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DISSERTATION

VIRUS VECTORS AS TOOLS TO STUDY HERPES SIMPLEX VIRUS TYPE-1 IN NEURONS

Submitted by

Donald Lloyd Traul

Department of Microbiology

In Partial Fulfillment of the Requirements for

the Degree of Doctor of Philosophy

Fort Collins, Colorado

Fall, 1999

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ABSTRACT OF DISSERTATION

VIRUS VECTORS AS TOOLS TO STUDY HERPES SIMPLEX TYPE-1 IN NEURONS

Herpes simplex viruses are ubiquitous pathogens of humans. The hallmark of these viruses is their ability to establish and maintain a latent infection in the neurons of the host throughout its lifetime, creating a reservoir from which reactivation and transmission can occur. What neuronal or viral component(s) allow the virus to establish, maintain, or reactivate from a latent infection is not known. Potentially, the viral immediate early proteins ICP0, ICP4, and ICP27, which regulate gene expression during the productive infection, are important during the latent stages of the virus lifecycle as well.

Virus vectors remain the best way to deliver and express genes in the nervous system. This is, in part, due to the unique characteristics of neurons to be in a post mitotic state and recalcitrant to other methods of introducing genes. Recombinant adenoviruses were used to deliver and express herpes simplex virus (HSV-1) immediate early proteins ICP0, ICP4, and ICP27 fused to the green fluorescent protein in cell lines or neurons. ICP4 or ICP27 fusion proteins showed fluorescence in the nucleus of cell lines or neurons, while the ICP0 fusion protein showed punctate cytoplasmic fluorescence in cell lines and was not detectable in neurons. These studies showed there might be differential expression of an HSV-1 immediate early protein in neurons.

Virus vectors have also been used to ablate gene expression by expressing the complement of a gene's coding region, or anti-sense RNA. Anti-sense RNA produced from the complementary strand can then block the expression of its targeted protein by binding to the mRNA, which normally would be translated to produce a functional protein. We used an RNA

virus vector and a DNA virus vector to produce anti-sense RNA targeted to ablate gene expression of two essential HSV-1 immediate early genes ICP4 or ICP27, and of one non-essential HSV-1 immediate early gene ICP0. A Sindbis (RNA) virus vector and an adenovirus (DNA) virus vector were able to express anti-sense RNA targeted against the 5' end of the ICP4 coding region. As ICP4 is essential for viral replication, we used the anti-sense expressing virus vectors to attempt to inhibit HSV-1 growth in cell lines or neurons. The Sindbis virus or adenovirus vectors, expressing anti-sense ICP4, did not inhibit HSV-1 growth in a specific manner. Control virus vectors, expressing RNA not specific for ICP4, inhibited HSV-1 growth to the same or greater extent. Control adenovirus vectors, interestingly, were able to inhibit 4-5 logs of HSV-1 growth. These studies suggest that using virus vectors to express anti-sense RNA to interfere with HSV-1 growth may be more difficult than expected.

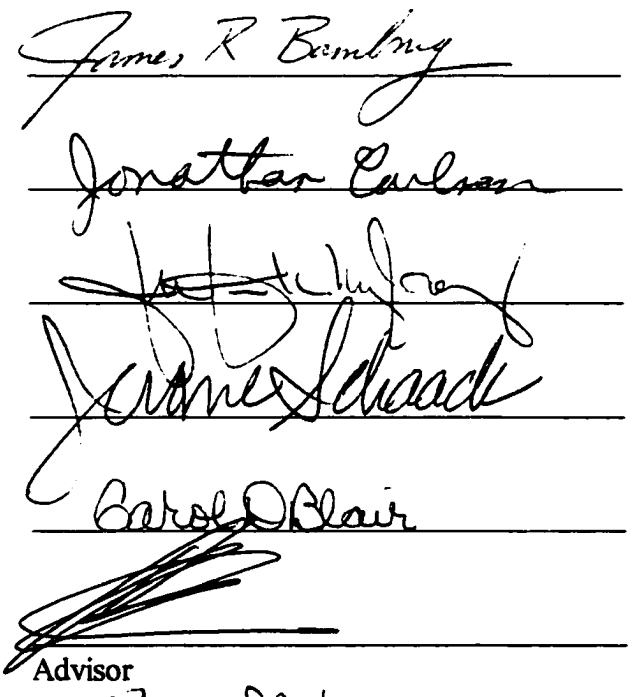
Donald L. Traul
Microbiology Department
Colorado State University
Fort Collins, CO 80523
Fall, 1999

COLORADO STATE UNIVERSITY

August 3, 1999

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY DONALD L. TRAUL ENTITLED *VIRUS VECTORS AS TOOLS TO STUDY HERPES SIMPLEX VIRUS TYPE-1 IN NEURONS* BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work



Advisor

Department Head

ACKNOWLEDGEMENTS

"To be who we are and to become what we are capable of becoming is the only end in life."

