CONSTRUCTION AND CHARACTERIZATION OF AN ADENOVIRUS VECTOR CONTAINING A BICISTRONIC IL-12 EXPRESSION CASSETTE

CONSTRUCTION AND CHARACTERIZATION OF AN ADENOVIRUS VECTOR CONTAINING A BICISTRONIC IL-12 EXPRESSION CASSETTE

by

DEREK S. KUNSKEN, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

November, 1996

MASTER OF SCIENCE (1996) McMASTER UNIVERSITY
(Biology) Hamilton, Ontario

TITLE : Construction and Characterization of an Adenovirus Vector Containing a Bicistronic IL-12 Expression Cassette

AUTHOR : Derek S. Künsken, B.Sc. (University of Guelph, Ontario)

SUPERVISOR: Professor Frank L. Graham

NUMBER OF PAGES : ix, 127

ABSTRACT

Gene therapy, although a young field, has become an intense area of study for the potential treatment of many inborn or acquired diseases, including cancer. The systemic or intratumoural delivery of cytokines and other immune system modulators has shown that protective, specific immunity to tumours is possible and effective. Adenovirus is an extremely useful vehicle for the transfer of genes, due to the ease of its preparation, its ability to be grown to high concentrations, and its capacity to infect a wide variety of cell types, including nonproliferating cells. Although a great deal of research has been done, there are a number of possible improvements, specifically, the consistency and range of transgene expression. The HCMV IE promoter/enhancer, used in a wide range of vector systems, has proven to drive lower levels of transgene expression in murine cells as compared to human cells. The use of the MCMV IE promoter/enhancer within the context of an adenovirus vector system has been investigated to see if more consistent transgene expression is possible in murine models. Studies with a luciferase reporter gene have demonstrated that in all cell types, the MCMV IE promoter/enhancer drives transgene expression to equivalent or higher levels than the HCMV IE promoter/enhancer. Our lab has investigated adenovirus as an intrtumoural gene delivery vehicle for various cytokines, including IL-12. However, the current IL-12-expressing vector carries the IL-12p35 subunit in E1 and the IL-12p40 subunit in E3. A homologous recombination event between E1 sequences in the viral genome and those that are integrated into 293 cells, would produce a replication-competent adenovirus expressing high levels of the p40 subunit, which is a potential antagonist to IL-12. A bicistronic adenovirus vector using the poliovirus IRES was constructed containing all IL-12 coding sequences in E1 to avoid this potential hazard. This virus expressed between 60 ng to 2.5 μg of IL-12/10^6 cells, depending on the cell type. These levels of expression, however are 7-30-fold lower than those driven by previous IL-12 vector. Although this new virus is not appropriate for tumour immunotherapy, the shuttle plasmids developed during its construction have many potential uses.

ACKNOWLEDGEMENTS

I wish to thank Dr. F.L. Graham for his guidance, support, and patience throughout this work, as well as the occasional spot on the benchpress. As well, I would like to thank my labmates who made these past two years fun as well as educational: Dan Pereira, Andy Bett, Bob Marr, Jonathan Bramson, David Matthews, Liane Chen, Christina Addison, Mary Hitt, Valeri Krougliak, Afra van der Beek, Uma Sankar, John Rudy, Nathalie Louis, John Wesseling, Brigitte Puetzer, Martina Anton and Wai Yin Quan. I can't think of any lab filled with more fun, interactive people, or even a lab full of more people. Several people have been especially and proactively helpful and deserve special mention. These are Mary, Dan, Andy, Jon, Martina and David. I must also thank Jonathan and Mary especially for doing the IL-12 ELISA and IL-12 bioassay experiments. Finally, I must thank my various friends and family who have been supportive and patient during this time.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1 : Introduction

Gene Therapy

It has long been the hope of science to cure inherited and acquired diseases by means of gene therapy. Advances in molecular biology and an increasing understanding of cancer and inherited disorders brought this dream closer to reality, although it can be argued that gene therapy is still in its infancy.

Advances in the early 1990s that support the principle of gene therapy include the treatment of adenosine deaminase deficiency (Culver *et al.*, 1994). Transplants of retrovirus transduced autologous lymphocytes has lead to nearly normal lives for the treated children, to the point where their immune systems have responded to vaccinations (Siegfried, 1993).

Vectors

The techniques to genetically modify a cell are many, including coprecipitation of DNA with calcium phosphate, electroporation, DEAE-dextran, liposome-mediated transfection (Rosenberg, 1992), or infection with a virus, such as adenovirus (Hitt *et al.*, 1994), vaccinia virus (Meko *et al.*, 1995) or Moloney leukemia virus (Li *et al.*, 1994). The viral vector systems are among the most attractive because of the efficiency with which they insert DNA into mammalian cells.

Recombinant retroviruses represent a powerful, replication-

incompetent viral vector system. Most of the coding sequences have been removed for the purposes of safety and to accomodate a greater size of transgene. Infections are generally done *ex vivo*, which is advantageous in that cells can be washed free of any residual virus and unintended infection in the host is avoided (Siegfried, 1993), but *ex vivo* manipulations are often technically involved and sometimes unfeasible. The infecting virus efficiently integrates its genetic information into the host cell genome, which is often an advantage, but sometimes a disadvantage. Long term expression is possible, but integration carries the potential danger of activating an oncogene or of inactivating an anti-oncogene (Siegfried, 1993). A last point is that retroviruses generally only transduce dividing cells (Siegfried, 1993), although recent developments have generated vectors that can transduce non-dividing cells (Naldini *et al.*, 1996).

Adenovirus is one of the most useful vectors for gene transfer and will continue to occupy a central place in the field. Adenoviruses infect a variety of cell types, independent of their proliferation rate (Siegfried, 1993) and generally do not integrate into the host cell genome, as more than 99% of adenovirus DNA is observed to function extrachromosomally (Horwitz, 1990). Preparation and purification of large quantities of recombinant adenovirus vector is technically simple and expression of intratumourally administered recombinant virus has been reported to be localized to the tumour by several investigators (Tang *et al.*, 1994; Bramson and Graham, unpublished results). From the

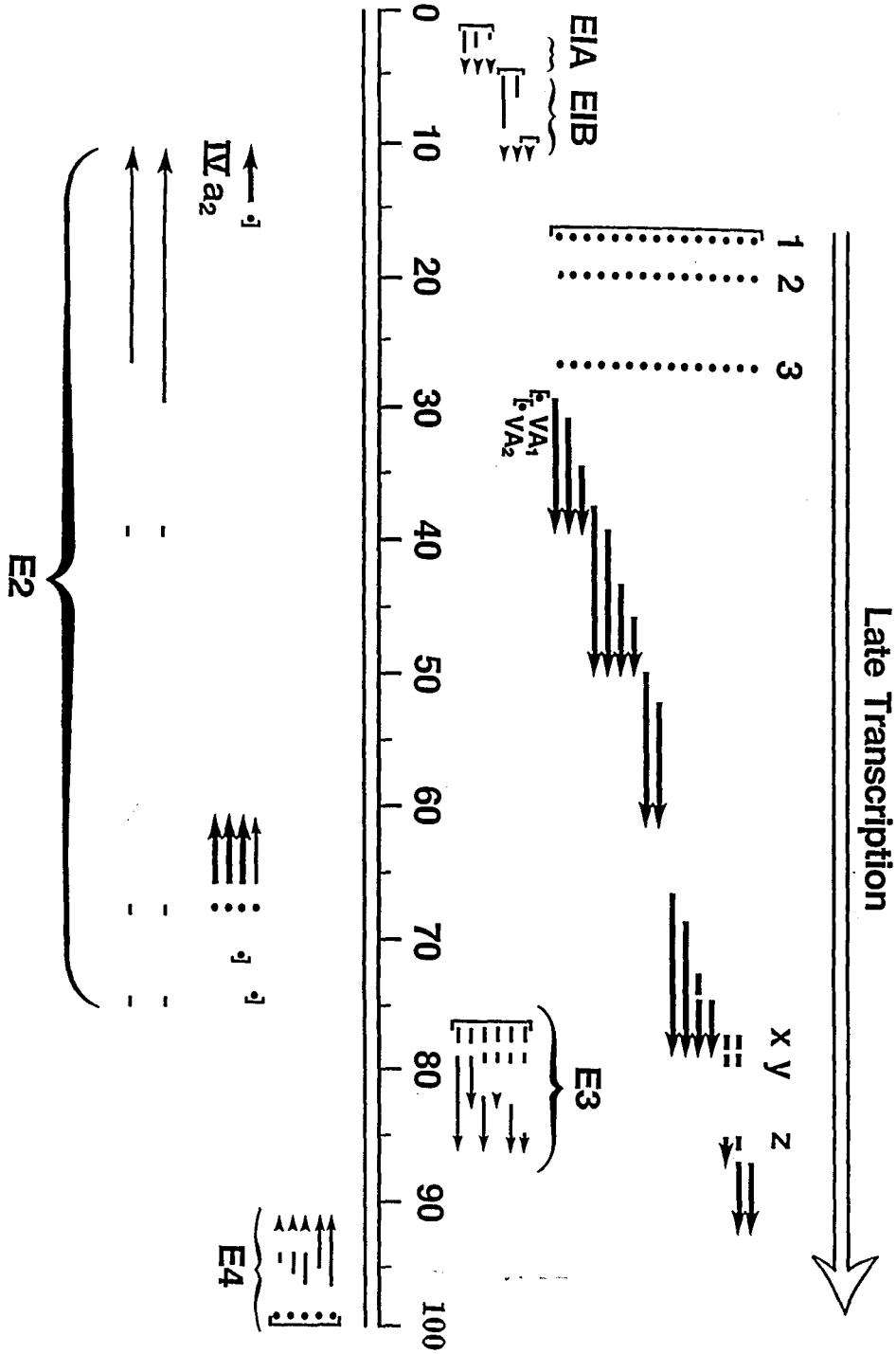
perspective of safety, some adenoviruses are tumorigenic in newborn rodents, but no adenovirus DNA has yet been observed in human tumours (Green *et al.*, 1980). Furthermore, the tumorigenic genes of the virus, E1A and E1B, are typically deleted in recombinant adenoviruses designed for use in gene therapy. A brief discussion of the molecular biology of adenoviruses follows.

Adenoviruses

The adenoviridae are a well-characterized family with over 100 members identified to date, 49 serotypes of which are human in origin (Horwitz, 1996). A major criterion used in adenovirus classification is a comparison of neutralization of infectivity by the antisera of other known serotypes. Other characters that are used to collect the serotypes into subgroups include their capacity to agglutinate red blood cells and their oncogenicity in rodents (Shenk, 1996).

Structurally, an icosahedral protein shell, the capsid, measuring 70 to 100 nm in diameter, surrounds a double-stranded, linear DNA genome (Shenk, 1996). The genome possesses two *cis*-acting sequences that direct replication and packaging. These are the inverted terminal repeats and the packaging signal (Horwitz, 1990). Transcriptional analysis has identified five early units (E1A, E1B, E2, E3 and E4), two delayed early units (IX and IVa2) and one late unit that is processed post-transcriptionally into five families of mRNA (L1 to L5) (Shenk, 1996). Figure 1.1. shows a transcription map of adenovirus type 2.

Figure 1.1. Transcription map of the adenovirus type 5 genome. The genome is measured in map units (mu) with each map unit equal to 360 bp. The thin lines indicate early transcripts while heavy lines demarkate the late transcripts. The single 29,000 nucleotide primary transcript driven by the major late promoter is shown by the large, open arrow. Differential polyadenylation yields the five families of late transcripts. Arrow heads indicate the 3′ end of each transcript. Adapted from Ginsberg, 1984.


Late Transcription
1 2 3
x y z
EIA EIB
VA1
VA2
E3
E4
E2
IVa2
0 10 20 30 40 50 60 70 80 90 100

The E1A promoter is constitutively active and thus the first to be expressed upon infection. Two major proteins are encoded in this region. The first activates transcription by binding to cellular transcription factors. The second induces the host cell to proliferate by binding p105, p107 and p130 or p300 (Shenk, 1996). The antagonism of p105, p107 and p130 by the E1A 12S and 13S proteins prevents them from sequestering E2F, a transcriptional factor associated with cell cycle progression (Johnson *et al.*, 1993). S phase provides an optimal environment for viral replication. The E1B proteins contribute to cell growth by targetting p53, which regulates progression from G_1 to S phase and also by preventing E1A-induced apoptosis, although it is unclear how this is accomplished (Shenk, 1996).

E2 encodes three replication proteins including the 80 kD preterminal protein, the 140 kD DNA polymerase and the 59 kD single-stranded DNA binding protein. The preterminal protein primes replication and is proteolytically cleaved to a terminal protein found covalently bound to the 5′ terminus of each strand of virion DNA (Horwitz, 1990a). The virally encoded DNA polymerase has a 5′ to 3′ polymerase activity and a 3′ to 5′ exonuclease activity and forms a heterodimer with the preterminal protein in solution (Shenk, 1996). The DNA binding protein binds DNA in a cooperative and sequence-independent manner and contributes to chain elongation (Shenk, 1996).

The host immune response seems to be primarily directed against E1A peptides, which has implications for the use of

recombinant adenovirus as a vector. E3 products modulate the immune response to infection in two ways. Firstly, the 19K protein prevents the transport of major histocompatibility complex (MHC) class I molecules to the cell surface. Secondly, anti-tumour necrosis factor (TNF) genes encoded by E3 prevent TNF-α-mediated cytolysis (Horwitz, 1996).

E4 proteins mediate transcriptional regulation by binding cellular transcription factors as well as by contributing to DNA replication and mRNA transport (Horwitz, 1990).

While early and late refer to transcriptional units expressed before and after the commencement of replication, this division is not strict. Early genes are expressed at lower levels after replication has begun, while the major late promoter is observed to drive basal expression even in the early phase (Shenk, 1996)

After replication has begun, levels of mRNA driven by the major late promoter level increase several hundred-fold on a per template basis. Late coding regions are transcribed as a single primary transcript of 29,000 nucleotides, that is differentially polyadenylated to yield five families of mRNAs (Shenk, 1996).

In addition to the aforementioned advantages of their capacity to infect non-dividing cells and their inability to integrate the viral genome into the host cell genome, human adenovirus is capable of infecting (i.e.: able to adsorb, penetrate, and drive early transcripts) most other mammalian cells, although replication is not observed (Horwitz, 1996). This broad spectrum of infectable cell types becomes especially useful in the context of preclinical

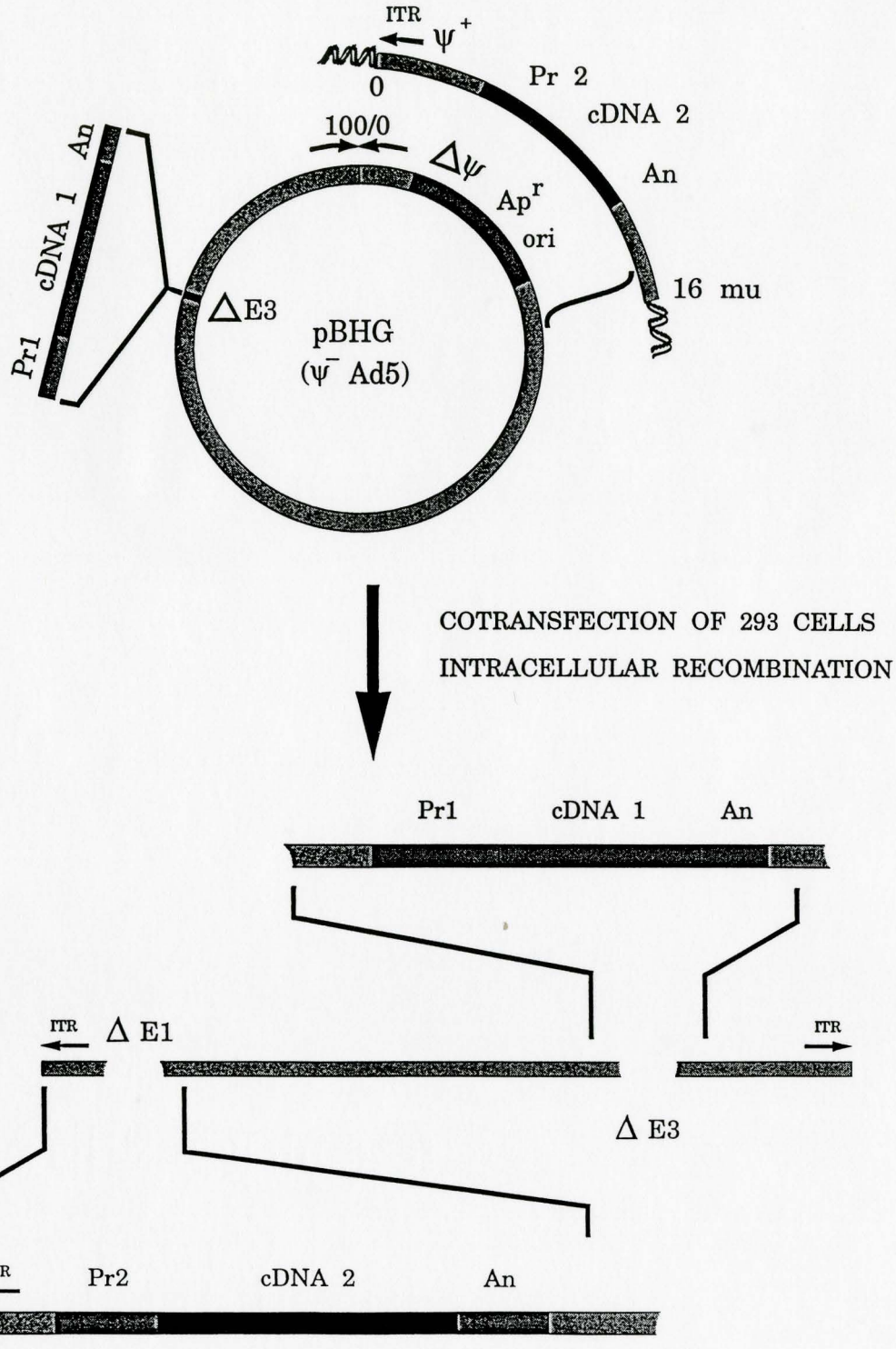
studies for gene therapy.

Construction of Recombinant Adenovirus Vectors

The maximum packageable genome size is approximately 105% of the wildtype genome (Bett *et al.*, 1993). To accomodate transgenes in the E1 region, recombinant adenoviruses are typically E1 deficient ($E1^-$) and E3 deficient ($E3^-$). In addition to increasing the potential insert size to approximately 8 kb, these viruses are incapable of replicating without the E1 proteins provided in *trans* (Hitt *et al.*, 1994). The genes of the E3 region are dispensible for replication *in vitro* (Horwitz, 1990a) and, as will be discussed below, E3-deficient viruses, incapable of downregulating MHC class I expression on the surfaces of cells, may act as adjuvants for some therapies.

The most convenient system for the construction of E1-replacement adenovirus vectors is that developed by Bett *et al.* (1994). Briefly, Bett *et al.* developed a series of plasmids (pBHG) that include most of the adenovirus type 5 genome, but lack the DNA packaging signal. Any of several sets of shuttle plasmids are capable of recombining *in vivo* with a pBHG plasmid to reconstitute a full-length molecule of DNA, including a DNA packaging signal. The shuttle plasmids contain the gene of interest and various control elements (promoter/enhancer and poly-adenylation signal) flanked by a portion of the left hand end of the genome (treated in more depth in the materials and methods), including the adenovirus

Figure 1.2. The strategy for constructing E1-replacement vectors using the pBGH10 system developed by Bett *et al.* (1994). The virus is rescued *in vivo* by recombination between a circular adenovirus genome plasmid and a shuttle plasmid with E1 sequences flanking the transgene and its control elements. The inverted terminal repeats are joined head to tail in the genomic plasmid and are shown by thick black arrows. Helical lines delineate plasmid sequences.



DEFECTIVE VIRAL VECTOR CONTAINING SUBSTITUTIONS IN E1 AND E3

DNA packaging signal (Hitt *et al.*, 1994). In 293 cells (Graham *et al.*, 1977), the adenovirus serotype 5 E1 genes are constitutively expressed, providing E1 proteins in *trans* to allow for the normal transcription, replication and packaging of the recombinant genome. A schematic of recombinant adenovirus construction is shown in Figure 1.2.

Previous Recombinant Adenovirus Vectors

Recombinant adenovirus vectors have been used for various purposes and in December, 1992, the National Institute of Health Recombinant DNA Advisory Committee gave approval to begin treating humans with a Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)-expressing adenovirus (Siegfried, 1993). Since then, adenovirus vectors have been engineered to express transgenes *in vivo* to treat many disorders including, among others, α-1-antitrypsin deficiency (Rosenfeld *et al.*, 1992), respiratory distress syndrome (Korst *et al.*, 1995), Parkinson's disease (Horellou *et al.*, 1994), hemophilia (Conelly *et al.*, 1995), anemia (Tripathy *et al.*, 1994) and various cancers (see below). Many transgenes have been engineered into adenovirus vectors for tumour therapy, including herpes simplex virus thymidine kinase (Chen *et al.*, 1994), human p53 (Drazan *et al.*, 1994), and for immunotherapy, human interleukin-2 (IL-2) (Addison *et al.*, 1995a), murine IL-2 (Haddada *et al.*, 1993; Cordier *et al.*, 1995), murine interleukin-4 (IL-4) (Addison *et al.*, 1995b) and murine interleukin-12 (IL-12) (Bramson *et al.*, 1995, 1996).

Cancer

Cancer may be defined as the aberrant proliferation of cells, free of normal growth restrictions. Cancer is the end result of an accumulation of genetic abnormalities that leads to deregulated clonal proliferation (Culver *et al.*, 1994). These cells are capable of invading and colonizing surrounding or distal tissues (Verbik *et al.*, 1995). Cancer can be treated by surgery, chemotherapy and radiation therapy, but these procedures are often ineffective with some tumour types and late stage cancers. By repressing the immune system or modulating their antigen presentation, tumour cells often escape the host defense system (Verbik and Joshi, 1995).

The new understandings that immune cells participate in protective immune responses against tumours have led to novel approaches to tumour therapy. Lymphokine activated killer cells (Lotze *et al.*, 1981; Grimm *et al.*, 1982), tumour infiltrating lymphocytes (Cameron *et al.*, 1990), cytotoxic T lymphocytes (Fearon *et al.*, 1990) and natural killer (NK) cells (Hackett *et al.*, 1986) are observed to mediated tumour regression. The strategy has become inducing these responses *in vivo*. By 1994, the National Institute of Health Recombinant DNA Advisory Committee had approved cancer gene therapy human trials for 10 different cancers with transgenes such as IL-2, IL-4, TNF, γ-interferon, B7 costimulatory molecule, granulocyte/macrophage-colony stimulating factor (GM-CSF) and others (Culver *et al.*, 1994). It should be emphasized that the principle underlying tumour immunotherapy is different from the

principle upon which other forms of gene therapy are based. In gene therapy, a transgene is most often supplied to complement a genetic deficiency in *trans*. Immunotherapy aims to induce an immune response against tumour antigens by the supplemental production of a cytokine or other immunoreactive protein. The former situation requires long term expression, while immunotherapy should be effective with more transient doses.

A challenge to either objective, long or short term transgene expression, is that Ad-infected cells are cleared by the immune system during the first infection. This limits transgene expression and neutralizing antibodies to the recombinant virus reduce or prevent expression of a transgene during a second administration (Yang *et al.*, 1995). Providing a higher initial dose of virus would not overcome this difficulty, since the inflammation, and presumably infected-cell clearance, is observed to be dose-dependent in cotton rat lung (Yei *et al.*, 1994). Of potential benefit is the recent observation that coadministration of IL-12 or interferon-γ (INF-γ) with recombinant adenovirus reduced neutralizing antibody production (Yang *et al.*, 1995a), which may point a way to future treatments involving multiple administrations of recombinant adenovirus.

Interestingly, in the case of tumour immunotherapy, transient expression combined with the adjuvant properties of the viral antigens could be beneficial. Zhang *et al.* (1996) observed a significant tumour growth delay in a murine breast cancer model treated with wild-type adenovirus control, although part of this

effect was thought to be due to lysis of infected cells.

Results with various immunotherapies have shown promise (discussed more extensively later) and this has prompted the testing of recombinant adenovirus vectors in the hopes of improving delivery. In addition to tumour regression at the site of intratumoural injection of an IL-2-expressing adenovirus, Addison and Graham (unpublished results) found that 30% of mice bearing tumours on the right and left flanks could be cured of both tumours by intratumoural injection at only one of the sites. This suggests that the mechanism of the antitumour response is effective at distal sites and possibly for treatment of metastases. An adenovirus expressing IL-12 induced initial regressions in greater than 75% of mice, with 40% demonstrating complete regression and protection from rechallenge (Bramson *et al.*, unpublished results). These themes of protection from rechallenge and the potential of treating metastatic tumour models are some of the most compelling in gene therapy. A more in depth examination of IL-12 follows.

Interleukin-12

IL-12 is a well-characterized, clinically relevant cytokine originally cloned from B lymphoblastoid lines (Kobayashi *et al.*, 1989). It will be dealt with in this text from the perspectives of : structure and production, biological effects and use in antitumour therapies.

IL-12 is a heterodimer composed of a 35 kDa (p35) subunit linked by a disulphide bridge to a 40 kDa (p40) subunit (Kobayashi

et al., 1989). The human p40 and the p35 genes are located on different chromosomes and hence are under separate transcriptional controls (Sieburth *et al.*, 1991). The mature p40 polypeptide is 306 amino acids (Mr = 34,699) with 10 cysteine residues and 4 potential N-glycosylation sites (Gubler *et al.*, 1991), being approximately 10% carbohydrate by mass (Podlaski *et al.*, 1992). The mature p35 polypeptide is 197 amino acids (Mr = 22,513) with 7 cysteine residues and 3 potential N-glycosylation sites (Gubler *et al.*, 1991), being approximately 20% carbohydrate by mass (Podlaski *et al.*, 1992). A single intact disulphide bond per heterodimer, formed before secretion, is necessary for bioactivity (Podlaski *et al.*, 1992).

There is no sequence homology between the p35 and p40 subunits, but the p35 subunit is distantly related to interleukin-6 (IL-6), granulocyte-colony stimulating factor, chicken myelomonocytic growth factor (Merberg *et al.*, 1991) and ciliary neurotrophic factor receptor (Trinchieri, 1993). The p40 subunit shares amino acid homology with the extracellular domain of the IL-6 receptor (Gately *et al.*, 1992), which raises the interesting possibility that the IL-12 heterodimer evolved from a receptor and its cytokine that became covalently linked (Gearing *et al.*, 1991).

Between species, IL-12 is sufficiently conserved for the murine heterodimer to be bioactive to human cells. The murine p40 subunit has 70% identity to the human p40 subunit, while the p35 subunits share 60% identity. The human heterodimer is not active on murine cells, although a hybrid [murine-p35]-S-[human-p40]

heterodimer is capable of eliciting all of the characteristic IL-12 effects in murine cells (Schoenhaut *et al.*, 1992).

Parts of the IL-12 receptor (IL-12R) have been identified and cloned. The IL-12R complex is a high affinity (Km=100-600 pM) receptor with 1000-9000 sites per lymphoblast that are not inhibited by other cytokines (Chizzonite *et al.*, 1992). The cloned IL-12R subunit encodes a transmembrane protein of 662 amino acids. Sequence analysis places it in the hematopoietic receptor superfamily (Chua *et al.*, 1994). The IL-12R is expressed on mitogen or IL-2 activated T-cells (CD4+ or CD8+) and on CD56+ IL-2-activated NK cells (described below), but not on resting peripheral blood mononuclear cells (PBMCs) or B-Cells (Desai *et al.*, 1992).

