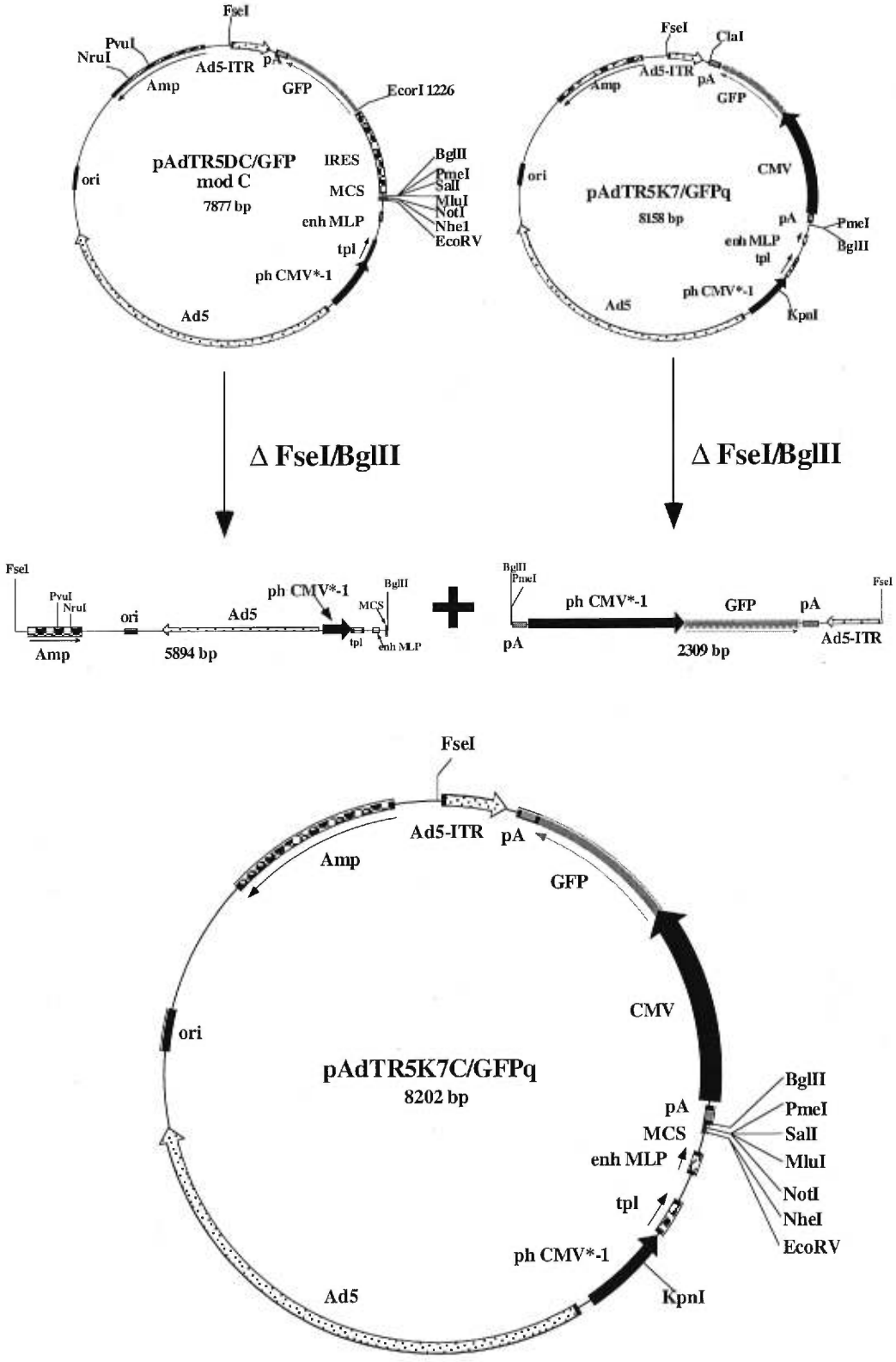
2.1 Construction of adenoviral E1 expressing transfer plasmids

Three transfer vectors were constructed in order to express independently the three proteins encoded by the adenovirus E1 gene. The transfer vector pAdTR5K7C-GFP^{Q} served as the backbone for the construction of these three recombinant vectors.

2.1.1 pAdTR5K7C/GFP^{Q}

The transfer vector pAdTR5K7C/GFP^{Q} was constructed by ligating fragments which were isolated from the transfer vectors, pAdTR5K7/GFP^{Q} and pAdTR5DC/GFP mod C (see Fig. 2.1). Both transfer vectors were digested with the restriction enzymes *Bgl*II and *Fse*I purchased from New England Biolabs (Beverly, Mass.), generating a large and small fragment in each case. The large fragment from the dicistronic (DC) vector as well as the small fragment from the cassette (K7) vector were isolated by cutting the corresponding bands from the agarose gel and purified using the "QIAEX II Agarose Gel Extraction kit" from Qiagen Inc. (Chatsworth, CA). Essentially, the QIAEX II principle involves the extraction and purification of DNA fragments based on the solubilization of agarose and selective, quantitative adsorption of nucleic acids to the QIAEX II silica-gel particles in the presence of high salt. Elution of the DNA was accompanied with a low salt solution such as Tris buffer or water. Once purified the two fragments were ligated using the "Rapid DNA Ligation Kit" from Boehringer Mannheim (Germany). Briefly, a 1 to 5

Figure 2.1: Construction of the pAdTR5K7C/GFPq adenoviral transfer plasmid



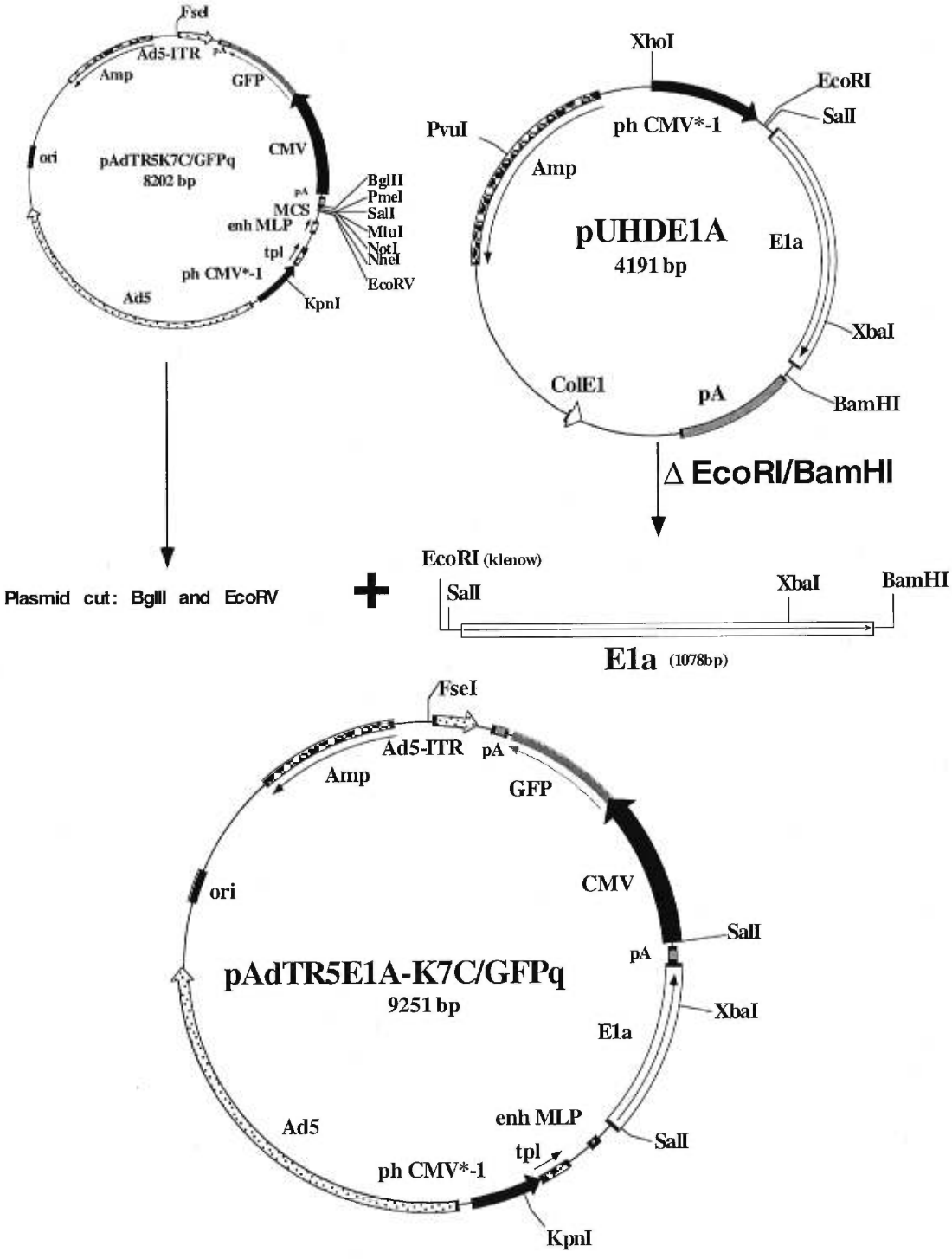
ratio of large to small fragment DNA was used to ligate the sticky ends and was left to incubate for 10 minutes at room temperature. The ligation mix was then transformed using the heat shock method into the competent cells *Escherichia coli* DH5∝. The transformed cells were then plated on an agar plate which was supplemented with ampicillin at a concentration of 100μg/ml. The addition of the ampicillin restricts the growth of any nontransformed cell or that of cells which may have been transformed with nonligated fragments. In theory, all of the colonies which appear after an incubation at 37°C should carry the desired recombinant plasmid since only these colonies have the antibiotic resistant gene present within them. However, in order to confirm that a colony does in fact possess the recombinant plasmid of interest a screening of many bacterial colonies was done. This systematic screening involves the growth of the bacterial culture, followed by the harvest and lysis of the bacteria and completed with the purification of the plasmid DNA. Individual bacterial colonies were picked from an agar plate containing ampicillin and were inoculated in Luria-Bertani liquid medium also containing 100 μg/ml of ampicillin. Each culture was harvested by centrifugation and then lysed for purification of the recombinant plasmid DNA. Extraction of plasmids were done using the QIAGEN plasmid purification kit from Qiagen Inc. (Chatsworth, CA). Briefly, this kit is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to the QIAGEN Anion-Exchange Resin under appropriate low-salt and pH conditions. Impurities, such as RNA, proteins, and dyes, were removed by a medium-salt wash. The desired plasmid was then eluted in a

high-salt buffer and concentrated and desalted by isopropanol precipitation.

2.1.2 pAdTR5E1A-K7C/GFP^{Q}

The transfer vector pAdTR5E1A-K7C/GFP^{Q} which expresses the adenovirus E1A protein was constructed by splicing the E1A gene into the tetracycline-regulated transfer vector pAdTR5E1A-K7C/GFP^{Q} (see Fig. 2.2) using the standard cloning procedures described in materials and methods section 2.1.1. The pUHE1A plasmid contains the coding sequence for the expression of both 12S and 13S E1A proteins and was isolated by digesting the plasmid with the enzymes *Eco*RI and *Bam*HI. However, the *Eco*RI digest was rendered blunt ended by using the DNA Polymerase I, large (Klenow) fragment from New England Biolabs (Beverly, MA.) This manipulation generated a fragment of ~1 kilobase in length and was ligated into the transfer vector pAdTR5K7C/GFP^{Q} which was digested with *Bgl*II and *Eco*RV. This construction generated a new plasmid whereby all of the multiple cloning sites from the transfer vector pAdTR5K7C/GFP^{Q} were deleted and oriented the E1A gene in the same direction as the tetracycline-regulated promoter.

Figure 2.2: Construction of the pAdTR5E1A-K7C/GFPq adenoviral transfer plasmid



2.1.3 pAdTR5E1B55K-K7C/GFP^{Q}

The transfer vector pAdTR5E1B55K-K7C/GFP^{Q} which expresses the adenovirus E1B55K protein was constructed by splicing the E1B55K gene into the tetracycline-regulated transfer vector pAdTR5K7C/GFP^{Q} (see Fig. 2.3) using the standard cloning procedures (described in: Materials and Methods, section 2.1.1). The plasmid, pCE1B58, contains the gene encoding E1B55K and was isolated by digesting the plasmid with the enzymes *Bam*HI and *Not*I. This generated a fragment that was ~1.5 kilobase in length and was cloned into the transfer vector, pAdTR5K7C/GFP^{Q}, which was digested with the enzymes, *Bgl*II and *Not*I. This generated a new plasmid and oriented the spliced E1B55K gene in the same direction as the tetracycline-regulated promoter.

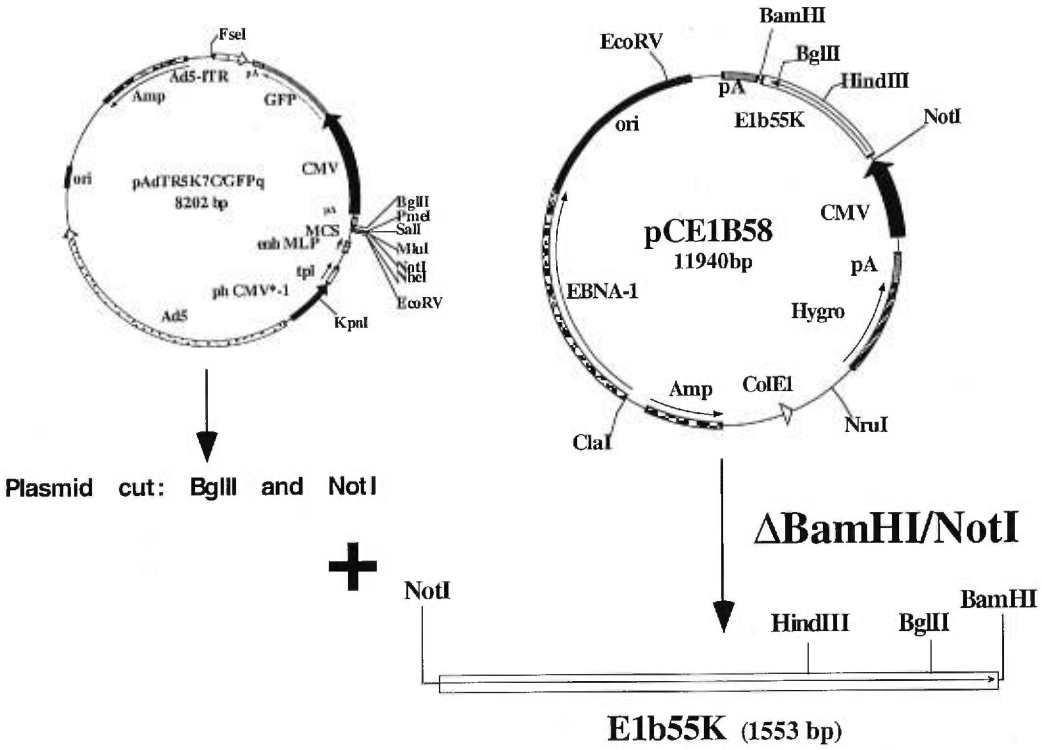
2.1.4 pAdTR5E1B19K-K7C/GFP^{Q} and pAdTR5E1B19K-K7/GFP^{Q}

The two transfer vectors, pAdTR5E1B19K-K7C/GFP^{Q} and pAdTR5E1B19K-K7/GFP^{Q}, both express the adenovirus E1B19K protein. They differ, however, by the fact that pAdTR5E1B19KK7C/GFP^{Q} has a multiple cloning site (MCS) sandwiched between the tetracycline-regulated promoter and the E1B gene whereas the pAdTR5E1B19K-K7/GFP^{Q} does not have a MCS separating its transgene from the inducible promoter. A PCR fragment (see Fig. 2.4) containing the E1B19K

coding region from pUHE1B19K with *Bam*HI ends and an optimized Kozak consensus sequence flanking the ATG initiation codon was cloned into the transfer vectors, K7 and K7C, which were both digested with *Bgl*II using the standard cloning procedures described in materials and methods section 2.1.1. (see Fig. 2.5A and Fig. 2.5B).

In addition, to facilitate the cloning of E1 proteins, the MCS was added to the pAdTR5 plasmid (Massie et al., 1998), in both orientations, in order to subclone the green fluorescent protein (GFP) so that the GFP would be flanked by the polylinker at the 5' end, 3' end or not flanked at all (see Fig. 2.6).

FIGURE 2.3: Construction of the pAdTR5E1B55K-K7C/GFPq adenoviral transfer plasmid



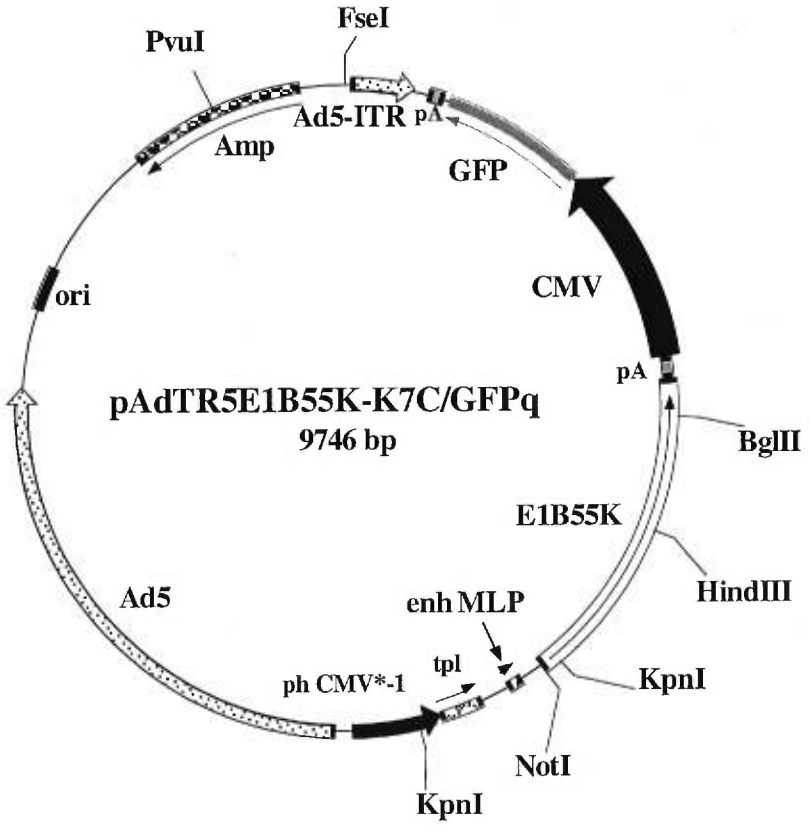


Figure 2.4: Isolation of the E1B19K coding region

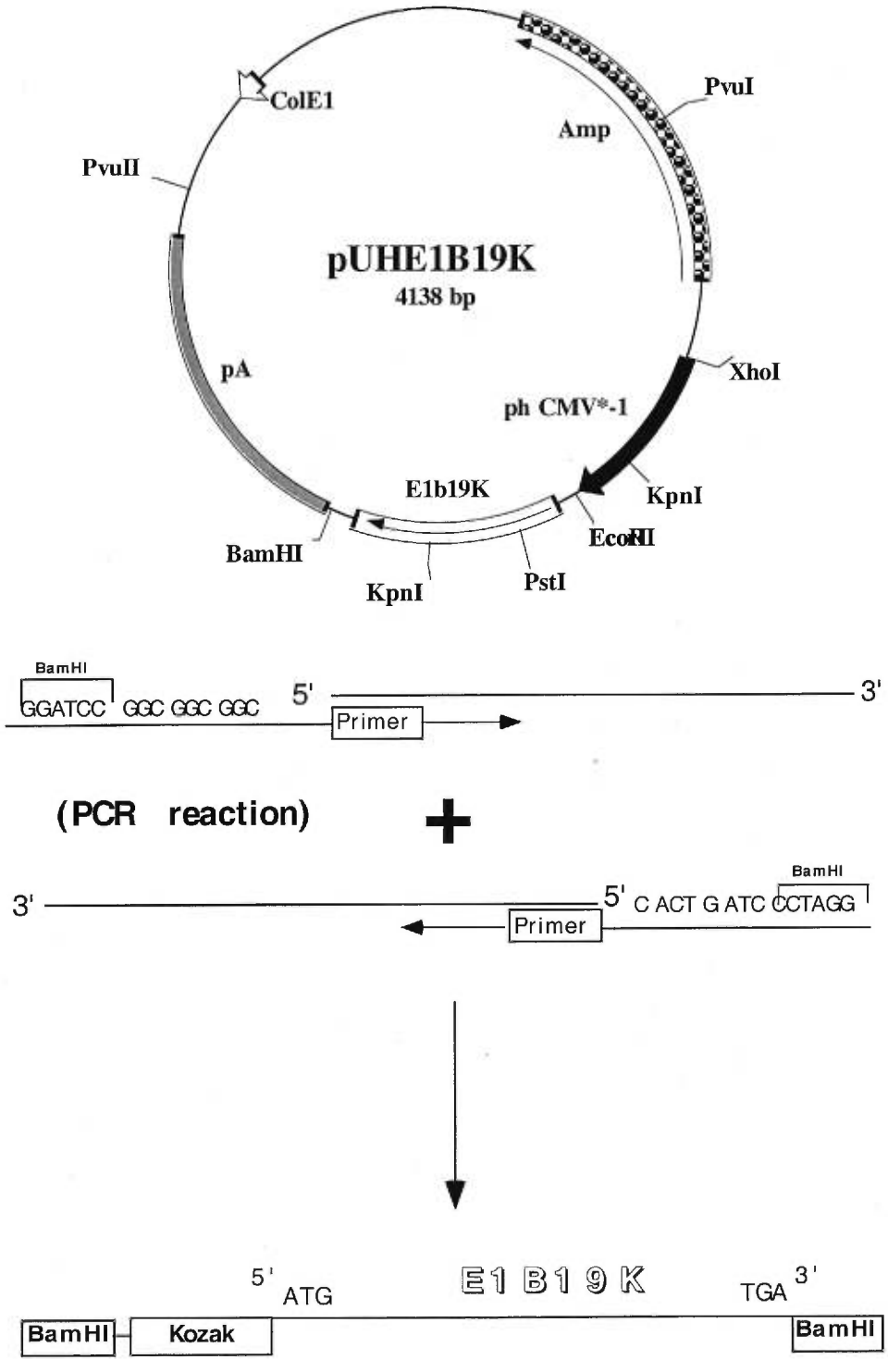


Figure 2.5: Schematic representation of the two E1B19K adenoviral transfer plasmids