-Robert Louis Stevenson

It is often interesting to speculate on the events that have led one to any point in their life at any particular moment. Was it my first chemistry set? Was it my Mr. Wizard science book? Was it my sitting in front of the television set and drawing with my crayons as Neil Armstrong took his small step for mankind? I have yet to make a firm conclusion as to how I am here, at this moment, writing these words, other than to know I would not have gotten here without the help of many others along the way. I hope that these pages do some justice to those who went before me and to those who dedicated so much to the success of my academic career. I hope you all know of my deep gratitude for your help, support and guidance.

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Chapter 1.

INTRODUCTION

The presented research can best be described as an amalgamation of many different disciplines (virology, neurobiology, molecular biology, gene therapy, anti-sense technology, etc.) with the ultimate goal of employing virus vectors to understand the pathogenesis of herpes simplex virus (HSV) and for developing better ways to treat neurons infected with HSV. While this background review is not all-inclusive, it is intended to give enough detail about the salient features of each discipline used to form the basis for this research.

A. Herpes simplex viruses and the nervous system

1. Historical considerations

Herpes simplex viruses have probably tormented humans since before their first descriptions by Hippocrates in ancient Greece (for reviews see 132, 133). The term herpes (ερεπιν, to creep), however, was used to describe the spreading cutaneous lesions caused by many different illnesses (for review see 131). The Roman Herodotus is (probably) the first physician who described the specific herpetic eruptions around the mouth, which have come to be associated with herpes simplex viruses (for reviews see 132, 133). Two thousand years later, in 1736, the French physician Astruc described genital herpes in men and women (for reviews see 132, 133). During the 19th and 20th centuries herpetic conditions were further characterized to separate "herpes febriles" and "herpes genitalis" from other herpes viruses and poxviruses. The German physician Lipschutz around 1920 claimed that herpes febrilis and genitalis were related but etiologically distinct (132, 133). This was confirmed in the 1960s by Schneweis (132, 133) in West Germany and Plummer (146) in England, who found antigenic differences among HSV strains. Nahmias and Dowdle (131) finally correlated HSV-2 with genital and newborn infections and HSV-1 with most non-genital infections, relating these clinical findings to the usual mode of transmission of the two virus types.

2. HSV infection of the nervous system

Alphaherpesviruses HSV-1 and HSV-2 infect humans through inoculation into the skin or on mucosal membranes where active viral replication occurs (10-12, 145, 148, 209). Subsequently, virions or nucleocapsids enter the nerve terminals and are transported through the axon of neural crest-derived sensory and/or sympathetic ganglia to the nucleus of the cell body, where a latent infection can be established (34, 148, 185). The easiest way to visualize latency is

to envision the viral genome remaining in the nucleus of the infected cell in a "quiescent" state where no new virus is produced. Latently established virus can then reactivate throughout the lifetime of the host. After reactivation, virus travels anterogradely within the axon back to the site of infection, producing a recurrent lesion (35, 84, 111). Shedding of virus from lesions, therefore, is the essential route of transmission. The specific virus-host interactions that cause reactivation from the latent state are not yet known.

3. HSV pathogenesis

HSV can potentially infect any organ in the body with severe consequences; however, disease is usually self-limiting and confined to those afflicted with episodes of recurrent painful herpetic eruptions and lymphadenopathy. Approximately 1 in 200,000 individuals succumb to a necrotizing encephalitis (for review see 159). Herpes simplex encephalitis is the most common cause of sporadic, fatal encephalitis in the United States (for review see 213). Even when treated with anti-viral drugs, such as acyclovir, there is a 50% mortality rate, or severe neurologic impairment. There are 300,000 cases of herpes simplex keratoconjunctivitis reported yearly in the United States, which is the second leading cause of blindness (for review see 213). HSV disease in neonates is probably the most devastating. Neonates are by far the most vulnerable to encephalitis, disseminated disease, and death due to HSV infection with an estimated 3,000-5,000 cases per year (132, 133, 214). Immunocompromised patients also manifest severe forms of HSV (for review see 213). While anti-viral drugs such as acyclovir have done much to improve the prognosis of patients with HSV diseases, advanced disease and resistant viral strains do not improve with treatment (for review see 213).