Interleukin-12 Production

IL-12 production is induced by various stimuli, including bacteria, bacterial products or parasites (Trinchieri, 1993), or phorbol esters in proximity to antigen (Stern *et al.*, 1990). IL-12 production seem to be restricted to B-Cells and antigen presenting cells (APCs) (Tahara *et al.*, 1994; Aragane *et al.*, 1994). Constitutive, low level IL-12 p35 mRNA has been detected in several T-Cell lines, as well as myeloid or solid tumour cell lines (D'Andrea *et al.*, 1992; Wolf *et al.*, 1992), but free p35 is not secreted (Yoshida *et al.*, 1994). Upon induction, p40 is expressed at high levels, resulting in secreted heterodimers and free p40, although it should be noted that free p40 or even p40 homodimers do not possess IL-12 bioactivity (Podlasky *et al.*, 1992; D'Andrea *et*

al., 1992).

In general, stimulators of the T_H2 response will downregulate IL-12 production, while stimulators of the T_H1 response will upregulate it. Interleukin-10 (IL-10) blocks the production of the heterodimer and free p40, to the point of downregulating the accumulation of p40 mRNA induced by *Staphylococcus aureus* or lipopolysaccharide (LPS), although levels of p35 mRNA are not significantly affected (D'Andrea *et al.*, 1993). IL-4, Interleukin-13 (IL-13) (D'Andrea *et al.*, 1995) and prostaglandin-E2 (van der Pouw Kraan *et al.*, 1995) all inhibit the production of IL-12. Finally, while other cytokines are unaffected, human immunodeficiency virus-infected patients produce only 10% of normal IL-12 levels. Since monocytes infected *in vitro* with human immunodeficiency virus (HIV) experience the same deficit, reduced IL-12 levels are thought to be a direct effect of HIV infection (Chehimi *et al.*, 1994).

Interleukin-12 : T Helper Type 1 and Type 2 Switching

The biological effects of IL-12 are numerous. At the centre of these various activities is IL-12's role as a critical link between innate and specific immunity and its balancing effect between the non-specific inflammation and the specific immunity necessary to clear pathogens (Tripp *et al.*, 1994). The key to this role is the balance between the activities of T_H1 and T_H2 cells. Intracellular and viral pathogens as well as tumours are the primary targets of the T_H1 subset, which is characterized by

the dominance of cell mediated immunity, IL-2, IFN-γ, and lymphotoxin production (Hsieh *et al.*, 1993). Humoral (antibody-dependent) immunity is the domain of the T_H2 subset and the cytokines primarily associated with this phenomenon are IL-4, interleukin-5 (IL-5) and IL-6 (Hsieh *et al.*, 1993). The decision to produce either a T_H1 or T_H2 response is dependent on a balance between IL-12, IL-4 and IL-10. IL-4 is produced by antigen-stimulated T-cells and acts directly on other T-cells to promote T_H2 development and overrides IL-12 signals (Hsieh *et al.*, 1993). IL-10 indirectly influences the decision by suppressing promoters of T_H1 development, like IL-12-producing cells (Hsieh *et al.*, 1993). However, *in vitro* and *in vivo*, IL-12 strongly inhibits the development of IL-4-producing T-cells (McKnight *et al.*, 1994). It was thought that the effect was mediated through NK cells and their production of IFN-γ, but NK cell depletion did not restore IL-4-production to inhibited cells and it is now thought that IL-12 may directly affect $CD4^+$ differentiation (McKnight *et al.*, 1994). Evidence supports this idea, but IL-12 and IFN-γ are both needed for the T_H1 development of naive CD4+ T cells (Schmitt *et al.*, 1994), which gives IL-12 both a direct and indirect role. As will be discussed below, this is not the first depletion experiment that has had difficulty in finding the effectors of IL-12 action.

Interleukin-12 Biological Effects : Cytokine Production

IL-12 is a relatively poor inducer of cytokines, except for IFN-γ, for which it is sufficient and necessary for dose-dependent,

high level expression by NK cells (Kobayashi *et al.*, 1989), some PBLs (Andrews *et al.*, 1993), and T-cells (Chan *et al.*, 1991). IL-2 also stimulates production of IFN-γ, but anti-IL-2 antibodies only reduce IL-12-induced IFN-γ levels by 22%, reaffirming the suggestion of largely independent pathways (Chan *et al.*, 1991). Other IFN-γ inducers, without antigen costimulus, synergize with IL-12 (listed more extensively in Chan *et al.*, 1991; Murray *et al.*, 1989; and Orange *et al.*, 1994). IL-4 (Kiniwa *et al.*, 1992), IL-10 (D'Andrea *et al.*, 1993) and tumour growth factor-ß (TGF-ß) (Wu *et al.*, 1993) reduce IL-12-induced IFN-γ production.

Interleukin-12 Biological Effects : The Proliferative Response of T and NK Cells

Several sets of cells proliferate in response to IL-12, in keeping with it's role as a determinant of the T_H1 response, namely T-cells, NK cells and miscellaneous PBMCs (Brunda, 1994). IL-12 is not a mitogen for resting T-cells (Pignata *et al.*, 1994), but the proliferative response of activated T-cells, including $CD4^+$, $CD8^+$, and T-cells clones, and sometimes alloantigen activated T-cell receptor (TCR) (αß+/γδ+) lymphoblasts is increased by IL-12 (Gately *et al.*, 1991; Perussia *et al.*, 1992). These observations support IL-12's role as an initiator and determinant of the specific, cellular arm of immunity.

The other side of this immunity includes NK cells, macrophages and phagocytes. NK cells are a subpopulation of peripheral blood lymphocytes that kill virally-infected cells and tumour cells without previous sensitization. Their ability to kill cells is

independent of MHC and these cells do not rearrange immunoglobin or T cell receptor (TCR) genes (Verbik and Joshi, 1995). IL-12 induces the proliferation of activated NK cells, although the response is only 10% of the response illicited by IL-2 (Naume *et al.*, 1992). Immature NK cells do not proliferate under IL-12 induction, but mature NK cells become cytotoxic in a manner regulated by tumour necrosis factor (TNF-α) and IFN-γ (Jewett *et al.*, 1994). This cytotoxicity is demonstrated *in vitro*, where IL-12-stimulated NK cells kill NK-sensitive, NK-resistant, antibody-coated tumour target cells, virus-infected fibroblasts or virus-infected T-cells (Robertson *et al.*, 1992). Neither anti-IL-2 receptor antibodies nor IL-4, inhibitors of NK activation by IL-2 (Robertson *et al.*, 1992), nor antibodies to IFN-α, IFN-ß, IFN-γ and TNF-α (Chehimi *et al.*, 1993) inhibit IL-12-induced activation of NK cells. However, IL-12 does inhibit IL-2-induced proliferation of NK cells, which strengthens the argument for separate signaling pathways (Pignata *et al.*, 1994). The proliferative response of resting PBMCs is modest (Gately *et al.*, 1992) unless they have been previously activated (Gately *et al.*, 1991). The proliferative effects of IL-12 seem somewhat limited compared to powerful mitogens like IL-2, but it will be shown that IL-12 has other, more potent effects that make it a valuable clinical tool.

Interleukin-12 Biological Effects : Induction of Cytotoxicity

Low concentrations (0.4 pM) of IL-12 will enhance the cytotoxicity of peripheral blood lymphocytes (PBLs) against various

tumour-derived and virus-infected targets and the observed enhancement is even greater when NK cells are exposed to IL-2 or IL-12 before the NK-target cell conjugate is formed (Chehimi *et al.*, 1992) . This effect is thought to be due to an enhanced ability of the exocytic machinery to respond to intracellular second messengers (Bonnema *et al.*, 1994). The mechanism of NK cell cytotoxicity is either direct cell-mediated cytotoxicity or antibody-dependent cellular cytotoxicity. Between the two is the common event of receptor-stimulated secretion of granule-derived proteins (perforins, serine esterases, proteoglycans and lysozymes). IL-12 (and IL-2) will enhance these activities and render NK cells capable of killing targets resistant to resting NK cells (Bonnema *et al.*, 1994). Whether these cytokines qualitatively affect the composition of cytoplasmic granules is still debatable, but it is known that preexposure to IL-12 increases the number of granules undergoing exocytosis after stimulation (Bonnema *et al.*, 1994).

Interleukin-12 Biological Effects : Effectors of IL-12 Activities

As will be discussed more extensively below, IL-12 has potent antitumoural activities. But prior to preclinical and clinical studies, it was necessary to understand the exact mechanisms of IL-12 activity. IL-12's proliferative effects on T-cells are thought to be both direct, by action on T-cells and their precursors, and indirect, through the induction of IFN-γ by NK and T-cells (Trinchieri, 1993). However, the antitumoural mechanisms were

often unclear.

IL-12 is not directly toxic to tumour cells because *in vitro* administration of IL-12 did not halt cell proliferation, and nude mice (mice that are athymic and therefore lacking cells that mature in the thymus, i.e. T-cells) are less responsive to IL-12 than wildtype mice which implies the existence of effectors that are absent in nude mice (Brunda and Luistro, unpublished observations, from Brunda, 1994) and *in vitro*.

To eliminate some possible candidates, TNF-α can probably be ruled out as a main effector since IL-12 is a relatively poor inducer of TNF-α (Naume *et al.*, 1992). Next, beige mice (mice lacking NK cells) and NK cell-depleted mice maintain some of their antitumour capacities, which indicates that the primary effects of IL-12 are probably not mediated by NK cells (Brunda *et al.*, 1993). Antitumour efficacy was maintained in mice depleted of CD4+ cells, which is surprising in light of IL-12's effects on T_H1 cell differentiation and cytokine production (Brunda *et al.*, 1993) and in light of the observation that CD4+ cells infiltrate IL-12-treated tumours (Zou *et al.*, 1995). However, in these mice, NK cells probably assume the cytokine production responsibilities of CD4+ cells (Brunda *et al.*, 1993), which implies that CD4+ cells have a role in the wildtype immune response, but that this role can be supported by other secretors.

Positive clues to the identities of the effectors are observations that CD4+ and CD8+ cells (Zou *et al.*, 1995) and Mac-1+ mononuclear cells (Tannenbaum *et al.*, 1996) infiltrate IL-12

treated tumours. Furthermore, the lytic effector molecules perforin and granzyme B, the products of cytotoxic T-lymphocytes (CTLs), were found to be associated with tumour regression in IL-12 treated mice (Tannenbaum *et al.*, 1996).

These observations leave CD8+ cells as the best candidates as effectors of the IL-12 antitumour-response. They are observed by immunohistochemistry to infiltrate tumour tissue (Nastala *et al.*, 1994) and CD8+ cell-depletion decreases antitumoural efficacy, although it does not eliminate it. The residual effect may be due to a small remaining population of CD8+ cells or minor effectors such as macrophages (Brunda *et al.*, 1993). An interesting side note that demonstrates the redundant and multivariate nature of the effect is that the antitumoural effects are noticed even in severe combined immunodeficiency (SCID) mice, which lack both B- and T-cells (O'Toole *et al.*, 1993).

A necessary, but insufficient part of the mechanism that must complement the CD8+ effectors is the induction of IFN-γ. Systemic administration of anti-IFN-γ prior to IL-12 treatment completely abrogated the antitumour effects (Zou *et al.*, 1995), but recombinant IFN-γ alone, administered intraperitoneally to Renca tumour bearing mice showed a lower antitumour effect than IL-12 alone (Brunda *et al.*, 1995). Also nude mice have higher IFN-γ levels, but still have lower antitumoural efficacy following IL-12 administration (Brunda, 1994).

Thus, in a wildtype immune response, the major antitumoral effector is likely the CD8+ subset, with ancillary, but significant

effects contributed by cytokine production in CD4+ and NK cells.

Immunotherapy with Interleukin-12

In the past, delivery systems have included systemic or local administration of recombinant IL-12 (rIL-12), implantation of IL-12-cDNA-transfected cells (fibroblastic or tumoural) or infection with an IL-12-expressing recombinant virus.

Systemic administration of rIL-12 has had positive, dose-dependent (Brunda *et al.*, 1993) results with many tumour models. Daily administration of rIL-12 1-14 days after model adenocarcinoma or sarcoma inoculation yielded prolonged survival or complete regression, including a reduction in metastases (Nastala *et al.*, 1994). Mice bearing sub-cutaneous (s.c.) tumours experienced complete regression at early, intermediate or late stages of tumour growth in response to five intra-peritoneal (i.p.) injections of rIL-12 (Zou *et al.*, 1995). Another murine study resulted in nearly complete regression of s.c. RENCA or CT26 tumours with daily doses of rIL-12 (Tannenbaum *et al.*, 1996). In humans, neuroblastoma, a solid extracranial tumour in children, was targetted for cell lysis by activating peripheral NK cells with IL-12 and IL-2. IL-2 or IL-12 alone were insufficient, but in combination induced large granular lymphocytes (PBMCs) to lyse neuroblastoma cells. Interestingly, the results were unaffected by intensive previous chemotherapy (Rossi *et al.*, 1994).

Intrinsic Toxicity of Interleukin-12

A brief discussion of toxicity would be useful at this point. With respect to dosage and regimen, pre-clinical experiments showed few to no toxicities associated with IL-12. Specifically, for established tumours, maximal antitumoural effects were found with five weekly i.p. doses of 1 μg each (Brunda *et al.*, 1993). No toxic effects were mentioned in an experiment involving 50-120 units IL-12 locally/48 hours (Tahara *et al.*, 1994) and effective daily doses of 0.1 μg - 1.0 μg had a minimal associated toxicity (Nastala *et al.*, 1994). A dose study of IL-12 on infection in the brain using 0.01 μg, 0.1 μg, 1.0 μg, 2.5 μg or 5.0 μg/day for 10 days demonstrated no obvious toxicities at any dose and the optimal dose for reducing infection was 1 μg/d (Clemons *et al.*, 1994). However, clinical trials revealed a unique priming character to IL-12. During IL-12 clinical trials in June 1995, most of the 17 renal cell carcinoma patients became sick and two died as a result of toxicity (Cohen, 1995). A clinical hold imposed by the Federal Drug Administration (FDA) was lifted in October after the deaths and sicknesses were explained. It was shown that a single dose of IL-12 followed by a rest period characterized by systemic clearance of IL-12 before multiple high doses results in a safe regimen. However, high multiple doses without the priming and rest steps resulted in serious toxicity in mice and cynomologus monkeys. Low multiple doses without the priming step appear to be safe (Cohen, 1995).

Interleukin-12 Immunotherapy : Local Administration

In light of these observations, local production or injection of rIL-12 is an even more attractive method that has achieved equivalent results and sidesteps the potential of systemic toxicity. Autologous or allogeneic fibroblasts engineered to secrete high levels of IL-12 have slowed or stopped the growth of an established MCA207 sarcoma in mice in a dose-dependent manner and have produced long-term protective immunity (Zitvogul *et al.*, 1995), meaning that such a treatment is appropriate for metastatic conditions. This protective immunity has been observed with other tumours treated with IL-12 (Brunda *et al.*, 1993; J. Bramson personal communication), which has very important implications for metastatic conditions. A recombinant vaccinia virus expressing IL-12 infected a variety of murine and human tumour lines. The infected cells expressed high levels of IL-12 and their administration significantly reduced tumour growth (Meko *et al*,. 1995). However, using transfected or infected cells as vectors requires *ex vivo* manipulations with dividing cells that can often be inconvenient or technically difficult. These manipulations involve the removal, culture and infection or transfection of tumour cells in the hopes of inducing immune recognition of the tumour. A potential block to this approach is the fact that tumour cells sometimes resist culturing efforts and may be difficult to transfect, and selection strategies may demand prolonged culture which may alter tumour antigen expression (Tahara *et al.*, 1994). Transfected fibroblasts offer a method that circumvents some of

these problems. Autologous fibroblasts can be readily cultured, transfected and selected to express relevant levels of the transgene and tend to migrate less than reimplanted tumour or lymphoid cells (Tahara *et al.*, 1994).

Interleukin-12 : Other Effects

IL-12 possesses some other, clinically relevant, but tumour independent functions, including stem cell manipulation, antimicrobial chemotherapy enhancement and cell-mediated immunity (CMI) enhancement in acquired immune deficiency syndrome (AIDS) patients, should be noted. Firstly, the use of hematopoietic stem cells as gene therapy vectors is often difficult due to complications inherent in the culturing of stem cells. IL-12, in combination with interleukin-3 (IL-3) and steel factor, can enhance the recovery of hematopoietic stem cells in liquid culture (Ploemacher *et al.*, 1993). Secondly, pathogens of the central nervous system can be difficult to treat, often requiring chemotherapy. IL-12 entered clinical trials in 1994 as an enhancer of suboptimal antimicrobial chemotherapy against opportunistic central nervous system pathogens (Clemons *et al.*, 1994). Lastly, IL-12-treatment of PBLs from HIV-infected patients enhanced the cytotoxicity of NK cells (Chehimi *et al.*, 1992). IL-12 has also been found to restore *in vitro* HIV-specific cell-mediated immunity, which suggests potential treatments for the reduced immunological capabilities of AIDS patients (Clerici *et al.*, 1993). Finally, T-cell loss in HIV-infected individuals seems to be due to the

process of activation-induced apoptosis of CD4+ T_H1 cells. IL-12 is observed to upregulate T_H1 cell types and inhibit apoptosis (Estaquier *et al.*, 1995).

Considerations for an Adenovirus Expressing Interleukin-12

An IL-12-expressing adenovirus (AdmIL-12.1) has been previously produced by this lab (Bramson *et al.*, 1996), with the cDNA for the IL-12p35 subunit in E1 and that of the IL-12p40 subunit in E3. Although it expresses well and has marked antitumoural properties *in vivo* (J. Bramson, Human Gene Therapy, in press), it is possible for the E1 sequences in the virus to homologously recombine with the E1 sequences in 293 cells, where E1-deleted recombinant adenovirus is propagated. As the p40 subunit alone possesses biological effects (Mattner *et al.*, 1993; Ling *et al.*, 1995), such a recombination event would produce a replication competent virus expressing the IL-12p40 subunit. Furthermore, such a virus could potentially act as a helper for replication incompetent recombinant virus.

To circumvent this possibility, it was decided to construct a recombinant adenovirus with both subunits in E1. However, the expression of two cDNAs in E1 presented a novel challenge. A genome driving expression of two cDNAs from two promoters in E1 may exceed the 105% of wildtype genome size space limitation during packaging (Bett *et al.*, 1993). Production of bicistronic messages from a single promoter reduces the size problem, but the second cistron of a eukaryotic mRNA is often expressed up to 100-fold less

than the first cistron (Kaufman *et al.*, 1991). Previous work (Nicholson *et al.*, 1991, Kaufman *et al.*, 1991) has demonstrated the utility of using a *cis*-acting viral sequence called the internal ribosome entry site (IRES) for the expression of polycistronic recombinant systems. A description of the IRES follows.

The Internal Ribosome Entry Site

The initiation of protein synthesis usually occurs at the first AUG codon of an mRNA after ribosome binding at the 5′ cap structure (5′-m^7-GpppN) and downstream scanning (Belsham, 1992). Cap-binding eukaryotic initiation factors (eIFs) and 5′-untranslated region (UTR) unwinding factors create a stretch of single-stranded RNA to which the small ribosomal subunit binds (Scheper *et al.*, 1994). Secondary structure or extra AUG codons 5′ of the initiator AUG (AUG^i) can negatively influence translation by interfering with scanning (Belsham, 1992).

Picornavirus genomes consist of positive-sense, single-stranded RNA molecules of approximately 7000 nucleotides (Meerovitch *et al.*, 1991). They possess a single open reading frame (ORF) that encodes a polyprotein that is proteolytically processed by a complex cascade mechanism into the final, functional, viral proteins. The ORF is always preceeded by a long (approximately 600-1100 nucleotide) leader sequence that possesses multiple AUG codons and extensive secondary structure. No picornavirus RNA has a 7-methyl-guanine cap, but instead each RNA genome possesses a short peptide called Vpg covalently bound to the

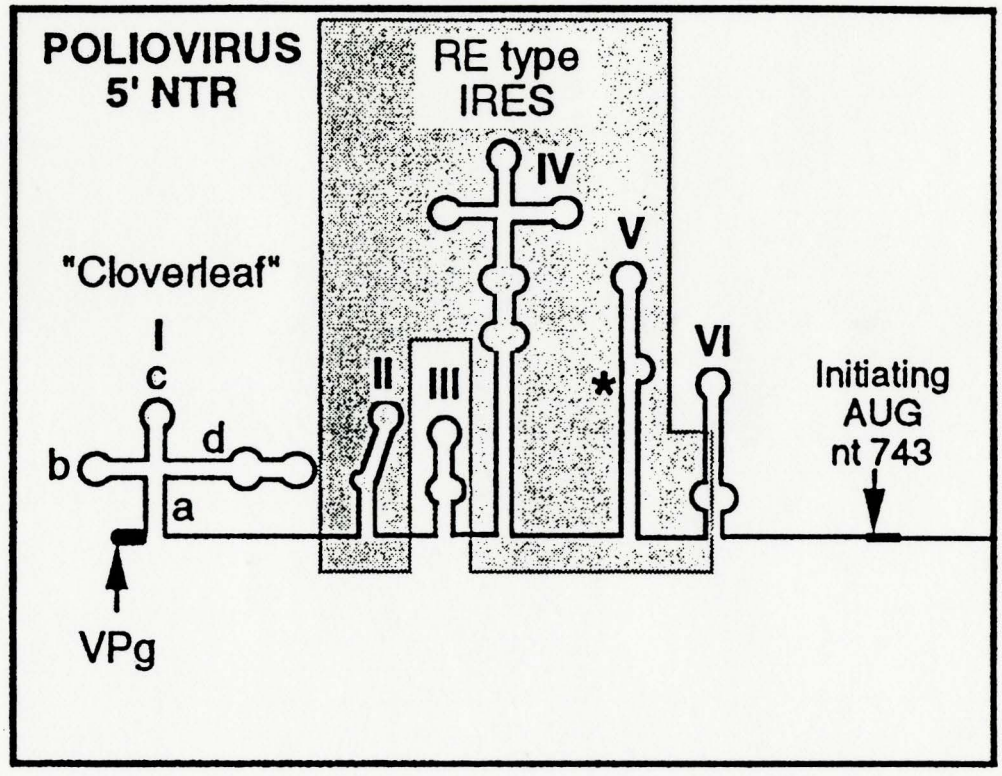
5′ terminus. Vpg has no cap-like activity and picornaviruses must therefore rely on a cap-independent mechanism of translation initiation (Bandyopadhyay *et al.*, 1992). This cap-independent mechanism is called internal initiation and is mediated by a large (approximately 400-500 nucleotide), non-terminal, *cis*-acting element within the 5′-UTR called an IRES. The IRES is defined as the segment of RNA conferring the capacity for efficient cap-independent initiation of translation on engineered templates (Pilipenko *et al.*, 1994). A schematic of the poliovirus IRES is shown in Figure 1.3.

The general structure of the IRES is of alternating stretches of stem-loops of various sizes and stretches of unstructured single-stranded RNA. Interspersed within both conformations are multiple (7-10) AUG codons, something not seen in cap-dependent messages (Meerovitch *et al.*, 1991).

Although the diversity and the subtleties of the mechanisms of the different picornaviral families are interesting, this text will restrict its discussion to the poliovirus IRES and the encephalomyocarditis virus (EMCV) IRES.

Internal initiation in poliovirus requires *cis*- and *trans*-acting elements. There are 6-8 AUG codons upstream of the AUG^i, 3 of which are conserved among all serotypes. 22-23 nucleotides, depending on the serotype, upstream of AUG-7 is a stretch of pyrimidines (5′UUUCCUUUU3′), called the pyrimidine-tract (PyT). Seven stem-loops of varying size are distributed from nt 10 to 610. AUG-7 is not recognized as an AUG^i, but its presence is essential.

Figure 1.3. The folding pattern of the poliovirus 5′-untranslated region. The IRES is shaded and recruits ribosomes independent of 5′ or 3′ sequences. From Rueckert (1996).


POLIOVIRUS
5' NTR
RE type
IRES
IV
V
VI
"Cloverleaf"
I
c
b
d
a
II
III
*
Initiating
AUG
nt 743
VPg

Models predict that the PyT region is unstructured, which would allow ribosomes and other *trans*-acting factors to interact here. There is no direct evidence of tertiary structure, but the amount of evidence for secondary structure and the nature of some *trans*-acting factors support a tertiary structure hypothesis (Meerovitch *et al.*, 1991).

Various experiments have been conducted to characterize the IRES within the 5'-UTR. The conclusions from these studies are summarized below. Altering the capping characteristics of poliovirus RNA had no effect on translation, which implies that initiation is independent of the 5'-terminus structure (Meerovitch *et al.*, 1991). The core IRES of poliovirus consists of at most 400 nts (134-155) to (556-585), consistent with the idea that ribosomal recognition involves secondary structure. However, not all core sequences are essential (Nicholson *et al.*, 1991) and mutations that alter the primary, but not the predicted secondary structure support the secondary structure recognition model as well. Sequences extending 3' to nt 612 are not essential, but enhance translational activity (Meerovitch *et al.*, 1991). Additional support for the independence of the IRES on sequences 3' of it is found when the IRES element is fused to heterologous mRNA, it functions in *cis* to render translation cap-independent (Meerovitch *et al.*, 1990; Meerovitch *et al.*, 1991)

The 43S preinitiation complex is postulated to bind the RNA upstream of an AUG in the vicinity of the PyT (AUG^{Y}). The interaction of the PyT and the 43S complex is thought to be

analogous to the interaction between the Shine-Dalgarno sequence and the 16S rRNA in bacteria. Here, base-pairing is hypothesized to occur between the PyT and a purine-rich stretch (-AAAGGAA-) which is conserved near the 3′ end of all 18S rRNA. Supporting this model is the conserved PyT-AUG^y distance and its intolerance to change (Pilipenko *et al.*, 1994). The complex would then translocate, possibly by scanning to the AUG^i. This system bypasses the 5′-terminal structure and any other obstacles like upstream AUG codons (Meerovitch *et al.*, 1990). Ribosome landing probably requires unstructured RNA, so some unwinding might be needed (Sonenberg and Meerovitch, 1990). Despite some subtle variations, this model of poliovirus translation remains the dominant paradigm.

Internal initiation is a phenomenon that is independent of viral proteins and therefore probably represents a eukaryotic system that has been usurped by viruses, although no such eukaryotic system has yet been found. The phenomenon is widely distributed in nature, counting among its members the Potato Mosaic Virus M, Cowpea Mosaic Virus (Gramstat *et al.*, 1994) and all of the Picornaviridae.

The major functional differences between the poliovirus and EMCV IRES's is that in poliovirus, the ribosome binds, then scans to the AUG^i, so that changes in distance between the ribosome binding site and the AUG^i are tolerated (Meerovitch *et al.*, 1990). In the EMCV system, the AUG^i must be at a specific location with respect to the ribosome landing site (Borman *et al.*, 1992) and

there is already an AUG^i there, so that any cDNA placed under the translational control of an EMCV IRES must fuse the cDNA AUG^i with the viral AUG^i. For this reason, the poliovirus IRES was chosen to support translation of the various second cistrons of this thesis.

Promoters

The site of RNA polymerase binding on the DNA is called a promoter. Promoters are *cis*-acting and are typically contain several sequence motifs that are conserved across broadly differing species. These sequence motifs include GC regions, a TATA-box and a CCAAT-box. Although these elements in combination may direct transcription, for inducible regulation and high level constitutive control, another *cis*-acting element is required. This second type of control element is called an enhancer. Enhancers are structured much in the same way as prokaryotic promoters, with multiple repetitive units that increase the transcription initiation in a position-independent, orientation-independent manner. Promoters and their enhancers constitute an important element of any engineered high-level expression system. Given adenovirus as the vector of choice for effective transient expression of IL-12 in and around tumour tissue, it may be advantageous to choose the most potent transcriptional control available. The reason is that, although adenovirus construction and preparation are relatively straightforward and that adenovirus will infect most cell types, there is an immune response raised against a viral vector that theoretically will limit the efficacy of successive treatment. Thus higher transgene production per treatment may be necessary.