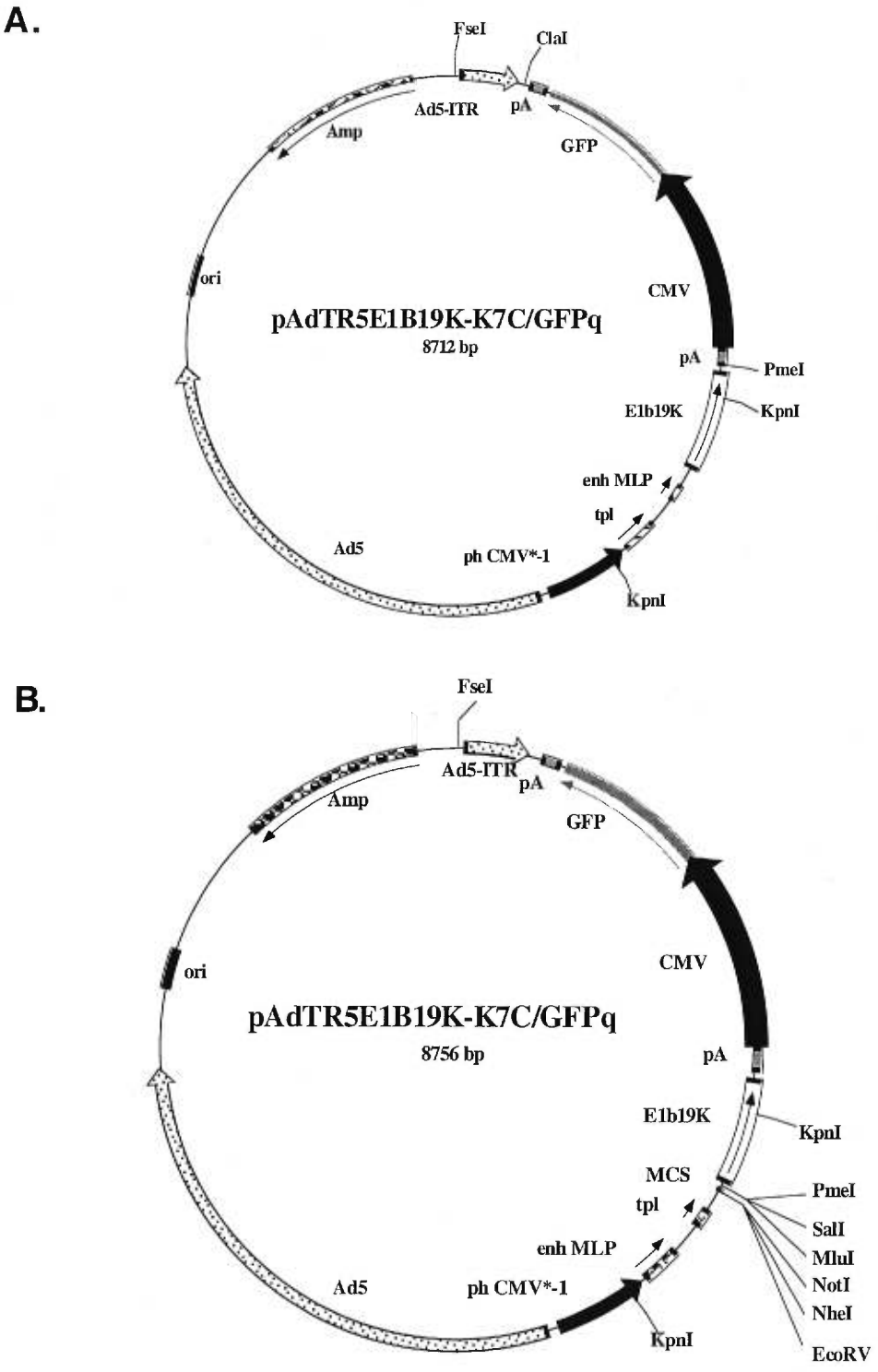
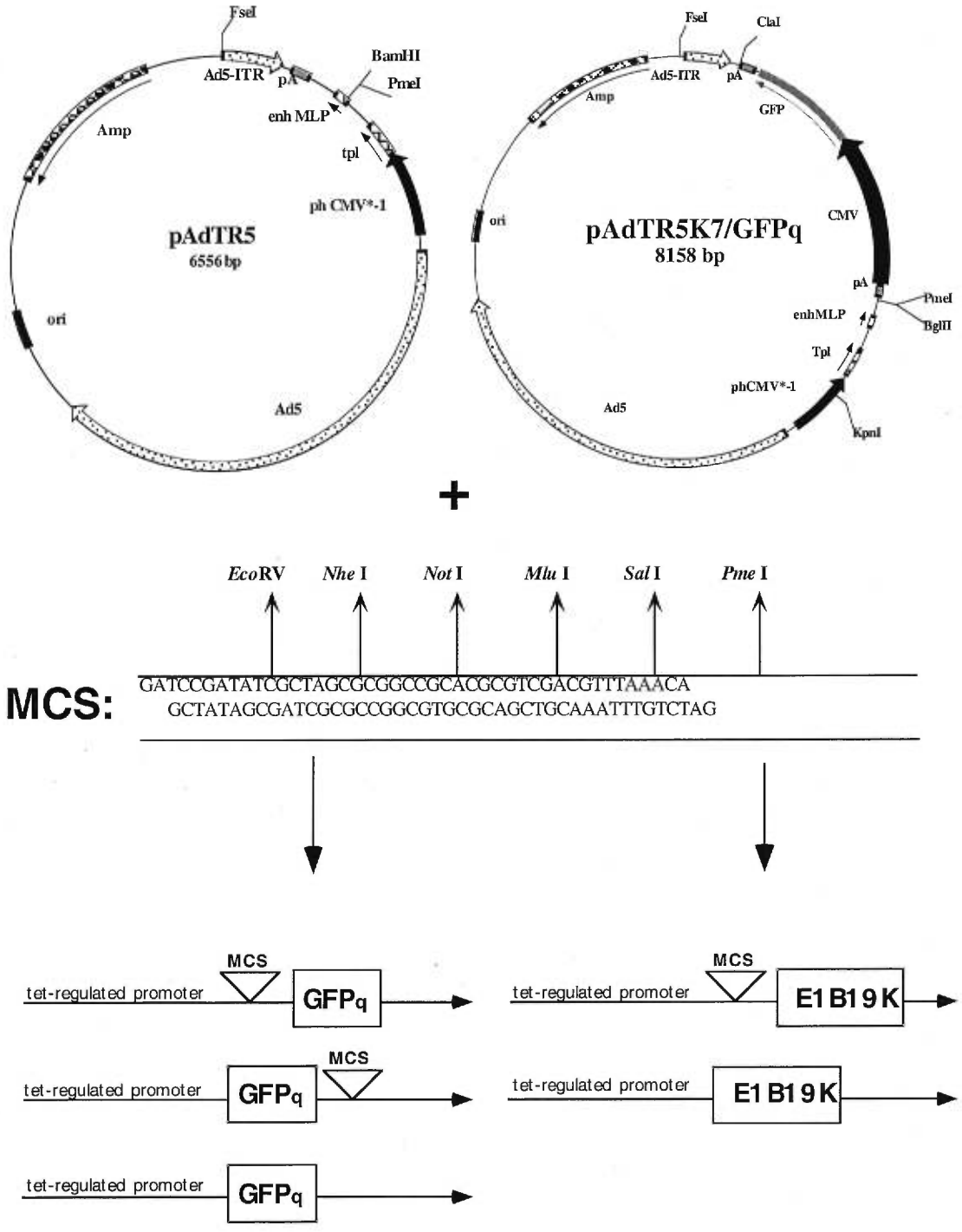


Figure 2.6: **Schematic representation of the plasmids used to study the impact of a typical MCS polylinker on gene expression**



2.2 Sequencing of transfer plasmids

Sequencing of the transfer vectors was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit from Perkin Elmer (Foster City, CA). Briefly, this method is a modified version of the classic Sanger sequencing technique (Sanger et al., 1986) whereby the sequence is labeled with four different fluorescent dye terminators and extended by the thermal stable enzyme AmpliTaq DNA Polymerase.

2.3 Construction of adenoviral transfer vectors

2.3.1 DNA transfection

E1-deleted recombinant adenoviruses were made of the four transfer vectors described in section 2.1 of materials and methods. All of the methods relating to the construction of recombinant adenoviruses have been well documented in Lamarche et al. (Lamarche et al., 1990), and Jani et al. (Jani et al., 1997). Briefly, 10 μg of the transfer vectors were linearized with *Fse*I and co-transfected with 10 μg of *Cla*I digested Ad5/ΔE1ΔE3 viral DNA into 293A cells using a modified version of the calcium phosphate technique (Graham and van der Eb, 1973).

2.3.2 Screening for recombinant adenovirus plaque isolates

The primary advantage in using the K7 transfer vectors is that it facilitates the screening and selection of recombinant viruses expressing inducible gene products. As such, recombinant viruses were easily identified as green plaques, on monolayer cultures of 293 cells that were overlaid with agarose, by direct observation with a standard fluorescence microscope. However, individual plaques are further investigated for proper expression of the transgene of interest. Therefore, the selected plaques were eluted in 1ml of media, amplified on 293A cells and then with the addition of micromolar concentrations of doxycycline tested on 293rtTA cells followed by western blotting (see section 4 of material and methods) to verify the presence of the recombinant protein (Jani et al., 1997).

2.3.3 Purification of selected plaques

The purification of selected plaques was crucial to the isolation of a pure recombinant virus (see Introduction, section 1.7). Briefly, after plaques were eluted from the 293A cell line a serial dilution of the eluted virus from 10^{-1} to 10^{-3} was done from which 1ml was placed on a subconfluent monolayer of BMAdE1 cells (Table 2.1) and left on a rocker at 37° C for 6 hours. Afterwards, the media was removed and the cells were overlaid with agarose. Green plaques from the highest dilution were visible within one week and ready to be picked within two weeks post-

infection. This procedure was repeated three times for optimal purity of the recombinant virus.

2.3.4 Viral production

Once the recombinant virus has gone through the three rounds of purification, the next step was to amplify the virus. For the purpose of this project a pre-stock of each of the recombinant viruses made was sufficient. Briefly, 200 µl from the eluted virus (obtained after the third round of purification) was used to infect 10^5 BMAdE1 monolayer cells in 1ml complete 10% DMEM medium. The cells were harvested after the appearance of a complete cytopathic effect, as indicated by rounding up of the cells, and freezed/thawed three times in order to release the virus from the cells. A volume of 500 µl, from the previous step, was then used to infect 5 x 10^6 BMAdE1 monolayer cells in 10ml of media. The cells were again harvested and freezed/thawed three times after the appearance of a complete cytopathic effect was evident. This time, 9ml was used to infect 3 x 10^7 BMAdE1 cells in 75ml. The cells were again harvested and freezed/thawed three times harvested after the appearance of a complete cytopathic effect. The pre-stock was obtained by infecting 5 x 10^8 293S cells (these cells have the capacity to grow in suspension) with 50ml of the recombinant virus in 1liter of complete DMEM- medium. In fact, the 293S were concentrated to 10 x 10^6 cells/ml and infected with the 50ml of virus in a 250ml

Spinner Flask from Bellco Biotechnology (USA). The cells were stirred gently and left at 37°C for one hour. Afterwards, the cells were diluted to 1liter and transferred to a 1750cm^2 Tissue Culture Roller Bottle from Corning and placed on a Modulator Cell Production Roller Apparatus from Wheaton Instruments. Twenty-four hours post-infection the cells were supplemented with glucose to feed the cells and bicarbonate to raise the pH. Forty-eight hours post-infection the cells were harvested by centrifugation and resuspended in 60 ml of complete DMEM media.

2.4 Titration of adenoviral vectors

A virus titer is an expression of the number of infectious units per unit volume. This calculation was essential to all subsequent work with the virus. For this project, the viral titer was used to infect different cell lines with the same multiplicity of infection (MOI). Two methods of titration were used in this project. The first was the plaque assay method (see Materials and Methods, section 2.4.1) whereby the number of plaques counted is divided by the dilution and the volume of inoculum. This method has been extensively used for the last thirty years but has recently been shown to underestimate the number of infective units (Mitterder et al., 1996). A new method of quantification, which was developed in this laboratory (Couture et al., in preparation), is based on the quantification of GFP expression by the adenoviral vector by fluorometric methods (see Materials and Methods, section

2.4.2). This method has the advantage of calculating the gene transfer of GFP by the recombinant virus as an indicator of viral infection which leads to a more accurate determination of the viral titer.

2.4.1 Plaque assay

A minimal volume (1ml) of complete DMEM containing virus was used to infect a subconfluent monolayer of 3 x 10^5 293A cells. Serial dilution of a factor of 5 which ranged between 10^{-7} and 10^{-10} were used. The cells were left rocking with the virus for 16 hours for maximal adsorption after which time the medium was removed and replaced with agarose. The plaques usually appear within four days with wild-type adenovirus and ten days for the recombinant viruses. The plaques were counted daily from day ~7 post-infection for wild-type adenoviruses and ~14 days post-infection for recombinant viruses. Plates which have between twenty to one-hundred plaques present were counted with the unaided eye and titered using the formula:

$$\frac{\text{Average number of plaques}}{\text{Volume of inoculum x dilution}} = \text{PFU/ml*}$$

*PFU - number of plaque-forming units per milliliter of original suspension

2.4.2 Gene transfer unit (GTU)

This method of titration makes use of the reporter gene, GFP, which was present on all of the recombinant adenoviral vectors used in this project. The presence of the GFP molecule within a cell was an indication that the cell has been infected in opposition to the cytopathic effect required in the plaque assay method. A total of 6 x 10^5 293A cells were plated on 60mm plates and infected the next day with 1ml of complete DMEM containing virus. Five serial dilution varying from 10^{-2} to 10^{-6}, depending on the viral production, were used to infect the cell monolayers. However, a dilution factor of 5 and under were always used for highest precision. Several plates were counted the day of infection while the others were infected and left on a rocker overnight. The next day, cells were harvested and fixed with paraformaldehyde. Briefly, cells were resuspended in 1ml of PBS and fixed by adding 1ml of paraformaldehyde(4%)-PBS from Fisher Scientific (Pittsburgh, PA). The cells were then incubated at 4°C for 30 min., centrifuged and resuspended in 1ml of cold PBS.

2.4.2.1 Flow Cytometry

The percentage of GFP emission by an infected population was measured using an EPICS® XL-MCL flow cytofluorometer (FCM) from Coulter (Hialeah, FL). The FCM is equipped with a 15mW argon-ion laser and the following filters: 488 nm laser blocking, 488 nm long-pass dichroic, 550 nm long-pass dichroic and 525 nm band-pass. Three parameters were acquired with the System II™ software (version 1,0) in listmode namely, forward angle light scatter (FS or cell size), side light scatter (SS or granularity) and green fluorescence (FL1). FS and SS were processed in linear and area mode, and debris and dead cells were excluded from the analysis by FS and SS gating. In order to differentiate the autofluorescent population from the GFP-expressing one, a logarithmic mode was used to acquire green fluorescence. For each analysis, 10,000 gated events were collected and uninfected 293A cells fixed in the same manner (Section 2.4.2.2) were used as a reference for fluorescence intensity measurements.

2.4.2.2 Statistical evaluation

Mathematical modeling evaluation were performed by using Microsoft® Excel software (version 5.0) for Macintosh. The LINEST function, which uses the method of "least squares", was used to calculate a straight line that best fits the data. The equation for the line is: $y = mx + b$, where the dependent y-value, which was the

percentage of positive green cells, is a function of the independent x-values, which was the viral dilution divided by number of cells at the time of infection. The slope of the line was then calculated and represents the titer of the virus expressed as GTU/ml.

2.5 Western Blotting

Infected and noninfected cells were lysed by sonication in 100-150 µl of Laemmli disruption buffer (80mM Tris-HCL, pH 8.0, + 2% sodium dodecyl sulfate + 10% glycerol). The concentration of protein in each sample was determined by using the DC Protein Assay microplate protocol from Bio-Rad Laboratories (Hercules, CA.). The absorbances were read using a microplate reader from Dynatech MR5000 at a wavelength of 590 nm. Generally, samples were boiled for 5 min. and equivalent amounts, 15-30 µg, of protein were loaded onto 10-14% acrylamide Tris-glycine gels from Novex. Following electrophoresis, the gels were transferred onto Hybond cellulose membranes from Amersham (Oakville, Ont.) using a TE Series Transphor Electrophoresis Unit from Hoefer Scientific Instruments (San Francisco, CA.). The transfer buffer consisted of 25mM Tris-HCL (pH 8.5), 192mM glycine and 20% (vol/vol) methanol. Following transfer, the blotted membranes were blocked by incubating the blot overnight at 4°C on a rocker in a blocking solution containing 5% nonfat dry milk and 0.1% Tween® 20. The membranes were then incubated with the

desired primary antibody diluted to the specified dilution in blocking solution for ~2 hours on a rocker at room temperature. The membranes were then washed (3 x 10 min.) with PBS/0.1% Tween® 20 and then incubated with either a horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG secondary antibody from Amersham (Oakville, Ont.) which were diluted 1:10,000 in blocking solution. Following a ~2 hour incubation with the second antibody on a rocker at room temperature, the membranes were again washed (3 x 10 min.) with PBS/0.1%Tween®20. Specific anti-primary antibody signals were detected using the ECL™ chemiluminescent detection kit from Amersham (Oakville, Ont.) followed by exposure to Kodak BIOMAX™ MR film from Eastmen Kodak (Rochester, NY.).

2.6 Northern Blotting

Northern hybridization of RNA samples was achieved by using a modified procedure from Current Protocols In Molecular Biology section 4.9.1. (Ausubel et al., 1987).