There are an estimated 500,000 new cases of HSV-2, 40-60 million total infected individuals (for review see 213), and at least as many new cases of HSV-1 in the United States

each year. This translates to 1 in every 5-10 individuals in the United States being infected with HSV-1 and/or HSV-2. It is estimated that between 50-70% of any population worldwide is seropositive for HSV-1 or HSV-2 and harbors the latent virus (for review see 213). These high numbers of infected individuals harboring latent virus signify the need for better anti-viral therapies to reduce or eliminate the latent infection and prevent continued transmission due to reactivation. Prevention and eradication will depend on a complete understanding of how HSV establishes and reactivates from a latent infection.

B. Herpes simplex virus and the immediate early proteins ICP0, ICP4, and ICP27

1. The herpes simplex virion

The virion structure and productive life cycle of the HSV have been well characterized through genetic and biochemical studies in cell culture. The enveloped virion has a diameter of 150-200 nm (for review see 132). An amorphous tegument layer, containing viral proteins, is directly beneath the envelope. Innermost is the icosahedral capsid containing the double stranded linear DNA genome. The genome consists of 77 known genes and several more open reading frames, which may be found to encode functional proteins.

Upon infection of a permissive cell by receptor mediated uptake (for review see 160), the capsid is released into the cytoplasm and actively transported to the nuclear membrane (for review see 160). The capsid binds to the nuclear membrane and releases the linear genome into the nucleus, where there is a temporal cascade of gene expression (for review see 160). Gene expression can most easily be divided into three major classes (31, 91, 104). First, the immediate early or alpha genes are transcribed, which are translated to produce proteins that regulate the viral life cycle. Second, the early or beta genes are transcribed and translated into proteins necessary for viral genome replication. Lastly, the late or gamma genes are transcribed, and are

translated into structural proteins necessary for the icosahedral capsid and envelope to form the completed virion.

2. Regulation of the immediate early proteins

The immediate early proteins are well known as "gatekeepers" of the productive viral lifecycle. The role they have (alone or in combination) during the establishment, maintenance, or reactivation from latency in neurons, however, is not yet completely understood. This stems from a lack of understanding of the regulation and interactions of these proteins in neurons.

In permissive cells, the cellular Oct-1 protein binds with host cell factor (HCF) and the viral protein VP16 carried in the virion (for review see 160). Normally Oct-1 regulates transcription through binding to the octamer motif ATGCAAAT found in a variety of promoters (83). Binding of VP16 to Oct-1, however, alters the protein recognition site so that the Oct-1/HCF/VP16 complex now recognizes the TAATGARAT motif found in herpes simplex viral promoters. The complex allows transcription of the immediate early genes needed to induce other viral genes for a productive infection. Intriguingly, in mature neurons Oct-1 expression is downregulated and replaced by the expression of other octamer binding proteins including Oct-2 (80). Oct-2 has been shown to repress HSV immediate early genes necessary for viral replication (120). Furthermore, VP16 is not expressed in latently infected neurons (for review see 160). It has recently been found that there are multiple forms of HCF (HCF-1 and HCF-2) and that neurons express HCF-2 (102). VP16 complexes with HCF-1 but not HCF-2 (102). These findings suggest that reactivation in latently infected neurons requires either upregulation of HCF-1 and Oct-1, or the use of different transcription factors to induce viral genes for replication.

The immediate early proteins

Much of our understanding of the immediate early proteins arises from intensive studies of their functions during the productive viral lifecycle in permissive cells. There are generally considered to be 5 immediate early, infected cell proteins (ICPs), ICP0, ICP4, ICP22, ICP27 and ICP47 (see figure 1.1). Genes encoding ICP0 and ICP4 are diploid, while genes encoding ICP22,