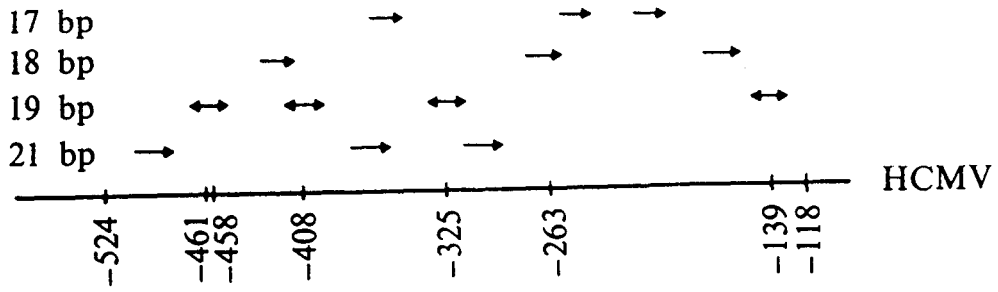
A well-characterized promoter/enhancer that achieves high level expression in many cell types (Schmidt *et al.*, 1990) is the human cytomegalovirus (HCMV) immediate early (IE) promoter/enhancer (Boshart *et al.*, 1985). Fragments of this promoter/enhancer have previously been shown to be sufficient to drive high levels of transgene expression in an enhancer trap assay (fragment from -118 to -458) (Boshart *et al.*, 1985) and in adenovirus backgrounds across several cell types (-299 to +72) (Wilkinson and Akrigg, 1992; C.L. Addison, personal communication).

Following the observation that the HCMV IE promoter/enhancer expressed transgenes 10-50 lower in murine cells, compared to human cells (Addison *et al.*, 1995), the murine cytomegalovirus (MCMV) IE promoter/enhancer (see Figure 1.4) was tested and found to reproducibly express similar or higher transgene levels than the HCMV IE promoter/enhancer (C.L. Addison, personal communication). These results were determined with the lacZ gene under the transcriptional control of either of two fragments of the MCMV promoters, one of which was 900 bp larger than the other. It became relevant to see if these results could be confirmed with a second reporter, the firefly luciferase cDNA (DeWet *et al.*, 1987) under the transcriptional control of the two promoters.

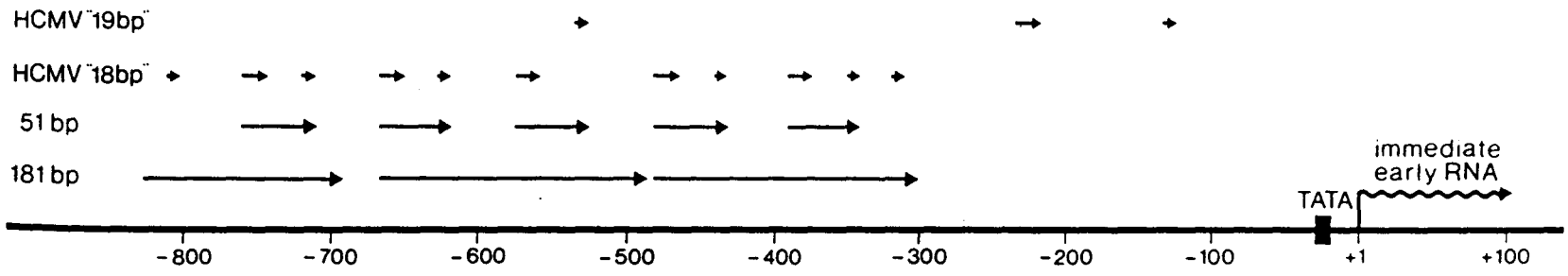
Thus, there were two main aims to this thesis. The first was to examine luciferase expression under the transcriptional control of the three promoter/enhancer fragments: the short MCMV IE promoter/enhancer fragment, the long MCMV IE promoter/enhancer fragment and the HCMV IE promoter/enhancer fragment. Once the

Figure 1.4. Panel A. A schematic of the HCMV IE promoter/enhancer region from Fickenscher *et al.* (1989). Numbers below the solid line correspond to nucleotide position 5′ of the transcriptional start site (+1), while arrows indicate repetitive elements of sizes 17 bp, 18 bp, 19 bp and 21 bp. Panel B. Structural organization of the MCMV IE promoter/enhancer region from Dorsch-Häsler *et al.* (1985). Numbers below the solid line correspond to nucleotide position 5′ of the transcriptional start site (+1), while arrows indicate repetitive elements of sizes 18 bp, 19 bp, 51 bp and 181 bp.

A. HCMV IE promoter/enhancer.



B. MCMV IE promoter/enhancer



transcriptional activities were compared, the most active would be used for the transcriptional control element of a bicistronic IL-12-expressing adenovirus for tumour immunotherapy. A secondary project was the construction of the same bicistronic system, but with reporter genes in place of the IL-12 cDNAs. This would allow for more precise measurements of expression from each cistron.

Chapter 2 : Materials and Methods

1. Materials

1.a. Bacterial Strains

Escherichia coli (*E. coli*) DH5-α (Hanahan, 1983; genotype: *sup*E44, δlacU169 (Δ80*lac*ZΔM15), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*1, *rel*A1) was obtained from Bethesda Research Laboratories and was used for all cloning procedures.

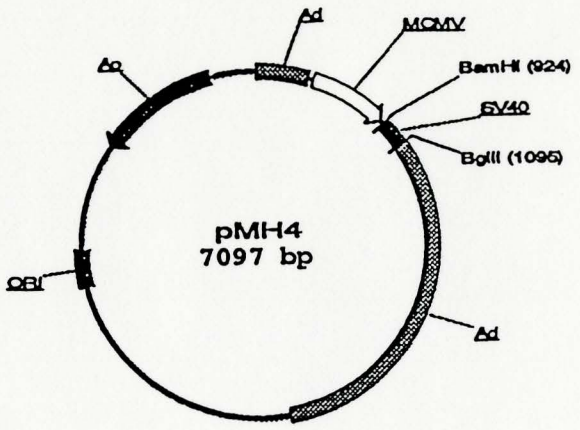
1.b. Plasmids

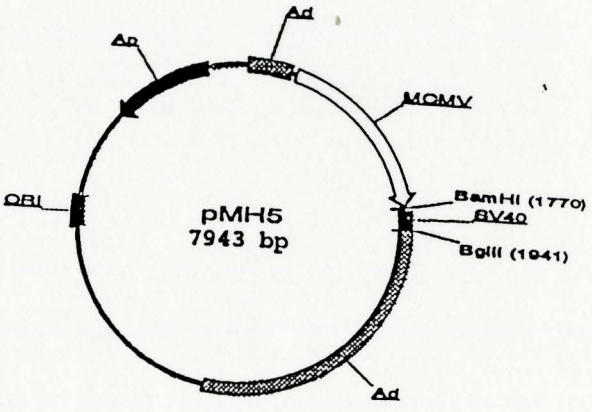
The parental plasmids of the cloning steps to be described in this text are : pMH4, pMH5, pP2CAT, p548FL, pMH1lacZ, pCMVp40 and pVL2IL12p35.

pMH4 and pMH5 are shuttle plasmids that contain the Ad5 genomic sequences from map units (mu) 0 to 15.8, including the DNA packaging signal. These two plasmids are shown in Figure 2.1. The E1 sequences between mu 1 and 9.8 have been replaced by either the 0.53 kb fragment (-491 to +36) or the 1.5 kb fragment (-1464 to +36) respectively of the MCMV IE promoter/enhancer from pHind33 (gift of Dr U. Kozinowski, Institute for Medical Virology, University of Heidelberg) (Dorsch-Hasler *et al.*, 1985). The promoter/enhancer is oriented parallel to the direction of transcription of the E1 genes that have been deleted and is followed by a multicloning site and the polyadenylation signal from SV40.

pP2CAT is a plasmid containing the 5'-UTR of poliovirus type

Figure 2.1. The shuttle plasmids pMH4 and pMH5. These plasmids are derived from pBR322 and contain the adenovirus type 5 genomic sequences from map units (mu) 0 to 15.8, including the packaging signal, where each mu corresponds to 360 bp. The E1 sequences have been deleted between mu 1 and mu 9.8. They have been replaced by either the .5 kb (pMH4) fragment or the 1.4 kb (pMH5) fragment of the MCMV IE promoter. A multicloning site exists 3' of the promoter, followed by the polyadenylation signal from SV40.


Ad
MCMV
Ap
BamHI (924)
SV40
BglII (1095)
pMH4
7097 bp
ORI
Ad


Ad
Ap
MCMV
ORI
BamHI (1770)
SV40
pMH5
7943 bp
BglII (1941)
Ad

II, kindly provided by Y. Svitkin from the laboratory of Dr N. Sonenberg, Department of Biochemistry, McGill Univeristy, Montreal, Canada.

p548FL is a plasmid containing the complete, intronless firefly luciferase cDNA (constructed by Dr. M. Hitt, Department of Biology, McMaster University, Hamilton, Canada). The luciferase cDNA was isolated from pSVOA/L, a gift kindly provided by Dr. D.R. Helinski, University of California, San Diego, La Jolla, CA.

The lacZ cDNA was isolated from pMH1lacZ, constructed by Dr. M. Hitt by subcloning the lacZ cDNA from pHCMVsp1lacZ (Morsey *et al.*, 1993).

The plasmids pVL2IL12p35 and pMEIL12p40#4 carry the cDNAs for the p35 and p40 subunits of IL-12, respectively. They are a gift from A. O'Garra, at DNAX. The cDNA for the p40 subunit used by this thesis was actually taken from pCMVp40 (Bramson *et al.*, 1995), which derives the p40 cDNA from pMEIL12p40#4, but which offered more convenient restriction sites for this project.

For the purposes of virus generation, the plasmids pFG140 and pBHG10 were used. pFG140 (Graham, 1984) is an infectious plasmid in single transfections of 293 cells, providing a quantitative positive control for cotransfection experiments. It was derived from the *dl*309 viral genome described by Jones and Shenk (1979). It contains nearly the entire *dl*309 genome with an ampicillin resistance gene and a bacterial origin of replication (*ori*) inserted at bp 1339 (3.7 mu) in the Ad5 sequences. pBHG10 is a plasmid that contains the Ad serotype 5 genome with deletions in E1

and E3 and an ampicillin resistance gene, but lacks the DNA packaging signal (Bett et al., 1994). When pBHG10 is cotransfected with appropriately designed shuttle plasmids, *in vivo* recombination occurs, generating recombinant adenovirus.

1.c. Antibodies

Three antibodies were used during this study, one for immunoprecipitations, two for western immunoblotting (one primary, one secondary). The antibody used for the immunoprecipitation of IL-12 was a polyclonal rabbit antiserum, kindly provided by A. O'Garra at DNAX. The primary antibody for the western immunoblot of luciferase was a polyclonal anti-luciferase antibody available from Promega, PN# E4191. The secondary antibody for the western immunoblot was Immunopure Mouse-α-Rabbit (H+L) IgG, Peroxidase Conjugated, from Pierce, PN# 31464.

1.d. Enzymes

All enzymes were purchased from Boehringher Mannheim, Bethesda Research Laboratories, New England Biolaboratories or Pharmacia and used according to the manufacturers' recommendations.

1.e. Mammalian Cell Lines

The mammalian cells used during this project were 293, MRC5, a primary rat lung fibroblast explant, MDCK, SV329 and MT1A2. They are described below. 293 is a transformed human embryonic kidney cell line, constitutively expressing the Ad serotype 5 E1 genes (the left 12% of the genome; Graham *et al.*, 1977) and was used for generation of recombinant Ad5 viruses following cotransfection as well as for propagation and titration of such viruses. MRC5 is a

primary human fetal lung fibroblast (ATCC CCL 171), was used for *in vitro* viral expression studies. A primary primary lung fibroblast explant isolated from a Sprague-Dawley rat was kindly provided by J. Gauldie for *in vitro* viral expression studies. MDCK is a canine kidney cell line (ATCC CCL 34) that was used for *in vitro* viral expression studies. SV329 is an SV40-transformed murine fibroblast line that was used for *in vitro* viral expression studies. MT1A2 is a murine mammary adenocarcinoma cell line derived from polyoma middle T antigen (PyMidT) expressing transgenic mice (Guy et al., 1992).

1.f. Oligonucleotides

Four oligonucleotides were used during this project, all of which were produced in the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Canada. Two (AB5352 and AB5353) were annealed to each other to produce the multicloning site inserted into pDK3 and pDK4. They are shown in Figure 2.2.

The other two oligonucleotides (AB3339 and AB4724) were used for sequencing. AB3339 was used for sequencing leftward (antiparallel to E1 transcription) in Ad serotype 5 and hybridizes from nucleotides 3584 to 3564 of Ad. AB4724 was used for sequencing downstream from the MCMV IE promoter/enhancer and hybridizes to nucleotides -82 to -66 of that sequence. They are shown in Figure 2.2.

1.g. Viruses

Add170-3 is a previously described recombinant adenovirus

Figure 2.2. A. Oligonucleotides AB5352 (top strand) and AB5353 (bottom strand, shown in the complementary orientation), shown as they are annealed together with restriction sites indicated. B. Oligonucleotides AB3339 and AB4724, used for sequencing shuttle plasmids.

A.

```
    EcoRI  BglII  HpaI  HindIII PmlI  BamHI  EcoRV  SalI
5'- AATTC AGATCT GTTAAC AAGCTT CACGTG GGATCC GATATC GTCG-3'
3'-     G TCTAGA CAATTG TTCGAA GTGCAC CCTAGG CTATAG CAGCT -5'
```

B.

AB3339 5'-GAT ACA AAA CTA CAT AAG ACC-3'

AB4724 5'-CCT GGA AAT TGC ATA TT-3'

(Bett *et al.*, 1994). Nucleotides 339-3533 from E1 and 28133-30818 from E3 are deleted, which renders this virus $E1^-$, $E3^-$.

AdCA18 is a recombinant adenovirus constructed by C. Addison. It was rescued using pJM17 (McGrory *et al.*, 1988) instead of pBHG10 as the plasmid carrying the majority of the adenovirus genome. It possesses the same E1 deletions as Add170-3, but expresses a functional E3 19K protein. An expression cassette is oriented rightwards in E1, containing the HCMV IE promoter/enhancer driving transcription of the full-length, intronless luciferase cDNA followed by the polyadenylation signal from SV40.

2. DNA Manipulations

2.a. DNA Restriction Enzyme Digests

Restriction enzymes were used as cloning and analytical tools. Typical reactions were carried out for 2-4 hours for plasmid DNA, 4-16 hours for viral DNA preparations, at the appropriate temperature for the enzyme in the buffer supplied by the manufacturer. 3-10 units (U) of enzyme per µg of DNA were used. When appropriate, enzymes were inactivated according to the manufacturer's recommendations.

2.b. Producing Non-Cohesive Ends After Restriction Digests

When it was necessary to obtain blunt-ended DNA fragments after a restriction enzyme digest that produced a 5′ overhang, the large fragment of *E. coli* DNA polymerase I (the Klenow enzyme (Gibco)) was used. The Klenow enzyme has a 5′-3′ polymerase activity and a 3′-5′ exonuclease activity, but lacks the 5′-3′ exonuclease activity possessed by the complete DNA polymerase I.

Reactions typically contained 1-5 μg of DNA, 0.25 mM of each of the dNTPs and 1-5 U of the klenow enzyme. The reactions incubated for 15 minutes at room temperature (RT) before heat inactivation at 65°C for 20 minutes.

2.c. DNA Dephosphorylation

Removing the 5'-terminal phosphates from the vector fragment of a ligation was often useful, preventing self-ligation of the vector and regeneration of the parental plasmid. This reduced the number of colonies to be screened. Calf intestinal alkaline phosphatase (CIP) was purchased from Boehringer-Mannheim. After appropriate digestion, the restriction enzyme was heat inactivated at 65°C for 20 minutes. 1 unit of CIP was added per microgram of DNA, then 10 μl of the 10 X dephosphorylation buffer provided and the whole mixture volumized to 100 μl with water. For 5'-overhangs, the reactions were incubated for 1 hour at room temperature. For blunt ended dephosphorylations, the reactions were incubated for 1 hour at 50°C. The reactions were inactivated by the addition of 1 μl of 0.5 M EDTA and incubated at 75°C for 20 minutes. Samples were phenol extracted, ethanol precipitated and resuspended in a volume appropriate for ligation.

2.d. DNA Ligation

T4 DNA ligase (Gibco) was used to catalyze the formation of phosphodiester bonds between the 5' phosphate and 3' hydroxyl termini of abutting duplex DNA. Ligation reactions contained 1 X T4 DNA Ligase Buffer (10 mM $MgCl_2$, 10 mM DTT, 50 mM Tris, pH 7.6, 1 mM ATP) and 1 unit of T4 DNA Ligase per μg or less of DNA.

Ligation reactions were incubated at RT for 2-16 hours before electroporation.

2.e. Agarose Gel Electrophoresis and Isolation of Fragments

DNA fragments generated by restriction enzyme digests were separated by electrophoresis through 1% (w/v) agarose (Gibco) gels containing 2.5 µg ethidium bromide per 100 ml. Following electrophoresis, DNA bands were visualized using a UV transilluminator. If required, bands were excised from the gel using a clean scalpel and placed in a fresh Eppendorf tube. The Promega Wizard PCR kits (Cat# 7170) were used according to the manufacturer's specifications to isolate the DNA from the agarose. DNA was resuspended in 1 X TE and used in subsequent cloning procedures.

2.f. Plasmid DNA Sequencing

Plasmid DNA samples (200 ng/µl) and primers (0.2 pM/µl) were provided to the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University, where sequencing reactions were carried out using a cycle sequencing procedure. Annealing, elongation and denaturation temperatures were 50°C, 60°C, and 96°C respectively.

3. Bacterial Manipulations

3.a. Bacterial Culture Techniques

E. coli (strain DH5α) was grown in either Lennox L (LB) broth (0.5% (w/v) yeast extract (Difco), 1% (w/v) bacto tryptone (Difco), 0.5% (w/v) NaCl (BDH), 0.1% (w/v) glucose (Benton Dickinson), sterlized by autoclaving) or Super-Broth (SB) (2% LB premix (1%

select pancreatic digest of casein, 0.5% select yeast extract, 0.5% NaCL (Benton Dickinson), 2.2% bacto tryptone, 1.5% yeast extract, and 0.1% glucose, sterilized by autoclaving) or on solid LB agar (20 g bacto-agar per litre of LB) at 37°C. Bacteria carrying plasmids with the ampicillin resistance gene were grown in media supplemented with ampicillin (60 μg/ml, Boehringer-Mannheim). For long term storage, 3 ml of a fresh overnight culture was diluted with 1 ml of sterile glycerol and frozen -80°C.

2.b. Transformation of E. coli with plasmid DNA

2.b. Part I : Preparation of Competent Bacterial Cells

To produce competent *E. coli* (strain DH5α) for electroporation, fresh 500 ml cultures were grown to an absorbance (600nm) of 0.5 to 1.0 and harvested by chilling the cultures on ice for 15 minutes prior to a 10 minute centrifugation at 4000 rpm. Cells were resuspended twice in cold, sterile 10% glycerol (500 ml and 20 ml) and centrifuged as above. The pellets were resuspended in a final volume of 2-3 ml 10% glycerol, to a final concentration of approximately $3x10^{10}$ cells/ml. Aliquots of 50 μl were flash frozen in liquid nitrogen and stored at -70°C.

2.b. Part II : Transformation of Bacteria with Plasmid DNA

Aliquots of competent cells were mixed with 2-100 ng of plasmid DNA in 0.2 cm electroporation cuvettes (Biorad), and electroporated at 2.25 kV and resuspended in 1 ml LB. After 30 minutes at 37°C, cells were plated at appropriate dilutions on LB agar plates containing 0.8 mg/ml ampicillin and incubated at 37°C overnight.

2.c. Purification of plasmid DNA from E. Coli

2.c. Part I : Small-Scale Analytical Preparation

Small scale preparations of plasmid DNA were made following the alkaline lysis method outlined by Sanbrook *et al.* (1989) with a few modifications. Briefly, 3ml aliquots of LB supplemented with 0.8 mg/ml ampicillin, innoculated with transformed bacteria and incubated on a shaker (225 rpm) at 37°C for 14-20 hours. Half of each culture were dispensed in 1.5 ml Eppendorf tubes and pelleted by microcentrifugation. After aspiration of the supernatants, the cell pellets were resuspended in 100 µl 50mM Tris/HCl, 10 mM EDTA, pH 8.0. The suspensions were lysed on ice for 5 minutes with 200 µl 200 mM NaOH, 1% SDS. Chromosomal DNA and proteins were precipitated for 20 minutes on ice by the addition of 150 µl 3 M sodium acetate, pH 5.3. Cellular debris and chromosomal DNA were pelleted during 10 minutes of microcentrifugation. The plasmid DNA in the supernatants was precipitated with 2 volumes of isopropanol, pelleted with 5 minutes of microcentrifugation, rinsed with 70% ethanol, dried and resuspended in 100 µl of 10 mM Tris, 1 mM EDTA, pH 8.0, 100 ng/ml RNaseA.

2.c. Part II : Large-Scale Plasmid Purification

Large scale plasmid preparations were done essentially as described by Birnboim and Doly (1979), with a few modifications. Log phase cultures of 500 ml were grown at 37°C overnight in SB containing 0.8 mg/ml ampicillin. These cultures were harvested at 4°C by centrifugation for 10 minutes at 6000g. The pellets were resuspended in 100 ml of a resuspension buffer (supplemented with

200 mg/ml lysozyme (Boehringer-Mannheim)) and lysed for 5 minutes on ice with 200 mM NaOH, 1% SDS. Each sample received 40 ml of 3M potassium acetate and was incubated for 20 minutes on ice to precipitate chromosomal DNA and proteins. Precipitates were pelleted by centrifugation at 6000g and the supernatants filtered through cheesecloth into a fresh centrifuge tube containing 100 ml 2-propanol. Low molecular weight nucleic acids were pelleted by a 10 minute centrifugation at 6000g after a 30 minute, room temperature incubation. The pellets were dried, resuspended in 7 ml 10 mM Tris, 1 mM EDTA, pH 8.0 and incubated at 4°C with 8.6 g CsCl. After 30 minutes, precipitates were pelleted by a 15 minute centrifugation at 3200g at 4°C. The supernatants were transferred to 3"x5/8" heat-sealable, polyallomer ultracentrifuge tubes. Light parafin oil filled the remainder of each tube after the addition of 200 µl of 10 mg/ml of ethidium bromide (Sigma) per sample. The tubes were heat-sealed and plasmid DNA purified by equilibrium ultracentrifugation for 16-20 hours at 55,000 rpm in a VTi 65.1 rotor (Beckman). Plasmid bands were collected in syringes fitted with 16 gauge needles and transferred to 15 ml corning polypropylene tubes. The ethidium bromide was removed with three to five extractions of CsCl-saturated 2-propanol. The aqueous phase was diluted with 3 volumes of 10 mM Tris, 1 mM EDTA, pH 8.0 and 8 volumes of 96% ethanol. Purified plasmid DNA was pelletted by centrifugation at 4000 rpm at room temperature for 5 minutes. Pellets were rinsed with 70% ethanol, dried and resuspended in 0.3-1.0 ml of 10 mM Tris, 1 mM EDTA, pH 8.0. DNA was quantified by

fluorimetry with a Hoefer TKO 100 Fluoremeter (Hoefer Scientific Instruments, San Fransisco).

4. Mammalian Tissue Culture Techniques

4.a. Growth and Passaging of Mammalian Cells

The MRC5, primary rat and MDCK cells were cultured in monolayers on 60mm or 150mm plastic tissue culture dishes (Nunclon) and maintained in α-minimum essential medium (α-MEM; Gibco), supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco), 2 mM L-glutamine (Gibco), 100 units/ml penicillin G (Gibco), 100 µg/ml streptomycin sulphate (Gibco) and 2.5 µg/ml fungizone (Squibb Canada). 293 cells were maintained in minimal essential medium (MEM) F11 supplemented with 10% (v/v) Newborn Calf Serum (NCS; Gibco), 2 mM L-glutamine, 100 units/ml penicillin G, 100 µg/ml streptomycin sulphate and 2.5 µg/ml fungizone.

To passage mammalian cells, medium was removed from confluent or near-confluent monolayer cultures and the cells rinsed with 1-3 ml of PBS (137 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 0.027 mM KCl). Rinsed cells were detached from culture dishes with 0.5-2 ml of 0.5% trypsin-EDTA (Gibco), appropriately diluted with fresh growth media and seeded onto new dishes. 293 cells were passaged similarly, except that citrate saline (134 mM KCl and 15 mM sodium citrate) was used to rinse and detach cells.

4.b. Generation of Recombinant Adenovirus by Cotransfection

One or two days prior to cotransfection, early passage 293 cells (passage 28-40) were seeded onto 60 mm dishes at a ratio of 1x150 mm dish to 8-16x60 mm dishes. At 70-90% confluency, the

cells were considered ready to cotransfect.

A volume of 1 X HEPES-buffered saline (HeBS) (21 mM HEPES, 0.137 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.5 mM glucose, pH 7.1) equal to 0.5 ml per dish of 293 cells was aliquotted into a 50 ml Corning tube, supplemented with 10 µg/ml salmon sperm DNA (1 mg/ml stock solution (Boehringer-Mannheim)) as a carrier. This mixture was vortexed to shear the salmon sperm DNA and divided into 1-2 ml aliquots, with each aliquot receiving 5 or 10 µg of each plasmid. To the HeBS, carrier DNA and experimental DNA, 50 µl/ml of 2.5 M $CaCl_2$ was added to a final concentration of 125 mM. This solution was mixed immediately and allowed to incubate for 30 minutes at RT. The mixture was added dropwise to the cells without removing the medium. Cells were incubated at 37°C in a 5% CO_2 incubator for 4-16 hours. The medium was then removed and the cells overlayed with F-11 MEM supplemented with 0.5% agarose, 5% (v/v) horse serum, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2.5 µg/ml fungizone. The cells were incubated in a 5% CO_2 incubator at 37°C for 14-21 days, with plaques usually appearing between 5-14 days.

4.c. Plaque Isolation

Viral plaques generated from cotransfections were isolated by punching out agar plugs with a sterile Pasteur pipet and stored in 1 ml 1 X PBS^{++} (PBS with 0.1% $CaCl_2$ and 0.1% $MgCl_2$) + 10% glycerol. After one cycle of freeze-thaw at -70°C, 200 µl of the plaque suspension was used to infect (see below) a dish of 293 cells for analysis of adenoviral DNA.

4.d. Adenovirus Infection

Infections of 80-90% confluent monolayers were conducted at various multiplicities. Viral particles were diluted from concentrated stocks or plaque isolates in 0.2 ml PBS^{++} for infection of 60 mm dishes, 0.5 ml for 150 mm dishes. After removing the medium from cells, diluted virus was dispersed dropwise over the monolayer and allowed to adsorb at room temperature for 20-30 minutes after which fresh media supplemented with 5% horse serum (Gibco) was added and the cells returned to 37°C. Cells were subsequently monitored for cytopathic effect (cpe).