2.6.1 Isolation of total RNA from animal cells

Total RNA was isolated from cells infected with recombinant viruses using the RNeasy Mini Kit from QIAGEN (Valencia, CA.). Briefly, 1 x 10^7 cells were

infected with various recombinant viruses at a multiplicity of infection of 20. The cells were then harvested 48 hours post-infection and disrupted in the presence of highly denaturing guanidinium isothiocyanate (GITC)-containing lysis buffer which immediately inactivates RNases to ensure isolation of intact RNA. The cells were then homogenized by centrifuging through the QIAshredder spin columns which sheared the genomic DNA and reduced the viscosity of the lysate. Ethanol was then added to adjust binding conditions and the samples were applied to the RNeasy mini spin columns where the total RNA bound to the membrane and contaminants were washed away. The RNA was then eluted in DEPC-treated water.

2.6.2 Electrophoresis of RNA samples

RNA samples were electrophoresed under denaturing conditions in order to break up intermolecular base pairing which prevent proper separation of the samples. Denaturing was achieved by heating samples at 55°C for 15 min. and renaturating was prevented by adding formaldehyde to the gel and to the samples. RNA samples were prepared by adding 10X MOPS (400 nM, pH 7.5, 500 mM NaCl, 100mM $MgCl_2$ in double distilled water), 12.3M (37%) formaldehyde and formamide to 20 µg of total RNA. Samples were then loaded onto a 1% agarose/formaldehyde gel and electrophoresed at 5 V/cm in 1X MOPS running buffer until the bromophenol blue dye had migrated two-thirds the length of the gel. The gel, which was stained with ethidium bromide, was then photographed with a ruler and rinsed in DEPC-treated water.

2.6.3 Transfer of RNA and Hybridization analysis

RNA was transferred from the gel to a positively charged HybondN+ nylon membrane from Amersham (Oakville, Ont.) by upward capillary transfer in 20X SSC (standard saline-citrate: 17.53 g NaCl and 8.82 g sodium citrate) transfer buffer. Following an overnight transfer, the nylon membrane was UV cross-linked with a UV light box in order to covalently attach the RNA to the membrane.

DNA probes were prepared using the T7 Quick Prime Kit from Pharmacia. A specific activity of greater than 10^7 dpm/μg was obtained after unincorporated nucleotides were removed. The membrane was then placed in a glass tube and incubated in ~8ml formamide prehybridization/hybridization solution with rotation for 3 hours at 42°C in a commercial hybridization oven. The DNA probe, which was denatured by heating for 10 min. at 100°C, was then added to the prehybridization/hybridization solution and left overnight in the hybridization oven at 42°C with rotation. The next day, the prehybridization/hybridization solution was poured off and the membrane was washed to reduce the background signal. The first wash was with 2X SSC with rotation for 15 min. at room temperature followed by 0.5% SSC and 0.1% SDS with rotation for 15 min. at room temperature. After each wash the membrane was checked with a Geiger for radioactivity intensity and after the second wash it was revealed that no further washing was necessary. Finally, specific binding of the probe to the membrane was revealed by autoradiography with Kodak BIOMAX™ MR film from Eastmen Kodak (Rochester, NY.).

Chapter 3
Results

3.1 Construction of E1 transfer plasmids

The adenoviral expression system (AES) is routinely used for the transfer of foreign genes into cells in order to study their effects *in vivo*. This approach was used to study the role that each of the adenovirus E1 proteins might play in the production of E1-deleted recombinant adenoviruses.

The adenoviral E1 proteins were individually subcloned into the adenoviral transfer plasmid, pAdTR5K7C/GFP^{Q}, which has the unique feature of allowing for the inducible expression of the transgene, placed under the control of a tetracycline-regulated promoter, as well as having a reporter gene, the green fluorescent protein (GFP) gene from the jellyfish *Aequorea victoria*, controlled by the constitutive cytomegalovirus (CMV) promoter.

3.1.1 pAdTR5K7C/GFP^{Q}

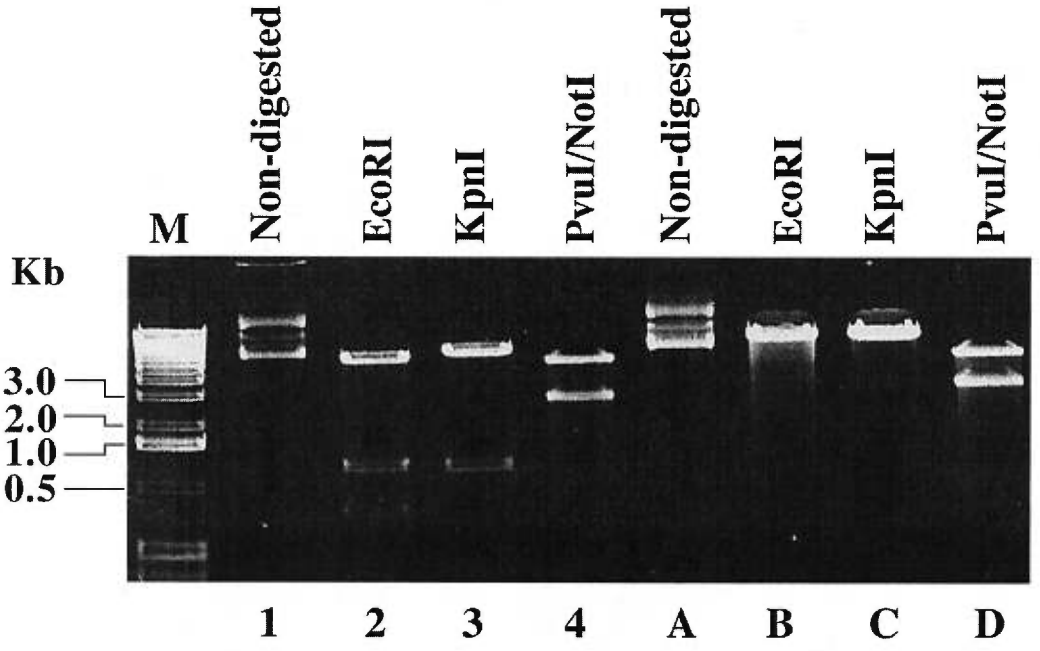
The pAdTR5K7C/GFP^{Q} adenoviral transfer plasmid was used for the construction of three E1 expressing recombinant adenoviruses. This plasmid has a multiple cloning site (MCS), which was taken from the pAdTR5DC/GFP mod C transfer plasmid and placed into the pAdTR5K7/GFP^{Q} transfer plasmid (Fig. 2.1). The inclusion of the MCS was desired in order to facilitate the subcloning of other E1 genes into the pAdTR5K7/GFP^{Q} plasmid while maintaining the features of this plasmid. The construction was achieved by digesting the pAdTR5K7/GFP^{Q} and

pAdTR5DC/GFP mod C with *Fse*I and *Bgl*II. Ligation of the desired fragments generated the pADTR5K7C/GFP^{Q} transfer plasmid (Fig. 2.1). Verification of the desired construction was done by comparing the digestion patterns of pAdTR5K7C/GFP^{Q} with that of pAdTR5DC/GFP mod C. The restriction enzymes *Eco*RI and *Kpn*I are unique in the pAdTR5K7C/GFP^{Q} plasmid and consequently linearized the plasmid (Fig. 3.1, lanes B and C). However, these sites are not unique in the pAdTR5DC/GFP mod C plasmid (lanes 2 and 3) and consequently display a multiple band pattern. Furthermore, the *Pvu*I and *Not*I double digest confirmed the presence of the MCS in the pAdTR5K7C/GFP^{Q} since the *Not*I site is not found in the pAdTR5K7/GFP^{Q} plasmid (lanes 4 and D). A positive clone, identified from the screening, was selected and the nucleotide sequence of the modified region was confirmed by DNA sequencing (data not shown).

Figure 3.1

Verification of the pAdTR5K7C/GFP^{Q} adenoviral transfer plasmid by restriction enzyme digestion.

Both pAdTR5K7/GFP^{Q} and pAdTR5DC/GFP mod C transfer plasmids were digested with *Fse*I and *Bgl*II which generated a small (2300bp) and a large (5894bp) fragment, respectively. Ligation of the two fragments generated the pAdTR5K7C/GFP^{Q} transfer plasmid. Digestion of the plasmid with either *Eco*RI or *Kpn*I showed that, as expected, these sites are unique in the pAdTR5K7C/GFP^{Q} plasmid (lanes B and C) but not in the pAdTR5DC/GFP mod C plasmid (lanes 2 and 3). Furthermore, the *Pvu*I and *Not*I double digest (lanes 4 and D) confirmed the presence of the multiple cloning site, since the *Not*I site is absent from the plasmid pAdTR5K7/GFP^{Q}.



M - 1 kb ladder (DNA molecular weight marker)

1
2
3
4 — pAdTR5DC/GFP mod C

A
B
C
D — pAdTR5K7C/GFP^{Q}

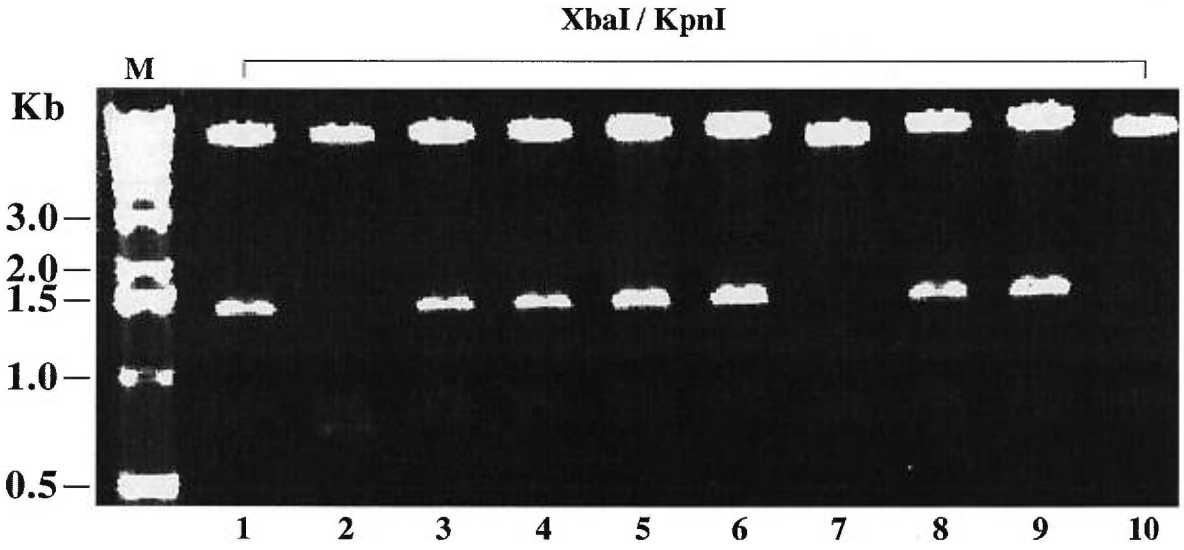
3.1.2 pAdTR5E1A-K7C/GFP^{Q}

The transfer plasmid, pAdTR5E1A-K7C/GFP^{Q}, contains the E1A gene subcloned into pAdTR5K7C/GFP^{Q} such that E1A is positioned in the sense orientation relative to the inducible promoter. The E1A gene was isolated from the plasmid pUHDE1A by digestion with *Bam*HI and *Eco*RI. The *Eco*RI end was rendered blunt to facilitate ligation of this fragment into the plasmid pAdTR5K7C/GFP^{Q} which had been digested with *Bgl*II and *Eco*RV (Fig. 2.2). Confirmation of the clone selected was done by digesting the recombinant plasmid, pAdTR5E1A-K7C/GFP^{Q}, with the unique restriction enzymes *Xba*I and *Kpn*I, simultaneously. Since each one these enzymes originated from a different fragment, the presence of both restriction enzyme sites on one plasmid could only occur if the ligation between the two fragments took place, thus generating a double band pattern when digested together, as could be seen in Fig. 3.2, lanes 1, 3, 4, 5, 6, 8 and 9. Sequencing of the plasmid confirmed that the DNA sequence was as expected (data not shown).

Figure 3.2

Verification of the pAdTR5E1A-K7C/GFP^{Q} adenoviral transfer plasmid by restriction enzyme digestion.

Ten putative clones were screened by simultaneously digesting the plasmids with *Xba*I and *Kpn*I. The pAdTR5E1A-K7C/GFP^{Q} plasmid was constructed by digesting pAdTR5K7C/GFP^{Q} with *Bgl*II and *Eco*RV and introducing a ~1Kb fragment of genomic DNA from *Bam*HI and *Eco*RI digested pUHDE1A. The ligation of the fragments generated two unique sites, one originating from pAdTR5K7C/GFP^{Q}, *Kpn*I, and the other from the coding fragment, *Xba*I. The results show that seven clones (lanes 1, 3, 4, 5, 6, 8 and 9) were positive, whereas two were not (lane 7 and 10) and one clone (lane 2) resulted in a digestion product but of the wrong size.



M - 1 kilobase ladder (DNA molecular weight marker)
1 -10: putative clones of pAdTR5E1A-K7C/GFP^{Q}

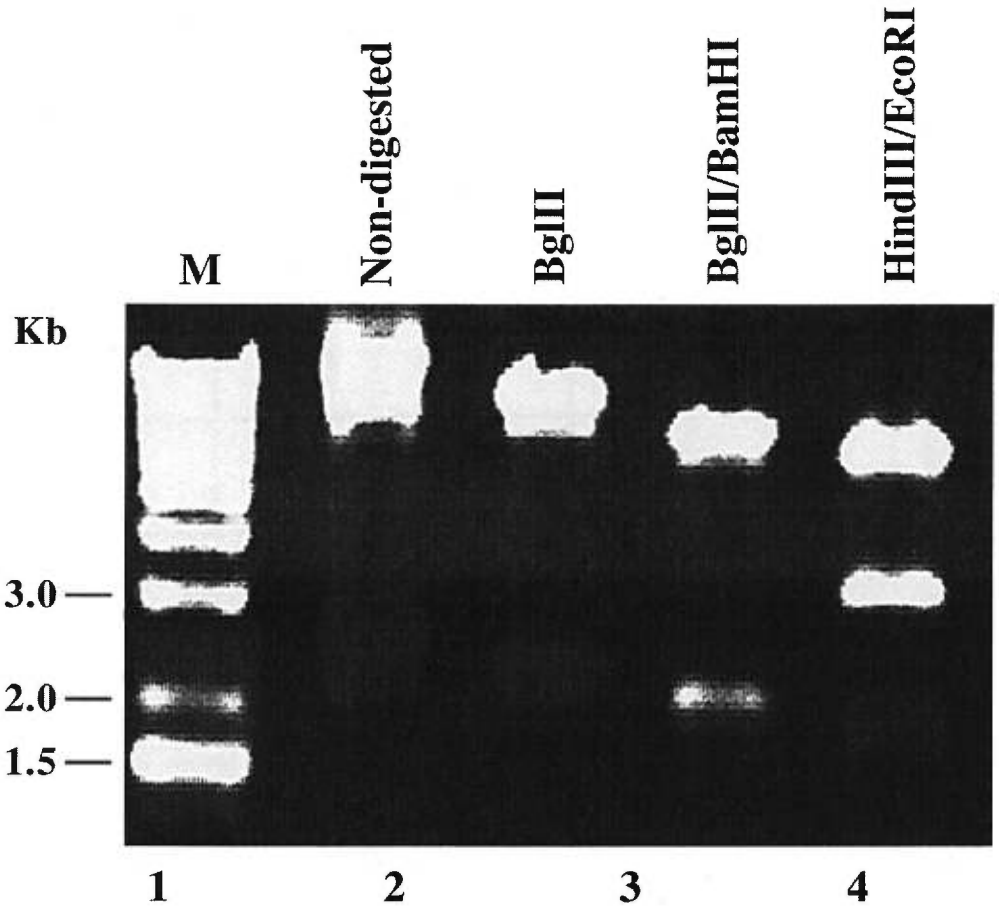
3.1.3 pAdTR5E1B55K-K7C/GFP^{Q}

The transfer plasmid pAdTR5E1B55K-K7C/GFP^{Q} was constructed by the insertion of the E1B55K gene, from the plasmid pCEIB58, into pAdTR5K7C/GFP^{Q} (Fig.2.3). The ligation of the fragments generated a new plasmid, pAdTR5E1B55K-K7C/GFP^{Q}, with four unique sites. Two of which originated from the pCE1B58 fragment, *Bgl*II and *Hind*III, and two originating from pAdTR5K7C/GFP^{Q}, *Bam*HI and *Eco*RI. Confirmation of the newly formed plasmid was done by either single digestion (Fig. 3.3, lane 2) or double digestion (lanes 3 and 4). Sequencing of the selected clone also confirmed that the desired inserted DNA sequence was correct (data not shown).

Figure 3.3

Verification of the pAdTR5E1B55K-K7C/GFP^{Q} adenoviral transfer plasmid by restriction enzyme digestion.

The pAdTR5K7C/GFP^{Q} plasmid was digested with *Bgl*II and *Not*I. The E1B55K coding sequence was isolated from the pCE1B58 plasmid as a ~1.5Kb *Bam*HI and *Not*I fragment. The ligation of the fragments generated a new plasmid with four unique sites. Two of which originated from the E1B55K genomic DNA fragment, *Bgl*II and *Hind*III, and two originating from pAdTR5K7C/GFP^{Q}, *Bam*HI and *Eco*RI. Single digestion (lane 2) or double digestions (lanes 3 and 4) confirmed the identity of the newly formed plasmid.



M- 1 kilobase ladder
(DNA molecular weight marker)

1
2
3
4 — **pAdTR5E1B55K-K7C/GFP^{Q}**

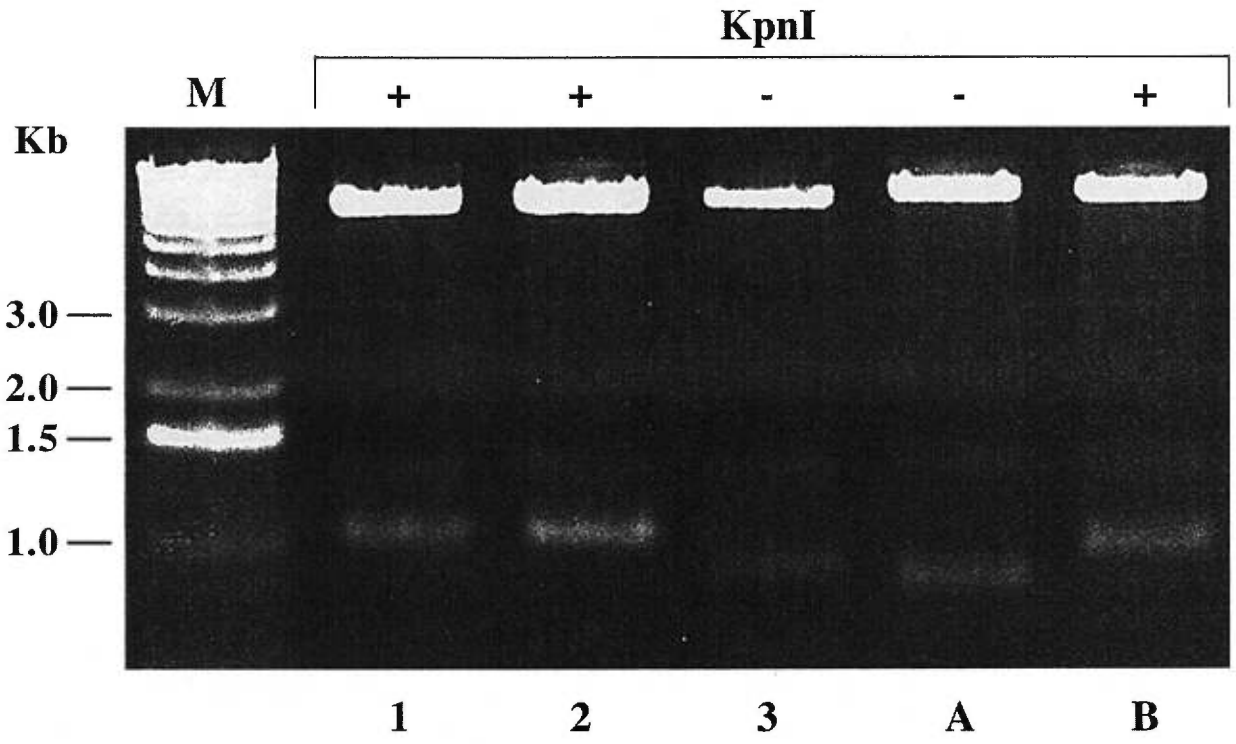
3.1.4 pAdTR5E1B19K-K7/GFP^{Q} and pAdTR5E1B19K-K7C/GFP^{Q}

Two recombinant transfer plasmids were made for the expression of the E1B19K protein. Each of these plasmids have the E1B19K gene placed under the control of the tetracycline-regulated promoter and the GFP gene under the control of the constitutive promoter, CMV. However, the pAdTR5E1B19K-K7C/GFP^{Q} plasmid differs from the pAdTR5E1B19K-K7/GFP^{Q} solely by the fact that it has a MCS located at the 5' end of the E1B19K gene (Fig. 2.5). This construct, in addition to determining the role E1B19K plays in the production of E1-deleted recombinant adenoviruses, was done so that a study could be done of the impact (if any) that the MCS has on expression of the encoded proteins. The cloning was done by inserting a PCR generated *Bam*HI fragment from pUHE1B19K into either the pAdTR5K7/GFP^{Q} or pAdTR5K7C/GFP^{Q} transfer plasmids which were previously digested with *Bgl*II. Since the insert can be ligated in either sense or anti-sense orientation, both plasmids had to verified by a screening which could discriminate between the two orientations. This was accomplished by digesting the plasmids with *Kpn*I which is unique in the E1B19K fragment and in both of the original transfer plasmids. As such, two successful clones were generated (Fig. 3.4, lane 1 and 2 for pAdTR5E1B19K-K7C/GFP^{Q}; and lane B for pAdTR5E1B19K-K7/GFP^{Q}) when the *Kpn*I sites were found closer to each other generating a smaller band than if the gene were in the anti-sense orientation (lane 3 for pAdTR5E1B19K-K7C/GFP^{Q} and lane A for pAdTR5E1B19K-K7/GFP^{Q}). DNA sequencing of both plasmids confirmed that

Figure 3.4

Verification of the two E1B19K adenoviral transfer plasmids by restriction enzyme digestion.