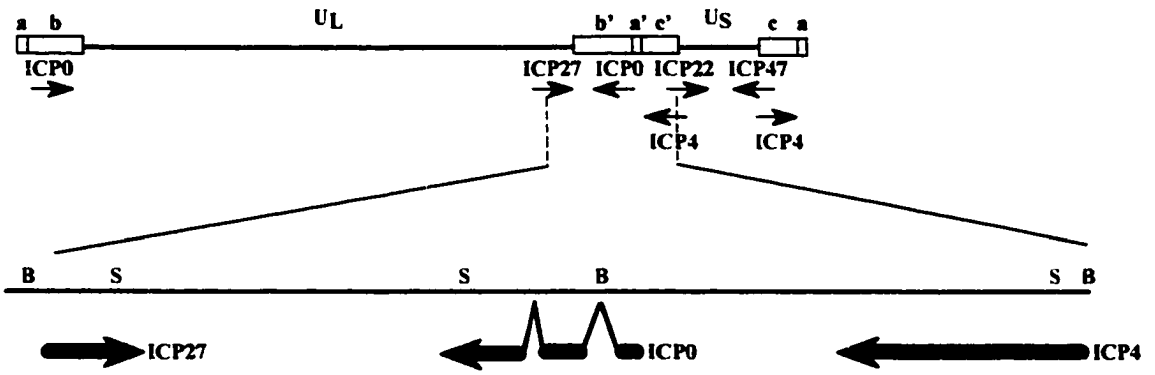

Figure 1.1 The HSV-1 genome and locations of the immediate early genes. The genome is comprised of a unique long and a unique short coding region with terminal repeats indicated by lower case letters between them. Arrows indicate the direction of the coding regions. The exploded view shows how ICP0, ICP4 and ICP27 are oriented to each other in the unique long and terminal repeat region. Splice sites are also indicated for ICP0 (B, *Bam*HI; S, *Sal*I).

ICP27, and ICP47 are single copies. ICP4 and ICP27 are essential proteins, so viral mutants not expressing these proteins will not produce progeny virus. ICP0 is a non-essential protein for viral growth in cell culture, but may be important in regulating viral latency (216). ICP22 is also a non-essential protein for viral growth, and is the least well characterized of the immediate early proteins. ICP47 is the only protein that appears not to regulate viral gene expression and is non-essential in cell culture. However, *in vivo*, it is potentially involved in abrogating the host immune

response against the virus (160). Each of these immediate early proteins has a multitude of effects on the virus lifecycle, and the host cells where the proteins are expressed.

Our research has focused on the two essential proteins, ICP4 and ICP27, and one non-essential protein, ICP0. There is a large body of literature on each of these proteins and I will briefly summarize some of their important characteristics here (see figure 1.2).

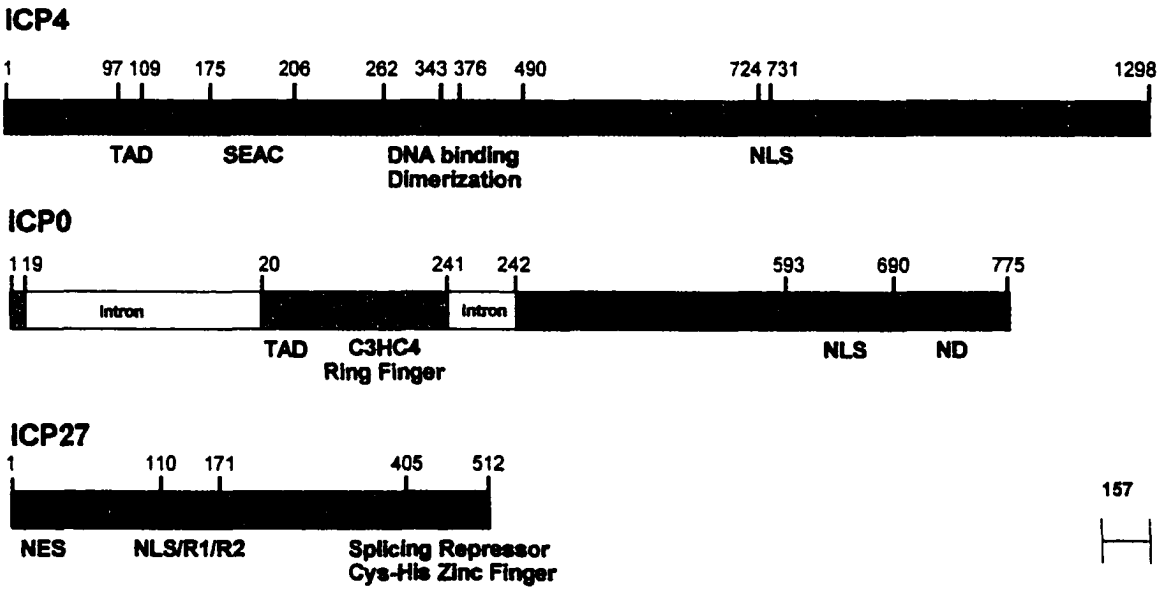

Figure 1.2 Diagram of the ICP0, ICP4 and ICP27 primary transcripts. The figure shows the locations of the some of the known functional regions including transactivation domains (TAD), serine/acidic residue-rich domain (SEAC), arginine rich domains (R1/R2), ring finger motifs, nuclear localization and/or export signals (NLS or NES), introns, and regions that interact with nuclear domains (ND). Transcript numbering refer to amino acid residues.