4.e. Analysis of Adenoviral DNA

Isolation of adenoviral DNA occured at a point in the infection when almost all the 293 cells were rounded and very few were still attached to the dish. Dishes were left undisturbed at RT to allow the cells to sediment out of the media. The medium was gently aspirated, leaving the majority of the cells in the dish. Addition of 0.5 ml of digestion buffer (1 mg/ml pronase in 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% SDS) was followed by a 4-16 hour incubation at 37°C. The mixture was extracted once with buffer-saturated phenol (Boehringher Mannheim) and the DNA precipitated with 0.1 volumes of 3M sodium acetate (pH 5.3) and 2 volumes of 96% ethanol and centrifugation. The dried pellet was resuspended in 50 µl of TE and 15 µl used for a given restriction digest. If the desired recombinant was obtained, the sample was plaque purified and reanalyzed.

4.f. Plaque Purification

For the purification of adenoviral stocks, serial dilutions of 10^{-1} to 10^{-4} were infected onto 60 mm dishes of 293 cells that were 90-95% confluent at the time of infection. These were overlayed with MEM F-11 containing 0.5% agarose, 5% (v/v) horse serum, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2.5 µg/ml fungizone. Plaques appeared within 4-8 days and appropriate plaques were isolated and reanalyzed.

4.g. Generation of High Titre Adenoviral Stocks

To produce high titre crude lysates, 8-10 near-confluent 150 mm dishes of 293 cells were infected with 200-500 µl of the supernatant from a viral DNA preparation, left for 30 minutes, then refed with F11 medium that differs from the passaging medium in that it is supplemented with 5% (v/v) horse serum (Gibco) instead of newborn bovine serum. After the majority of the cells had rounded (3-4 days), the dishes were scraped with a rubber policeman and the infected cells pelleted by a low speed (1000 rpm) centrifugation for 10 minutes. After aspirating the supernatant, the pellets were resuspended together in 4 ml PBS^{++} + 10% glycerol, subjected to three cycles of freeze-thawing and stored at -70°C. Quantitation of plaque forming units (pfu) is described below.

4.h. Titration of Adenoviral Stocks

For the titration of adenoviral crude lysates, serial dilutions of 10^{-5} to 10^{-8} were infected in duplicate onto 60 mm dishes of 293 cells that were 90-95% confluent at the time of infection. These were overlayed with MEM F-11 (containing 0.5%

agarose, 5% (v/v) horse serum, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2.5 µg/ml fungizone). Plaques appeared within 5-10 days. Titres were expressed as the number of pfu per ml of viral preparation and were calculated by the formula below.

$$\frac{\text{number of plaques}}{\text{volume of dilution used}} \times \text{dilution factor}$$

5. Protein Techniques

5.a. Immunoprecipitation Analysis

Prior to ^{35}S-methionine labelling, 60 mm dishes (approximately 10^6 cells/sample) of the appropriate cell type were infected at an MOI of 10. At the designated time points, the medium was removed and replaced with 0.5 ml 199 medium lacking methionine and cysteine, supplemented with 50 µCi ^{35}S-translabel (ICN) per sample. After a 4 hour incubation at 37°C, the medium was removed, the cells washed with cold PBS before receiving 0.5 ml of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% (w/v) SDS, 1% (v/v) Triton X-100, 10 mg/ml) plus protease inhibitors (aprotinin (Sigma) and phenylmethylsulfonyl fluoride) (PMSF)). Cell extracts were collected by scraping, transferred to Eppendorf tubes, vortexed for 5 seconds and incubated on ice for 30 minutes. Samples were centrifuged for 30 minutes at 4°C and 30,000g and the clarified extracts transferred to new tubes and stored at -80°C or immunoprecipitated immediately.

To each sample of clarified extract, 2-5 µl of appropriately

diluted antibody was added with 100 µl of protein A sepharose suspension (0.5 g in 5 ml of RIPA buffer with inhibitors) and gently rotated at 4°C for 2-3 hours. Immunoprecipitates were recovered by centrifugation and washed 4 times with 0.5 ml RIPA buffer (unsupplemented). The final pellets were resuspended in 45 µl of 2 X SDS-PAGE loading buffer (1 mM urea, 83 mM Tris, pH 6.8, 2.5% SDS, 12.5% ß-mercaptoethanol, 27% glycerol, bromphenol blue) and boiled for 3 minutes prior to loading on SDS-PAGE gels.

5.b. Protein Electrophoresis

Discontinuous SDS-Polyacrylamide gel electrophoresis using a ratio of acrylamide:N,N'-methylenebisacrylamide of 30:0.8 was used to separate cell extracts and immunoprecipitates. The concentration of acrylamide in the gels ranged from 10-12%, depending on the size of the proteins being analyzed. The separating gels contained the acrylamide solution in 0.375 M Tris, pH 8.8, 0.1% SDS and the stacking gels contained 5% acrylamide in 0.12 M Tris, pH 6.8, 0.1% SDS. The addition of 0.05% ammonium persulphate and 0.5 µl/ml of N,N,N',N'-tetramethylethylenediamine (TEMED) served to polymerize the gels. Electrophoresis buffer contained 25 mM Tris, 192 mM glycine and 0.1% SDS. Either [^{14}C]-methylated protein molecular weight markers (Amersham Canada Ltd.) ranging from MW 14,300-200,000 or prestained protein markers (Amersham Canada Ltd.) from MW 14,300-200,000 were used as marker proteins as appropriate. Gels were run in a BioRad Protean II Slab cell gel apparatus at 5 mAmps or 18 mAmps.

Fluorography was used to enhance the detection of labelled

proteins. After electrophoresis, the gels were soaked in two changes of 300 ml dimethylsulphoxide (DMSO) for 30 minutes, 400 ml of DMSO containing 22% 2,5-diphenyloxazole (PPO) for 1 hour and then in distilled water for 15 minutes. Gels were then transferred to Whatman chromatography paper, dried using a gel drier and exposed to Kodak X-OMAT AR film at -80°C for 3-7 days.

5.c. Western Immunoblotting

After electrophoresis, a BioRad transfer tank was used to transfer proteins from acrylamide gels to nylon membranes. The gels were presoaked in transfer buffer (20 mM Tris, 150 mM glycine, pH 8.0, 20% (v/v) methanol) for 15 minutes. Simultaneously, a piece of Immobilon P nylon membrane (Millipore) was cut to the size of the gel, wet with methanol, soaked in distilled water for 2 minutes and then in transfer buffer for 10 minutes. A gel/membrane "sandwich" was then assembled containing two pieces of Whatman chromatography paper soaked in transfer buffer, the gel, the membrane and finally two more pieces of Whatman paper. Air bubbles were removed using a 10 ml pipet as a rolling pin and the sandwich placed in the plastic support and then into the transfer tank with the membrane facing the positive electrode. Proteins were transferred overnight at 25V or 50V for 4 hours at 4°C.

After the transfer, the membranes were blocked by incubating in PBS containing 5% skim milk powder with shaking for 2 hour at RT. Membranes were then incubated for 1-3 hours at RT in PBS-5% skim milk containing the appropriately diluted primary antibody. Three washes of 10 minutes each in large volumes of PBS were

performed before incubating the membrane for 1 hour at RT in PBS-5% skim milk containing the appropriate secondary antibody conjugated to horseradish peroxidase. The membranes were washed as above and the proteins detected by enhanced chemiluminescence (ECL) (Amersham). Briefly, the membranes were placed on Saran Wrap, protein side up, and covered with 0.125 ml/cm^2 of an equal volume mixture of detection reagent one and detection reagent two (Amersham Canada Ltd.) for 1 minute. The detection reagents are a mixture of hydrogen peroxide and luminol, which is oxidized by the horseradish peroxidase conjugated to the secondary antibody. Luminol is left in an excited state following this oxidation reaction which decays to the ground state with the resultant emission of light. The detection reagents were removed from the blot and the blot placed between two overhead transparencies for exposure to Kodak X-OMAT AR film at RT.

5.d. Luciferase Assay

To perform luciferase assays, cells in 60 mm dishes were counted and infected with a luciferase reporter or an appropriate control at a multiplicity of infection (MOI) of 10. The cells were harvested at various time points by the following procedure. The supernatant was aspirated, and the cells washed three times with PBS. 500 µl of 0.1 M potassium phosphate (pH 7.8) with 1mM dithiothreotol (DTT), was added to each dish before the monolayers were scraped with a rubber policeman. The cells and solution were transferred to an Eppendorf tube and kept on ice until they were pelletted by centrifugation at 4°C. The supernatants were

aspirated and the pellet resuspended in 100 µl of 0.1 M potassium phosphate (pH 7.8), 1 mM DTT, by brief vortexing. Samples were stored at -80°C until the day of the assay. At this point, samples were subjected to two freeze-thaw to release the luciferase from inside the cells. The cellular debris was pelleted by centrifugation at 4°C and discarded. The supernatants were appropriately diluted.

The assay was performed as follows. 20 µl of the diluted sample was added to 200 µl of luciferase assay buffer (25 mM glycyl glycine, pH 7.8, 5 mM ATP, 15 mM magnesium sulphate) in a luciferase assay tube (Sarstedt Inc., St-Laurent, Québec, Canada). The tube was placed in the luminometer chamber of a Lumat 9501 luminometer (Berthold Systems Inc., Pittsburg, PA, USA) where 100 µl of luciferin solution (1 mM luciferin (Boehringer-Mannheim Inc., Laval, Québec, Canada)) in .1 M potassium phosphate buffer, pH 7.8). This addition of the substrate initiated the enzyme reaction which evolved light that was read by the luminometer. The luminometer reading of dilution buffer alone typically resulted in a reading of 100-200 relative light units, which was subtracted from the samples before quantitating expression. The amount of luciferase in the samples was quantified by comparing relative light unit readings to a standard curve generated using known amounts of purified luciferase (Boehringer-Mannheim Inc., Laval, Québec, Canada). All values are expressed as time point means ± standard error of the mean.

5.e. ß-Galactosidase Assay

To perform ß-galactosidase assays, cells in 60 mm dishes were counted and infected with a ß-galactosidase reporter at an MOI of 10 and the cells were then harvested at various times post infection. The cells were harvested in Promega 1 X Reporter Lysis Buffer according to the manufacturer's specifications. Serial dilutions of the cell extracts were made in 1 X Reporter Lysis Buffer (typically 10^{-1} and 10^{-2}). 40 µl of each dilution in the series was added to 350 µl of mercaptoethanol solution (10 mM KCl, 1 mM $MgSO_4$, 100 mM sodium phosphate, pH 7.5, 50 mM 2-mercaptoethanol) and incubated at 37°C for 5 minutes. 132 µl of ONPG solution (13.3 mM o-nitrophenol ß-D-galactopyranoside (ONPG) (Sigma) in 100 mM sodium phosphate, pH 7.5) was then added and the samples incubated at 37°C for 1 hour. ß-galactosidase hydrolyzes colourless ONPG to o-nitrophenol which is yellow. Reactions were terminated by adding 172 µl of 1 M Na_2CO_3 and the absorbance measured at 420 nm in a Beckman spectrophotometer. To quantitate ß-galactosidase expression, 1 mg/ml commercial ß-galactosidase was serially diluted and assayed as above. Activity is expressed as µg ß-galactosidase produced per 10^6 cells.

6. Enzyme-Linked Immunosorbent Assay (ELISA) for Interleukin-12

Flat-bottom 96 well microtitre plates (NUNC Maxicorp) were coated with 18 µg/ml (in dilution buffer : PBS, 0.0005% Tween-20, 2% FCS) anti-IL-12 polyclonal rabbit antiserum at 4°C overnight. The following day, the wells were washed three times with PBS. Wells were blocked by the addition of 2% BSA in PBS for 90 minutes

at 37°C to prevent nonspecific binding. The wells were washed three times with PBS prior to the addition of the samples and a 60 minute incubation at 37°C. The wells were washed 5 times with PBS. Biotinylated anti-IL-12 antibody (18 µg/ml dilution buffer) was added and the wells incubated for 45 minutes at 37°C. The wells were washed three times with PBS and the avidin-conjugated horseradish-peroxidase (Bio-Rad) was added for a 30 minute incubation at 37°C. Following three more washes with PBS, the substrate for peroxidase O-phenyldiamine dihydro chloride (Pierce) was added. The reaction was terminated after 5 minutes by the addition of 50 µl 3N H_2SO_4. Serially diluted recombinant protein served as a positive control in each analysis.

7. Proliferative Bioassay for Interleukin-12

The IL-12 proliferative bioassay was performed as described by Schoenhaut *et al.* (1992). Splenocytes (10^6 cells/ml) were cultured in RPMI 1640 medium, supplemented with 10% (v/v) FCS, 100 units/ml penicillin G, 100 µg/ml streptomycin sulphate, 50 mM ß-mercaptoethanol, 20 U/ml human rIL-2 and 2 µg/ml of concavilin A (ConA) (Sigma). At 48 to 72 hours, the splenocytes were harvested, washed in PBS and resuspended at 4 x 10^5 cells/ml. An aliquot of 50 µl of this suspension was added to each well of a flat bottomed 96 well plate and mixed with 50 µl aliquots of serial dilutions of supernatants of infected cells. These cultures were incubated at 37°C for 24 hours in a CO_2 incubator prior to the addition of 0.05 µCi of [^{3}H]thymidine (Amersham) in 50 µl of RPMI. The cultures were again incubated for 24 hours in a CO_2 incubator. The

following day, a cell harvester was used to harvest the cells onto glass fibre filters prior to the quantitation of [^{3}H]TdR incorporation into cellular DNA by liquid scintillation counting.

Chapter 3 : HCMV/MCMV Promoter/Enhancer Study

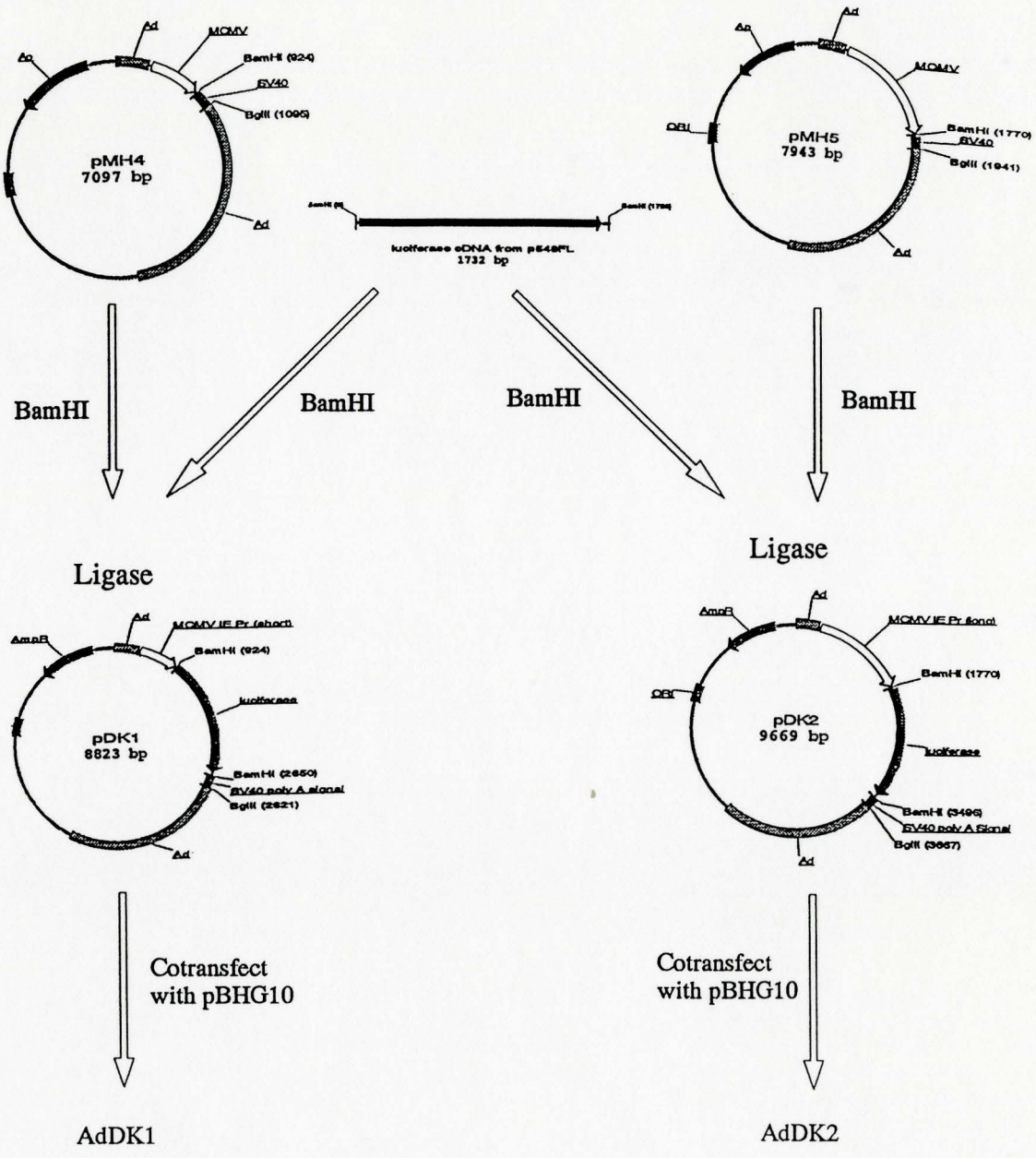
In adenovirus vectors, the HCMV promoter/enhancer was observed to express 10-50-fold lower levels of transgene in murine cells lines compared to human cell lines. To determine whether this difference in expression levels between the species was due to the infection efficiency of the adenoviral vectors or due to some species preference of the promoter, two fragments (0.5 kb and 1.4 kb) were derived from the MCMV IE promoter/enhancer and utilized in adenovirus vectors.

Previous comparisons used the *lacZ* cDNA as a reporter under the transcriptional control of the three aforementioned promoter/enhancers (C. Addison, personal communication). To extend and generalize this previous study, the firefly luciferase cDNA was used as a reporter gene under the control of the same three promoter/enhancer fragments.

To generate a reporter virus expressing the luciferase cDNA driven from each of two different fragments of the MCMV IE promoter, two plasmids were constructed (pDK1 and pDK2) and then cotransfected into 293 cells with pBHG10. A schematic is given in Figure 3.1 and the construction is described below. The plasmid p548FL was digested with BamHI, and the fragment containing the full-length, intronless cDNA was gel-purified from the parental plasmid. pMH4 was digested with BamHI and gel-purified from uncut circular plasmid to reduce parental contamination in later

Figure 3.1. Construction of plasmids pDK1 and pDK2 and the rescue of AdDK1 and AdDK2.

The BamHI-restricted luciferase cDNA was inserted into the shuttle plasmids pMH4 and pMH5 which represent the short (.5 kb) and long (1.4 kb) fragments of the MCMV IE promoter, respectively. The plasmids produced, pDK1 and pDK2, were cotransfected with pBGH10 into 293 cells. In vivo recombination between the shuttle respective shuttle plasmid and pBHG10 yielded the viruses AdDK1 and AdDK2.


pMH4
7097 bp
MCMV
BamHI (924)
SV40
BglII (1095)
Ad
Ap
luciferase cDNA from pS48FL
1732 bp
pMH5
7943 bp
MCMV
BamHI (1770)
SV40
BglII (1941)
ORI
BamHI
BamHI
BamHI
BamHI
Ligase
Ligase
pDK1
8823 bp
AmpR
MCMV IE Pr (short)
BamHI (924)
luciferase
BamHI (2650)
SV40 poly A signal
BglII (2821)
pDK2
9669 bp
MCMV IE Pr &ont
BamHI (1770)
luciferase
BamHI (3496)
SV40 poly A Signal
BglII (3667)
Cotransfect
with pBHG10
Cotransfect
with pBHG10
AdDK1
AdDK2

analyses. This fragment was ligated to the luciferase cDNA to produce pDK1. pDK2 was produced similarly except that the vector was pMH5 instead of pMH4.

pDK1 and pDK2 DNA were each purified by CsCl gradient ultracentrifugation and combined with pBHG10 DNA and cotransfected, as described in Chapter 2, into 293 cells. Recombination between pDK1 and pBHG10 generated the recombinant adenovirus AdDK1 while the recombination between pDK2 and pBHG10 generated the recombinant adenovirus AdDK2. The correct recombinants were plaque purified prior to the preparation of crude lysates as detailed in the Materials and Methods. Figure 3.2. shows restriction enzyme digests of AdDK1 and AdDK2 and a schematic of the left termini of the genomes.

Reporter Expression Experiments

Once the construction of AdDK1 (-1336 to +36 of the MCMV IE promoter/enhancer) and AdDK2 (-491 to +36 of the MCMV IE promoter/enhancer) was complete, it was possible to produce high titre crude lysates and infect different cell types *in vitro* to assess the levels of luciferase produced. The positive control in all cases was AdCA18 (constructed by C. Addison, Department of Biology, McMaster University, Hamilton, Canada), an E1-replacement virus with the -299 to +72 fragment of the HCMV IE promoter/enhancer directing transcription of the luciferase cDNA rightward. The negative control was a non-infected series of dishes that received 200 µl of PBS^{++} instead of 200 µl of virus. As described in the materials and methods, the five cell lines used

Figure 3.2. Restriction enzyme digests of AdDK1 and AdDK2 with appropriate diagnostic enzymes.

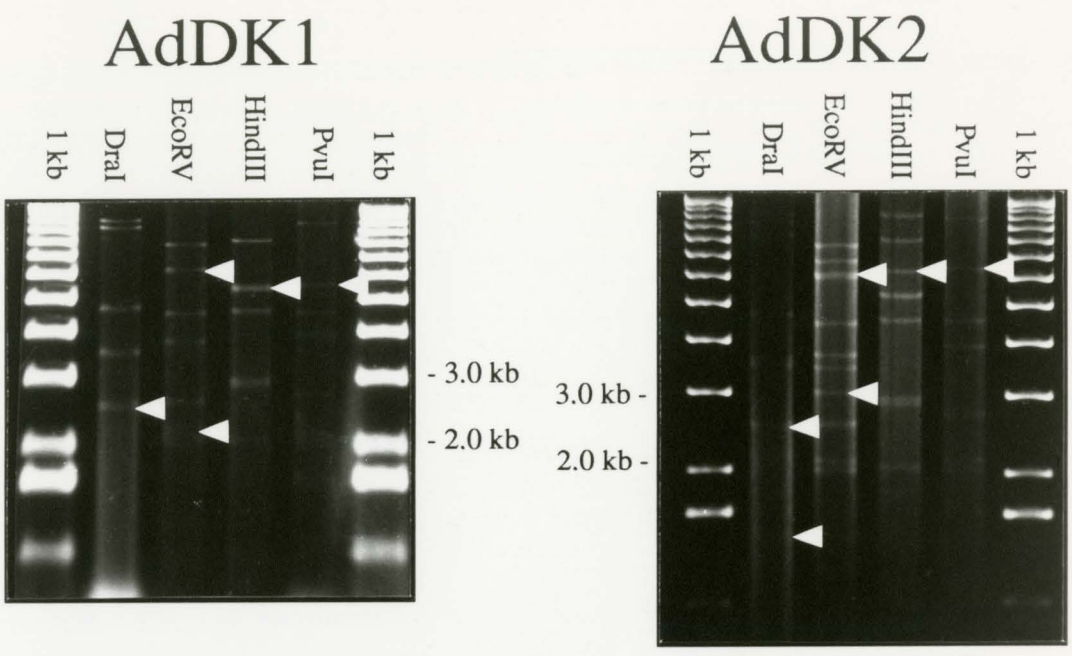
Viral DNA was prepared and digested as previously described. The enzymes DraI, EcoRV, HindIII and PvuI were used and the expected patterns are indicated below. The fragment sizes accompanied by asterisks (*) indicate fragments containing part of the expression cassette (from the 5′ end of the promoter to the 3′ end of the SV40 polyadenylation signal). These same bands are marked on the photographs with small white arrows.

		AdDK1		
Fragment	DraI	EcoRV	HindIII	PvuI
A	10045	7637	8010	9774
B	9227	6208*	5541*	5620*
C	4796	4546	5322	4553
D	3575	3808	4597	3942
E	2655*	2623	3012	2782
F	815	2289*	2937	2174
G	789	2179	2081	2062
H	674*	2052	1010	1669
I		1238	75	

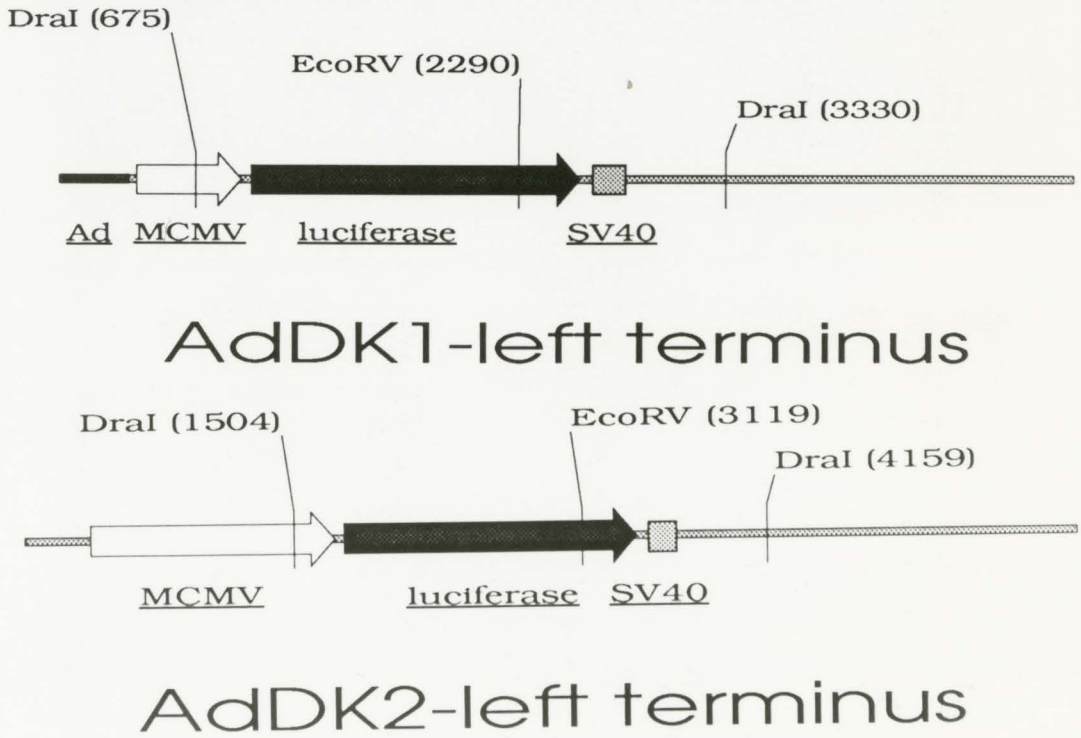
		AdDK2		
Fragment	DraI	EcoRV	HindIII	PvuI
A	10045	7637	8010	9819
B	9227	6208*	6385*	6464*
C	4796	4546	5322	4553
D	3575	3808	4597	3942
E	2655*	3133*	3012	2782
F	1518*	2623	2937	2174
G	834	2220	2081	2062
H	815	2052	1010	1669
I		1238	75	

Repetitive Cellular Bands		
8000	6800	10000
	3400	

Panel A. AdDK1 and AdDK2.



Panel B. Left termini of AdDK1 and AdDK2.



were SV329 and MT1A2 (transformed murine), 1° Rat (untransformed rat), MDCK (untransformed canine) and MRC5 (untransformed human).

The data for each cell line are plotted as a four-day time course of nanograms of luciferase per million cells plus or minus the standard error of the mean. Shown in Figure 3.3 are five sample graphs, one for each cell line. Raw data from day 2 post infection are also presented with the ratios of AdDK1/AdCA18 and AdDK2/AdCA18 in Table I. Day 2 was chosen for these comparisons as it is at this day that luciferase expression for most infected cell lines (MRC5 excepted) reaches its peak and is therefore also the timepoint at which the difference in transcription activity is most pronounced.