Two transfer plasmids, each expressing the E1B19K protein were constructed in order to evaluate the impact that the multiple cloning site (MCS) had on the expression of the downstream protein coding sequence when introduced into the pAdTR5K7C/GFP^{Q} plasmid. pAdTR5E1B19K-K7C/GFP^{Q} differs from pAdTR5E1B19K-K7/GFP^{Q} in that the former has an MCS (symbolized by the "C") separating the inducible promoter, TR5, from the E1B19K coding sequence. A PCR generated E1B19K (not sequenced) fragment of approximately 500 bp containing *Bam*HI ends was cloned into the unique *Bgl*II site within each of the two plasmids. The screening was done by digesting the plasmids with *Kpn*I which is unique in the E1B19K fragment and in both of the original transfer plasmids. Two positive clones, containing the E1B19K gene in the sense orientation, were obtained for pAdTR5E1B19K-K7C/GFP^{Q} (lanes 1 and 2) and one was obtained for pAdTR5E1B19K-K7/GFP^{Q} (lane B).



1
2
3 — pAdTR5E1B19K-K7C/GFP^{Q}

A
B — pAdTR5E1B19K-K7/GFP^{Q}

+ → Sense orientation

- → Anti-sense orientation

the plasmid contained the desired DNA sequence in the sense orientation relative to the tetracycline-regulated promoter (data not shown).

3.2 Transient transfection of E1 transfer plasmids

The new transfer plasmid pAdTR5E1A-K7C/GFP^{Q} (Fig. 2.2) was tested to verify that it was capable of expressing the E1A proteins in transiently transfected Hela-rtTA cells. These cells were chosen for two reasons: i) they do not express E1A proteins and ii) they express the tetracycline-regulated transactivator protein, rtTA, therefore these cells can induce the tetracycline-regulated promoter present on the transfer plasmid by the addition of micromolar concentrations of doxycycline. As a preliminary step, three clones of pAdTR5E1A-K7C/GFP^{Q} (A, B and C), selected on the basis of their expected digestion pattern, were observed 24 hours post-transfection for GFP expression using a fluorescence microscope. This observation would confirm the success of the transfection, since only cells which had incorporated a plasmid would be seen as green. As such, transient transfection of Hela-rtTA cells with 10 μg of DNA by the three different clones of pAdTR5E1A-K7C/GFP^{Q}, were all found to be positive for GFP expression with the exception of the non-transfected cells (data not shown). The presence of E1A was then revealed by western blotting using the monoclonal antibody M73 (graciously provided by Dr. P. Branton, McGill University) which specifically recognizes the adenoviral E1A gene products. Transfected and non-transfected cells were harvested 48 hours post-infection and

extracts containing 15 μg of proteins were separated on a 12% denaturing SDS-PAGE gel. Western blot analysis showed that all three clones (A, B and C) displayed two bands (Fig. 3.5, lanes 2, 3 and 4) within the molecular weights of approximately 40-50 kDa (Harlow et al., 1986) which likely corresponded to the two most abundant transcripts of E1A, 13S and 12S mRNA, respectively. Conversely, the non-transfected cells did not have any proteins that were recognized by the monoclonal antibody M73 (lane 1) which strongly suggested that the bands that appeared in the lanes containing extracts from transfected cells were proteins transcribed from the E1A plasmids.

The band which appeared slightly below the 36 kDa region (lanes 2, 3, and 4) was also suspected to represent a protein transcribed by the E1A plasmids for several reasons. First of all, the primary E1A transcript is differentially spliced to produce at least five mRNAs (13 S, 12 S, 11 S, 10 S and 9 S) four of which differ in size and number of introns (Hitt and Graham, 1990). Secondly, the 289 and 243 residue E1A proteins, corresponding to the 13S and 12S mRNAs' respectively, are post-translationally modified by phosphorylation and thus give rise to an upper, slower migrating hyperphosphorylated protein band, and a lower faster migrating hypophosphorylated protein band resulting in the anomalous migration of the E1A proteins (Boulanger et al., 1991; Bayley and Mymryk, 1994). Thirdly, gel conditions used for electrophoresis could give rise to different band patterns. Therefore, any combination of the aforementioned reasons could explain the appearance of the band at the ~36 kDa region by the three clones of pAdTR5E1A-K7C/GFP^{Q}. What's more,

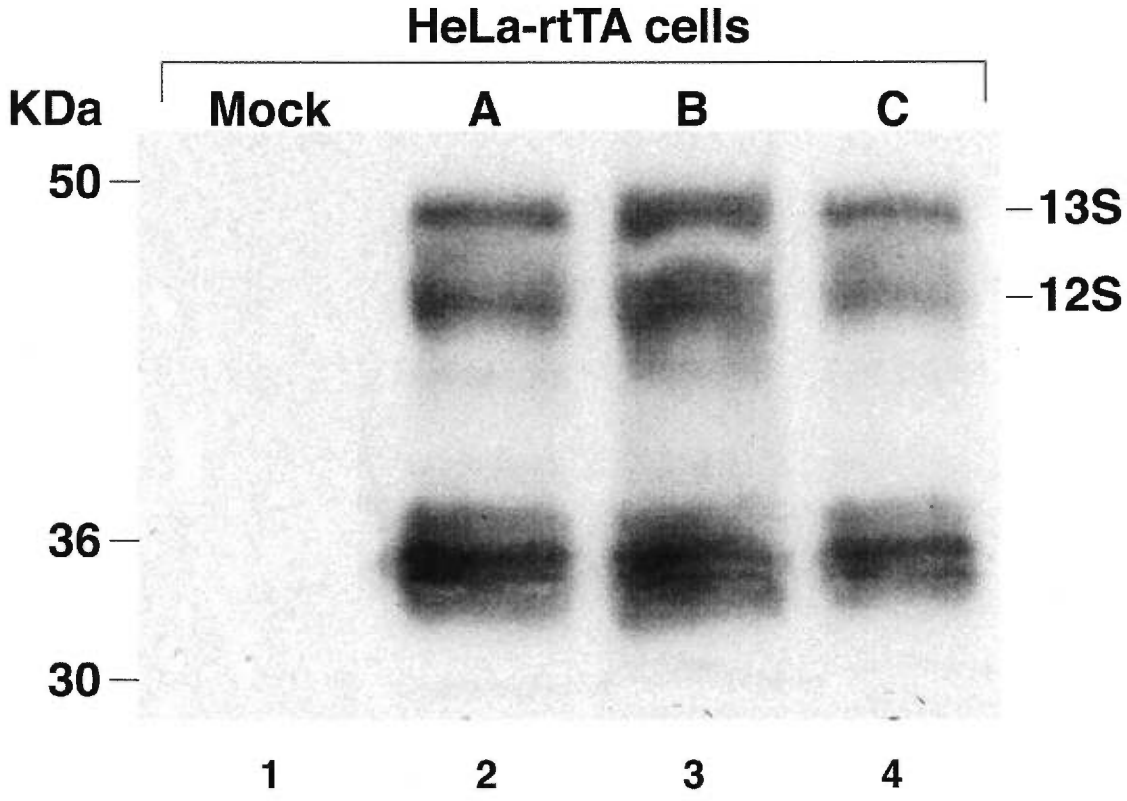
since the mock transfection (lane 1) did not have a band appear in the ~36 kDa region it was assumed that the band that did appear in lanes 2, 3 and 4 around the ~36 kDa was most likely of plasmid origin. However, a possibility which was not given much consideration is that this ~36 kDa band was the product of aberrantly spliced mRNAs. If this were the case, an investigation to determine why the E1A plasmids produce this truncated protein would have been warranted (i.e. northern blotting) particularly since E1A producing viruses were to be made from these plasmids.

In numerous transient transfections with the E1B encoding plasmids the expression of E1B could not be detected by western blotting. Different cell lines (i.e. Hela-rtTA and Kb-rtTA) were used as well as different transfection techniques (calcium phosphate and electroporation) but with no success. Still, we were encouraged by the fact that the cells transfected by the E1B plasmids were found to be positive for GFP expression when observed 24 hours post-transfection under the inverted fluorescence microscope. Therefore, we pursued the construction of the recombinant E1B viruses with the confidence that GFP was being expressed in the cells and the expectation that the levels of E1B would be higher and sufficient to detect when expressed by a recombinant virus.

Figure 3.5

Expression of E1A proteins by the pAdTR5E1A-K7C/GFP^{Q} adenoviral transfer plasmids.

HeLa-rtTA cells were transiently transfected with 10 μg of DNA from three different clones (A, B and C) of the plasmid pAdTR5E1A-K7C/GFP^{Q} (lanes 2, 3 and 4). Each of these clones were previously screened by restriction enzyme digestion and selected on the basis of displaying the expected digestion pattern. Doxycycline (1 μg/ml) was added to all transfected cells as well as to non-transfected cells (mock, lane 1). The cells that were transfected with the pAdTR5E1A-K7C/GFP^{Q} plasmids were found to be positive for GFP expression at 24 hours. Cells were harvested after 48 hours and extracts containing 15 μg of protein were separated on a 12% denaturing SDS-PAGE gel. Western blot analysis for the detection of E1A was performed using a specific anti-E1A monoclonal antibody (M73) at a dilution of 1/3000.


HeLa-rtTA cells
KDa
Mock
A
B
C
50
36
30
13S
12S
1
2
3
4

3.3 Expression of E1 proteins in AdTR5K7C/GFP^{Q} derived viral recombinants

Recombinant adenoviral vectors expressing each of the E1 proteins were obtained by co-transfection of 293A cells with various *Fse*I-cut transfer vectors and the large right-end fragment of the Ad5/ΔE1ΔE3 genome. Positive plaques were selected based on their ability to express the reporter gene, GFP. In fact, the presence of the red shifted GFP gene, referred to as GFP^{Q} (Quantum Biotechnologies Inc., Montreal, Canada), simplified the screening and selection of all the recombinant viruses expressing inducible gene products (Massie et al, 1998d). Finally, positive plaques were purified and expanded in 293 cells as described by Jani et al. (1997) so that the problem of replication-competent adenoviruses would be kept to a minimum. Finally, large scale virus stocks were prepared by infecting 3 x 10^{9} 293S cells in suspension and titers were determined using a fluorometric method.

Recombinant viruses expressing inducible E1 proteins were analyzed by western blotting. Total extracts of viral inoculates corresponding to estimated multiplicities of infection (MOI's) of 50 gene transfer units (GTU) per cell were used to infect subconfluent noncomplementing cells at a density of 5.0 x 10^{5} cells per dish. Infected cells were harvested 48 hours post-infection, lysed and sonicated to shear the DNA.

3.3.1 Expression of E1A by the AdTR5E1A-K7C/GFP^{Q} recombinant virus

Western blot analysis was used in order to verify the proper expression of the E1A proteins by the recombinant E1A virus, AdTR5E1A-K7C/GFP^{Q}. Three non-complementing and non-E1A expressing cell lines (Kb-rtTA, Hela-rtTA and A549-tTA) were infected by AdTR5E1A-K7C/GFP^{Q} either in the induced state ("on"), where expression of the transgene was induced, or in the noninduced state ("off"), where the transgene was expressed at a basal level which corresponded to the MOI used. The absorption conditions for infection were done in a minimal volume (ex., 1 ml in 60 mm plates) for 16 hours in order to ensure optimal entry of the virus (Mittereder et al., 1996). Subsequently, the volume of the plates was raised (ex., one additional ml in the 60 mm) and cells were observed for GFP expression. All of the various cell lines infected with AdTR5E1A-K7C/GFP^{Q} displayed green fluorescence when observed under the inverted fluorescence microscope which indicated GFP expression in the cells whereas non-infected cells did not show any fluorescence (data not shown). Cells were then harvested (48 hours post-infection) and total cell extracts containing 15 μg of protein were separated on a denaturing SDS-PAGE gel. Western blotting was performed with a 1:10,000 dilution of the M73 monoclonal antibody (Yee et al., 1985) which binds specifically to E1A proteins. Results showed that infection of 293A (Fig. 3.6, lane 3), Kb-rtTA (lane 4 and 5), Hela-rtTA (lane 8 and 9) and A549-tTA (lane 11 and 12) cell lines with the E1A virus, AdTR5E1A-K7C/GFP^{Q}, resulted in the expression of two closely migrating E1A-immuno-

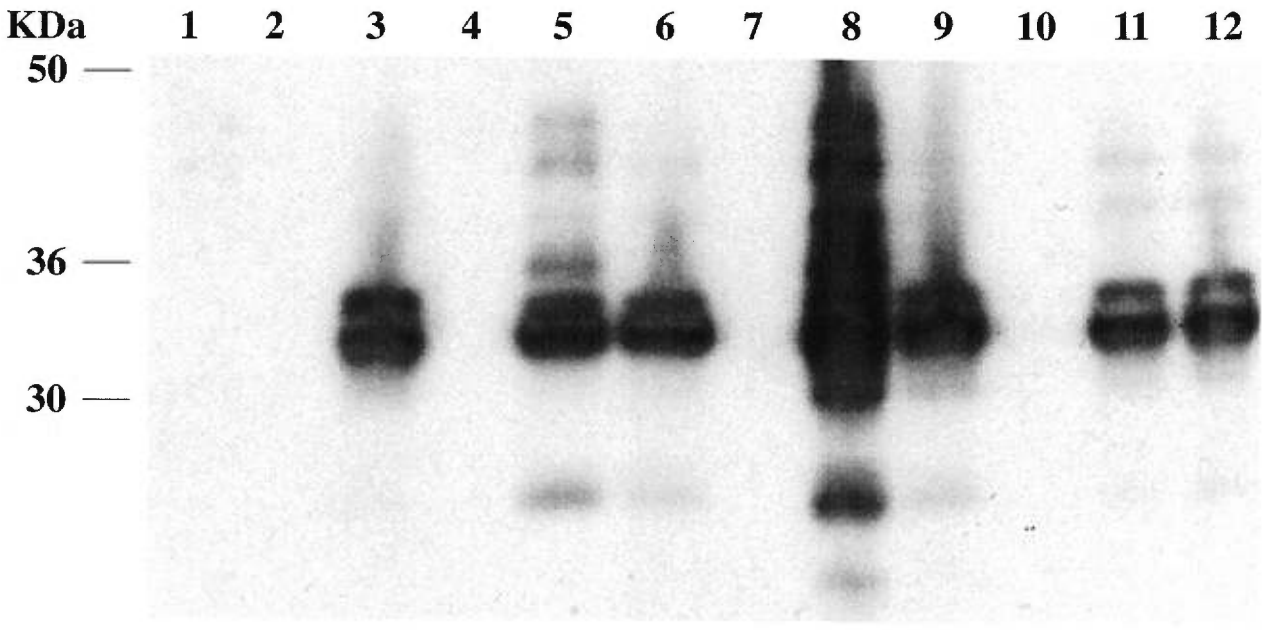
reactive proteins of approximately 36 kDa in both the "on" and "off" state, with the lower of the two bands being slightly more intense. A doublet migrating in the expected range of 40-50 kDa was observed only in Kb-rtTA (lane 5) and Hela-rtTA cells (lane 8) infected with the E1A-expressing virus. However, at much longer exposures the 40-50 kDa doublet could be seen in all of the infections with the E1A virus as well as with the 293A cells (lane 1) (data not shown). The only exception was the infected A549-tTA cells in which a doublet that migrated to position lower than that of the others was observed (lanes 11, 12). None of the three cell lines, Kb-rtTA (lane 4), Hela-rtTA (lane 7) and A549-tTA (lane 10) expressed E1A proteins. Neither did an infection of Kb-rtTA with the AdTR5K7C/GFP^{Q} virus, a virus which only expresses GFP, show transgene expression which could be recognized by the M73 antibody (lane 2). The induction of the E1A gene was significant in Kb-rtTA cells (lane 8 vs. lane 9), marginal in Hela-rtTA cells (lane 5 vs. lane 6) and was not detected with the A549-tTA cell line (lane 11 vs. lane 12). In order to verify the proper transactivation by the -tTA or the -rtTA system, all of the various cell lines used in this experiment were infected with the AdTR5K7C/GFP^{Q} virus. This virus has the unique feature of having the GFP gene under the control of the same tet-regulated promoter that is present in the E1A virus and thus was used to compare the GFP expression both in the induced and non-induced state. For this experiment, cells were infected, at an MOI of 50, either in the presence of doxycycline(1 μg/ml) in the Kb-rtTA and Hela-rtTA cell lines or in the absence of doxycycline in the A549-tTA cell line. These conditions allow transgene expression. The virus was also used to infect cells in the non-induced state in which case doxycycline was omitted from the

Kb-rtTA and Hela-rtTA cell lines and added to the A549-tTA cell line. Relative assessment of GFP expression, 24 hours post-infection, by observation under an inverted fluorescence microscope revealed a significant difference (at least ~10 fold) between the "on" and "off" state in both the Kb-rtTA and Hela-rtTA cell lines but not with the A549-tTA cell line (data not shown). These results, which are not in accordance with those previously reported (Massie et al., 1998b), could simply reflect the choice of a A549-tTA clone which did not function properly. Nevertheless, they did at least offer a possible explanation for the absence of induction of the E1A proteins in the A549-tTA cells (lane 11 vs.12).

Figure 3.6

Expression of E1A proteins following infection of various cell lines with the recombinant AdTR5E1A-K7C/GFP^{Q} virus.

None of the three cell lines, Kb-rtTA, HeLa- rtTA or A549- tTA, express the E1A proteins (lanes 4, 7 and 10). Kb-rtTA cells infected with the K7C virus did not show transgene expression (lane 2). E1A was overexpressed in 293 cells infected with the E1A virus (lane 3). E1A was expressed following infection with the AdTR5E1A-K7C/GFP^{Q} virus (lanes 5, 8 and 11) in the presence of doxycycline (1 μg/ml) in the -rtTA cell lines (lanes 5,8) or in the absence of doxycycline in the A549-tTA cell line (lane 11). The reverse was done to turn "off" the expression (lanes 6, 9 and12). The expression of E1A by the 293 cells alone (lane 1) could not be seen at this exposure due to the much lower abundance of E1A protein in these cells relative to the virus-infected cells. Cell extracts containing 15 μg of protein were separated on a 12% denaturing SDS-PAGE gel. Western blotting was done using a 1:10,000 dilution of the specific anti-E1A (M73) monoclonal antibody.