ICP0 (Vmw 110)

The immediate early 1 (IE1) gene produces, from translation of a transcript spliced of two introns, the full length 775 amino acid ICP0 of 110-kD (141). This phosphorylated nuclear protein is a transcriptional activator on its own, but more importantly is synergizes with ICP4 to transactivate viral transcription (58, 59). Viral mutants deleted in the IPC0 gene produce smaller

plaque sizes at low multiplicities of infection (190). ICP0 can bind DNA and is associated with chromatin in infected cell nuclei, giving a characteristic punctate nuclear staining pattern with immunohistochemistry (1, 79, 140). The protein contains multiple regions (see figure 1.2) important for a variety of virus-host interactions. The amino terminal portion contains the transactivation domain and ring finger motif common to transcriptional activators (57, 62, 121). Regions both on the amino and carboxyl ends have been demonstrated to be important for synergy with ICP4 (57). The carboxyl end contains a nuclear localization signal (56). More recently ICP0 has been shown to have pleiotropic effects on infected cells including disruption of nuclear domains associated with transcriptional activity, association with the ubiquitin-proteosome pathway, and destruction of kinetochores essential to cell division (61, 63, 64, 122).

ICP4 (Vmw175)

ICP4 has been the most studied of these three transcription factors. ICP4 is the product of the immediate early 3 (IE3) gene and produces from translation of a single transcript the 1298 amino acid protein of 175-kD. This essential phosphorylated nuclear protein is the predominant IE transcriptional regulator of HSV-1, and viral mutants deleted of this protein cannot replicate (45, 139). ICP4 is a promiscuous activator that can induce the expression of both HSV and non-HSV promoters (58, 115, 170, 198). ICP4 also can repress transcription including its own promoter (155). Transcriptional regulation is in part due to the ability of ICP4 to bind as a dimer and bend specific DNA sequences (e. g. ATCGTC) found in many promoter regions (60, 107, 127, 174, 217). ICP4 also contains multiple regions important for its functions. The amino terminal region contains a transactivation domain, a serine rich domain, and the DNA binding and dimerization domains (218). More toward the carboxyl end of the protein are the nuclear localization signal and repressor domain (46). ICP4 has numerous effects on both viral replication

and host cell functions. The protein is localized to the nucleus during viral replication and associates with other proteins including ICP8 and DNA polymerase (28, 149, 150).

ICP27 (Vmw 63)

The immediate early gene 2 (IE2) encodes ICP27 and, from a single transcript, is translated to produce the 512 amino acid protein of 63-kD (125). ICP27 is an essential, phosphorylated nuclear protein (124, 163). As with ICP0 and ICP4, ICP27 regulates transcription of viral genes. ICP27 has been shown to transactivate and transrepress promoter functions (58, 170). ICP27 deletion mutants do not appear to inhibit viral DNA replication (124). These mutants do not express late viral proteins and immediate early proteins are overexpressed (124, 154, 163, 179). ICP27 is a strong repressor of ICP0 and ICP4 transactivation (170). Other viral functions that have been elucidated for ICP27 are its ability to shuttle HSV RNAs between the nucleus and cytoplasm during the viral lifecycle, the ability to redistribute other viral proteins such as ICP0 and ICP4, and its ability to self-interact and form dimers or multimers (165, 227, 229, 230). The effects of ICP27 on the host cell include its ability to associate with the snRNPs and inhibit host cell mRNA splicing, and its required presence during viral infection to inhibit apoptosis (7, 77, 166, 167).

While it is not clearly known what specific effects ICP0, ICP4 or ICP27 have on HSV latency in neurons, it is known they have profound positive and negative feedback on each other during the viral lifecycle in permissive cells. Besides ICP0 synergism with ICP4, and ICP27 repression and redistribution of ICP0 and ICP4 mentioned above, the three proteins can interact in different complexes with each other that alter their expression patterns at different times throughout the virus lifecycle (43, 129, 228). All of these co-localization studies have been done in cell lines, and not in primary neuronal cells.

Latency- ICP0, ICP4 and ICP27

No discussion of herpes simplex viruses would be complete without some mention of latency. The ability to establish latency and remain latent in neurons is probably the defining pathology of herpesviruses that separates these viruses from any other. It is still debated whether HSV in neurons is truly latent, and not simply in a persistent or chronic state. It is also considered that there may be other tissues where the virus can remain latent. It appears, however, from all criteria that the virus is latent in neurons. During HSV latency in neurons there is no detectable virus production or viral protein expression, and only the production of one set of viral RNA transcripts called the latency associated transcripts (LATs) (187). There have been a number of experiments done to define the transcripts, determine if there is a protein product, and define if they have a function during latency. Many of the results are contradictory or are confusing because the results are compared between studies using different latency models.