The kinetics of luciferase expression and the ratios of AdDK1/AdCA18 and AdDK2/AdCA18 are reasonably reproducible from experiment to experiment and in this, the data presented are representative. However, as is obvious from the raw data, interexperimental variance within each cell line is high.

To convincingly show that the variance from experiment to experiment is not due to any of the assay reagents, eight separate standard curves were plotted in Figure 3.4.

This Figure shows that, although there are small differences in the standard curves, these represent less than an order of magnitude, which is too small to account for the differences from experiment to experiment. This will be considered in the discussion.

Figure 3.3. Graphs of timecourse experiments of nanograms of luciferase expression per million cells infected by AdDK1, AdDK2 or AdCA18.

The cell lines SV329 (panel A), MT1A2 (panel B), 1° Rat (panel C), MDCK (panel D) or MRC5 (panel E) were grown to 70-95% confluence and infected at an MOI of 10 with either AdDK1 (.5 kb fragment of the MCMV promoter), AdDK2 (1.4 kb fragment of the MCMV promoter) and AdCA18 (HCMV promoter). Extracts were prepared at various times post infection and assayed for luciferase activity as described in the materials and methods.

SV329 is an SV40 transformed murine fibroblast line and was infected between passages 52-58. MT1A2 is a polyoma middle T antigen-transformed murine epithelial line and was infected between passages 18 and 20. The 1° rat line is a primary rat lung fibroblast line explanted from Sprague-Dawley rats and was infected between passages 12 and 15. MDCK cells are a canine kidney cell line (ATCC CCL 34) and passages 18-25 were infected. MRC5 is a primary human fetal lung fibroblasts (ATCC CCL 171) and was infected between passages 38 to 46.

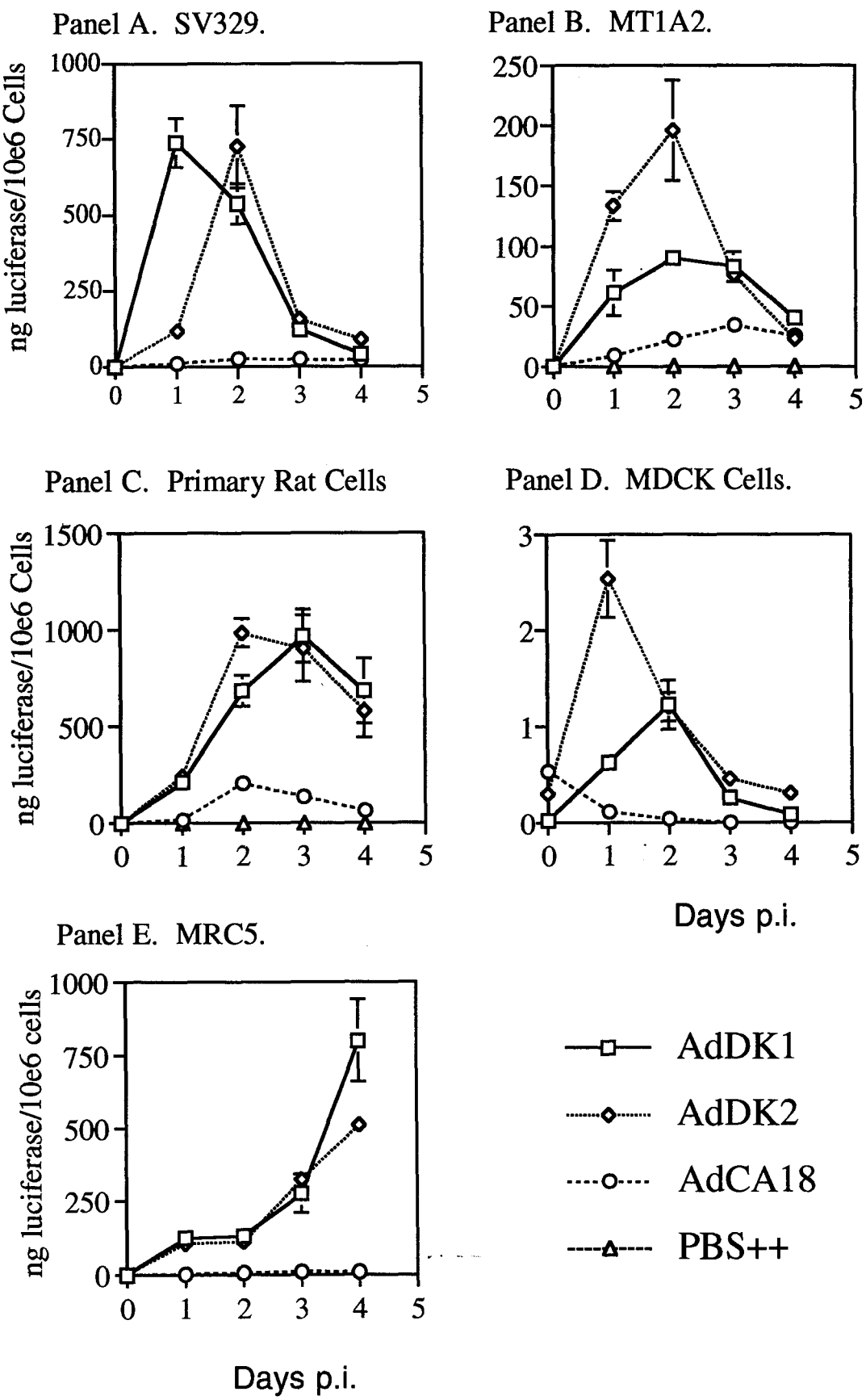

Panel A. SV329.
Panel B. MT1A2.
Panel C. Primary Rat Cells
Panel D. MDCK Cells.
Panel E. MRC5.
ng luciferase/10e6 Cells
ng luciferase/10e6 cells
Days p.i.
AdDK1
AdDK2
AdCA18
PBS++

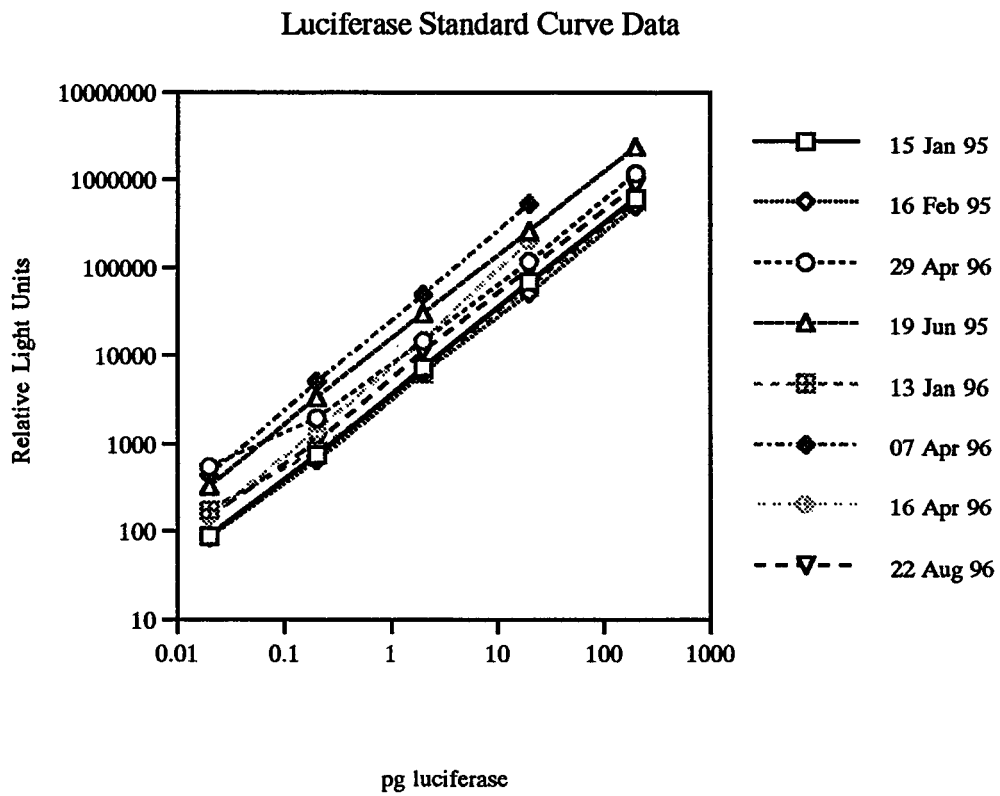
Table I. Absolute levels of luciferase and ratios of luciferase activities at day 2 post infection using adenovirus vectors containing the luciferase cDNA under the transcriptional control of the .5 kb (AdDK1) or 1.4kb (AdDK2) fragment of the MCMV IE promoter/enhancer or the HCMV promoter/enhancer (AdCA18).

	Luciferase Expression (ng/10^6 cells)[a]			Ratios	
Exp't #	AdDK1	AdDK2	AdCA18	AdDK1 / AdCA18	AdDK2 / AdCA18
SV329					
1	14	9	2.3	6	4
2	538±66	725±137	26±1.2	21±2	29±5
MT1A2					
1	160±15	761±200	25±4	6±.6	31±8
2	90±2	196±42	22±2	4±.4	9±2
1° Rat Cells					
1	683±80	983±73	208±16	3.3±.4	4.8±.4
2	610±97	853±108	36±2	17±3	24±3
3	66,000	37,000	1,660	39	22
MDCK Cells					
1	.47	.55	.12	3.9	4.6
2	5.5±.8	12.4±1.4	.23±.02	24±3	53±6
3	1.2±.25	1.2±.15	.4±.06	30±6	30±4
MRC5 Cells					
1	6250	2000	1250	7±3	2±1
2	453	111	65	7	2
3	170±17	110±24	7±.4	24±4	16±6

a The cell lines SV329, MT1A2, 1° Rat, MDCK or MRC5 were grown to 70-95% confluence and infected at an MOI of 10 with either AdDK1 (.5 kb fragment of the MCMV promoter), AdDK2 (1.4 kb fragment of the MCMV promoter) and AdCA18 (HCMV promoter). Extracts were prepared at various times post infection and assayed for luciferase activity as described in the materials and methods.

Figure 3.4. A comparison of a sample of standard curves of the luciferase assays.

Eight sample standard curves are plotted as picograms of luciferase versus relative light units. Standard curves were produced by ten-fold serial dilution of commercially available luciferase and were assayed as detailed in the materials and methods.


Luciferase Standard Curve Data
Relative Light Units
10000000
1000000
100000
10000
1000
100
10
0.01
0.1
1
10
100
1000
pg luciferase
15 Jan 95
16 Feb 95
29 Apr 96
19 Jun 95
13 Jan 96
07 Apr 96
16 Apr 96
22 Aug 96

Western Blot of Luciferase Expression in MRC5 and 1° Rat Cells

Due to the variability in absolute values of luciferase between the experiments, it seemed wise to seek corroborative evidence of luciferase expression.

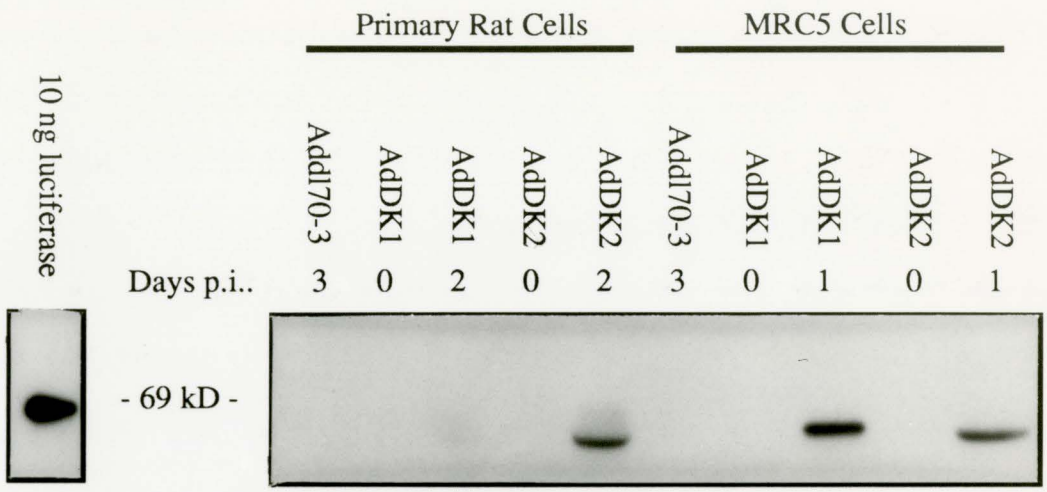
MRC5 cells or 1° Rat cells were infected at an MOI of 10 pfu per cell with either Add170-3 (an $E1^-E3^-$ adenovirus (Bett *et al.*, 1994) carrying no transgene that is often used as a control), AdDK1 (luciferase cDNA under the transcriptional control of the short MCMV IE promoter/enhancer) or AdDK2 (luciferase cDNA under the transcriptional control of the long MCMV IE promoter/enhancer). At the indicated timepoints, the cells were harvested as described in Chapter 2. Protein concentration quantification, polyacrylamide gel electrophoresis and protein immunoblotting were all performed as described in Chapter 2. Two autoradiographs (3 minute exposure and 20 minute exposure) are presented in Figure 3.5.

Although the enhancement system makes quantitation of band intensities very difficult, a few rough estimations indicate that the amounts of luciferase shown in Figure 3.5 correspond to the same range of values. Each dish was harvested in 500 µl RIPA buffer and between 5-15 µl (roughly 1/50th) of each sample was loaded into each well. Even at the 4 minute exposure, the 10 ng of recombinant luciferase is very dark, although not as dark as the 20 minute exposure. Compared to the luciferase control, AdDK1, day 1 of MRC5 cells appeared to be approximately 1/5th the intensity and therefore, by rough approximation, 2 ng of luciferase. As 1/50th of the sample, the equates to approximately 100 ng/10^6 MRC5 cells.

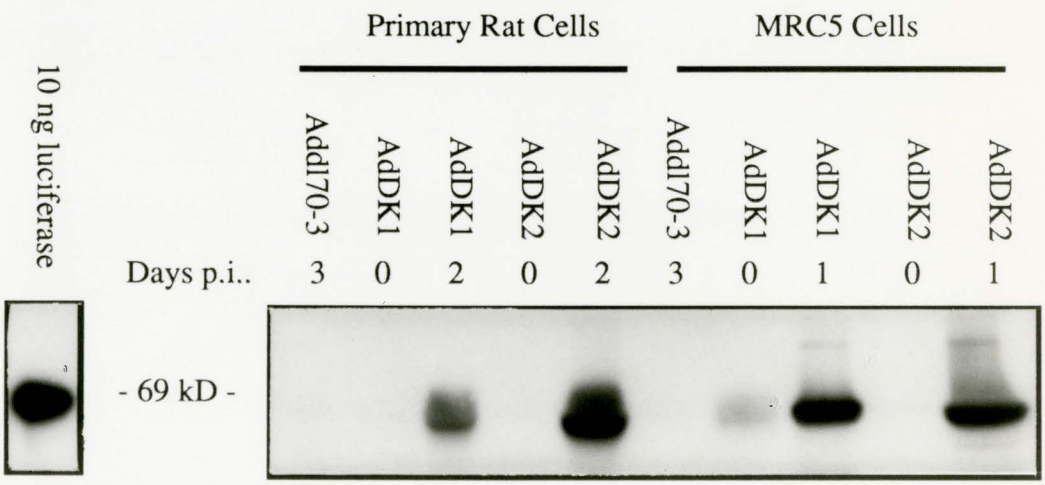
Figure 3.5. Autoradiograph of an immunoblot of infected cells probed with a polyclonal anti-luciferase antiserum.

MRC5 and 1° Rat cells were infected at an MOI of 10 pfu per cell with either AdDK1 (short MCMV IE promoter/enhancer), AdDK2 (long MCMV IE promoter/enhancer) or Addl70-3 ($E1^-$, $E3^-$ adenovirus negative control) and harvested at the indicated times in RIPA buffer. 25 μg of total protein were loaded in each lane.

Panel A. Three minute exposure.



Panel B. Twenty minute exposure.



This amount agrees with Figure 3.3, panel E. Compared to AdDK1, day 1 of MRC5 cells, AdDK2, day 2 of the 1° Rat cells appeared to be approximately the same intensity and therefore, by rough approximation, 2 ng of luciferase. This yields a rough estimate of approximately 100 ng/10^6 cells, which agrees with Figure 3.3, panel C.

The results of the luciferase assay and the anti-luciferase immunoblot clearly indicated the superior transcriptional activity of either MCMV promoter/enhancer over the HCMV promoter/enhancer, so construction of the bicistronic system was begun using the MCMV promoter/enhancer. However, it remained unclear which fragment of the MCMV promoter/enhancer was more active, so all cloning steps were carried out for both promoter/enhancer fragments.

Chapter 4 : A Bicistronic IL-12 Vector

The aim of this project was twofold. The first goal was the construction of a recombinant adenovirus expressing IL-12 in E1 from a single promoter. If this virus expressed high levels of IL-12 upon infection of mammalian cells, it was to be assessed for its potential for tumour immunotherapy. As this would be the first adenoviral system using a poliovirus IRES, the second aim was to design the shuttle plasmids so they would also be capable of accomodating the cloning of other cDNAs for any future work. This system would be characterized by the construction of viruses with reporter cDNAs in both cistrons. The construction strategy is shown in Figure 4.1 and is described below.

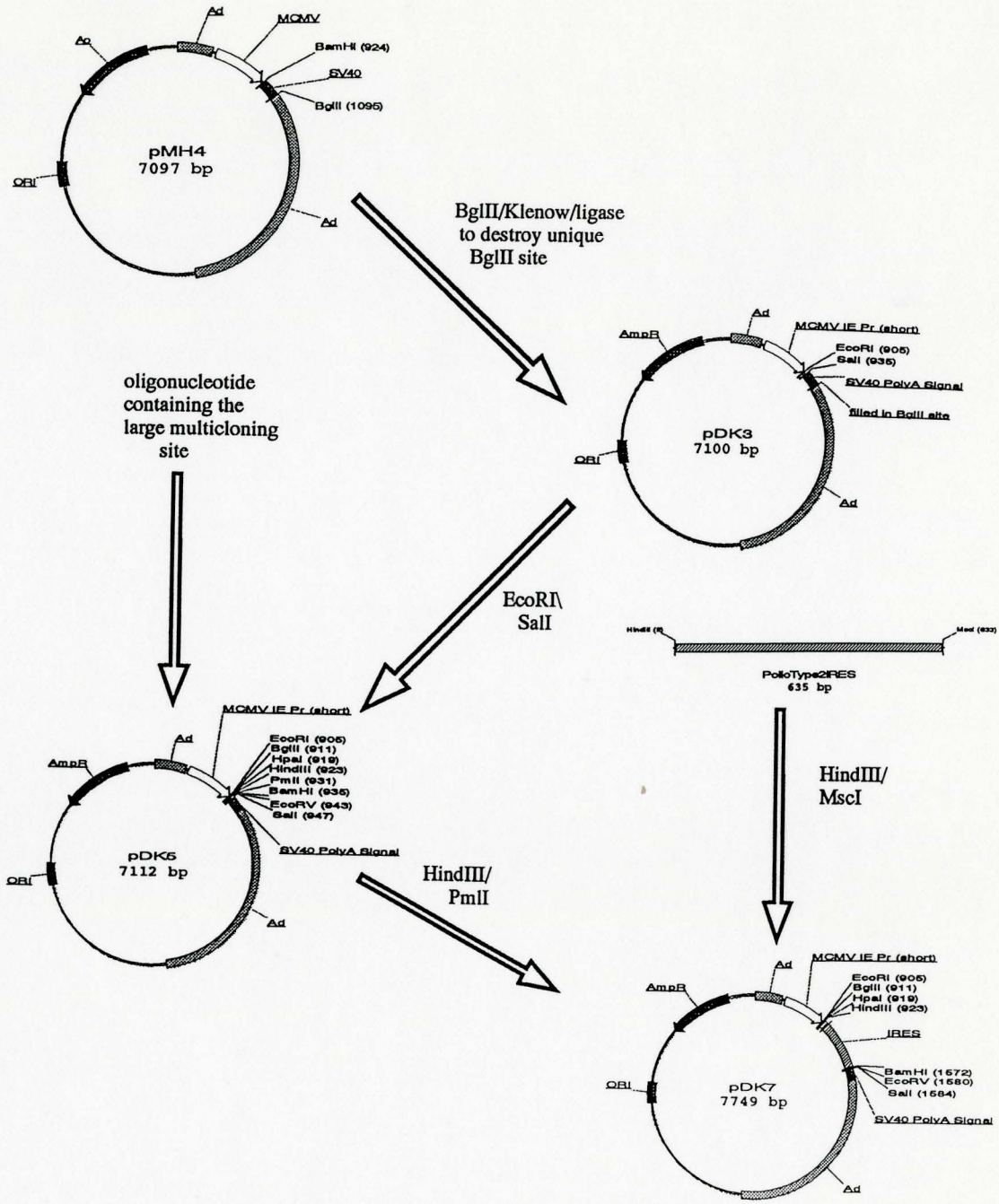
The restriction enzymes BglII and BamHI produce compatible cohesive ends, whereas restriction enzymes EcoRV and HpaI produce blunt ends. If one restriction enzyme from each of these two sets were included in each cistron of the mutlicloning site, then any given cDNA could be accomodated in either site (5′ or 3′ of the IRES). Additionally, the first cistron also possessed unique EcoRI and HindIII sites, while the second cistron contained a unique SalI site.

To accomplish this, the existing, unique BglII site in pMH4 and pMH5 was inactivated by digesting each plasmid with BglII, filling in the 3′ recessed terminus and religating each plasmid to itself. This process produced pDK3 (.5 kb fragment of the MCMV IE

Figure 4.1. Construction of pDK3, pDK5 and pDK7.

The unique BglII site in pMH4 was destroyed by treatment with Klenow after digestion with BglII. Upon insertion of an oligonucleotide (detailed in figure 2.2) containing the multicloning site, the poliovirus type II IRES was inserted, splitting the multicloning site in two, each smaller site capable of accepting inserts with blunt or BamHI compatible ends.

The plasmids pDK4, pDK6 and pDK8 are identical to pDK3, pDK5 and pDK7 respectively except that instead of the short MCMV IE promoter/enhancer, they carry the long MCMV IE promoter/enhancer.


Ad
MCMV
Ap
BamHI (924)
SV40
BglII (1095)
pMH4
7097 bp
ORI
Ad
BglII/Klenow/ligase
to destroy unique
BglII site
AmpR
Ad
MCMV IE Pr (short)
EcoRI (905)
SalI (935)
SV40 PolyA Signal
filled in BglII site
pDK3
7100 bp
ORI
Ad
oligonucleotide
containing the
large multicloning
site
EcoRI\
SalI
PolioType2IRES
635 bp
HindIII/
MscI
MCMV IE Pr (short)
Ad
AmpR
EcoRI (905)
BglII (911)
HpaI (919)
HindIII (923)
PmlI (931)
BamHI (935)
EcoRV (943)
SalI (947)
SV40 PolyA Signal
pDK5
7112 bp
ORI
Ad
HindIII/
PmlI
MCMV IE Pr (short)
Ad
AmpR
EcoRI (905)
BglII (911)
HpaI (919)
HindIII (923)
IRES
BamHI (1672)
EcoRV (1580)
SalI (1584)
SV40 PolyA Signal
pDK7
7749 bp
ORI
Ad

promoter/enhancer) and pDK4 (1.4 kb fragment of the MCMV IE promoter/enhancer).

To replace the existing multicloning site of pDK3 and pDK4 with one that would accomodate the poliovirus (type II) IRES and subsequent inserts 5′ and 3′ of the IRES, pDK3 and pDK4 were digested with EcoRI and SalI, removing the existing multicloning site. The oligonucleotides AB5352 and AB5353 were annealed to each other (see Figure 2.2) and ligated into each plasmid. These new plasmids were called pDK5 (.5 kb fragment of the MCMV IE promoter/enhancer) and pDK6 (1.4 kb fragment of the MCMV IE promoter/enhancer).

The poliovirus (type II) IRES was excised from the plasmid pP2CAT with the restriction endonucleases HindIII and MscI, producing a fragment 635 nucleotides long. This frgament was gel-purified from the parental plasmid fragment. pDK5 and pDK6 were each restricted with HindIII and PmlI and gel-purified from uncut parental vector plasmid to facilitate later analyses. The restriction enzymes MscI and PmlI both produce non-cohesive termini, and the IRES fragment and the vector fragments were ligated, generating the plasmids pDK7 and pDK8.

The products, pDK7 and pDK8, were the shuttle plasmids from which all the bicistronic constructs in this project were derived.

The Generation and Expression of AdDK6

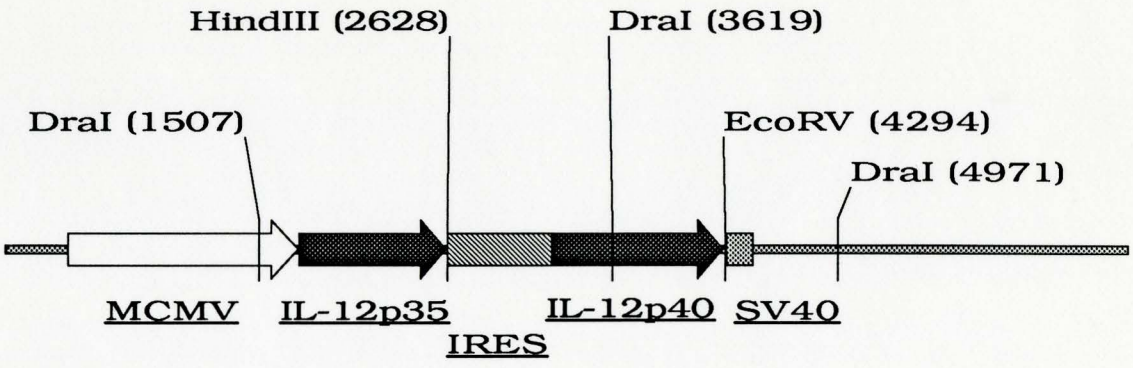
The plasmid pVL2IL-12p35, containing the IL-12p35 cDNA, was restricted with BamHI and EcoRV and gel-purified from the parental vector. The shuttle plasmids pDK7 and pDK8 were each restricted

with BglII and HpaI to open the first cistron (5' of the IRES) and gel-purified from any uncut plasmid to reduce parental contamination in later analyses. BglII and BamHI produce compatible cohesive termini, while HpaI and EcoRV produce compatible non-cohesive termini. The IL-12p35 cDNA fragment was ligated into each restricted shuttle plasmid to produce pDK13 (short MCMV IE promoter/enhancer) and pDK14 (long MCMV IE promoter/enhancer).

The plasmid pCMVp40, containing IL-12p40 cDNA, was restricted with BamHI, yielding the IL-12p40 cDNA which was gel purified from the parental plasmid. This fragment was subcloned into BamHI restricted pDK13 and pDK14. These ligations yielded the plasmids pDK15 and pDK16. pDK15 and pDK16 were each purified by CsCl gradient ultracentrifugation, combined with similary purified pBHG10 and cotransfected into 293 cells. The recombination of pDK15 with pBHG10 resulted in the virus AdDK5. The recombination between pDK16 and pBHG10 produced AdDK6. AdDK6 plaques were picked, analyzed by restriction enzyme digests and the correct recombinants were plaque purified before scaling up to produce crude lysates.

In screening potential AdDK5 recombinants, the viral DNA from four different plaques was not visible above the background of 293 cellular DNA. In an attempt to analyze viral DNA, four crude lysates were produced. Each grew slowly (5-7 days from infection to harvest) and the titres were $3.5x10^6$ pfu/ml, 10^5 pfu/ml, 10^5 pfu/ml, 10^4 pfu/ml. Figure 4.2 shows the left terminus of AdDK6

Figure 4.2. Diagram of the left terminus of AdDK6 showing some diagnostic restriction enzyme sites.