1 - 293A cells
2 - K7C virus / KB-rtTA
3 - E1A virus / 293A (+)
4 - KB-rtTA cells
5 - E1A virus / KB-rtTA (+)
6 - E1A virus / KB-rtTA (-)
7 - HeLa-rtTA cells
8 - E1A virus / HeLa-rtTA(+)
9 - E1A virus / HeLa-rtTA(-)
10 - A549-tTA cells
11 - E1A virus / A549-tTA (+)
12 - E1A virus / A549-tTA (-)

Abv. :
/ means - infection
+ means with induction
- means without induction

K7C virus - AdTR5K7C/GFP
E1A virus - AdTR5E1A-K7C/GFP^{Q}

3.3.1.1 The expression of E1A by an E1-deleted adenovirus induced a semi-replicative state in a non-complementing cell line

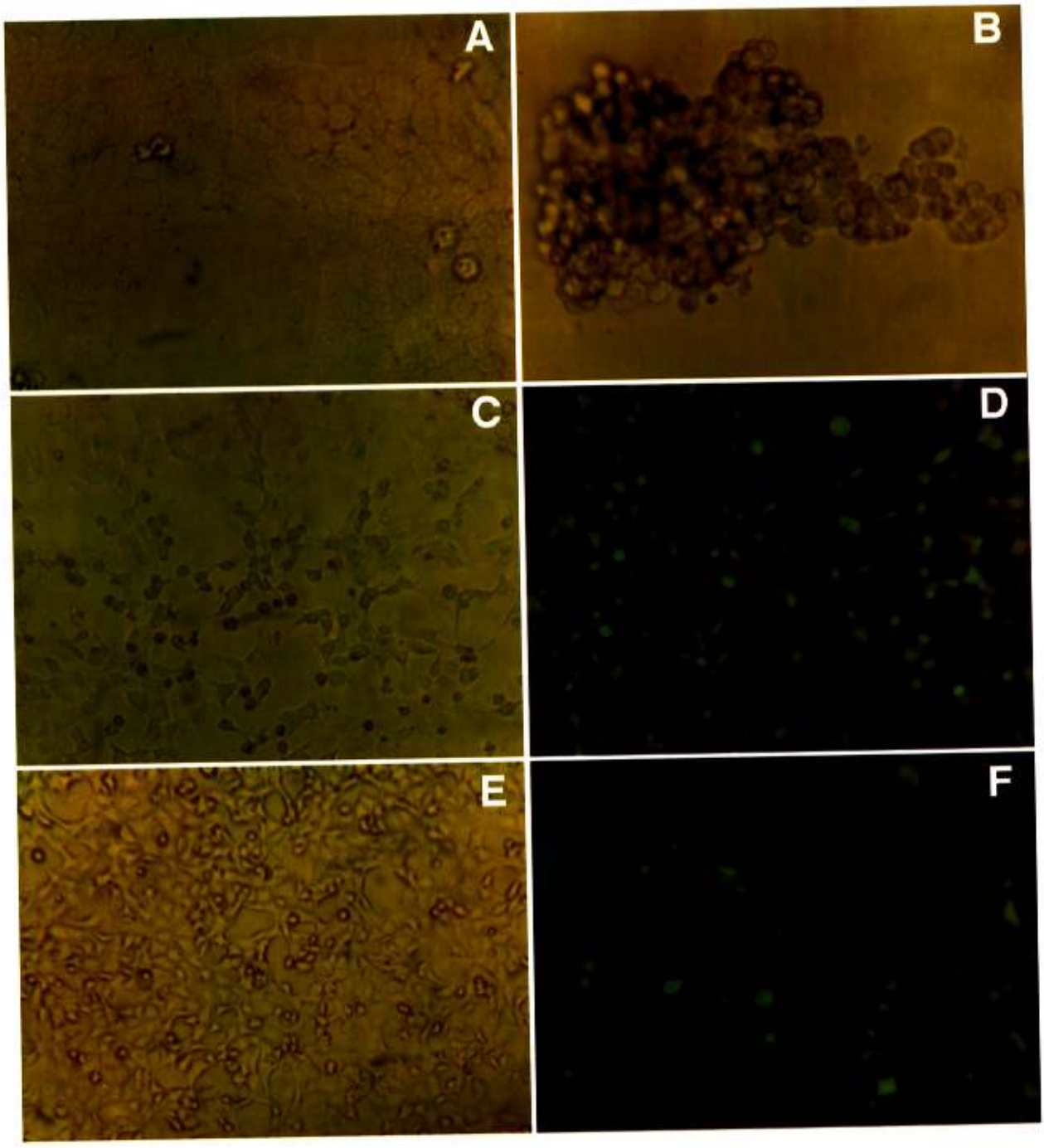
The construction of replication deficient adenoviruses is generally accomplished by co-transfecting complementing cell lines, which express the E1 proteins, with a transfer vector that has the gene of interest cloned into it. The resultant recombinant adenovirus, which has its E1 region replaced by the transgene, is obtained by in vivo homologous recombination between overlapping sequences on the linearized plasmid and the large right-end fragment of the Ad5 genome. These replication-deficient, E1-deleted viruses are unable to replicate in cells that do not provide the E1 proteins in trans. The 293cell line expresses the E1 proteins and can complement E1-deleted viruses. Since the E1 transcript encodes both E1A and E1B proteins we were interested in testing whether E1A alone was sufficient to allow for the replication of an E1-deleted virus in a non-complementing cell line. The recombinant E1A-expressing adenovirus, AdTR5E1A-K7C/GFP^{Q}, that did not express E1A proteins in a manner that is generally seen in adenovirus infected cells (Fig. 3.6), did nonetheless produce the doublet between the 40-50 kDa region associated with 13 and 12S and thus could be used for a preliminary study of the role of E1A in viral production. As such, Kb-rtTA cells were infected with either the AdTR5K7C/GFP^{Q} or the AdTR5E1A-K7C/GFP^{Q} recombinant viruses at an MOI of 30 and left rocking overnight for maximal absorption. Doxycycline (1 μg/ml) was added at the time of infection to induce the expression of the recombinant protein. Infected cells were photographed 24 hours post-infection both under visible light, to

monitor cell morphology, and ultraviolet light, to measure GFP expression (Fig. 3.7). As a general rule, adenovirus replication leads to a cytopathic effect (CPE) which is manifested by the rounding up and clumping of cells as seen by the infection of Kb-rtTA cells by the Ad5/dl309 (wild-type) adenovirus (Fig. 3.7, panel B). In contrast to the wild-type adenovirus infection which leads to a clear CPE, non-infected cells remain attached to the surface and maintain their morphology (panel A). E1-deleted viruses do not replicate in non-complementing cell lines but do cause a stress to the cell which is amplified if associated with some viral replication. One indicator of cell stress is cell morphology. Both of the E1-deleted viruses used to infect the Kb-rtTA cells namely, AdTR5K7C/GFP^{Q} and AdTR5E1A-K7C/GFP^{Q}, did not induce a CPE which was observed with the wild-type adenovirus. Nevertheless, cell stress could be observed and the effect was substantially more pronounced with the AdTR5E1A-K7C/GFP^{Q} virus (panel C) where the Kb-rtTA cells were more detached and rounded than the same cells infected with the AdTR5K7C/GFP^{Q} (panel E). A second indicator of cell stress and viral replication was the level of GFP expression found in cells 24 hours post-infection. Cells with higher intensity levels of GFP expression could suggest that viral gene replication is occurring. When the two viruses, AdTR5K7C/GFP^{Q} (panel F) and AdTR5E1A-K7C/GFP^{Q} (panel D), were compared for GFP intensity, the cells in which E1A proteins were expressed (panel D) had a much higher intensity level of visible fluorescence than those where no E1A was expressed (panel F). Therefore, these results indicate that the expression of E1A during the infection of an E1-deleted adenovirus did not lead to a productive cycle like what is observed with wild-type viruses, but may lead to a semi-replicative state

Figure 3.7

Semi-replicative state of an E1-deleted adenoviral vector induced by the expression of E1A, in a non-complementing cell line.

Three viruses, dl309 (panel B), AdTR5E1A-K7C/GFP^{Q} (panels C and D) and AdTR5K7C/GFPQ (panels E and F), were used at an MOI of 30 to infect the Kb-rtTA cell line. Doxycycline (1 μg/ml) was added at the time of infection to the cell monolayer. At 24 hours post-infection, non-infected cells (panel A) and infected cells (panel B) were photographed under visible and UV light. The infection with the wild-type adenovirus, dl309, displayed the typical rounding and detachment of cells (panel B), commonly associated with a complete cytopathic effect (CPE). The mock virus, AdTR5K7C/GFP^{Q}, which has no transgene expression also showed some ability to induce morphological changes (panel E) to the cells characteristic of a cell infection, but had very limited gene amplification once inside the cell (panel F). On the other hand, the AdTR5E1A-K7C/GFP^{Q} virus, which was induced to express E1A at the time of infection, displayed a more pronounced CPE (panel C), than the mock. Furthermore, once penetration of the virus had occurred there was clear evidence of gene amplification as could be seen by the higher percentage of GFP fluorescence in these cells (panel D) relative to that of the mock infected cells (panel F).


A
B
C
D
E
F

where the gene of interest could be amplified without displaying the full spectrum of a cytopathic effect.

3.3.2 Aberrant expression of E1B55K by the AdTR5E1B55K-K7C/GFP^{Q} recombinant virus

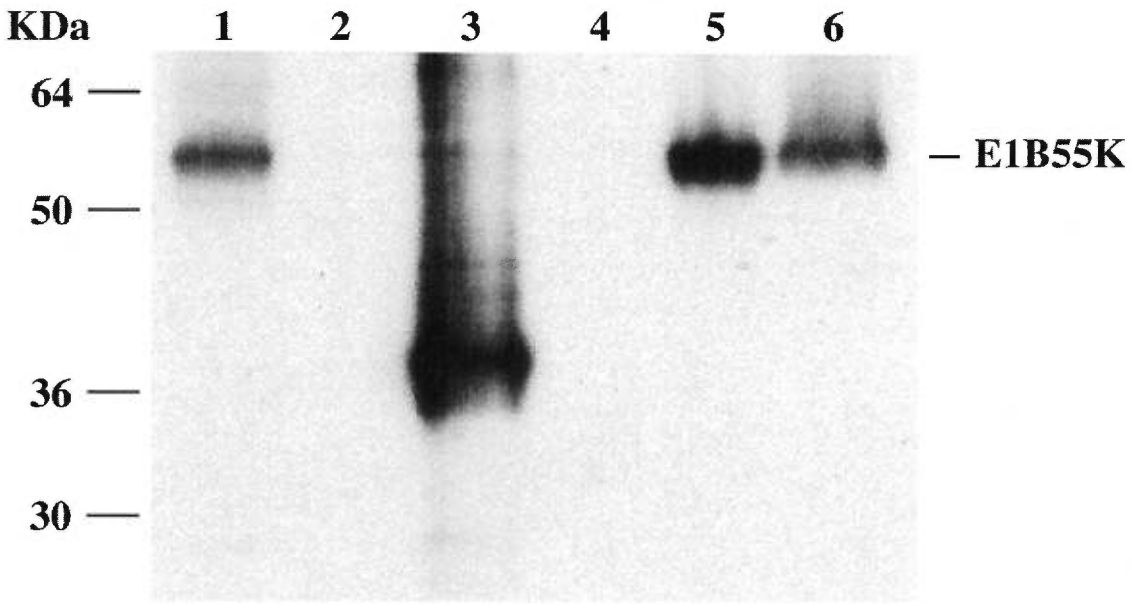
Generation of the E1B55K virus, AdTR5E1B55K-K7C/GFP^{Q}, was carried out even though transient transfection with the pAdTR5E1B55K-K7C/GFP^{Q} vector did not show any expression of the E1B55K protein. The decision to pursue this was based on the fact that the vector displayed the expected restriction enzyme digestion pattern and sequencing of the vector confirmed that the desired DNA sequence had been inserted. The construction of the virus was done by co-transfecting 293A cells with *Fse*I-digested pAdTR5E1B55K-K7C/GFP^{Q} and the large right-end of the Ad5/ΔE1ΔE3 genome as was previously described (section 3.3). The resulting virus , AdTR5E1B55K-K7C/GFP^{Q} (Fig. 3.8, lane 3), as well as AdTR5K7C/GFP^{Q} (lane 4), and the wild-type adenovirus dl309 (lane 5) were next used to infect separate cell cultures of the non-complementing, non-E1 expressing cell line Kb-rtTA. Cells were infected, with minimal volume, at an MOI of 50 overnight with slow rocking to maximize absorption of the virus by the cells. Doxycycline (1μg/ml) was added at the time of infection in all of the cultures. 24 hours post-infection the volume of the media was raised to 2 ml and cells were observed for GFP expression. As expected, only the recombinant virus constructed, AdTR5K7C/GFP^{Q} and AdTR5E1B-

K7C/GFP^{Q}, displayed the green fluorescence characteristic of GFP expression (data not shown). Cells were then harvested after another 24 hour incubation period. Extracts containing 15 μg of proteins were separated on a 12% denaturing SDS-PAGE gel and western blot analysis of the E1B55K was done using the specific anti-p55 monoclonal antibody 2A6 (Sarnow et al., 1982). Results showed that the non-infected Kb-rtTA cells (lane 2) as well as infection with the AdTR5K7C/GFP^{Q} virus (lane 4), a virus which has the GFP gene under the control of tet-regulated promoter, did not have any proteins which were recognized by the 2A6 antibody. On the other hand, two clones of 293S, 293A (lane 1) and 293-rtTA (lane 6), and cells infected with the wild-type virus, dl309 (lane 5), all displayed proper expression of E1B55K, however, this was not the case for the recombinant E1B55K virus, AdTR5E1B55K-K7C/GFP^{Q} (lane 3). The last displayed an aberrant band pattern with several faint distinct bands ranging from 40-55 kDa with a major one appearing slightly above the 36 kDa mark. Although proteins of a size less than 55 kDa are sometimes observed with the 2A6 antiboby (Teodoro et al., 1994), they are generally associated with a wild-type infection and are found around the 21 kDa region and not 36 kDa as was the case here.

Figure 3.8

Expression of the E1B55K protein by the recombinant AdTR5E1B55K-K7C/GFP^{Q} virus in Kb-rtTA cells.

Three different viruses, AdTR5E1B55K-K7C/GFP^{Q} (lane 3), AdTR5K7C/GFP^{Q} (lane 4), and dl309 (lane 5) were used to infect separate cultures of Kb-rtTA cells. Infection of Kb-rtTA cells with the K7C virus showed no transgene expression (lane 2). Doxycycline (1 μg/ml) was added at the time of infection and cells were harvested 48 h post-infection. Two clones of 293 (lanes 1 and 6) and cells infected with the wild-type virus, dl309 (lane 5), displayed proper expression of E1B55K, however, this was not the case for the recombinant E1B55K virus (lane 3). Extracts containing 15 μg of proteins were separated on a 12% denaturing SDS-PAGE gel. Western blot analysis of E1B55K was done using a 1:1,500 dilution of the specific anti-p55 monoclonal antibody 2A6.



1 - 293A cells
2 - KB-rtTA cells
3 - E1B55K virus / KB-rtTA (+)
4 - K7C virus / KB-rtTA
5 - d1309 / KB-rtTA
6 - 293-rtTA cells

Abv. :
/ infection
+ with induction

K7C virus → AdTR5K7C/GFP^{Q}
E1B55K virus → AdTR5E1B55K-K7C/GFP^{Q}

3.4 Proper expression of a transgene by a recombinant adenovirus with or without a multiple cloning site (MCS)

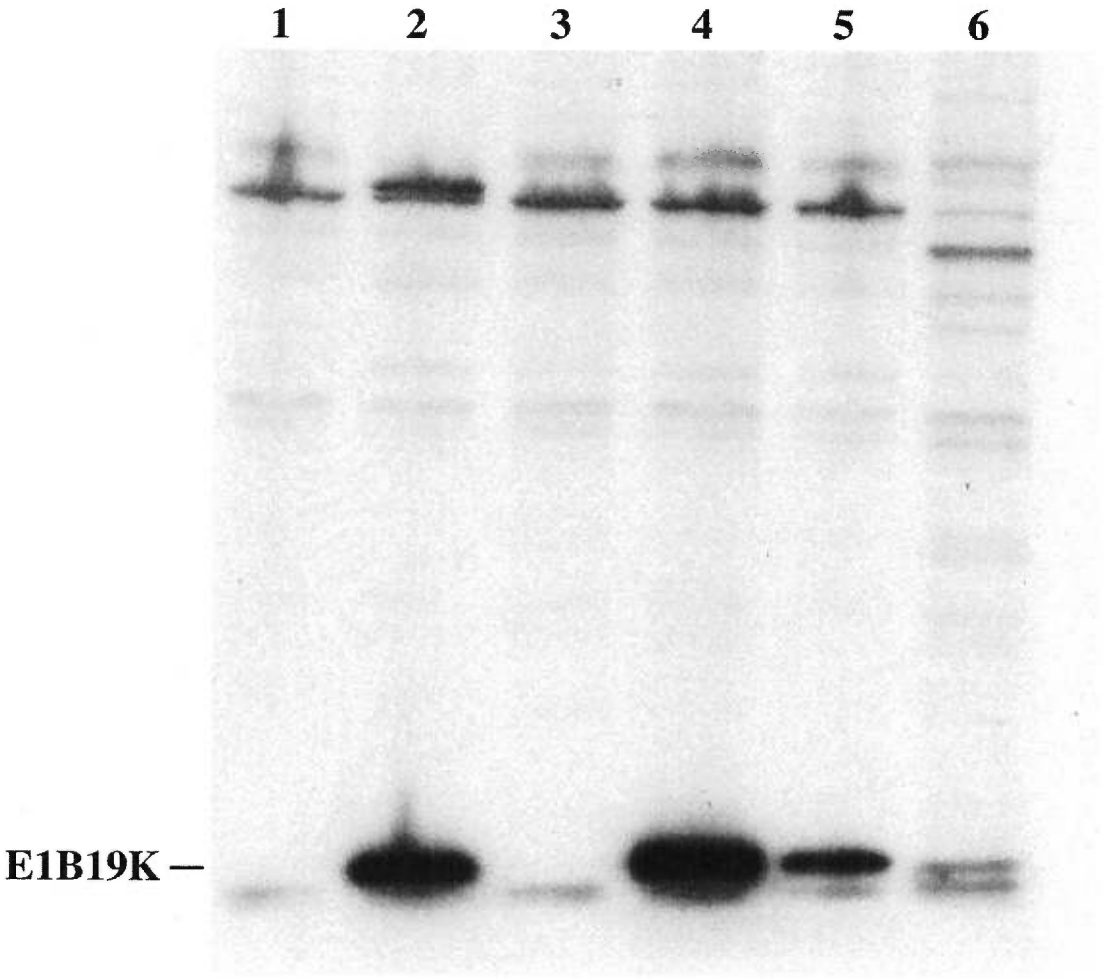
The adenoviral E1B19K gene was subcloned into two plasmids, pAdTR5K7/GFP^{Q} and pAdTR5K7C/GFP^{Q} generating two new recombinant adenoviral vectors, pAdTR5E1B19K-K7/GFP^{Q} and pAdTR5E1B19K-K7C/GFP^{Q}. The only difference between the pAdTR5E1B19K-K7C/GFP^{Q} vector and pAdTR5E1B19K-K7/GFP^{Q} is that the former has a MCS (symbolized by the "C") separating the inducible promoter, TR5, from the E1B19K fragment (see Fig. 2.5). These constructions were done in order to evaluate the impact that the MCS would have, if any, on the expression of downstream coding regions. After failing to observe transgene expression by transient transfection assays the construction of the E1B19K viruses was nonetheless pursued since the clones selected displayed the expected digestion pattern of both vectors (Fig. 3.4) and sequencing confirmed that the correct DNA sequence was present. Viruses were generated by co-transfecting each of the linearized vectors, together with the large right-end genome of Ad5/ΔE1ΔE3, into separate cultures of 293A cells . Purified and amplified viruses were then tested for proper expression of the gene of interest. Kb-rtTA cells, which do not express E1B19K, were separately infected by the two E1B19K viruses, namely, AdTR5E1B19K-K7/GFP^{Q} and AdTR5E1B19K-K7C/GFP^{Q}. In order to maximize absorption of the viruses onto the cells, infections were left overnight in minimal volumes, with slow rocking. Both infections were positive for GFP

expression 24 hours post-infection. Cells were harvested 48 hours post-infection and extracts containing 15 μg of protein were separated on a denaturing SDS-PAGE gel. Detection of the E1B19K protein was done by western blotting using a polyclonal E1B19K antibody (graciously donated by Dr. P. Branton, McGill University). Results showed that both of the E1B19K viruses, AdTR5E1B19K-K7/GFP^{Q} (Fig. 3.9, lane 4) and AdTR5E1B19K-K7C/GFP^{Q} (lane 5), expressed the E1B19K protein at the expected size of 19 kDa. On the other hand, both the mock infected Kb-rtTA cells (lane 1) and an infection with the AdTR5K7C/GFP^{Q} (lane 3), a virus which has the GFP gene under the control of the tet-regulated promoter, did not show expression of the E1B19K protein. Finally, an infection with the wild-type adenovirus, dl309 (lane 2), and the extract of cells which constitutively expresses E1B19K, 293A (lane 6), both confirmed the specificity of the antibody by displaying the presence of a sharp band around the 19 kDa, representing the E1B19K protein.