In spite of this, several observations have been made which may have important implications for the function of this region of the viral genome. The LAT region produces two RNA transcripts, which resemble introns (for reviews see 88, 186, 208). The predominant LAT transcript is a 2.0 kb intron that is extremely stable (224). It has been suggested that this stable intron is an antisense molecule or even a hammerhead ribozyme (96, 224). A second minor intron of 1.5 kb is also produced from splicing of the major 2.0 kb LAT. There have been reports of an 8.3 kb mRNA transcript that could potentially be translated into a protein, but these results have been difficult to confirm (47). The LAT coding region completely overlaps the ICP0 gene and the 3' end of the ICP4 coding regions on the opposite genomic DNA strand. It is possible that transcription of the LAT gene blocks the ability of the opposite DNA strand to transcribe the ICP0 or ICP4 genes. Many studies have been done to investigate the cellular events that

upregulate the LATs or the immediate early proteins, by characterizing the promoter regions of these genes. It is hard to believe that the LAT region, the only one actively expressed during latency, is not involved in some aspect of regulating the latent state. However, straightforward experiments have not defined a function for the LAT region. New techniques and understandings of gene expression may help define what role it does play in latency and/or reactivation.

Deletion mutants in the immediate early genes have been used to study their effects on latency. One study used the mouse ocular model and looked at mutant viruses deleted in ICP0, ICP4, or ICP27 (117). They concluded that the ICP4 and ICP27 mutants could not establish latency, whereas the ICP0 mutant could (117). This makes intuitive sense, since viruses deleted of ICP4 or ICP27 genes cannot replicate and it would be very difficult for the virus to reach nerve terminals without an increase in viral progeny at the site of inoculation. When similar deletion mutants for ICP0 and ICP4 were directly infected onto neurons, however, in an *in vitro* latency model, the ICP4 deletion mutant was able to establish latency, while the ICP0 deletion mutant was less efficient in the establishment of a latent infection (216). No studies have directly looked at ICP27 deletion mutants for their ability to establish latency, although the viruses do exist. Using HSV-1 mutant viruses deleted of these immediate early genes co-infected with adenoviral vectors expressing the immediate early proteins, we hopefully can address if any of these proteins are necessary for establishment, maintenance or reactivation of latency in neurons.

C. Viral vectors, gene transfer and the nervous system

We have learned that "errors" or "mistakes" in genes are the underlying cause of many disorders and diseases that afflict humans. If we can correct these mistakes in the genetic code, it may be possible to cure or reverse the pathology these mistakes cause. A timeline for the advent of gene therapy begins with a concept described by the chemical pathologist Sir Archibald

Garrod. Around 1908 he described that human genetic diseases and other disorders and defects result from the disruption of the normal metabolic pathways by inherited mistakes in the factors that regulate those pathways (for review see 65). He called these mistakes "inborn errors of metabolism". During the 1960s and 70s the scientific knowledge and technologies to prove this idea were developed. DNA was found to be the code of life and within this code were the factors where the mistakes occurred. These factors we now know are "genes", which can be normal or abnormal. It would take approximately 30 more years before virus vectors would actually be used to carry genes into mammalian cells.

Viral vectors

A series of concepts in virology and molecular biology were needed before the idea that viruses could be "engineered" to transfer and express genes. The first use of the term "genetic engineering" appeared in an essay by R. D. Hotchkiss entitled "Portents for a Genetic Engineering" in the Journal of Heredity (94). Renato Dulbecco, during the 1960s, was the first to show that an animal virus, simian virus 40, could efficiently transform a cell into a tumor by integrating the viral genome into the host cell (50-53, 78, 164). These studies suggested that if viruses could be modified to contain suitable genetic material they might be used to complement genetic defects found in patients. Stanfield Rogers was the first to show that a naturally occurring poly(A)-modified tobacco mosaic virus caused infected plants to have increased poly-lysine (158). This showed that virus-mediated transduction was very efficient and engineered viruses might be able to introduce wild type alleles of deleterious mutant genes into defective human cells. At the beginning of 1970 Theodore Friedmann discovered that papovavirus contains "pseudovirions", which have random fragments of cellular DNA (6, 66). This extended the idea of transferring genes using animal viruses. At this point, however, it was still not possible to isolate normal

copies of the disease-related genes and produce recombinant DNA or produce recombinant virus vectors. In 1973 Stanfield Rogers used the Shope papillomavirus, which contains a viral arginase, to treat two young German girls who lacked the arginase enzyme (157). There has never been any rigorous follow-up biochemical studies to convincingly show that the girls were helped or harmed by the experiment. This experiment showed again that a naturally occurring virus could modify a genetic phenotype; however, not until 1972 were the methods for isolating, cloning, and characterizing disease-related genes finally developed.