AdDK6-left terminus

with the restriction enzymes used in Figure 4.3 where pDK15, pDK16 and AdDK6 were digested with appropriate diagnostic enzymes.

The expression of AdDK6 was analyzed by several methods. Firstly, the supernatants and extracts of ^{35}S-labelled, infected cells were immunoprecipitated with a polyclonal antiserum to IL-12 and the immunoprecipitate electrophoresed of an polyacrylamide gel. Secondly, supernatants from infected cells were used in an ELISA, using the same antiserum. Lastly, supernatants from infected cells were mixed with IL-2/ConA-stimulated splenocytes, which were then labelled with [^{3}H]-thymidine and evaluated for incorpoartion of the label into cellular DNA, which is a measure of proliferation, a hallmark of IL-12. The results of these analyses are described below.

Subconfluent 293, MRC5, 1° rat or MT1A2 cells were infected at an MOI of 10 pfu per cell with either AdDK1 (luciferase cDNA under the transcriptional control of the MCMV IE promoter/enhancer), AdmIL-12.1 (IL-12p35 cDNA and IL-12p40 cDNA under the transcriptional control of two separate HCMV IE promoter/enhancers), AdDK6 (bicistronic IL-12 cassette under the transcriptional control of the MCMV IE promoter/enhancer), or 200 µl of PBS. At 48 hours, 1 ml of supernatant was removed and frozen at -70°C for the ELISA and proliferative bioassay. The cells were labelled and the supernatants (sup) and cell extracts (ext) harvested as described in the materials and methods.

The autoradiograph of the 1° Rat cell infection is shown in Figure 4.4. AdDK1 sup and AdDK1 ext are a negative control for IL-

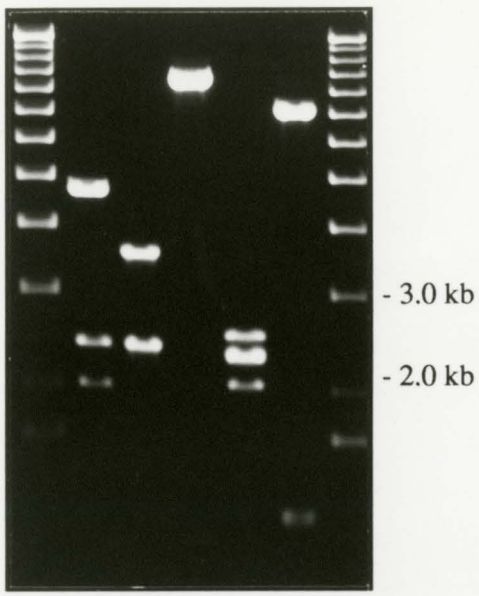
Figure 4.3. Restriction enzyme digests of pDK15 (Panel A), pDK16 (Panel B) and AdDK6 (Panel C). pDK16 plasmid DNA and AdDK6 viral DNA was prepared and digested as previously described. The expected patterns are indicated below. The fragment sizes accompanied by asterisks (*) indicate fragments containing part of the expression cassette (from the 5′ end of the promoter/enhancer to the 3′ end of the SV40 polyadenylation signal). These same fragments are marked on the photograph with a small white arrow.

		pDK15			
Fragment	AflIII	AvaI	BamHI	BglI	XbaI
A	4789*	3678*	8622*	2748*	7316*
B	2218*	2442	1023*	2319	1122*
C	2054	2218*		2275*	953*
D	585*	1127*		2070	255*
E		180		234	

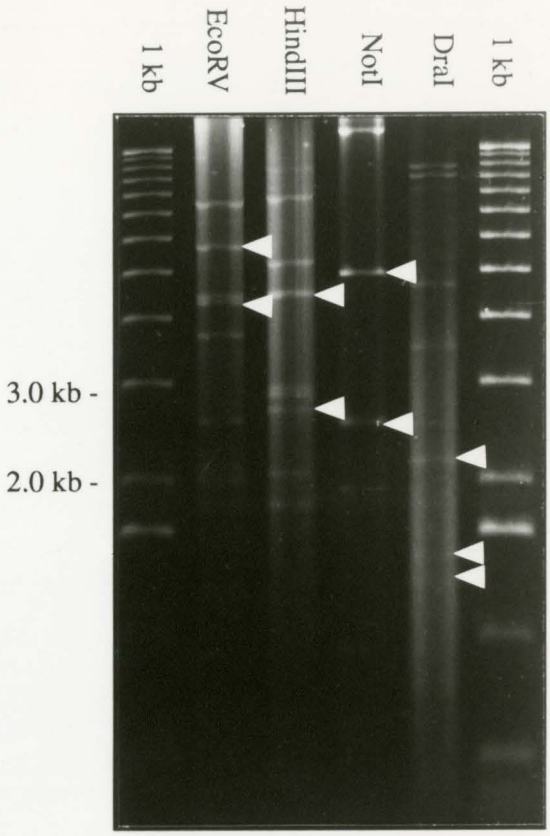
		pDK16			
Fragment	AflIII	AvaI	BamHI	BglI	XbaI
A	4789*	4524*	9455*	3580*	7316*
B	2054*	2442	1023*	2319	1968*
C	1431*	2204*		2275*	939*
D	1416*	1128*		2070	255*
E	788*	180		234	

		AdDK6		
Fragment	EcoRV	HindIII	NotI	DraI
A	7637	8010	15997	10054
B	5845*	5322	4999	9227*
C	4546	4597	4833*	4796
D	4282*	4555*	2589	3575
E	3808	3012	2554*	2112*
F	2623	2937	1931	1495*
G	2204	2616*	960	1352
H	2052	2081	326	818
I	1238	1010	46	815
J		75		

Panel A. pDK15.



Panel C. AdDK6.



Panel B. pDK16.

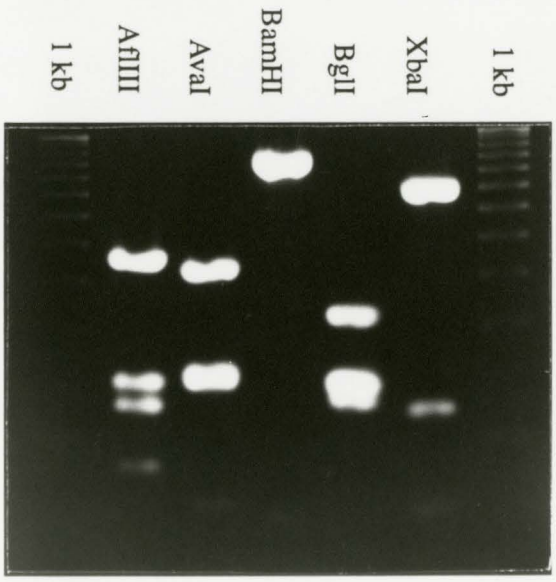
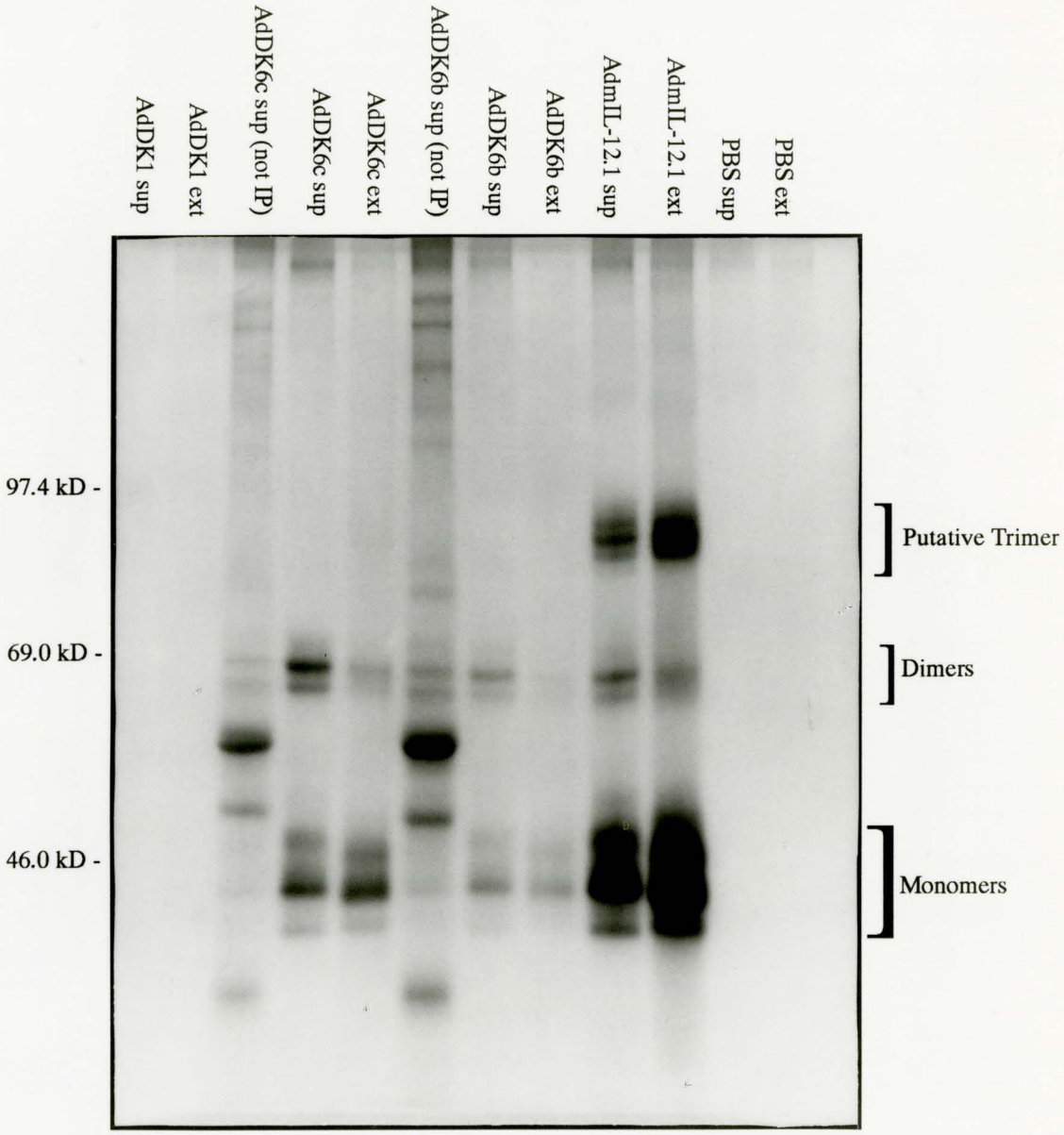


Figure 4.4 Autoradiograph of an immunoprecipitation of infected, ^{35}S-labelled 1° Rat cells with polyclonal anti-IL-12 antiserum.

1° Rat cells were grown to 80-90% confluence and infected at an MOI of 10 pfu per cell. At 48 hours post-infection, the cells were washed, labelled with ^{35}S-translabel for 2 hours and harvested in RIPA buffer plus inhibitors. The immunoprecipitated lanes represent 1/5th of each 500 µl sample, while the unprecipitated lanes (marked with not IP) represent 15 µl of the 500 µl samples, being untreated with antiserum or sepharose.


AdDK1 sup
AdDK1 ext
AdDK6c sup (not IP)
AdDK6c sup
AdDK6c ext
AdDK6b sup (not IP)
AdDK6b sup
AdDK6b ext
AdmIL-12.1 sup
AdmIL-12.1 ext
PBS sup
PBS ext
97.4 kD -
69.0 kD -
46.0 kD -
Putative Trimer
Dimers
Monomers

12 and also control for any proteins associated with the virus itself or the infection that may interact with the anti-IL-12 antiserum. PBS sup and PBS ext are also negative controls for IL-12, and control for any cellular feature that may interact with the anti-IL-12 antiserum. AdmIL-12.1 sup and AdmIL-12.1 ext are positive controls for Il-12, although it should be noted that the putative trimer (seen to be running below the 97.4 kD marker) is a feature so far observed only with this virus (Bramson *et al.*, 1995). AdDK6b and AdDK6c are two different clones of AdDK6, rescued from the same cotransfection experiment. As can be seen in those lanes, AdDK6 expressed an appropriately sized monomers and dimers.

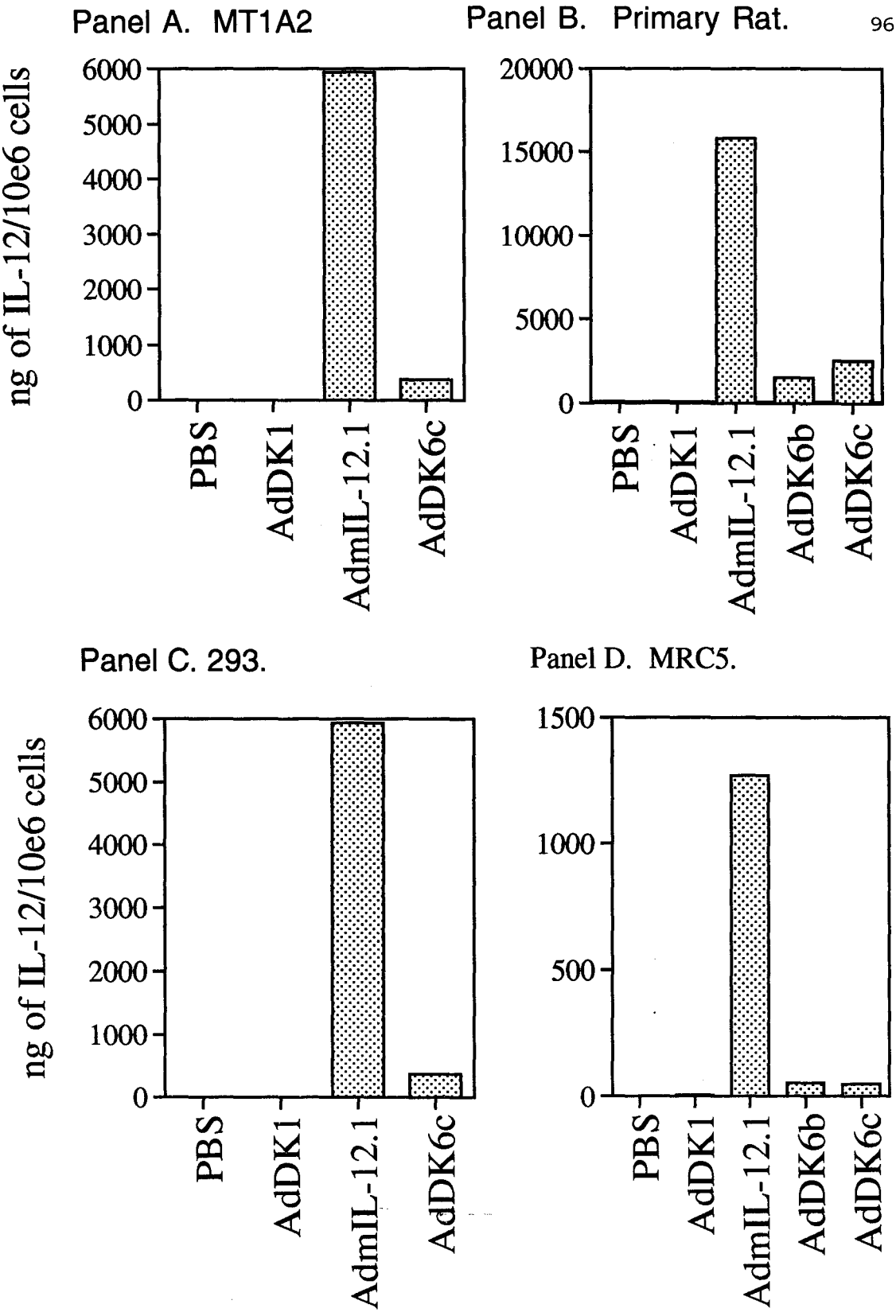
The autoradiograph of the immunoprecipitation of the 1° rat cell infection indicated that AdDK6 expressed proteins that interact with the anti-IL-12 antiserum and migrated with the monomers and dimers that are seen in the positive control lanes, but not the negative control lanes. The absence of the putative trimer from the AdDK6 lanes will be treated in the discussion.

The unlabelled supernatants were serially diluted and assayed by ELISA as described in the materials and methods. This produced 4 data sets that are shown in Figure 4.5. These figures clearly show that, although AdDK6 expressed levels (60-1540 ng) of an immunoreactive material that were significantly above the levels of the negative control and the background, AdmIL-12.1 (the positive control) expresses 7-30 fold more IL-12 than does AdDK6.

The proliferative bioassay, as measured by [^{3}H]-thymidine

Figure 4.5. Measurement of IL-12 production by ELISA of cells infected with AdDK1 (luciferase under the transcriptional control of the short fragment of the MCMV IE promoter/enhancer), AdmIL-12.1 (IL-12p35 cDNA and IL-12p40 cDNA under the transcriptional control of two HCMV IE promoter/enhancers) or AdDK6 (bicistronic IL-12 cassette under the transcriptional control of the MCMV IE promoter/enhancer).

Cells, either MT1A2, 1° Rat, 293 or MRC5, were infected at an MOI of 10 pfu per cell. At 24 hours, samples of supernatants were taken and analyzed by ELISA as detailed in Chapter 2, using a polyclonal anti-IL-12 antiserum.


Panel A. MT1A2
Panel B. Primary Rat.
ng of IL-12/10e6 cells
6000
5000
4000
3000
2000
1000
0
20000
15000
10000
5000
PBS
AdDK1
AdmIL-12.1
AdDK6b
AdDK6c
Panel C. 293.
Panel D. MRC5.
1500
500

The proliferative bioassay, as measured by [^{3}H]-thymidine incorporation into cellular DNA indicated that a factor in the supernatants of cells infected with AdDK6 induced proliferation of IL-2/ConA-activated splenocytes. Proliferation was also induced by the supernatants of cells infected with another IL-12-expressing adenovirus, AdmIL-12.1. There was no proliferative response to supernatants from cells infected with AdDK1, a recombinant adenovirus expressing luciferase. These results indicate that the IL-12 produced by the virus AdDK6 is bioactive.

Thus, AdDK6 has been shown by to express immunoreactive, biologically active, appropriately-sized IL-12, although the levels are 7-30 fold less than those expressed by AdmIL-12.1.

Appendix to the Results

I. A Bicistronic Reporter System

To characterize the poliovirus IRES system in the adenoviral context in greater detail, a reporter virus, AdDK3, was constructed. Its construction and its difficulties are described below, as well as useful data that was recovered.

The intronless luciferase cDNA was isolated from p548FL by restricting with BamHI and gel-purifying it from the parental plasmid. The first cistron of the shuttle plasmid pDK7 was opened by restricting with BglII, a restriction enzyme that produces termini that are cohesive and compatible with those produced by BamHI restriction. The two fragments were ligated to produce pDK9. The second cistron of pDK9 was opened with BamHI and SalI. The *lac*Z cDNA was isolated from the plasmid pMH1lacZ by digestion with the restriction enzymes BamHI and XhoI. XhoI produces termini that are cohesive and compatible with those produced by SalI. The two fragments were ligated to produce pDK11, which was cotransfected with pBHG10 to produce the virus AdDK3. However, BamHI was a mischoice of restriction enzyme as it cut several nucleotides 3′ of the initiator codon. This was not discovered until after the virus was rescued.

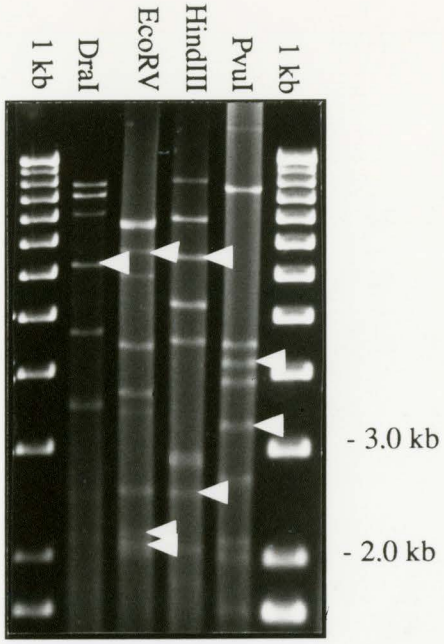
The left terminus of AdDK3, with appropriate diagnostic restriction enzyme sites, is shown in Figure A.1 with an electrophoretic gel of AdDK3 viral DNA digests with several

Appendix Figure 1. A. Restriction enzyme digests of AdDK3. AdDK3 viral DNA was prepared and digested as previously described. The enzymes DraI, EcoRV, HindIII and PvuI were used to analyze the viral DNA. The expected pattern is indicated below. The fragment sizes accompanied by asterisks (*) indicate fragments containing part of the expression cassette (from the 5′ end of the promoter/enhancer to the 3′ end of the SV40 polyadenylation signal). The same bands are marked on the photograph with small white arrows.

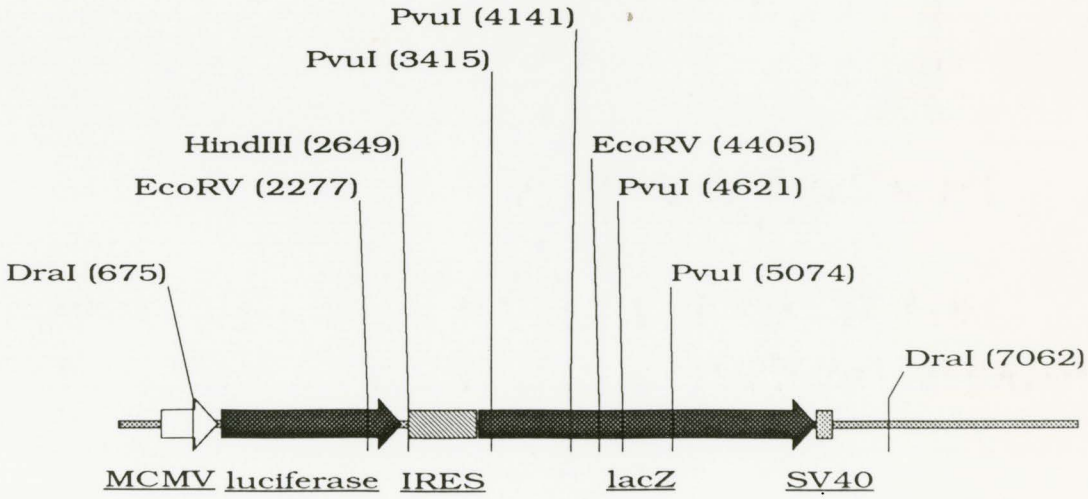
		AdDK3		
Fragment	DraI	EcoRV	HindIII	PvuI
A	10045	7637	8010	13541*
B	7489	6608*	7187*	4553
C	6387*	4546	5322	3942
D	4796	3808	4597	3390*
E	3575	3430	3012	2782
F	2566	2623	2937	2174
G	815	2252*	2624*	2062
H	650*	2128*	2081	1669
I		2052	1010	726*
J		1238	478	551
K			75	480*
L				453*
Repetitive Cellular Bands				
	8000	6400	10200	
		3400		

Panel B. The left terminus of AdDK3 with appropriate diagnostic restriction sites indicated.

Panel A. AdDK3.



Panel B. Left terminus of AdDK3.



AdDK3-left terminus

diagnostic enzymes. Despite AdDK3's inability to produce ß-galactosidase, luciferase was translated from the first cistron of a bicistronic mRNA that was transcribed from an adenoviral genome.

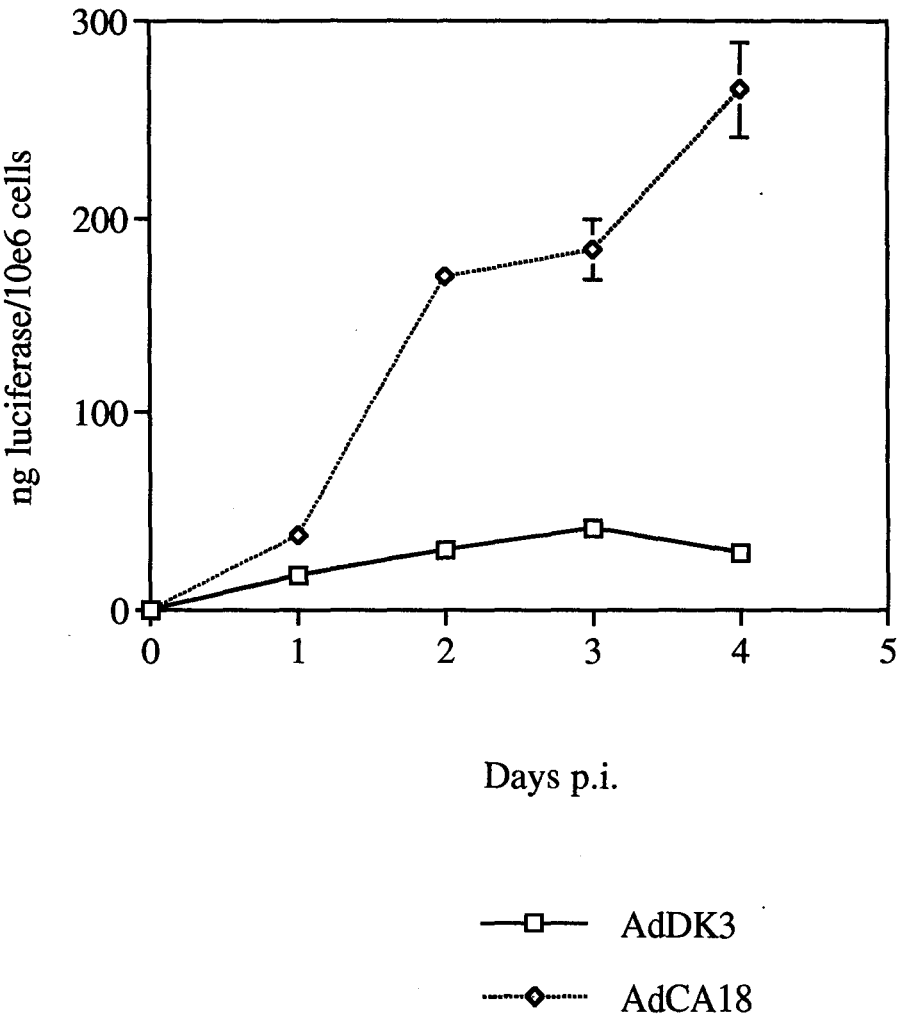
The luciferase expression data are presented in Figure A.2. MRC5 cells were infected at an MOI of 10 pfu per cell with either AdDK3 (bicistronic luciferase expression cassette under the transcriptional control of the short MCMV IE promoter/enhancer) or AdCA18 (luciferase cDNA under the transcriptional control of the HCMV IE promoter/enhancer) in triplicate and harvested at the appropriate timepoints as previously described. Luciferase assays were performed as detailed in Chapter 2.

The levels of luciferase expression from cells infected with AdDK3 correspond well with the levels of IL-12 produced from AdDK6, the other bicistronic virus in this study. However, expression of both viruses was far below what was expected for a transcript under the transcriptional control of the MCMV IE promoter/enhancer and this will be addressed in the discussion.

II. The Construction of Plasmids pDK17, pDK18, pDK19 and pDK20

At the time the study was begun, the kinetics of translation of the first and second cistrons of an adenovirally-derived mRNA bearing a poliovirus IRES were unknown. Moreover, although it was known that the IL-12p35 and IL-12p40 subunits covalently combine in a 1:1 ratio, the p40 subunit is overexpressed during wildtype production of IL-12 and it was possible that this was a requirement for proper dimerization. Thus, it was decided that a bicistronic

Appendix Figure 2. A timecourse of luciferase expression from MRC5 cells infected with AdDK3, AdCA18 or PBS^{++}. MRC5 cells were infected at an MOI of 10 in triplicate with either AdDK3 (bicistronic luciferase expression cassette under the control of the MCMV IE promoter/enhancer), AdCA18 (luciferase cDNA under the transcriptional control of the HCMV IE promoter/enhancer) or PBS^{++}. Cells were harvested and extracts assayed as detailed in Chapter 2.