Figure 3.9

Expression of the E1B19K protein in cells infected with the AdVs, AdTR5E1B19K-K7C/ (with MCS) or K7/GFP^{Q} (without MCS).

Kb-rtTA cells were infected at an MOI of 30 with the dl309 virus (lane 2), the K7C virus (lane 3), the 19K virus (no MCS) (lane 4) and the C19K virus (with MCS) (lane 5). All of the viruses express the E1B19K protein at the expected size of 19KDa. Mock infections were done with Kb-rtTA cells (lane 1) as well as 293A cells (lane 6). Extracts containing 15 μg of protein were separated on a 12% denaturing SDS-PAGE gel. The E1B19K protein was detected by western blotting using a rabbit polyclonal E1B19K antibody provided by Dr. Philip Branton (McGill University).



1 - KBrtTA cells
2 - d1309 virus
3 - K7C virus / KBrtTA cells
4 - 19K virus / KBrtTA cells
5 - C19 virus / KBrtTA cells
6 - 293A cells

Abv. :
/ → infection
K7C → AdTR5K7C/GFP^{Q}
19K → AdTR5E1B19K-K7/GFP^{Q}
C19 → AdTR5E1B19K-K7C/GFP^{Q}

3.4.1 Insertion of a MCS reduces expression levels of recombinant proteins

The impact that a MCS may have on the expression of a protein was first observed with the expression of E1B19K (Fig. 3.9), in which two identical viruses, differing only by the insertion of a polylinker upstream of the cDNA, displayed proper expression of the E1B19K protein but with levels that were not equal. In fact, the virus, AdTR5E1B19K-K7C/GFP^{Q}, which had the MCS in its 5'-untranslated region (UTR) (Fig. 3.9, lane 5), showed a significant decrease in protein expression when compared to the virus, AdTR5E1B19K-K7/GFP^{Q}, without the MCS (Fig. 3.9, lane 4), even though cells were infected by both viruses in the same conditions and with the same MOI of 50. These observations led to a more in-depth analysis of this phenomenon. Therefore, the same MCS used to clone all three of the E1 proteins was also subcloned into the pAdTR5 plasmid. This was done in order to see if the same MCS would lower the expression of a different protein. For this purpose the green fluorescent protein (GFP) of *Aequorea victoria* was selected. The GFP gene was subcloned into the pAdTR5 plasmid downstream of the MCS or simply in the original pAdTR5 plasmid without the MCS (see Fig. 2.6). Subsequently, two recombinant GFP-adenoviral viruses, Ad/GFP and Ad/MCS-GFP were constructed, purified, amplified and titered following the same methods used for the recombinant E1-adenoviral viruses. These GFP-viruses were used to infect different cultures of exponentially growing 293-rtTA cells in the presence of doxycycline (1 μg/ml). Infected cells were then harvested 48 hours post-infection and fixed with

paraformaldehyde(1%)-PBS. Flow cytometric analysis of the fixed cells showed that the fluorescence index (FI), which is calculated by multiplying the percentage of GFP positive cells by the mean fluorescence value, was much higher in cells which were infected by the Ad/GFP virus than in cells infected by Ad/MCS-GFP virus (Table 3.1). In fact, a ~21 fold decrease in GFP level was seen with the virus that had the MCS placed at the 5'-end of the GFP gene. These results with the GFP-viruses reaffirmed the observations seen with the E1B19K virus. Infections with the E1B19K virus were repeated in order to quantitatively know the extent to which the presence and position of the MCS lowered E1B19K protein expression. Western blotting of exponentially growing 293-rtTA cells infected with the two E1B19K viruses was done. Infected and non-infected cells were harvested 48 hours post-infection and probed with the E1B19K antibody and the human anti-actin antibody. The relative intensity of each E1B19K band was calculated by the NIH Image software and then normalized by calculating the intensity of its corresponding actin band. This experiment once again showed a lowering of protein expression of E1B19K protein by the virus that had a MCS placed at the 5'-end of the E1B19K gene (Fig. 3.10 lane 3). In fact, AdTR5E1B19K-K7C/GFP^{Q}, expressed its transgene 17 times less than the E1B19K virus without the MCS, AdTR5E1B19K-K7/GFP^{Q} (lane 5). What's more, similarly to what was seen in Fig. 3.9, the former (lane 3) produced less protein in the induced state than the latter (lane 4) in the non-induced state.

Table 3.1

Lower levels of GFP protein expression in cells infected with E1-deleted adenoviral vectors (AdVs) containing a multiple cloning site (MCS) located at the 5' end of the transgene.

Two AdVs, Ad/GFP and Ad/MCS-GFP expressing the same GFP were used to infect 293-rtTA cells in the presence of doxycycline (1 μg/ml). Infected cells were harvested 48 h post-infection and fixed with paraformaldehyde(1%)-PBS. The percentage of GFP positive cells and the fluorescence intensity of the cells were measured using an EPICSXL-flow cytofluorometer (Coulter). The fluorescence index (FI) was calculated by multiplying the percentage of positive cells by the mean fluorescence value. The factor calculated represents the ratio between the vector with no MCS and the MCS located at the 5'-end of the GFP gene.

Figure 3.10

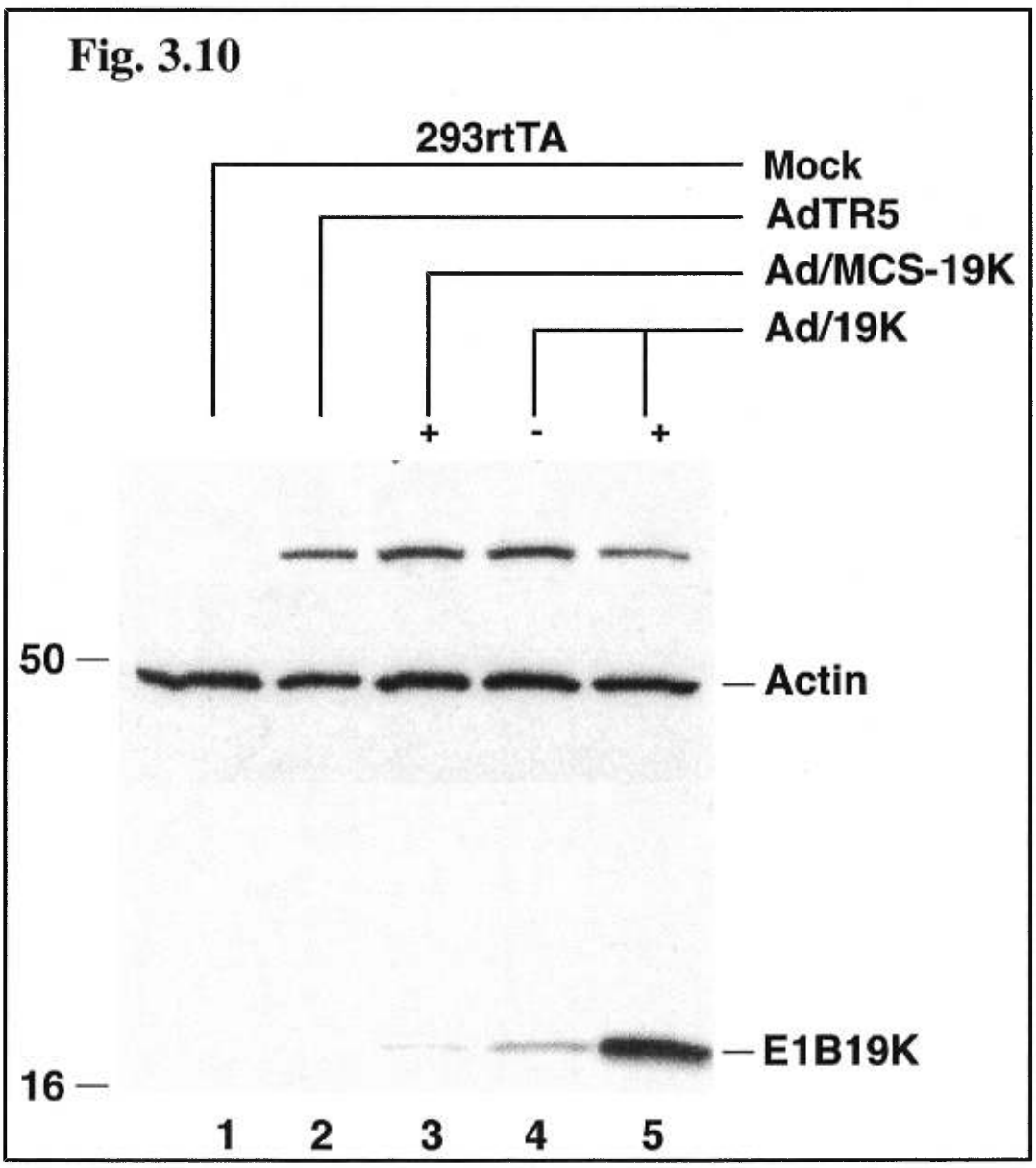
Comparison of E1B19K protein levels produced in cells infected with an adenovirus containing a MCS relative to that of one lacking the MCS.

Twelve million 293-rtTA cells were infected with either the AdTR5, Ad/MCS-19K or Ad/19K viruses at an MOI of 20 and harvested 48 h post-infection. Doxycycline (1 μg/ml) was added at the time of infection for the induction of E1B19K. After harvesting the cells were divided to provide two million cells for western blotting of total protein and ten million cells for northern blotting of total RNA (see Figure legend Fig. 3.11 and 3.12). The figure shows an immunoblot of total protein extracted from cells infected with various E1B19K AdVs and probed with the anti-E1B19K rabbit polyclonal and the human anti-actin mouse monoclonal antibody. The relative intensity of the E1B19K bands was calculated, following capture with a scanner, using the NIH Image software and normalized with the calculated intensity of the actin band (see text for details). The use of the complementing cell line, 293-rtTA, assured that differences in expression levels could not be attributed to minor differences in the titration of viral stocks.

Table 3.1

AdVs	F1	Factor
Ad/GFP	12682.1	-
Ad/MCS-GFP	3108.3	21.44

Fig. 3.10



3.4.2 The presence of a MCS polylinker reduces message levels of both GFP and E1B19K viruses

The results described above with the GFP and E1B19K viruses strongly suggested that the presence of this MCS in these vector plasmids would lower protein expression levels obtained by recombinant adenoviruses. However, the reasons why this was occurring were not known. One possibility was that the MCS was reducing message stability, thus shortening the half-life of the mRNA and ultimately lowering protein levels. Another possibility, was that the MCS was reducing the efficiency with which the mRNA could be translated leading to a lowered protein output. The latter possibility, however, would only lead to an accumulation of mRNA in the cytoplasm an would therefore not be seen by a reduction in message levels. To test these propositions, mRNA levels were evaluated of both E1B19K and GFP by way of northern hybridization. Initially, 10×10^6 exponentially growing 293-rtTA cells were infected in independent cultures by all of the E1B19K and GFP viruses at a MOI of 20. Infected and non-infected cells were then harvested 48 hours post-infection and total RNA was isolated using the RNeasy Mini kit. Northern hybridization was performed and band intensities were quantitated with a Phosphoimager using the ImageQuantNT software. The results showed that the message level of GFP was reduced 40-fold when the MCS was placed at the 5'-end of the GFP gene (Fig. 3.11, lane 1) when compared to the message level of the GFP virus which had no MCS (Fig. 3.11, lane 3). The introduction of a MCS also reduced the message level of the E1B19K virus, AdTR5E1B19K-K7C/GFP^Q (Fig. 3.12, lane 3), by 16-fold when

Figure 3.11 and 3.12

Northern hybridization showing message levels of GFP (Fig. 3.11) and E1B19K (Fig. 3.12) in extracts from 293-rtTA cells infected with adenovirus vectors that either contain a MCS or lack the MCS.

Each lane contains 10 μg of total RNA extracted from the infected cells (see Figure 3.10 for details). The band intensities on the film were quantitated with a Phosphoimager using ImageQuantNT software (both from Molecular Dynamics). The blots were also hybridized to an actin probe and the band intensity for actin was used to normalize the measured GFP and E1B19K band intensities.

Fig. 3.11

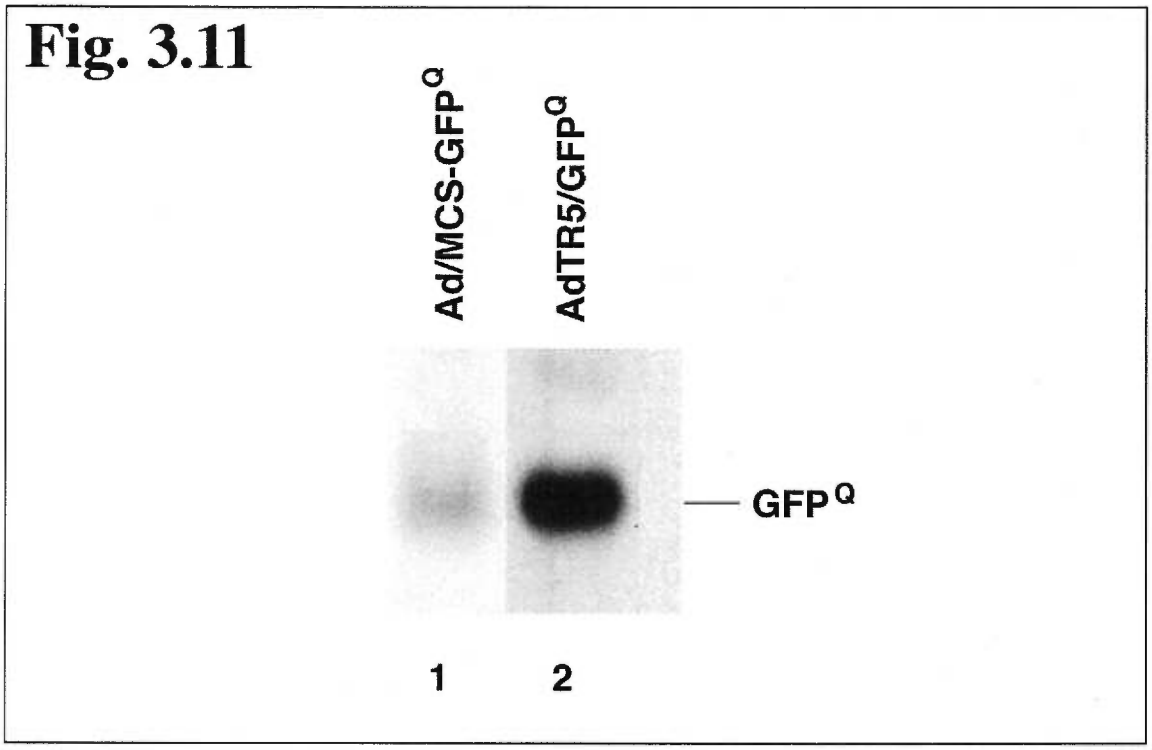
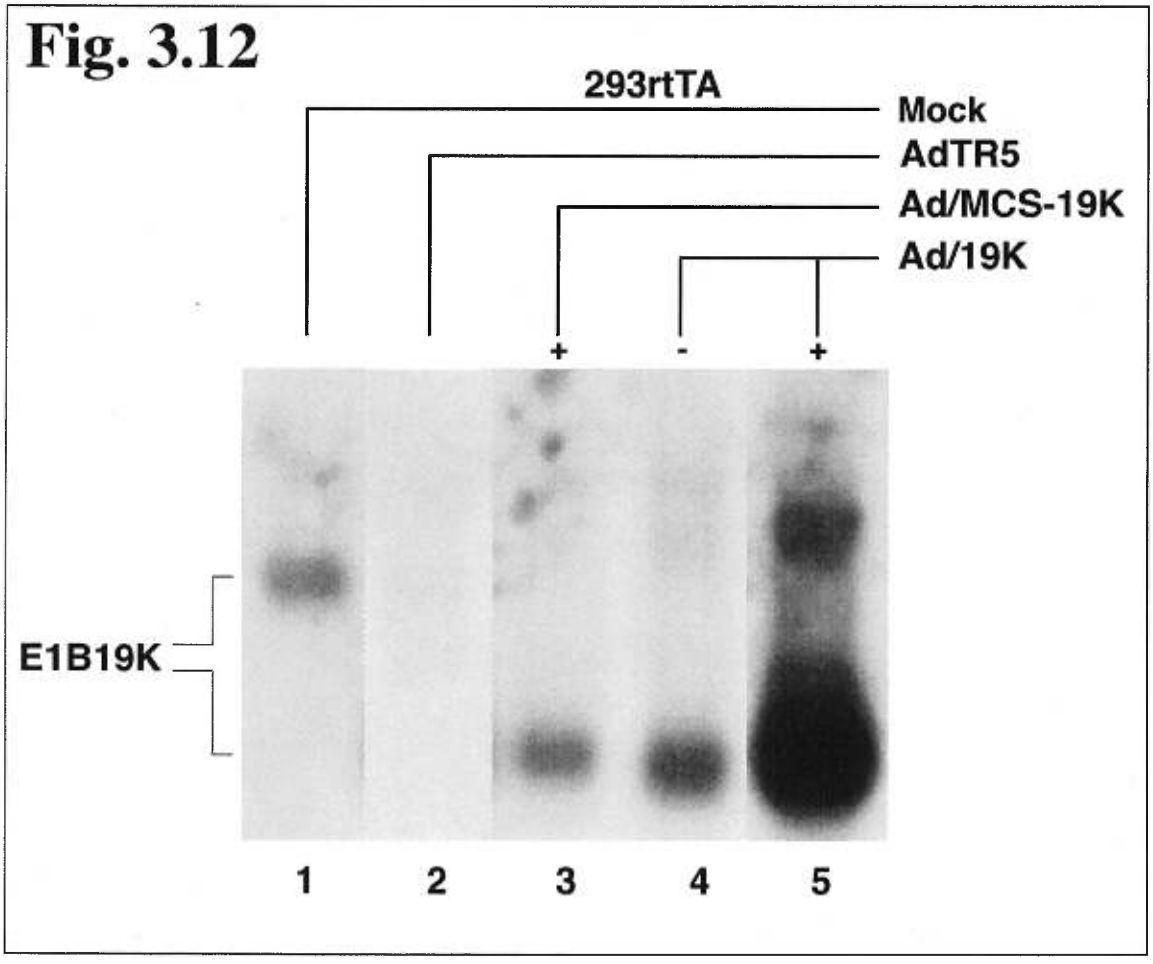


Fig. 3.12



compared to the virus, AdTR5E1B19KK7C/GFP^{Q} (Fig. 3.12, lane 5), which had no MCS separating the gene from its promoter. Additionally, in accordance to what was observed for E1B19K protein levels (Fig. 3.10), the E1B19K virus that had no MCS produced more mRNA in the non-induced state (Fig. 3.12, lane 4) than the MCS containing E1B19K virus in the induced state (Fig. 3.12, lane 3).