Defects of the β-globin gene were some of the first to be shown to cause important human disease, such as sickle cell anemia and forms of β-thalassemia. Martin Cline reported that mouse bone marrow cells could be transfected with foreign genes using, calcium phosphate transfection, and selected for to allow partial repopulation of the marrow by genetically modified cells (32). These studies led to bone marrow cells of two thallasemia patients being transfected with the β-globin gene, selecting the transduced cells, and re-infusing the cells into the patients (32). Critics of these experiments suggested that stem cells of the bone marrow are poorly transduced using calcium phosphate transfection and therefore these experiments would not succeed. United States regulatory committees did not approve these studies, which were carried out in Italy and Israel. While Cline was severely punished for these experiments, there was never any follow-up study of these patients to demonstrate that the normal globin gene was produced or that the patients were helped by the experiment.

The first truly efficient viral vectors for potentially introducing therapeutic genes to mammalian cells were reported by several groups in 1981. All three groups used retrovirus vectors to deliver and stably transduce cells with the HSV-1 thymidine kinase (tk) gene (175, 194, 210). A tool was finally available making it possible to efficiently introduce genes into

mammalian cells with the potential of correcting genetic defects. Retrovirus vectors were limited, however, by low titers, instability, and the inability to infect non-dividing cells. A host of other viral systems were developed to transfer genes into mammalian cells including vectors derived from human adenovirus, herpes simplex virus, and adeno-associated virus (16, 130, 177). Many clinical trials have begun; however, gene therapy using virus vectors is still in its infancy and whether it will succeed has yet to be proven. One prominent concern has been over the safety of using viral vectors. A status report of ongoing clinical trials found, however, that there were no wild-type viral contaminants in any of the viral vector based systems being used (161). The concept of gene therapy is a driving force in modern medicine and many of the limitations and technical problems are being addressed and overcome.

Gene transfer and the nervous system

Neurons pose unique challenges for gene transfer in that they are post-mitotic and recalcitrant to most conventional methods of transfecting DNA into cells. The transfer and expression of genes to produce functional proteins in the nervous system can still only be efficiently accomplished with viral vectors. Viral vectors have become important tools to study and understand the molecular biology of the nervous system. These studies could potentially lead to the treatment of diseases in the nervous system using virus vectors.

Viral vectors based on herpes simplex viruses (HSV) were the first vectors used to manipulate genes in the nervous system (49, 89, 90, 138). These obviously were used because of their neurotropic nature and the fact that HSV can establish a latent state for the lifetime of the host. Vectors based on HSV, however, have been extremely problematic because of the lack of understanding of the mechanisms involved in this latent state, as well as the toxicity of many of the viral genes that remain in the vector. A switch away from HSV based vectors came in 1993

when it was discovered that adenoviruses could infect and express genes in neurons with high efficiency (4, 9, 41, 116). This work was extended in 1994 when adeno-associated viruses were also found to be able to infect and express foreign genes in the nervous system (106). The most recent viral vector to be shown to transduce neurons is based on the human immunodeficiency virus (HIV) (134). Retroviral vectors had been used for a long time to transduce non-neuronal cells, but an absolute requirement was for the cells to divide. During cell division the nuclear membrane fragments and the proviral DNA is able to enter into the nucleus and integrate into the cell genome. Lentiviruses, such as HIV, have the unique ability to transport the preintegrative DNA complex through the host cell nucleus of non-dividing cells, such as neurons, where it can then integrate. This ability stems from the karyophilic qualities of two virion proteins, matrix and Vpr (20, 128, 151). While these four viral vectors based on DNA viruses and a retrovirus have received the main focus for gene therapy in the nervous system, many new RNA based viral vectors are now being considered as well. Our studies have included development and use of a DNA adenoviral vector, as well as an RNA Sindbis viral vector. These vectors were used to express specific HSV immediate early genes for ICP0, ICP4 and ICP27, as well as anti-sense RNAs targeted against these genes.