300
200
100
0
ng luciferase/10e6 cells
0
1
2
3
4
5
Days p.i.
AdDK3
AdCA18

IL-12 system was to be made with the IL-12p40 cDNA in the first cistron and the IL-12p35 cDNA in the second, in contrast to AdDK6 where the order is reversed.

To produce the IL-12/IRES viruses with the p35 and p40 cDNAs in the opposite order, pDK7 and pDK8 were restricted with BamHI and EcoRV to open the second cistron (3′ of the IRES). The IL-12p35 cDNA was isolated from pVL2IL-12p35 by restricting with BamHI and EcoRV and gel-purifying from the parental plasmid. The insertion of the IL-12p35 cDNA 3′ of the IRES in pDK7 and pDK8 produced pDK17 and pDK18 respectively. The IL-12p40 cDNA was isolated from pCMVp40 by BamHI restriction and gel-purification. This fragment was inserted 5′ of the IRES in pDK17 and pDK18 restricted with BglII. The daughter plasmids are pDK19 and pDK20, respectively.

Although two cotransfection attempts were made, no virus was rescued from pDK19 or pDK20 before the end of the allotted time for this project.

Chapter 5: Discussion

As mentioned in the introduction, recent advances in immunology show that the immune system can function as a powerful mediator of tumour regression. The significant advantage of tumour immunotherapy is that circulating anti-tumour immune effectors that have become activated at one site will be capable of mediating tumour regression at other sites, making immunotherapy effective for metastatic cancers. The best currently available vector for the purposes of immunotherapy is probably adenovirus. Recombinant adenvirus has a relatively large cloning capacity, does not integrate into the host cell genome, is capable of mediating high local levels of transgene expression and infects a wide variety of cell types, including non-proliferating cells. IL-12 represents one of the most promising cytokines for tumour immunotherapy and has proven capable of inducing tumour regression and long term protective immunity from rechallenge in several studies. This project involved each of these fields and this chapter will discuss the results of a comparison of two different promoter/enhancer types and the construction and characterization of a bicistronic IL-12-expressing adenovirus.

Comparison of the HCMV and MCMV IE Promoter/Enhancers

The HCMV IE promoter/enhancer in the adenoviral context was observed to express 10-50-fold lower levels of transgene in murine compared to human cell lines (Addison *et al.*, 1995). Two fragments

(0.5 kb and 1.4 kb) were derived from the MCMV IE promoter/enhancer and utilized in adenovirus vectors to determine whether this difference in expression was due to infection effiency of the adenovirus vectors or some species preference by the promoter/enhancer elements themselves.

This question had been previously examined (C. Addison, personal communication) using the three aforementioned promoter/enhancers driving the *lacZ* cDNA. The question was also addressed using luciferase as the reporter gene, both to extend these results and to further establish the utility of the short (0.5 kb) and long (1.4 kb) fragments of the MCMV IE promoter/enhancer in a second reporter system.

In all cell lines, either fragment (0.5 kb or 1.4 kb fragment) of the MCMV IE promoter/enhancer directed higher levels of reporter gene expression than did the HCMV IE promoter/enhancer fragment. A comparison of the ratios of expression is given in Table II.

There are no clearly defined sequences that indicate whether one promoter/enhancer would direct higher transcript levels than others, but there are some commonalities. Enhancers and promoters of the type examined here have modular structures composed of repetitive elements which are thought to be binding sites for *trans*-acting proteins. The HCMV matches this modular model (Stamminger *et al.*, 1990), possessing four sets of direct repeat elements that are hypothesized to be binding sites for cellular *trans*-acting proteins (Fickenscher *et al.* 1989). The MCMV IE promoter/enhancer is structured similarly, with many repeated

Table II. A comparison of the ratios[a] of luciferase expression to the ratios of ß-galactosidase expression.

Cell Line	ß-galactosidase Ratio Range[b]	Luciferase Ratio Range[c]
SV329	10-30	4-29
MT1A2	10-30	4-30
1° Rat	10-50	3-39
MDCK	10-50	4-53
MRC5	10-50	2-24

a. Ratios were calculated by dividing the expression of the MCMV IE promoter/enhancer at day 2 post infection by the levels of expression of the HCMV IE promoter/enhancer at day 2 post infection.
b. data from C.L. Addison (personal communication).
c. data from Chapter 3, Table I.

sequences in the far upstream region. This large upstream region (-1330 to -488) of the MCMV IE promoter/enhancer displayed as much enhancer activity as MCMV IE promoter/enhancer from -487 to -147 (Dorsch-Häsler *et al.*, 1985), which agrees with data presented here. In some experiments, the virus with the long MCMV IE promoter/enhancer fragment (1.4 kb) expressed higher luciferase levels than the virus bearing the short MCMV IE promoter/enhancer (0.5 kb). In other experiments the situation was reversed, but in very few cases were the differences statistically significant.

To account for the higher expression levels of luciferase with the MCMV IE promoter/enhancer, there are four factors that can be removed from consideration. Firstly, the sequences surrounding the initiation codon are the same in all three viruses, as are the polyadenylation signals. The only sequence difference in the mRNAs driven from the HCMV IE promoter/enhancer as compared to those from the MCMV IE promoter/enhancers might be in the 5'-UTR. However, this region is relatively short (less than 100 nucleotides) and contains no obvious regions of secondary structure that would interfere with ribosomal scanning. Therefore, one would expect translation efficiencies to be comparable. Secondly, the high titre viral stocks used in this study were produced by identical protocols and titrated in duplicate or quadruplicate, so initial titres are accurate. There may be a concern that repeated cycles of freezing and thawing may lower the titre of a stock, but when stocks were retitrated after several months of use, no change in titre was noted. Thirdly, any differences in infectivity between

the different cell lines may be due to structural differences or expression levels in the unidentified cell surface receptor that interacts with the fibre during adenoviral attachment. These species-specific differences in levels of measured reporter protein may also be accounted for by differences in the integrins ($\alpha_v ß_5$ or $\alpha_v ß_3$ integrins) that participate in the internalization process (Shenk, 1996), but cannot account for differences in expression within a cell line, i.e.: the differences between the HCMV and MCMV IE promoter/enhancers. Fourthly, as can be seen from Figure 3.4, there is no obvious difference between a random sample of standard curves, which implies that the commercially available and locally produced reagents are not a source of variance. Therefore, the differences in luciferase expression levels between the MCMV and the HCMV IE promoter/enhancer enhancers are probably not due to differing translational efficiencies, inaccurate quantifications of viral titres, host-specific factors or the reagents used for the preparation or assaying of samples, but rathher are probably due to differential transcriptional activity of the promoters. This implies that the MCMV IE promoter/enhancer is the promoter/enhancer of choice for any system seeking to direct high levels of expression. This is true especially for gene therapy or immunotherapy with adenovirus, since the number of administrations will be limited by the immune response (Yang *et al.*, 1996). If higher transgene expression levels can be achieved, less virion need be administered initially. Less antigen will stimulate less of an immune response, so successive effective doses may be

possible.

The interexperimental differences in levels of luciferase expression still requires some accounting. While ß-galactosidase levels seemed consistent from experiment to experiment (C. Addison, personal communication), luciferase levels were seen to vary from 10- to 100-fold between experiments. Within experiments, the relative ratios between the luciferase expression levels remained similar (long MCMV IE promoter/enhancer, short MCMV IE promoter/enhancer and the HCMV IE promoter/enhancer).

Given that the viral titres, the translation efficiency, the reagents and the infectivity are controlled, the interexperimental differences must be a result of the infection process, a variability related to cell culture conditions, the luciferase extraction procedure or a characteristic of the luciferase protein itself. A possible variable in the infection is the confluence of the cells. The goal of 70-95% confluence was always achieved, but in many cell types, especially SV329 cells, there was obvious cell growth after the infection and the range from 70-95% is relatively broad. One would expect higher levels of transcription factors in cells that are proliferating, which could increase transcription. As cells become increasingly confluent, less transcriptional precursors and machinery would be available and luciferase levels would presumably decrease. Moreover, any variances of this kind would also be amplified by the stability of luciferase. Luciferase has a relatively short half-life (6-8 hours (Mittal *et al.*, 1993) compared to ß-galactosidase (>20 hours (Gonda *et al.*, 1989). These

two factors in combination, the difference in confluence and the relatively short halflife of luciferase could account for the variance between experiments.

With this luciferase data to extend previous ß-galactosidase results, it was possible to begin construction of the bicistronic shuttle system, using the MCMV IE promoter/enhancer, which in all cell lines tested in this study, was as transcriptionally active or more so than the HCMV IE promoter/enhancer.

2. IL-12 Vector

The antitumoural properties of IL-12 have been shown by several groups and one of the main challenges is to produce the most effective gene delivery system for this cytokine. A bicistronic, IL-12-expressing, E1-replacement adenovirus vector was constructed using the poliovirus IRES. This is the first report of a poliovirus IRES used to produce IL-12 in a gene therapy vector system.

There are three other viral systems designed to produce IL-12. In one report, Zitvogel *et al.* (1994) constructed a retroviral vector that expressed both subunits of IL-12 from a single promoter, but rendered translation of the second cistron independent of the first (Kaufman *et al.*, 1991) through the use of the EMCV IRES. Infected cells were observed to produce 15-40 ng IL-12/10^6 cells/24 hours, as measured by ELISA and a proliferative bioassay. A second recombinant retrovirus was constructed in the same report, one that bore an additional EMCV IRES and a neomycin

resistance gene. Cells infected by this vector and selected with G418 resulted in up to 120 ng/10^6 cells/24 hours. In a second report, Meko *et al.* (1995) constructed a recombinant vaccinia virus expressing the IL-12p35 and the IL-12p40 subunits under the transcriptional control of vaccinia promoters. This recombinant vaccinia virus infected a variety of murine and human tumour cell lines, producing 1.5 μg IL-12/10^6 cells/24 hrs. Finally, Bramson *et al.* (1995) constructed an adenovirus expressing the IL-12p35 subunit in E1 and the IL-12p40 subunit from E3, both under the transcriptional control of a fragment of the HCMV IE promoter/enhancer. Although this virus has been shown by bioassay to express up to 6 μg IL-12/10^6 cells/24 hours, any recombination event that restored the E1 region to this virus would result in a replication competent virus expressing high levels of IL-12p40, which can inhibit the normal interaction between IL-12 and the-IL-12 receptor (Mattner *et al.*, 1993; Ling *et al.*, 1995). For this reason, a bicistronic adenovirus was constructed employing the poliovirus IRES. The poliovirus IRES was used because the subcloning of the IL-12p40 cDNA into the second multicloning site does not require polymerase chain reaction primers for each different subunit. In the poliovirus IRES, the distance from the pyrimidine tract to the AUG^i is flexible as the ribosome is recruited to the IRES and scans to the first AUG. Conversely, the EMCV IRES recruits the ribosome in such a way that it is localized to the AUG^i immediately. The AUG of any cDNA inserted 3' of the EMCV IRES is thus required to replace the wildtype viral AUG^i.

Complementary DNAs can be inserted 3' of the poliovirus IRES using standard procedures without the need for the design of PCR primers for each separate subcloning procedure. This is also an advantage for any future use of the bicistronic shuttle system.

The bicistronic recombinant adenovirus AdDK6 was shown to express IL-12. The IL-12 was immunoreactive by ELISA and the immunoprecipitated protein migrated appropriately compared to a positive control on a polyacrylamide gel. The IL-12 was also bioactive, as measured by a proliferative bioassay. However, the levels of IL-12 expression were lower than would be expected for a transcript driven by the MCMV IE promoter/enhancer. As measured by ELISA, AdDK6 expressed a maximum of 2540 ng of secreted IL-12/10^6 cells/48 hours in 1° Rat cells and a minimum of 60 ng of secreted IL-12/10^6 cells/48 hours in MRC5 cells. Interestingly, the unidentified putative trimer observed by Bramson *et al.* (1995) was not observed with AdDK6. This trimer has not been observed elsewhere, and the presence of an unexplained, immunoreactive protein from a gene therapy vector is not encouraging. Since the putative trimer interacts with a polyclonal anti-IL-12 antiserum, it can be argued that it must contain at least one subunit (either the p35 or p40). However, it may be that it is a full heterodimer bound to some cellular or adenoviral protein. Finally, the putative trimer may be exclusively composed of p35 and p40 subunits.

Although no exact quantitation of the expression of the second cistron was possible, the luciferase expression was measurable from

the first cistron of AdDK3 (0.5 kb fragment of the MCMV IE promoter/enhancer), the bicistronic reporter virus with luciferase in cistron one and an ATG-less *lac*Z cDNA in cistron two. As shown in the appendix, at day two post-infection, AdDK3 expressed between 22 and 30 ng of luciferase/10^6 MRC5 cells. Previous reports have suggested that the poliovirus IRES is capable of directing translation of the second cistron to approach 60-80% of the translation levels of the cap-dependent first cistron. Of the 60 ng IL-12/10^6 cells/48 hours, approximately 50% of this measurement is the IL-12p40 subunit. This value of approximately 30 ng compares well with the luciferase production from the first cistron of AdDK3 and agrees well with such previous characterizations of the poliovirus IRES.

A question remains : with the MCMV IE promoter/enhancer driving transcription, why is expression of the first cistron of a bicistronic message so low compared to the monocistronic controls? Since the promoters and other transcriptional elements are almost identical from control viruses to the bicistronic viruses (they differ in approximately 10 bp of the multicloning site in the 5'-UTR), transcription should be comparable and ribosomal scanning from the cap should be unaffected. This leaves mRNA conformation and mRNA stability as a possible explanation. Although neither feature has been observed to interfere with translation in previous poliovirus IRES studies, these have usually been done in the context of a transfected plasmid. Many adenoviral mRNAs are spliced (Shenk, 1996) and it is possible that the poliovirus IRES

contains a splice signal which may induce a change in mRNA stability. Polyadenylation is another element that contributes to mRNA stablity that may be compromised. The viral DNA coding for the IRES may assume some secondary structure while it is being read by the RNA polymerase. If this somehow interferes with processivity, the RNA polymerase may terminate transcription before transcribing the SV40 polyadenylation signal, resulting in reduced mRNA stability.

Regardless of the reason, the level of IL-12 expressed from AdDK6 is comparable to the IL-12 levels achieved by the recombinant retrovirus described by Zitvogel *et al.* (1995). However, AdDK6-IL-12 expression is approximately 10-30-fold lower than those produced by the adenovirus described by Bramson *et al. (1995)*. This is a drawback for the purposes of tumour immunotherapy, where the highest possible levels of local cytokine production are required.

The shuttle plasmid system developed in this study, specifically pDK7 (0.5 kb fragment of the MCMV IE promoter/enhancer) and pDK8 (1.4 kb fragment of the MCMV IE promoter/enhancer) remain as a flexible bicistronic shuttle plasmid system capable of expressing nanogram levels of biologically active protein in human cells and microgram levels of biologically active protein in rodent cells. Each multicloning site contains unique restriction sites capable of accommodating most inserts. Not all applications require maximal expression and for such systems, especially ones involving cell line construction or cDNA libraries. In such cases, where selection is necessary, these shuttle plasmids

could be useful, because expression of the selectable marker from the second cistron would assure expression of the cDNA or the transgene from the first.

This study has built upon previous studies of the MCMV and HCMV IE promoter/enhancers to generalize the results to a second reporter system. In all cell lines, the MCMV IE promoter/enhancer was shown to be a stronger transcriptional activator than the HCMV IE promoter/enhancer. Additionally, a bicistronic adenovirus, AdDK6, the first such adenovirus reported using the poliovirus IRES, was constructed and expresses bioactive IL-12. Although the levels of IL-12 expressed are insufficient for the purposes of gene therapy of cancer, the bicistronic shuttle system (pDK7 and pDK8) developed for the construction of the IL-12 virus may be used for other systems, especially for the development of cell lines and the construction of cDNA libraries.

References

Addison, C.L., T. Braciak, R. Ralston, W.J. Muller, J. Gauldie and F.L. Graham. Intra-tumoural Injection of an Adenovirus Expressing Interleukin-2 Induces Regression and Immunity in a Murine Breast Cancer Model. 1995. Proc. Natl. Acad. Sci. USA 92:8522-8526.

Addison, C.L., J. Gauldie, W.J. Muller, F.L. Graham. Intratumoural Injection of an Adenovirus Expressing Interleukin-2 Induces Regression and Immunity in a Murine Breast Cancer Model. 1995. Proc Natl Acad Sci USA 92:8522-8526.

Andrews, J.V.R., D.D. Schoof, M.M. Bertagnolli, G.E. Peoples, P.S. Goedegebuure and T.J. Eberlain. Immunomodulatory Effects of Interleukin-12 on Human Tumour-Infiltrating Lymphocytes. 1993. J. Immunother. 14:1-10.

Aragane, Y., H. Riemann, R.S. Bhardwaj, A. Schwarz, A. Sawada, H. Yamada, T.A. Luger, M. Kubin, G. Trinchieri and T. Schwarz. IL-12 is Expressed and Released by Human Keratinocytes and Epidermoid Carcinoma Cell Lines. 1994. J. Immunol. 153:5366-5372.

Bandyopadhyay, P.K., C. Wang and H.L. Lipton. Cap-Independent Translation by the 5' Untranslated Region of Thieler's Murine Encephalomyelitis Virus. 1992. J. Virol. 66:6249-6256.

Berk, A.J. and P.A. Sharp. Structure of Adenovirus 2 Early mRNAs. 1978. Cell 14:695-703.

Bett, A.J. W. Haddara, L. Prevec and F.L. Graham. An Efficient and Flexible System for Construction of Adenovirus Vectors with Insertions or Deletions in Early Regions 1 and 3. 1994. Proc. Natl. Acad. Sci. USA 91:8802-8806.

Bett, A.J., L. Prevec and F.L. Graham. Packaging Capacity and Stability of Human Adenovirus Type 5 Vectors. 1993. J. Virol. 67:5911-5921.

Birnboim, H.C. and J. Doly. A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA. 1979. Nucleic Acids Res. 7:1513-1523.

Bonnema, J.D., K.A. Rivlin, A.T. Ting, R.A. Schoon, R.T. Abraham and P.J. Leibson. Cytokine-Enhanced NK Cell-Mediated Cytotoxicity. 1994. J. Immunol. 152:2098-2104.

Borman, A. and R. Jackson. Initiation of Translation of Human Rhinovirus RNA: Mapping the Internal Ribosomal Entry Site. 1992. Virol. 188:685-696.

Boshart, M.F., F. Weber, G. Kahn, K. Dorsch-Hasler, B. Fleckenstein and W. Schaffner. A Very Strong Enhancer is Located Upstream of an

Immediate Early Gene of Human Cytomegalovirus. 1985. Cell-41:521-530.

Bramson, J., M. Hitt, W.S. Gallichan, K.L. Rosenthal, J. Gauldie and F.L. Graham. Construction of a Double Recombinant Adenovirus Vector Expressing a Heterdimeric Cytokine: *in vitro* and *in vivo* Production of biologically active Interleukin-12. 1996. Hum. Gene Ther. 7:333-342.

Brunda, M.J., L. Luistro, R.R. Warrier, R.B. Wright, B.R. Hubbard, M. Murphy, S.F. Wolf and M.K. Gately. Antitumour and Antimetastatic Activity of Interleukin-12 Against Murine Tumours. 1993a. J. Exp. Med. 178:1223-1230.

Brunda, M.J., L. Luistro, R. Warrier, B. Hubbard, S.F. Wolf and M.K. Gately. Antitumour Activity of Interleukin-12 (IL-12) in Murine Tumour Models. 1993b. Proc. Am. Ass. Cancer Res. 34:464

Brunda, M.J., L. Luistro, A. Hendrzak, M. Fountoulakis, G. Garotta and M.K. Gately. Role of Interferon-γ in Mediating the Antitumour Efficacy of Interleukin-12. 1995. J. Immunol. 17:71-77.

Brunda, M.J. Interleukin-12. 1994. J. Leukocyte Biol. 55:280-288.

Cameron, R.B., P.J. Spiess and S.A. Rosenberg. Synergistic Antitumour Activity of Tumour-Infiltrating Lymphocytes, Interleukin-2 and Local Tumour Irradiation. 1990. J. Exp. Med. 171:249-263.

Chan, S.H., B. Perussia, J.W. Gupta, M. Kobayashi, M. Pospísil, H.A. Young, S.F. Wolf, D. Young, S.C. Clark and G. Trinchieri. Induction of Interferon-γ Production by Natural Killer Cell Stimulatory Factor: Characterization of the Responder Cells and Synergy with Other Inducers. 1991. J. Exp. Med. April:869-879.

Chehimi, J., S.E. Starr, I. Frank, A. D'Andrea, X. Ma, R.R. MacGregor, J. Sennelier and G. Trinchieri. Impaired Interleukin 12 Production in Human Immunodeficiency Virus-Infected Patients. 1994. J. Exp. Med. 179:1361-1366.

Chehimi, J., S.E. Starr, I. Frank, M. Rengaraju, S.J. Jackson, C. Llanes, M. Kobayashi, B. Perussia, D. Young, E. Nickbarg, S.F. Wolf and G. Trinchieri. Natural Killer (NK) Cell Stimulatory Factor Increases the Cytotoxic Activity of NK Cells from both Healthy Donors and Human Immunodeficiency Virus-Infected Patients. 1992. J. Exp. Med. 175:789-796.

Chehimi, J., N.M. Valliante, A. D'Andrea, M. Rengaraju, Z. Rosado, M. Kobayashi, B. Perussia, S.F. Wolf, S.E. Starr, G. Trinchieri. Enhancing Effect of Natural Killer Cell Stimulatory Factor (NKSF/Interkeukin-12) on Cell-Mediated Cytotoxicity Against-Tumor-

Derived and Virus-Infected Cells. 1993. Eur. J. Immunol. 23:1826-1830.

Chizzonite, R., T. Truitt, B.B. Desai, P. Nunes, F.J. Podlaski, A.S. Stern and M.K. Gately. IL-12 Receptor. I. Characterization of the Receptor on Phytohemagglutinin-Activated Human Lymphoblasts. 1992. J. Immunol. 148:3117-3124.

Chua, A.O., R. Chizzonite, B.B. Desai, T.P. Truitt, P. Nunes, L.J. Minetti, R.J. Warrier, D.H. Presky, J.F. Levine, M.K. Gately and U. Gubler. Expression Cloning of a Human IL-12 Receptor Component. 1994. J. Immunol. 153:128-136.

Clemons, K.V., E. Brummer and D.A. Stevens. Cytokine Treatment of CNS Infection : Efficacy if IL-12 Alone and Synergy with Conventional Antifungal Therapy in Experimental Cryptococcus. 1994. Antimicro. Agents Chemother. 38:460-464.

Clerici, M., D.R. Lucey, J.A. Berzofsky, L.A. Pinto, T.A. Wynn, S.P. Blatt, M.J. Dolan, C.W. Hendrix, S.F. Wolf and G.M. Shearer. Restoration of HIV-Specific Cell-Mediated Immune Responses by Interleukin-12 *in vitro*. 1993. Science 262:1721.

Cohen, J. IL-12 Deaths: Explanation and a Puzzle. 1995. Science 270:908.

Cordier, L., M.-T. Duffour, J.-C. Sabourin, M.G. Lee, J. Cabannes, T. Ragot, M. Perricaudet and H. Haddada. Complete Recovery of Mice from a Pre-established Tumour by Direct Intratumoural Delivery of an Adenovirus Vector Harbouring the Murine IL-2 Gene. 1995. Gene Ther. 2:16-21.

Culver, K.W. and R.M. Blaese. Gene Therapy of Cancer. 1994. TIG 10:174-178.

D'Andrea, A., M. Aste-Amezaga, N.M. Valiante, X. Ma, M. Kubin and G. Trichieri. Interleukin 10 (IL-10) Inhibits Human Lymphocyte Interferon-γ Production by Suppressing Natural Killer Cell Stimulatory Factor/IL-12 Synthesis in Accessory Cells. 1993. J. Exp. Med. 178:1041-1048.

D'Andrea, A., X. Ma, M. Aste-Amezaga, C. Paganin and G. Trinchieri. Stimulatory and Inhibitory Effects of Interleukin-4 and Interleukin-13 on the Production of Cytokines by Mononuclear Cells: Priming for IL-12 and Tumour Necrosis Factor α Production. 1995. J. Exp. Med. 181:537-546.

D'Andrea, A., M. Rengaraju, N.M. Valiante, J. Chehimi, M. Kubin, M. Aste, S.H. Chan, M. Kobayashi, D. Young, E. Nickbarg, R. Chizzonite, S.F. Wolf and G. Trinchieri. Production of Natural killer Cell Stimulatory Factor (Interleukin-12) by Peripheral Blood Mononuclear Cells. 1992. J. Exp. Med. 176:1378-1398.

References

Addison, C.L., T. Braciak, R. Ralston, W.J. Muller, J. Gauldie and F.L. Graham. Intra-tumoural Injection of an Adenovirus Expressing Interleukin-2 Induces Regression and Immunity in a Murine Breast Cancer Model. 1995. Proc. Natl. Acad. Sci. USA 92:8522-8526.

Andrews, J.V.R., D.D. Schoof, M.M. Bertagnolli, G.E. Peoples, P.S. Goedegebuure and T.J. Eberlain. Immunomodulatory Effects of Interleukin-12 on Human Tumour-Infiltrating Lymphocytes. 1993. J. Immunother. 14:1-10.

Aragane, Y., H. Riemann, R.S. Bhardwaj, A. Schwarz, A. Sawada, H. Yamada, T.A. Luger, M. Kubin, G. Trinchieri and T. Schwarz. IL-12 is Expressed and Released by Human Keratinocytes and Epidermoid Carcinoma Cell Lines. 1994. J. Immunol. 153:5366-5372.

Bandyopadhyay, P.K., C. Wang and H.L. Lipton. Cap-Independent Translation by the 5' Untranslated Region of Thieler's Murine Encephalomyelitis Virus. 1992. J. Virol. 66:6249-6256.

Berk, A.J. and P.A. Sharp. Structure of Adenovirus 2 Early mRNAs. 1978. Cell 14:695-703.

Bett, A.J. W. Haddara, L. Prevec and F.L. Graham. An Efficient and Flexible System for Construction of Adenovirus Vectors with Insertions or Deletions in Early Regions 1 and 3. 1994. Proc. Natl. Acad. Sci. USA 91:8802-8806.

Bett, A.J., L. Prevec and F.L. Graham. Packaging Capacity and Stability of Human Adenovirus Type 5 Vectors. 1993. J. Virol. 67:5911-5921.

Birnboim, H.C. and J. Doly. A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA. 1979. Nucleic Acids Res. 7:1513-1523.

Bonnema, J.D., K.A. Rivlin, A.T. Ting, R.A. Schoon, R.T. Abraham and P.J. Leibson. Cytokine-Enhanced NK Cell-Mediated Cytotoxicity. 1994. J. Immunol. 152:2098-2104.

Borman, A. and R. Jackson. Initiation of Translation of Human Rhinovirus RNA: Mapping the Internal Ribosomal Entry Site. 1992. Virol. 188:685-696.

Boshart, M.F., F. Weber, G. Kahn, K. Dorsch-Hasler, B. Fleckenstein and W. Schaffner. A Very Strong Enhancer is Located Upstream of an Immediate Early Gene of Human Cytomegalovirus. 1985. Cell-41:521-530.

Bramson, J., M. Hitt, W.S. Gallichan, K.L. Rosenthal, J. Gauldie and F.L. Graham. Construction of a Double Recombinant Adenovirus

Vector Expressing a Heterdimeric Cytokine: *in vitro* and *in vivo* Production of biologically active Interleukin-12. 1996. Hum. Gene Ther. 7:333-342.