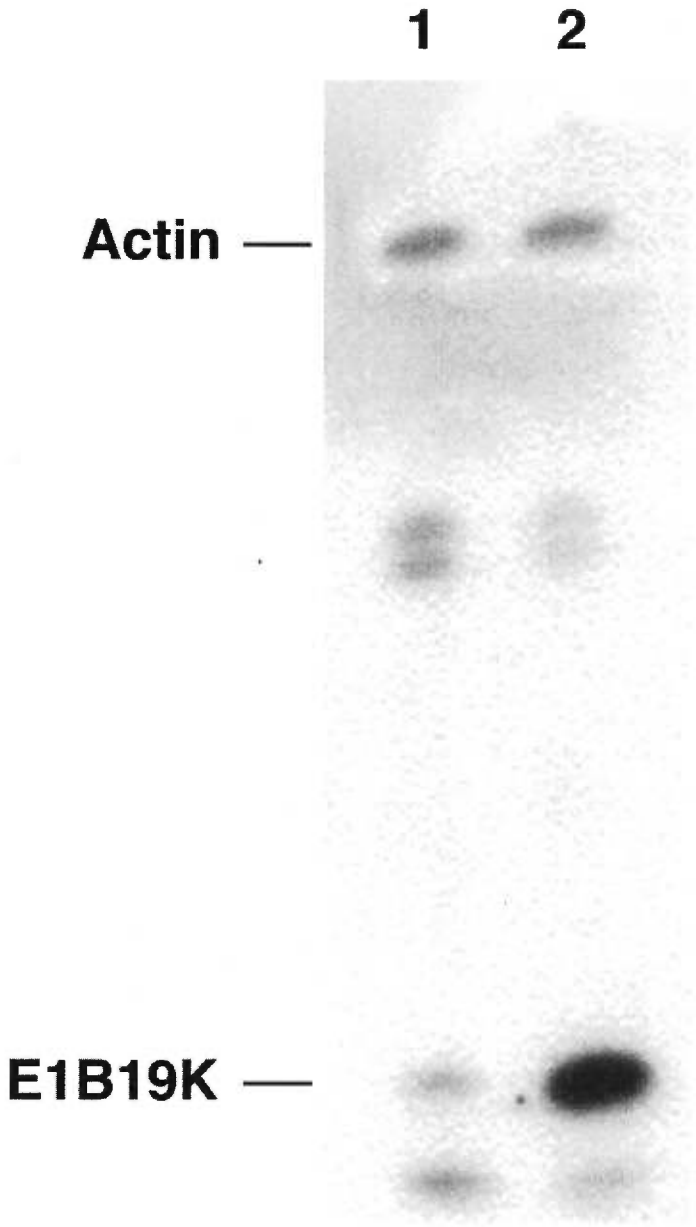
3.5 Expression of E1B19K is higher in dl309 infected cells than in the complementing cell line, 293A

One of the goals of this project was to explain the discrepancy found when calculating titers obtained by wild-type adenovirus, dl309, with that of E1-deleted recombinant adenoviral vectors. In theory, both wild-type and E1-deleted adenoviruses have the same genomic makeup except for the fact that the latter has its E1 region deleted in place of a gene of interest. These replication deficient viruses, nevertheless, have their replication deficiency taken care of by the constitutive expression of E1 proteins by the complementing cell line (i.e. 293), thus allowing for viral replication. Therefore, it was reasonable to believe that both wild-type adenovirus and E1-deleted adenoviruses should yield similar titers when both infected the complementing cell line, 293, but this was not the case. One of the proposed hypothesis, based on previous results (Massie, 1998a), was that E1 levels in the complementing cell line, 293, were suboptimal, thus limiting the replication potential of E1-deleted adenoviruses. For example, it was shown that in Ad5 virus

Figure 3.13

Comparison of the level of E1B19K in the 293A cell line (lane 1) and in 293A cells infected with the Ad5dl309 virus (lane 2).

Extracts containing 15 μg of total cellular protein were separated on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane for western blotting. The E1B19K protein was detected using a rabbit polyclonal E1B19K antibody provided by Dr. P. Branton (McGill University). The levels of a control protein, actin, were detected with a mouse monoclonal human anti-actin antibody (Amersham). The relative intensity of the E1B19K and actin bands were calculated, following capture with a scanner, using the NIH Image software. After normalization to the actin band the calculated levels of E1B19K in the dl309 infected cells were found to be 10 fold higher than that of the 293A cell line.


1
2
Actin
E1B19K
1 - 293A cells
2 - Ad5dl309 virus

infected cells the levels of E1A were two- fold greater in infected cells compared to what could be obtained by the constitutive expression of E1A by the 293 cell. Therefore, to further investigate this possibility, E1B19K levels in the 293A cell line and in 293A cells infected with the Ad5dl309 virus were compared (Fig. 3.13). As a result, exponentially growing 293A cells were infected with the dl309 virus and harvested 24 hours post-infection. Western blotting was performed using the E1B19K antibody and the relative intensity of the signal was calculated using the NIH Image software. After normalization with the actin band, it was observed that 293A cells infected with the dl309 virus (Fig. 3.13, lane 2) produced ~10-fold higher levels of E1B19K than what exponentially growing 293A cells constitutively produced (lane 1).

3.5.1 Overexpression of E1B19K at the time of infection lowers production of E1-deleted adenoviral vectors

It was believed that since the level of E1B19K was higher in dl309 infected cells, than in 293 cells (Fig.3.13) and since E1-deleted viruses were produced at roughly 30% the level of dl309, perhaps the two observations were correlated. Accordingly, if the levels of E1B19K were to be increased at the time of infection consequently, the titers of E1-deleted recombinant adenoviral vectors would also be increased. Thus, two recombinant adenoviruses, AdTR5E1B19K-K7C/GFP^{Q} and the AdTR5K7C/GFP^{Q}, and dl309 were used to infect separate cultures of exponentially

growing 293-rtTA cells in the presence or absence of the inducer doxycycline (1 μg/ml). Both of the recombinant viruses used are identical in that they are E1-deleted and have a GFP gene under the control of a CMV promoter. However, the AdTR5E1B19K-K7C/GFP^{Q} virus has the E1B19K gene under the control of tet-regulated promoter (see Fig. 2.5) whereas the AdTR5K7C/GFP^{Q} virus expresses no transgene (see Fig. 2.6). Infected cells were then collected and used to calculate the production of viral particles either by plaque assay, for dl309, or by gene transfer unit method, for the recombinant viruses used in this experiment. The results showed that although there was a slight increase in viral production in the second of two independent experiments (performed in triplicate), the viruses that were induced to overexpress E1B19K (Fig. 3.14, 19K "on") reached a titer that was about 30-fold lower than when the same virus was produced without the expression of E1B19K (i.e., in the non-induced state) (Fig. 3.14, 19K "off"). In fact, when the expression of E1B19K was repressed the amount of virus produced was similar to that of the AdTR5K7C/GFP^{Q} virus (Fig. 3.14, K7C), which does not even express a transgene protein. Finally, infection with the wild-type virus, dl309, yielded a 4-fold higher titer than the best recombinant virus in spite of the fact that the method used to calculate the dl309 titer, plaque assay, underestimates viral yield (data not shown).

These results seemed to imply that overexpression of E1B19K at the time of infection, negatively impacted viral production. In fact, when the E1B19K levels were lowered, as was the case in the non-induced state (19K "off"), the virus production level was restored to levels that are usually obtained with E1-deleted

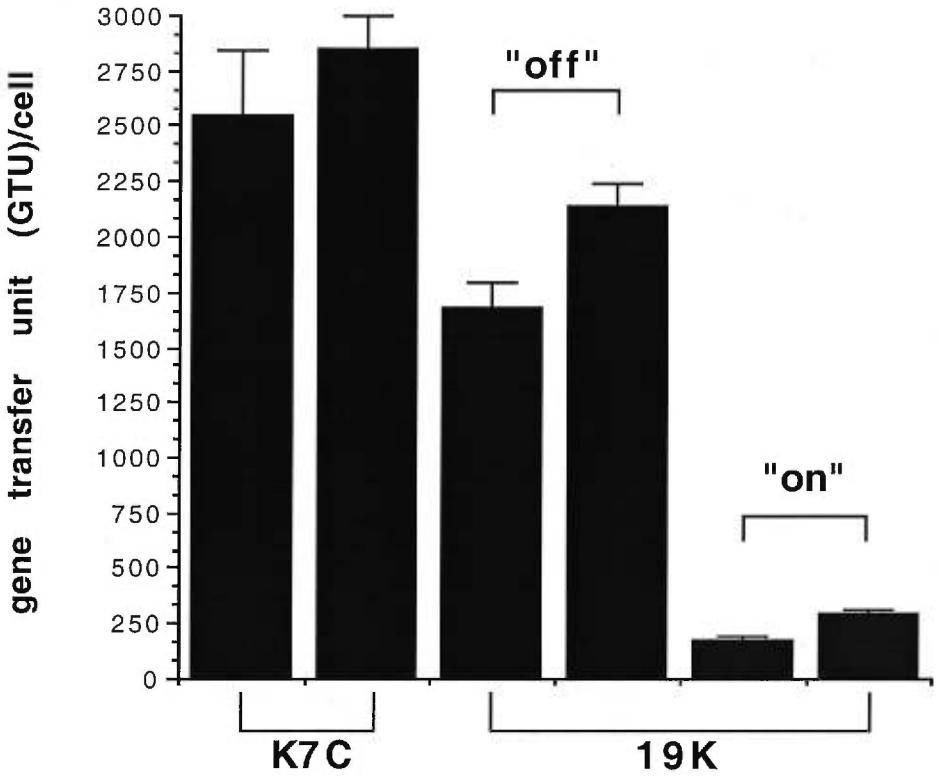
Figure 3.14

Overexpression of E1B19K at the time of infection dramatically lowers production of E1-deleted adenoviral vectors

The complementing cell line, 293-rtTA, was infected with the E1B19K virus, AdTR5E1B19K-K7C/GFP^{Q}, in the presence or absence of doxycyline (1 μg/ml). 293-rtTA cells were also infected with the E1-deleted GFP expressing virus, AdTR5K7C/GFP^{Q}, and the wild-type adenovirus, dl309. Infections were done using conditions that optimized viral absorption (minimal volume and overnight rocking) and were collected 48 hours post-infection. Three different levels of E1B19K were present in this experiment at the time of infection with the E1-deleted GFP expressing virus. High levels of E1B19K were obtained by infection with the E1B19K virus in the induced state, lower levels when cells were infected in the absence of doxycycline and finally basal levels were obtained in 293-rtTA cells that were not infected with the E1B19K virus prior to infection with the GFP-expressing virus.

Figure 3.14:

Overexpression of E1B19K at the time of infection dramatically lowers production of E1-deleted adenoviral vectors in 293-rtTA cells



Legend:

K7C: E1-deleted adenoviral vector AdTR5K7C/GFPq (does not express any transgene)

19K: E1B19K-expressing virus AdTR5E1B19-K7C/GFPq

"on": tet-regulated was induced to overexpress E1B19K

"off": basal level expression of E1B19K

viruses. As a result of these observations, it is conceivable that E1B19K is beneficial for virus production but up to a certain level after that threshold E1B19K may be cytotoxic to the cell and this would ultimately compromise viral output.

3.6 Production of E1-deleted viruses by 293 and BMAdE1

The appearance of replication competent adenoviruses (RCA) during the process of large scale production of E1-deleted recombinant AdV in 293 cells (Lochmüller et al., 1994) has created the need for new complementing cells lines. However, one of the problems associated with the generation of new complementing cell lines is the difficulty in maintaining stably transfected cell lines with E1 expression plasmids (Imler et al., 1996). The human lung carcinoma A549 cell line was chosen for its ability to support wild-type adenovirus replication and due to its ability to express all of the E1 proteins stably over many passages (Massie, 1998b). Yet the question remained whether this new complementing cell, which did not generate RCAs, could complement E1-deleted adenoviruses as efficiently as the 293 cell line. To examine this, six different cell lines, A549, 293A, 293-rtTA, and three BMAdE1 clones (220-8, 220-23 and 78-4), were infected with the AdCMV5GFP/dl309 virus. This recombinant adenovirus has the E1 region replaced with a green fluorescent protein (GFP) expression cassette. Each cell line was infected at a MOI of 10 with minimal volume and left overnight to rock to allow for maximum viral absorption. Only the infected cells were positive for GFP expression

24 hours post-infection. The cells were left to incubate for another 24 hours before harvesting. Infected and non-infected cells were collected and titrated using the gene transfer unit method using the 293A cell line.

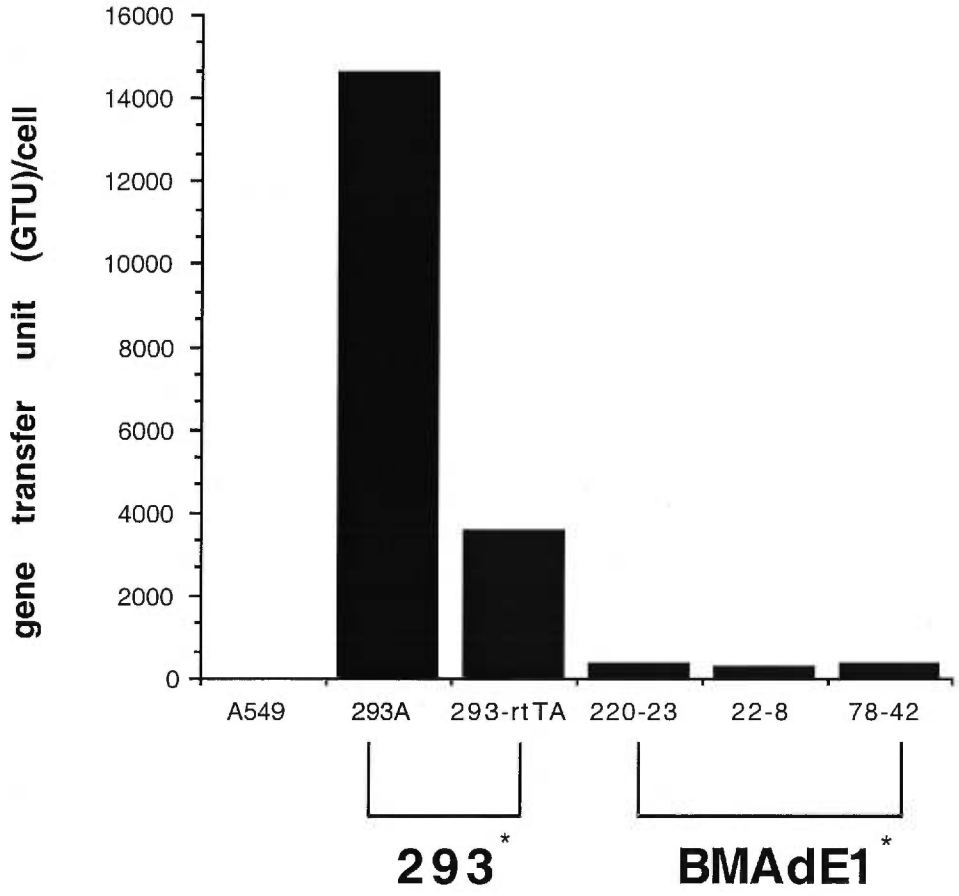
The results showed that the BMAdE1 clones were capable of transcomplementing E1-deleted viruses producing ~300-400 gene transfer units (GTU)/cell. This was obviously much greater than their parental counterpart, A549, which does not express the E1 proteins and thus cannot transcomplement E1-deleted adenoviruses. However, when comparing the BMAdE1 clones to the 293A cell line, the production of recombinant virus was significantly inferior. In fact, the best BMAdE1 clone (Fig. 3.15, column 220-23) produced 30-fold less recombinant virus GTU/cell than the adherent 293A cells (Fig. 3.15, column 293A). Furthermore, the results also revealed a 5-fold difference between the 293A (column 293A) and 293-rtTA (column 293-rtTA) cell lines. Both the 293A and 293-rtTA cell lines are identical except that the latter expresses a reverse transcriptional transactivator and is derived from the 293S cell line which was adapted for growth in suspension. Despite these minor differences, the recombinant viral output differed considerably and emphasizes the importance of establishing ideal conditions for viral production, perhaps even for each individual cell line considered. Finally, these differences between BMAdE1, 293 and amongst 293 cell derivatives could be attributed to factors other than the level of E1 expressed proteins (discussion).

Figure 3.15

Clones of BMAdE1 do not complement the E1-deleted adenoviral virus, AdCMV5GFP/dl309, as efficiently as the 293 cell

Six different cell lines, A549, 293A, 293-rtTA, BMAdE1 clone #220-23, BMAdE1 clone #220-8, BMAdE1 clone #78-42, were each infected with the AdCMVGFP/dl309 virus at an MOI of 10 using conditions that were optimal for viral absorption (i.e. minimal volume and overnight rocking). Infected and non-infected cells were collected 48 hours post-infection and titered on 293A cells. Flow cytometric analysis of GFP expression was done in order to determine the viral production by each cell line. These results show that the non-complementing cell line (column A549) did not produce E1-deleted viruses. Also the BMAdE1 clones (columns: 220-23, 220-8, 78-42) did not produce viruses as well as the clones (columns: 293A, 293-rtTA) of the 293 cells. The results shown are the average of three independent experiments.

Figure 3.15: **Viral production of an E1-deleted adenovirus (AdCMV5/dl309) by clones of the 293 and BMAdE1 cell lines**

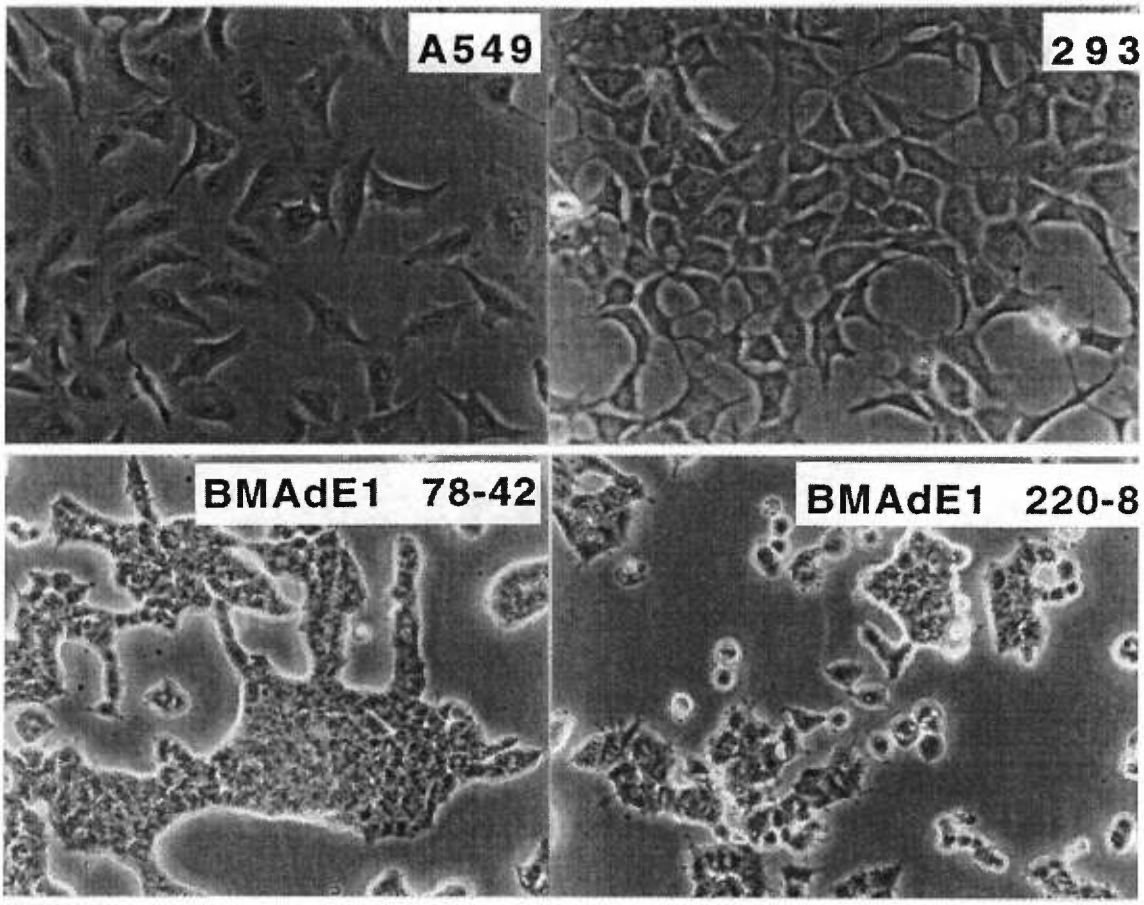


*** - cell lines expressing the adenoviral E1 proteins**

3.6.1 Expression of E1 proteins by 293 and BMAdE1 clones

One of the major factors influencing the cell's ability to produce viruses is its growth state at the time of infection. Even though E1 was tolerated by the BMAdE1 clones, they suffered physiologically from its expression (Fig. 3.16). The BMAdE1 cells, in contrast to the parental A549 cells, have a reduced size, were more rounded in shape, and tended to form aggregates. In light of these morphological differences, the level of E1 expression was compared in the BMAdE1 clones relative to 293 cells to determine whether the expression levels of the E1 proteins could be responsible for the altered morphology. This, however, was not the case. In fact, 293 and BMAdE1 clones made comparable levels of E1 proteins, with E1A levels about the same and E1B proteins being lower for BMAdE1 clones (data not shown). Surprisingly, the BMAdE1 clone with the lowest levels of the E1 proteins, 78-42 clone, still produced slightly higher levels of E1-deleted virus than the other BMAdE1 clones. It is noteworthy that these results were different from an independent experiment which showed that the 220-8 clones made 1.5-2 fold more virus than the 78-42 clones with production levels of around 1,400 gene transfer units (GTU)/cell (Massie, 1998a). Furthermore, the 293A cells were shown in those experiments to produce close to 10,000 GTU/cell and not the 14,000 GTU/cell production seen here. The difference in production levels between the BMAdE1 clones and 293 cells were not as high as presented here. Nonetheless, in agreement with what is reported here and in other reports (Imler et al., 1996), the BMAdE1 clones were found to be less efficient than the 293 cell line for the production of E1-deleted viruses.