Brunda, M.J., L. Luistro, R.R. Warrier, R.B. Wright, B.R. Hubbard, M. Murphy, S.F. Wolf and M.K. Gately. Antitumour and Antimetastatic Activity of Interleukin-12 Against Murine Tumours. 1993a. J. Exp. Med. 178:1223-1230.

Brunda, M.J., L. Luistro, R. Warrier, B. Hubbard, S.F. Wolf and M.K. Gately. Antitumour Activity of Interleukin-12 (IL-12) in Murine Tumour Models. 1993b. Proc. Am. Ass. Cancer Res. 34:464

Brunda, M.J., L. Luistro, A. Hendrzak, M. Fountoulakis, G. Garotta and M.K. Gately. Role of Interferon-γ in Mediating the Antitumour Efficacy of Interleukin-12. 1995. J. Immunol. 17:71-77.

Brunda, M.J. Interleukin-12. 1994. J. Leukocyte Biol. 55:280-288.

Cameron, R.B., P.J. Spiess and S.A. Rosenberg. Synergistic Antitumour Activity of Tumour-Infiltrating Lymphocytes, Interleukin-2 and Local Tumour Irradiation. 1990. J. Exp. Med. 171:249-263.

Chan, S.H., B. Perussia, J.W. Gupta, M. Kobayashi, M. Pospísil, H.A. Young, S.F. Wolf, D. Young, S.C. Clark and G. Trinchieri. Induction of Interferon-γ Production by Natural Killer Cell Stimulatory Factor: Characterization of the Responder Cells and Synergy with Other Inducers. 1991. J. Exp. Med. April:869-879.

Chehimi, J., S.E. Starr, I. Frank, A. D'Andrea, X. Ma, R.R. MacGregor, J. Sennelier and G. Trinchieri. Impaired Interleukin 12 Production in Human Immunodeficiency Virus-Infected Patients. 1994. J. Exp. Med. 179:1361-1366.

Chehimi, J., S.E. Starr, I. Frank, M. Rengaraju, S.J. Jackson, C. Llanes, M. Kobayashi, B. Perussia, D. Young, E. Nickbarg, S.F. Wolf and G. Trinchieri. Natural Killer (NK) Cell Stimulatory Factor Increases the Cytotoxic Activity of NK Cells from both Healthy Donors and Human Immunodeficiency Virus-Infected Patients. 1992. J. Exp. Med. 175:789-796.

Chehimi, J., N.M. Valliante, A. D'Andrea, M. Rengaraju, Z. Rosado, M. Kobayashi, B. Perussia, S.F. Wolf, S.E. Starr, G. Trinchieri. Enhancing Effect of Natural Killer Cell Stimulatory Factor (NKSF/Interkeukin-12) on Cell-Mediated Cytotoxicity Against-Tumor-Derived and Virus-Infected Cells. 1993. Eur. J. Immunol. 23:1826-1830.

Chizzonite, R., T. Truitt, B.B. Desai, P. Nunes, F.J. Podlaski, A.S. Stern and M.K. Gately. IL-12 Receptor. I. Characterization of

the Receptor on Phytohemagglutinin-Activated Human Lymphoblasts. 1992. J. Immunol. 148:3117-3124.

Chua, A.O., R. Chizzonite, B.B. Desai, T.P. Truitt, P. Nunes, L.J. Minetti, R.J. Warrier, D.H. Presky, J.F. Levine, M.K. Gately and U. Gubler. Expression Cloning of a Human IL-12 Receptor Component. 1994. J. Immunol. 153:128-136.

Clemons, K.V., E. Brummer and D.A. Stevens. Cytokine Treatment of CNS Infection : Efficacy of IL-12 Alone and Synergy with Conventional Antifungal Therapy in Experimental Cryptococcus. 1994. Antimicro. Agents Chemother. 38:460-464.

Clerici, M., D.R. Lucey, J.A. Berzofsky, L.A. Pinto, T.A. Wynn, S.P. Blatt, M.J. Dolan, C.W. Hendrix, S.F. Wolf and G.M. Shearer. Restoration of HIV-Specific Cell-Mediated Immune Responses by Interleukin-12 *in vitro*. 1993. Science 262:1721.

Cohen, J. IL-12 Deaths: Explanation and a Puzzle. 1995. Science 270:908.

Cordier, L., M.-T. Duffour, J.-C. Sabourin, M.G. Lee, J. Cabannes, T. Ragot, M. Perricaudet and H. Haddada. Complete Recovery of Mice from a Pre-established Tumour by Direct Intratumoural Delivery of an Adenovirus Vector Harbouring the Murine IL-2 Gene. 1995. Gene Ther. 2:16-21.

Culver, K.W. and R.M. Blaese. Gene Therapy of Cancer. 1994. TIG 10:174-178.

D'Andrea, A., M. Aste-Amezaga, N.M. Valiante, X. Ma, M. Kubin and G. Trichieri. Interleukin 10 (IL-10) Inhibits Human Lymphocyte Interferon-γ Production by Suppressing Natural Killer Cell Stimulatory Factor/IL-12 Synthesis in Accessory Cells. 1993. J. Exp. Med. 178:1041-1048.

D'Andrea, A., X. Ma, M. Aste-Amezaga, C. Paganin and G. Trinchieri. Stimulatory and Inhibitory Effects of Interleukin-4 and Interleukin-13 on the Production of Cytokines by Mononuclear Cells: Priming for IL-12 and Tumour Necrosis Factor α Production. 1995. J. Exp. Med. 181:537-546.

D'Andrea, A., M. Rengaraju, N.M. Valiante, J. Chehimi, M. Kubin, M. Aste, S.H. Chan, M. Kobayashi, D. Young, E. Nickbarg, R. Chizzonite, S.F. Wolf and G. Trinchieri. Production of Natural killer Cell Stimulatory Factor (Interleukin-12) by Peripheral Blood Mononuclear Cells. 1992. J. Exp. Med. 176:1378-1398.

Desai, B.B., P.M. Quinn, A.G. Wolitsky, P.K.A. Mongini, R. Chizzonite and M.K. Gately. IL-12 Receptor. II. Distribution and Regulation of Receptor Expression. 1992. J. Immunol. 148:3125-3132.

DeWet, J.R., K.V. Wood, M. DeLuca, D.R. Helinski and S. Subramani. Firefly Luciferase Gene: Structure and Expression in Mammalian Cells. 1987. Mol. Cell Biol. 7:725-737.

Estaquier, J., T. Idziorek, W. Zou, D. Emilie, C.M. Farber, J.M. Bourez and J.C. Ameisen. T Helper Type 1/T Helper Type 2 Cytokines and T Cell Death: Preventative Effects of Interleukin 12 on Activation-Induced and CD95 (FAS/APO-1)-mediated Apoptosis of CD4+ T Cells from Human Immunodeficiency Virus-Infected Persons. 1995. J. Exp. Med. 182:1759-1767.

Fearon, E.R., D. Pardoll, T. Itaya, P. Golumbek, H.I. Levitsky, J.W. Simmons, H. Karasuyama, B. Vogelstein and P. Frost. Interleukin-2 Production by Tumour Cells Bypasses T Helper Function in the Generation of an Antitumour Response. 1990. Cell 60:397-403.

Fickenscher, H., T. Stamminger, R. Rüger and B. Fleckenstein. The Role of Repetitive Palindromic Sequence Elements in the Human Cytomegalovirus Major Immediate Early Enhancer. 1989. J. Gen. Virol. 70:107-123.

Gately, M.K., B. Desai, A.G. Wolitzky, P.M. Quinn, C.M. Dwyer, F.J. Podlaski, P.C. Familletti, F. Sinigaglia, R. Chizzonite, U. Gubler and A.S. Stern. Regulation of Human Lymphocyte Proliferation by a Heterodimeric Cytokine, IL-12 (Cytotoxic Lymphocyte Maturation Factor). 1991. J. Immunol. 147:874-882.

Gately, M.K., A.G. Wolitzky, P.M. Quinn and R. Chizzonite. Regulation of Human Cytolytic Lymphocyte Responses by-Interleukin-12. 1992. Cell. Immunol. 143:127-142.

Gearing, D.P., and D. Cosman. Homology of the p40 Subunit of Natural Killer Cell Stimulatory Factor (NKSF) with the Extracellular Domain of the Interleukin-6 Receptor. 1991. Cell 66:9-10.

Graham, F.L., J. Smiley, W.C. Russell and R. Nairn. Characteristics of a Human Cell Line Transformed by DNA from Human Adenovirus Type 5. 1977. J. Gen. Virol. 36:59-77.

Gramstat, A., D. Prüfer and W. Rohde. The Nucleic Acid-Binding Zinc Finger Protein of Potato Virus M is Translated by Internal Initiation as well as by Ribosomal Frameshifting Involving a Shifty Stop Codon and a Novel Mechanism of P-Site Slippage. 1994. Nucleic Acids Res. 22:3911-3917.

Green, M., W.S.M. Wold, K.H. Brachmann. Human Adenovirus Transforming Genes: Group Relationships, Integration, Expression in Transformed Cells and Analysis of Human Cancers and Tonsils. Cold Spring Harbour Conference on Cell Proliferation Viruses in Naturally Occuring Tumours, Eds. M. Essex, G. Todaro and H. zur

Hausen. Cold Spring Harbour Laboratory, Cold Spring Harbour, NY, 1980, pgs 373-397.

Gubler, U., A.O. Chua, D.S. Schoenhaut, C.M. Dwyer, W. McComas, R. Motyka, N. Nabavi, A.G. Wolitsky, P.M. Quinn, P.C. Familletti and M.K. Gately. Coexpression of Two Distinct Genes is Required to Generate Secreted, Bioactive Cytotoxic Lymphocyte Maturation Factor. 1991. Proc. Natl. Acad. Sci. USA 88:4143-4147.

Guy, C.T., R.D. Cardiff and W.J. Muller. 1992. Induction of Mammary Tumours by Expression of Polyomavirus Mittle T Oncogene: A Transgenic Mouse Model for Metastatic Disease. Mol. Cell Biol. 12:954-961.

Hackett, J.Jr., M. Tutt, M. Lipscomb, M. Bennett, G. Koo and V. Kumar. Origin and Differentiation of Natural Killer Cells. II. Functional and Morphological Studies of Purified NK1.1 Cells. 1986. J. Immunol. 136:3124-3131.

Haddada, H., T. Ragot, L. Cordier, M.T. Duffour and M. Perricaudet. Adenoviral Interleukin-2 Gene Transfer into P815 Tumour Cells Abrogates Tumorigenicity and Induces Antitumoural Immunity in Mice. 1993. Hum. Gene Ther. 4:703-711.

Hanahan, D. Studies on the Transformation of *E. coli* with Plasmids. 1983. J. Mol. Biol. 166:557-580.

Hitt, M., A.J. Bett, L. Prevec and F.L. Graham. Construction and Propagation of Human Adenovirus Vectors. In Cell Biology: A Laboratory Handbook. J.E. Celis, ed. (Academic Press, San Diego) pp. 479-490 (1994).

Horwitz, M.S. 1990. Adenoviridae and Their Replication. *Virology*, Eds B.N. Fields and D.M. Knipe, 1679-1721.

Horwitz, M.S. 1996. Adenoviruses. *Virology*, Eds B.N. Fields, D.M. Knipe, P.M. Howley, Assoc Eds R.M. Cahnock, J.L. Melnick, T.P. Monath, B. Roizman and S.E. Straus, 2149-2171.

Hsieh, C-S., S.E. Macatonia, C.S. Tripp, S.F. Wolf, A. O'Garra and K.M. Murphy. Development of T_H1 CD4+ T Cells Through IL-12 Produced by *Listeria*-Induced Macrophages. 1993. Science-260:547-549.

Jewett, A. and B. Bonavida. Activation of Human Immature Natural Killer Cell Subset by IL-12 and Its Regulation by Endogenous TNF-α and IFN-γ Secretion. 1994. Cell. Immunol. 154:273-286.

Johnson, D.G., J.K. Schwarz, W.D. Cress and J.R. Nevins. Expression of Transcription Factor E2F1 Induces Quiescent Cells to Enter S Phase. 1993. Nature 365:349-352.

Kaufman, R.J., M.V. Davies, L.C. Wasley and D. Michnick. Improved Vectors for Stable Expression of Foreign Genes in Mammalian Cells by Use of the Untranslated Leader Sequence from EMCV. 1991. Nucleic Acids Res. 19:4485-4490.

Kiniwa, M., M. Gately, U. Gubler, R. Chizzonite, C. Fargeas and G. Delespesse. Recombinant Interleukin-12 Suppresses the Synthesis of IgE by Interleukin-4 Stimulated Human Lymphocytes. 1992. J. Clin. Invest. 90:262-266.

Kobayashi, M., L. Fitz, M. Ryan, R.M. Hewick, S.C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia and G. Trinchieri. Identification and Purification of Natural Killer Cell Stimulatory Factor (NKSF), a Cytokine with Multiple Biological Effects on Human Lymphocytes. 1989. J. Exp. Med. 170:827.

Li, M.-X., D. Banerjee, S.-C. Zhao, B.I. Schweitzer, S. Mineishi, E. Gilboa and J.R. Bertino. Development of a Retroviral Construct Containing a Human Mutated Dihydrofolate Reductase cDNA for Hematopoietic Stem Cell Transduction. 1994. Blood 83:3403-3408.

Ling, P., M.K. Gately, U. Gubler, A.S. Stern, P. Lin, K. Hollfelder, C. Su, Y.-C.E. Pan and J. Hakimi. Human IL-12 p40 Homodimer Binds to the IL-12 Receptor, but does not Mediate Biologic Activity. 1995. J. Immunol. 154:116-127.

Lotze, M.T., E.A. Grimm, A. Mazumder, J.L. Strausser and S.A. Rosenberg. Lysis of Fresh and Cultured Autologous Tumour by Human Lymphocytes Cultured in T-Cell Growth Factor. 1981. Cancer Res. 41:4420-4425.

Mattner, F., S. Fischer, S. Guckes, S. Jin, H. Kaulen, E. Schmitt, E. Rüde and T. Germann. The Interleukin-12 Subunit p40 Specifically Inhibits Effects of the Interleukin-12 Heterodimer. 1993. Eur. J. Immunol. 23:2202-2208.

McGrory, W.J., D.S. Bautista and F.L. Graham. A Simple Technique for the Rescue of Early Region Mutations into Infectuous Human Adenovirus Type 5. 1988. Virology 163:614-617.

McKnight, A.J., G.J. Zimmer, I. Fogelman, S.F. Wolf and A.K. Abbas. Effects of IL-12 on Helper T Cell-Dependent Immune Responses *in vivo*. 1994. J. Immunol. 152:2172-2179.

Meerovitch, K., R. Nicholson and N. Sonenberg. *In vitro* Mutational Analysis of *cis*-Acting RNA Translational Elements within the Poliovirus Type 2 5'-Untranslated Region. 1991. J. Virol. 65:5895-5901.

Meerovitch, K, N. Sonenberg and J. Pelletier. Translation in Eukaryotes. 1990. Chapter 11 : The Translation of Picornaviruses, pgs 273-291.

Meko, J.B., J.H. Kim, K. Tsung and J. Norton. High Cytokine Production of Effective Antitumour Activity of a Recombinant Vaccinia Virus Encoding Murine Interleukin-12. 1995. Cancer Res. 55:4765-4770.

Merberg, D.M., S.F. Wolf, and S.C. Clark. Sequence Similarity Between NKSF and the IL-6/G-CSF Family. 1992. Immunol. Today 13:77-78.

Mittal, S.K., M.A. McDermott, D.C. Johnson, L. Prevec and F.L. Graham. Monitering Foreign Gene Expression by a Human-Adenovirus-Based Vector Using a Firefly Luciferase Gene as a Reporter. 1993. Virus Res. 28:67-90.

Murray, J.S., J. Madri, J. Tite, S.R. Carding and K. Bottomly. MHC Control of CD4+ T cell Subset Activation. 1989. J. Exp. Med. 170:2135-2140.

Naldini, L., U. Blomer, P. Galley, D. Ory, R. Mulligan, F.H. Gaye, I.M. Verma and D. Trono. *In vivo* Gene Delivery and Stable Transduction of Nondividing Cells by a Lentiviral Vector. 1996. Science 272:263-267.

Nastala, C.L., H.D. Edington, T.G. McKinney, H. Tahara, M.A. Nalesnik, M.J. Brunda, M.K. Gately, S.F. Wolf, R.D. Schreiber, W.J. Storkus and M.T. Lotze. Recombinant IL-12 Administration INduces Tumour Regression in Assocaition with IFN-γ Production. 1994. J. Immunol. 153:1697-1706.

Naume, B., M.K. Gately, T. Espevik. A Comparative Study of IL-12 (Cytotoxic Lymphocyte Maturation Factor)-, IL-2- and IL-7-Induced Effects On Immunomagnetically Purified CD56+ NK Cells. 1992. J. Immunol. 148:2429-2436.

Nicholson, R., J. Pelletier, S-Y. Le and N. Sonenberg. Structural and Functional Analysis of the Ribosome Landing Pad of Polio virus Type 2 : *in vivo* Translational Studies. 1991. J. Virol.-65:5886-5894.

Orange, J.S., S.F. Wolf and C.A. Biron. Effects of IL-12 on the Response and Susceptibility to Experimental and Viral Infections. 1994. J. Immunol. 152:1253-1264.

Pignata, C., J.S. Sanghera, L. Cossette, S.L. Pelech and J. Ritz. Interleukin-12 Induces Tyrosine Phosphoryation and Activation of 44-kDa Mitogen-Activated Protein Kinase in Human T Cells. 1994. Blood 83:184-190.

Pilipenko, E.V., V.M. Blinov, B.K. Chernov, T.M. Dmitrieva and V.I. Agol. Conservation of the Secondary Structure Elements of the-5'-Untranslated Region of Cardio- and Aphthovirus RNAs. 1989. Nucleic Acids Res. 17:5701-5711.

Pilipenko, E.V., A.P. Gmyl, S.V. Maslova, G.A. Belov, A.N. Sinyakov, M. Huang, T.D.K. Brown and V.I. Agol. Starting Window, a Distinct Element in the Cap-Independent Internal Initiation of Translation on Picornaviral RNA. 1994. J. Mol. Bio. 241:398-414.

Pilipenko, E.V., A.P. Gmyl, S.V. Maslova, Y.V. Svitkin, A.N. Sinyakov and V.I. Agol. Prokaryotic-like *cis* Elements in the-Cap-Independent Internal Initiation of Translation on Picornavirus RNA. 1992. Cell 68:119-131.

Ploemacher, R.E., P.L. van Soest, H. Voorwinden and A. Boudewijn. Interleukin-12 Syngergizes with Interleukin-3 and Steel Factor to Enhance Recovery of Murine Hemopoietic Stem Cells in Liquid Culture. 1993. Leukemia 7:1381-1388.

Podlaski, F.J., V.B. Nanduri, J.D. Hulmes, Y.C.E. Pan, W. Levin, W. Danho, R. Chizzonite, M.K. Gately and A.S. Stern. Molecular Characterization of Interleukin-12. 1992. Arch. Biochem. Biophys. 294:230-237.

Perussia, B., S.H. Chan, A. D'Andrea, K. Tsuji, D. Santoli, M. Pospisil, D. Young, S.F. Wolf, G. Trinchieri. Natural Killer (NK) Cell Stimulatory Factor or IL-12 has Differential Effects on Proliferation of TCR-$\alpha\beta^+$, TCR-$\gamma\delta^+$ T Lymphocytes, and NK Cells. 1992. J. Immunol. 149:3495-3502.

Robertson, M.J., R.J. Soiffer, S.F. Wolf, T.J. Manley, C. Donahue, D. Young, S.H. Hermann, J. Ritz. Responses of Human Natural Killer (NK) Cells to NK Cell Stimulatory Factor (NKSF): Cytolytic Activity and Proliferation of NK Cells are Differentialy Regulated by NKSF. 1992. J. Exp. Med. 175:779-788.

Rosenberg, S.A. Gene Therapy of Cancer. 1992. Important Advances in Oncology, p 17-38.

Rossi, A.R., F. Pericle, S. Rashleigh, J. Janiec and J.Y. Djeu. Lysis of Neuroblastoma Cell Lines by Human Natural Killer Cells Activated by Interleukin-2 and Interleukin-12. 1994. Blood 83:1323-1328.

Rueckert, R.R. Picornaviridae: the Viruses and Their Replication. 1996. *Virology*, 3rd Ed., Eds B.N. Fields, D.N. Knipe, P.M. Howley, Assoc Eds R.M. Cahnock, J.L. Melnick, T.P. Monath, B. Roizman and S.E. Straus, 611-653.

Sanbrook, J., E.F. Fritsch and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Scheper, G.C., H.O. Voorma and A.A.M. Thomas. Basepairing with 18S Ribosomal RNA in Internal Initiation of Translation. 1994. FEBS Letters 352:271-275.

Schmitt, E., P. Hoehn, C. Huels, S. Goedert, N. Palm, E. Rüde and T. Germann. T Helper Type 1 Development of Naive CD4+ T Cells Requires the Coordinate Action of Interleukin-12 and Interferon-γ and is Inhibited by Transforming Growth Factor-ß. 1994. Eur. J. Immunol. 24:793-798.

Schoenhaut, D.S., A.O. Chua, A.G. Wolitsky, P.M. Quinn, C.M. Dwyer, W. McComas, P.C. Familletti, M.K. Gately and U. Gubler. Cloning and Expression of Murine IL-12. 1992. J. Immunol. 148:3433-3440.

Shenk, T. 1996. Adenoviridae: The Viruses and Their Replication. *Virology*, 3rd Ed., Eds B.N. Fields, D.N. Knipe, P.M. Howley, Assoc Eds R.M. Cahnock, J.L. Melnick, T.P. Monath, B. Roizman and S.E. Straus, 2111-2148.

Sieburth, D., E.W. Jabs, J.A. Warrington, X. Li, J. Lasota, S. LaForgia, K. Kelleher, K. Huebner, J.J. Wasmuth and S.F. Wolf. Assignment of Genes Encoding a Unique Cytokine (IL-12) Composed of Two Unrelated Subunits to Chromosomes 3 and 5. 1992. Genomics 14:59-62.

Siegfried, W. Perspectives in Gene Therapy with Recombinant Adenoviruses. 1993. Exp. Clin. Endocrin. 101:7-11.

Siegfried, W., M.A. Rosenfeld, L. Stier, L. Stratford-Perricaudet, M. Perricaudet, S. Jallat, A. Pavirani and R.G. Crystal. *Ex vivo* Transfer of the Human α-1-Antitrypsin Gene into Cotton Rat Lung Using a Recombinant Adenoviral Vector. 1991. Am. Rev. Resp. Dis., Suppl. 4, 143:A325.

Stamminger, T., H. Fickenscher and B. Fleckenstein. Cell Type-Specific Induction of the Major Immediate Early Enhancer of Human Cytomegalovirus by Cyclic AMP. 1990. J. Gen. Virol. 71:105-113.

Tahara, H., H.J. Zeh III, W.J. Storkus, I. Pappo, S.C. Watkins, U. Gubler, S.F. Wolf, P.D. Robbins and M.T. Lotze. Fibroblasts Genetically Engineered to Secrete Interleukin 12 Can Suppress Tumor Growth and Induce Antitumour Immunity to a Murine Melanoma *in vivo*. 1994. Cancer Res. 54:182-189.

Tang, D.C., S.A. Johnson and D.P. Carbone. Butyrate-Inducible and Tumour-Restricted Gene Expression by Adenoviral Vectors. 1994. Cancer Gene Ther. 1:15-20.

Tannenbaum, C.S., N. Wicker, D. Armstrong, R. Tubbs, J. Finke, R.M. Bukowski and T.A. Hamilton. Cytokine and Chemokine Expression in Tumours of Mice Receiving Systemic Therapy with IL-12. 1996. J. Immunol. 156:693-699.

Trinchieri, G. Interleukin-12 and its Role in the Generation of T_H1 Cells. 1993. Immunol. Today 14:335-338.

Tripp, C.S., M.K. Gately, J. Hakimi, P. Ling and E.R. Unanue. Neutralization of IL-12 Decreases Resistance to *Listeria* in SCID and C.B-17 Mice. 1994. J. Immunol. 152:1883-1887.

van der Pouw Kraan, T.C.T.M., L.C.M. Boeije, R.J.T. Smeenk, J. Wijdenes and L.A. Aarden. Prostoglandin-E2 Is a Potent Inhibitor of Human Interleukin-12 Production. 1995. J. Exp. Med. 181:775-779.

Werbik, D.J. and S.S. Joshi. Immune Cells and Cytokines: Their Role in Cancer Immunotherapy. 1995. Intl. J. Oncol. 7:205-223.

Wilkinson, G.W.G. and A. Akrigg. Constitutive and Enhanced Expression from the CMV major IE Promoter in a Defective Adenovirus Vector. 1992. Nucleic Acids Res. 20:2233-2239.

Wolf, S., D. Seiburth, B. Perussia, J. Yetz-Adalpe, A. D'Andrea and G. Trinchieri. Cell Sources of Natural Killer Cell Stimulatory Factor (NKSF/IL-12) Transcripts and Subunit Expression. 1992. FASEB J. 6:A1335.

Wu, C.-Y., C. Demeure, M. Kiniwa, M. Gately and G. Delespesse. -IL-12 Induces the Production of IFN-γ by Neonatal Human CD4 T-cells. 1993. J. Immunol. 151:1938-1949.

Yang, Y., Q. Li, H.C.J. Ertl and J.M. Wilson. Cellular and Humoral Responses to Viral Antigens Create Barriers to Lung-Directed Gene Therapy with Recombinant Adenoviruses. 1995. J. Virol. 69:2004-2015.

Yang, Y., G. Trinchieri and J.M. Wilson. Recombinant IL-12 Prevents Formation of Blocking IgA Antibodies to Recombinant Adenovirus and Allows Repeated Gene Therapy to Mouse Lung. 1995a. Nature Med. 1:890-893.

Yei, S., N. Mittereder, S. Wert, J.A. Whitsett, R.W. Wilmott and B.C. Trapnell. *In Vivo* Evaluation of the Safety of Adenovirus-Mediated Transfer of the Human Cystic Fibrosis Transmembrane Conductance Regulator cDNA to the Lung. 1994. Hum. Gene Ther. 5:731-744.

Yoshida, A., Y. Koide, M. Uchijima and T.O. Yoshida. IFN-γ Induces IL-12 mRNA Expression by a Murine Macrophage Cell Line, J774. 1994. Biochem Biophys Res Commun 198:857-861.

Zhang, J.F., C. Hu, Y. Geng, J. Selm, S.B. Klein, A. Orazi and M.W. Taylor. Treatment of a Human Breast Cancer Xenograft with an Adneovirus Vector Containing an Interferon Gene Results in Rapid Regression Due to Viral Oncolysis and Gene Therapy. 1996. Proc. Natl. Acad. Sci. USA 93:4513-4518.

Zitvogel, L., H. Tahara, P.D. Robbins, W.J. Storkus, M.R. Clarke,

M.A. Nalesnik and M.T. Lotze. Cancer Immunotherapy of Established Tumours with IL-12, Effective Delivery by Genetically Engineered Fibroblasts. 1995. J. Immunol. 155:1393-1403.

Zou, J.-P., N. Yamamoto, T. Fujii, H. Takenaka, M. Kobayashi, S.H. Herrmann, S.F. Wolf, H. Fujiwara and T. Hamaoka. Systemic Administration of rIL-12 Induces Complete Tumour Regression and Protective Immunity : Response is Correleated with a Striking Reversal of Suppressed IFN-γ Production by Anti-Tumour T Cells. 1995. Intl. Immunol. 7:1135-1145.