Figure 3.16: Altered morphology of different clones of A549 cells, 220-8 and 78-42, expressing E1 proteins.



Chapter 4
Discussion

The ability to deliver genes directly to human cells represents one of the great promises of the next century. Although genes are being discovered and elucidated on a daily basis, the ability to deliver their therapeutic potential to the desired cells remains an obstacle to its full development. Of the many vectors tested to date, adenoviral vectors represent one of the most important group of gene delivery vehicles for *in vivo* gene therapy. In fact, over twelve tissues have been targeted by adenoviral vectors for gene transfer with various transgenes with the gene of interest being expressed up to 180 days in some cases (Trapnell and Gorzilia, 1994). However, as the vector was being successfully used in various experiments certain problems arose in the production of the recombinant virus that required serious consideration. One of the most serious problems to be reported was the development of replication competent adenoviruses (RCAs) in large scale productions of adenoviral vectors (Lochmüller et al., 1994). Even though, no health related problems have been associated with RCAs (Yei et al., 1994) they still pose a serious health consideration and at the very least could be a detriment to the gene delivery success of the recombinant adenovirus by potentially lowering the titer of the latter as well as shortening the duration of transgene expression by stimulating an immune response to the infected area (Yang et al., 1994). To circumvent this problem a new complementing cell line, based on the A549 human lung carcinoma cell, was proposed (Massie, 1998b) which would eliminate RCAs from the production of adenoviral vectors. The novelty of this new cell line, called BMAdE1, is that the potential for recombination between the vector and the cell line which was the cause for the development of RCAs with the standard complementing cell line, 293, was

virtually eliminated. The strategy focused on limiting the overlapping sequences between the vector and the cell line to only 200 base pairs with an added insurance that if the remote recombination did occur the resulting recombinant would not be viable since it would not contain the packaging or ori sequences. Though BMAdE1 cells generated recombinant adenoviral vectors without the emergence of RCAs they appeared not to produce viruses with the same efficiency as was observed with 293. This observation was potentially attributed to the low tolerance that many cell lines have for the uncontrolled expression of adenoviral E1 proteins (Imler et al., 1996) and thus required an examination of the role that each E1 protein played in the production of adenoviral vectors.

The adenovirus early immediate 1 region (E1) encodes two transcripts, E1A and E1B, which are translated into the proteins, E1A, E1B19K and E1B55K. Stable cell lines expressing these proteins are used for the production of recombinant adenoviruses containing a deletion in the E1 region. Deletion of these sequences from the adenovirus genome permits the insertion of transcription units encoding desired gene products. These recombinant adenoviruses are capable of replicating only in these E1-expressing cells and are replication-defective in non-complementing cell lines. Although their role in a permissive infection by a wild-type adenovirus have been well characterized (reviewed in: Doefler and Bohm, "The molecular repertoire of Adenoviruses. book I and III", 1995) their influence on the production of an E1-deleted replication-defective adenovirus has yet to be fully elucidated. For example, Imler et al. (1996) have shown that E1-deleted viruses can replicate in cells

that do not express the E1B55K protein, but to what extent the production capacity was affected is not fully known. Therefore, a strategy was devised so that each protein would be expressed independently by an adenoviral vector in non-complementing cell lines. The observations were expected to shed more insight into the possible role(s) that each of the E1 proteins play in the production of E1-deleted recombinant adenoviral vectors (AdVs) in which case optimal expression levels of each protein could be obtained using a regulated expression of E1 proteins with the tetracycline inducible expression system.

The construction of the adenoviral vector expressing E1A, AdTR5E1A-K7C/GFP^{Q}, allowed for the inducible expression of E1A proteins. The E1A region of Ad5 produces five mRNAs as a result of multiple splicing (Branton et al., 1986; Boulanger and Blair, 1991). The two most abundant mRNAs, which also are the first viral products to be synthesized in the lytic cycle, are the major 289R and 243R transcripts, denoted 13S and 12S respectively. The encoded proteins differ by a 46 amino acid sequence, referred to as the unique region or conserved region 3 (CR3), which is missing from the 12S mRNA. The three other minor transcripts of E1A, known as 11S, 10S and 9S, are synthesized late in the cycle and do not have any known function during infection. Unfortunately, the results obtained by western blotting with the E1A virus, AdTR5E1A-K7C/GFP^{Q}, did not display the typical expression pattern which is usually observed with E1A (Fig. 3.6). In general, western blotting against E1A gives rise to a doublet between the 50-40 kDa region which represents the 13 and 12S transcripts with the smaller proteins seen only late in wild-

type adenovirus infection of permissive cells. However, in this experiment the infection of Kb-rtTA cells (a non E1-expressing cell line) in the induced and non-induced state gave rise to a doublet right below the 36 kDa region. Although the doublet expected between the 50-40 kDa region did appear especially at much longer exposures it did not diminish from the fact that the dominant protein being expressed by the recombinant virus was a doublet right below the 36 kDa and that its origin was not known. One possibility, albeit very unlikely, was that this doublet was the product of the minor transcripts of E1A, namely 11S, 10S and 9S. Yet this seemed unlikely since these proteins are seldom seen on western blots, migrate to a different position on the gel and are usually restricted to the late stages of a productive infection by wild-type adenoviruses (Ulfendahl et al., 1987). Another possibility was that this doublet around the ~36 kDa region represented a truncated version of the major proteins resulting from aberrant splicing of the mRNA. However, this latter result conflicted with results obtained by transient transfection with the E1A plasmid, pAdTR5E1A-K7C/GFP^{Q}, which displayed proper expression of full length E1A (Fig. 3.5). One explanation is that there was contamination in the transient transfection experiment and that the source of the E1A proteins were not from plasmid origin. This, however, seems unlikely for two reasons: i) the cell line used to perform the transient transfection, Kb-rtTA, does not express any of the E1 proteins and ii) the mock infection, which was the harvesting of cells treated the same way but with no plasmid transfected into the cells, did not reveal the presence of either full length or truncated E1A proteins (Fig. 3.5, lane 1). Possibly, the fact that these two

experiments represent two different types of expression, one by a plasmid the other by a virus, may be the reason for the inconsistency seen between plasmid and viral expression with the same expression cassette. In fact, this divergence has been previously reported (Emery et al., 1999; personal communication with Dr. Massie) and could possibly explain the difference seen between the results obtained by transient transfection and viral infection. Nevertheless, it is important to note that no positive controls were used in the transient transfection experiment which would have helped interpret these results with more accuracy.

Experiments were nonetheless conducted with the E1A virus for a preliminary investigation into the effect E1A proteins would have on replication of an adenoviral vector in a non-complementing cell line when no E1B proteins were present at the time of infection. Previous reports have shown that such a virus is replication deficient (i.e. that the virus will not complete the viral cycle) in non-complementing cell lines (Zhang et al., 1995) but to what extent was not evaluated since that was not the focus of this study. The replication capacity of the recombinant virus, AdTR5E1A-K7C/GFP^{Q}, was assessed by monitoring the cell's demise (CPE) and transgene amplification, which are both induced by viral replication and hence a preliminary indication of it. The experiments showed that both of these phenomena were observed with the virus expressing E1A (Fig. 3.7, panels C and D). Although far from conclusive, this result could have important implications for certain therapeutic applications. In fact, if the E1A virus is shown to be in a semi-replicative state, by more in depth experiments that can determine this type of result (i.e. DpnI

assay) it would represent a virus that is able to amplify the expression of the transgene in vivo without amplifying the virus in the process. Potentially, such a virus could be used for the development of vaccines, where the immune system would respond to the expression of an amplifying transgene without the presence of a viral load.

A similar phenomenon to that observed with the E1A virus was seen with the E1B55K-expressing virus, AdTR5E1B55K-K7C/GFP^{Q}. The protein expressed by this virus gave a protein that was specifically recognized by the 2A6 E1B55K antibody, but migrated to an unexpected position in SDS-PAGE gels. Instead of migrating to its usual position around the 55 kDa mark the protein expressed by the virus migrated to the 36 kDa position (Fig. 3.8). In fact, several other plaques were tested for E1B55K expression and all gave the same result (data not shown). The same explanations given for the truncated E1A protein apply here but in this case no protein at the expected position of 55 kDa, could be seen, which was not the case with the E1A experiment. Furthermore, contrary to E1A, transgene expression by the vector plasmid, pAdTR5E1B55K-K7C/GFP^{Q}, did not reveal the presence of any protein by western blotting. Still, like in the case with the E1A, the expression of GFP was detected by transient expression of the vector and was extremely helpful in selecting recombinant plaques during the purification process of the recombinant virus. Yet, it should not have been the sole criteria by which a successful transient transfection was determined or a desired recombinant virus was selected.

Of the three recombinant E1 viruses constructed only the E1B19K viruses, AdTR5E1B19K-K7C/GFP^{Q} and AdTR5E1B19K-K7/GFP^{Q} properly expressed their transgene (Fig. 3.9). These two viruses both expressed the E1B19K protein at the expected position of 19 kDa and both the negative and positive controls confirmed the accuracy of the result. However, a significant discrepancy in protein levels between the two E1B19K viruses was observed, notwithstanding, that the initial purpose of this experiment was only to reveal the proper expression of E1B19K. Thus, an examination into the viruses was undertaken and more specifically into the lone difference between the two: a multiple cloning site (MCS) which was placed 5' to the gene of interest. Therefore, more in depth experiments were performed to determine whether or not the inclusion of this MCS was impacting transgene expression by an adenoviral vector.

Initially, the MCS was desired to facilitate the cloning of various genes of interest into the pAdTR5K7/GFP^{Q} plasmid (Fig. 2.1). The MCS was cloned in the 5'-untranslated region (UTR) of this plasmid and thus extended the separation between the gene of interest and the TR5 promoter by as much as 44-base pairs. The E1B19K gene was subcloned either with or without the MCS. Likewise, an unrelated gene, the green fluorescent protein (GFP), was subcloned 5' of the MCS or in the absence of the MCS (Fig. 2.6). The addition of a second unrelated gene to the experimental setup, allowed to focus the results solely on the impact by the MCS and not on the type of protein that was being expressed. The results showed that the insertion of a polylinker upstream of the cDNA lowered the levels of both the

E1B19K and GFP proteins (Fig. 3.10, lane 3 and 5; Table 3.1). In fact, the E1B19K protein was lowered by 17-fold and the GFP protein by 21-fold. Interestingly, a virus which had the MCS placed at the 3'-end of the GFP gene did not display a reduction in protein expression (data not shown). These results thus confirm that the 5'-UTR had an important role to play, in this experiment, in the expression of heterologous proteins and that the sequence of the MCS itself did not seem to influence the reduction since protein levels remained the same when placed 3' of the gene (data not shown). Furthermore, extraction of total RNA from infected cells showed that message levels of both E1B19K and GFP were much higher, 20 and 40-fold respectively, in vectors that did not have a MCS when compared to message levels of vectors that possessed a MCS 5' to the gene of interest (Fig. 3.11 and 3.12). Such a result seems to indicate that a pretranslational mechanism was perturbing RNA levels in vectors which had a MCS, which ultimately lead to a reduction in protein expression levels.

These results thus strongly suggested that the 5'-UTR in the vector design has an important role to play in the expression of heterologous proteins. This result may therefore represent an important consideration when designing vectors where the goal is maximal protein expression. Such is often the case in the pharmaceutical industry where abundant quantities are needed of proteins that are normally available in only limited quantities from natural sources. Scale up of recombinant protein production can represent a significant cost to a pharmaceutical company. In this regard, optimization of the vector is of utmost importance and these results could indicate

that caution should be used when designing vectors for the purpose of maximizing protein levels in mammalian cells. Including polylinkers to the design may facilitate the cloning of future genes but as the results here showed it may come at the cost of reducing protein expression levels.

One of the goals of this thesis was to assess how the different E1 proteins would contribute to recombinant adenoviral replication. Since the AdTR5E1B19K-K7C/GFP^{Q} virus expressed E1B19K protein properly, it was hypothesized that increasing the cellular abundance of this protein could have a beneficial impact on the production of recombinant viruses. The idea was that since E1B19K's role during lytic infection is to protect the cell from premature apoptosis (Rao et al., 1992) perhaps if overexpressed during viral replication it would protect the cell and enhance viral output. Furthermore, it was observed that the levels of E1B19K produced by the wild-type virus, dl309, were much higher than what is present in the 293 cell line (Fig. 3.13), which was conceivably the explanation in the discrepancy between wild-type output and that of E1-deleted viruses. It was therefore conjectured that if the levels of E1B19K were raised in 293 cells this would improve the production of E1-deleted viruses to levels comparable to that of dl309 infected cells. However, this was not the case. On the contrary, the overexpression of E1B19K had a negative impact on recombinant viral production in that the output of the virus was reduced by 30-fold when it was induced to express E1B19K (Fig. 3.14). This result, albeit unexpected, may be explained in light of a recently published reports in which it was shown that overexpression of E1B19K (>2% of total cell protein) was very toxic,

killing the cell by necrosis (Massie et al., 1998c). Therefore, it is very likely that the overexpression of E1B19K was sufficiently (but not at toxic levels since no necrosis was observed) high enough to cause the cells to suffer thus lowering the output of recombinant virus by the cell.

The experiments that were performed with the E1B19K virus, AdTR5E1B19K-K7/GFP^{Q}, demonstrated that extremely high levels of E1B19K in the 293 cell was not beneficial to viral production. However, elevated levels of E1B19K in 293 cells may still be beneficial if the levels were closer to what is produced during infection by a wild-type virus. These levels of expression could possibly be attained by infection with the AdTR5E1B19K-K7C/GFP^{Q} virus in the non-induced state. Much lower levels of E1B19K protein was shown to be produced with this virus than by infection with the virus, AdTR5E1B19K-K7/GFP^{Q}, which lacks a multiple cloning site in its 5'-untranslated region. These experiments are currently being done and should shed more insight into the role that E1B19K has on viral production when transcomplementing E1-deleted recombinant adenoviruses. Furthermore, these results suggest that the basal levels produced by these AdVs are sufficiently high in order to investigate the impact that the E1 proteins play in viral replication of E1-deleted adenoviruses. For example, the induction of the E1B19K protein was so high that it could easily be detected in coomassie stained SDS-PAGE gels (data not shown) and as such represents a much higher level of protein than perhaps was at first desired. Therefore, future experiments do not necessarily need to be conducted in cells that express tTA or rtTA and consequently broadens the type

and number of cell lines which can be used to investigate the role of E1 proteins in viral production.

Finally, the new BMAdE1 cell line, constructed for the purpose of eliminating RCAs, was compared to the commonly used 293 complementing cell line for their ability to produce replication-defective adenoviral vectors. In general, it could be stated that the growth state of a cell at the time of infection influences the viral output. Since viruses are by definition absolute parasites, they depend on their hosts for their efficient replication and thus any malaise experienced by the cell before infection could dramatically affect the viruses ability to replicate within that cell. This seemed to be the case with BMAdE1. In fact, when different clones of BMAdE1 were compared to their parental origin, the human lung carcinoma A549 cell, a notable altered morphology was observed, which included a reduced size, and a more rounded shape which tended to form aggregates (Fig. 3.16). Therefore, when several BMAdE1 clones were compared to 293 clones in their ability to produce the same virus, AdCMV5GFP/dl309, it did not come to a great surprise to see the former produce significantly less virus than the latter (Fig. 3.15). Interestingly, a 5-fold difference in viral yield was measured even between clones of 293 (Fig 3.15, column 2 and 3) which highlights the point that not only are E1 levels important at the time of infection, since both of these clones are identical in their E1 region, but that other factors should be examined and used to predict a complementing cell line's ability to replicate adenoviral vectors.

The growth state of the host cell line can have a major impact on its ability to replicate an infecting virus. Virus production can be compromised if the cell is under stress or grown under suboptimal conditions. The reduced capacity of the BMAdE1 cells to serve as a host for viral production could be the result of the weakened state created by the expression of the E1 proteins. The E1 proteins have a wide variety of effects on cell physiology including affects on gene expression, DNA replication, cell cycle progression, and even cell death signaling (Debbas and White, 1993; Moran, 1994; Teodoro et al, 1995). The constitutive overexpression of these proteins must be such that the levels are sufficient to support the replication of E1 deleted AdV without significantly affecting the ability of the cell to serve as a host for protein production. The demands are enormous considering that during a productive infection a single cell can produce up to 30,000 infective viral particles and that only about 20% of the viral proteins produced are actually assembled into intact virions. This load is tremendous and requires absolute ideal conditions within the cell. It is conceivable that cell lines that have been developed to constitutively overexpress the E1 proteins have adapted to the consequences of their expression by altering the expression of specific cellular proteins. Different E1-expressing cell lines may differ in the adaptations that have occurred which might allow the cell to simply tolerate the expression of these proteins or to support high level protein expression in their presence. The "ideal conditions" mentioned above can be the presence or absence of many cellular and external factors which can cause stress to the cells and thus lower its potential for viral productivity. By way of illustration, certain cellular proteins within cells, such as heat shock proteins (HSPs), enhance a cell's ability to deal with

stress. A cell line which deals better with stress, by producing more HSPs for example, could conceivably produce more virus than a cell line that does not. This example and the countless other cellular factors which exist and varies from one cell line to the next could potentially explain why one cell line performs better than another at producing viruses. Furthermore, external factors, such as the culture conditions, can affect the capacity to support viral production. Different cell lines grow and respond differently to different media formulations, serum and glutamine concentrations, amino acids content etc..., such that determining the factor which will enhance viral productivity to an optimal level is a major task. Finally, potential cell lines for transcomplementation of E1 proteins must be able to deal well with the expression of E1 proteins. This is the case with the 293 cell line which appears to be physiologically in good health even though high levels of E1 proteins are present. One possible way to circumvent the problem of E1 toxicity is to use the tetracycline-regulated system. Therefore if the cells were capable of inducing the expression of E1 at the time of infection, instead of the constitutive expression of E1 within the cell, perhaps then cells would suffer less from the toxicity associated with E1 and consequently produce more virus. This strategy is currently being developed in the laboratory and should provide a better understanding of the influence E1 proteins have on an E1-expressing cell line and its ability to produce E1-deleted replication defective adenoviruses. Nevertheless, this strategy may be beneficial, since even modifications (i.e. adaptation to growth in suspension or the permanent introduction of a new gene) within the same cell line, as seen with the 293 adherent cell line, can lead to a lowered capacity to produce adenoviral vectors.

Chapter 5
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