Sindbis virus vectors

The Sindbis virus, named for the Egyptian village where it was first isolated, was first described by R. M. Taylor and H. S. Hurlbut in 1953 (97, 196). This RNA arbovirus isolated from mosquitoes and transmissable in mice was grouped with the genus Alphaviruses in the family *Togaviridae* (169).

The Sindbis virus particle (60-70 nm in size) contains an icosahedral nucleocapsid within which the RNA genome is complexed with capsid proteins. A lipid bilayer envelope with the E1

and E2 viral structural glycoproteins surrounds the nucleocapsid. Viral particles bind to cell receptors found on insect and mammalian cells and are taken into the cell by endocytosis. The RNA genome is then released into the cytoplasm where viral replication occurs. The Sindbis viral genome is a linear, plus-stranded RNA of 11,703 nucleotides that is capped and polyadenylated (see figure 1.3; 191). The 5' two-thirds of the genomic RNA acts directly as an mRNA upon release into the cytoplasm to be translated and processed into the four nonstructural proteins (nsP1-nsP4) comprising the RNA replication machinery (48, 109, 118, 176). The viral replicase copies the positive-strand RNA into a negative strand (105). This negative-strand serves as a template for full-length positive strand genomic RNA. The negative-strand also serves as a

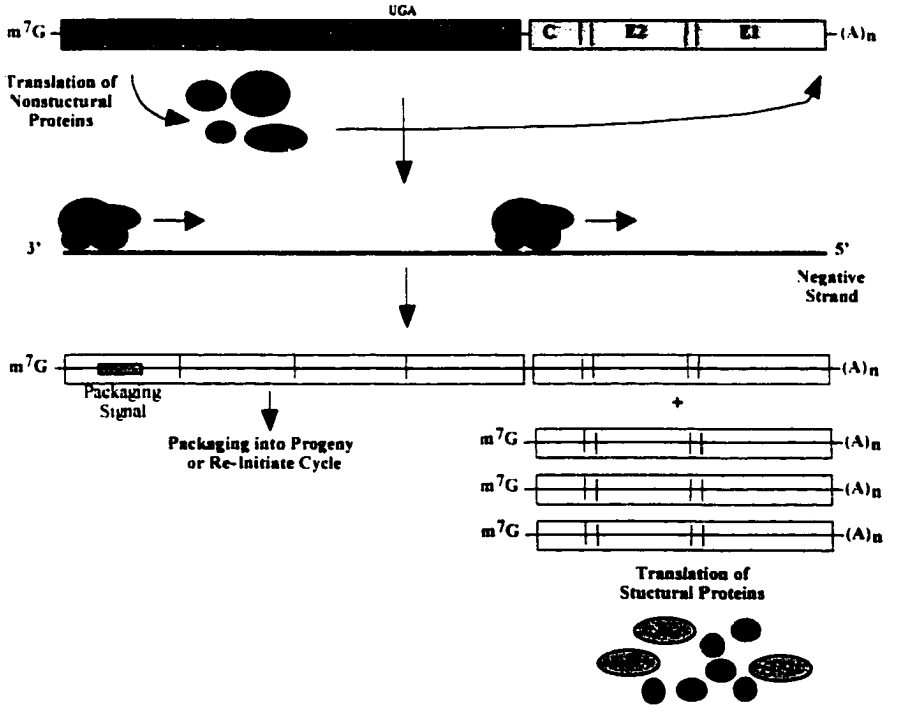

Figure 1.3 Genome and replication strategy of alphaviruses. The viral positive RNA genome is directly translated in the target-cell cytoplasm after the initial infection event (top lines). The translation products (nsp1-4) form the viral polymerase that synthesizes the negative strand (second line), and is followed by synthesis of both positive-strand genomic RNA and multiple copies of the structural protein subgenomic message.

template to produce a shorter RNA from an internal promoter (119). The shorter RNA is expressed at high levels and translated as a polyprotein that is proteolytically processed to produce the structural proteins including capsid, E1 and E2 (168). Viral particles form as mature glycosylated and acylated structural proteins accumulate in the cytoplasm (168). The RNA genome rapidly associates with the capsid protein to form the icosahedral nucleocapsid (211), which then associates with the viral spike glycoproteins to form an envelope that buds from the plasma membrane producing the completed viral particle.

Sindbis virus was one of the first alphaviruses to be cloned and from which infectious RNA transcripts were produced *in vitro* (153). The first descriptions of alphavirus vectors appeared in 1989 (219). Three types of vectors have been derived using these systems (see figure 1.4). Double-subgenomic vectors place a gene of interest behind a second subgenomic promoter

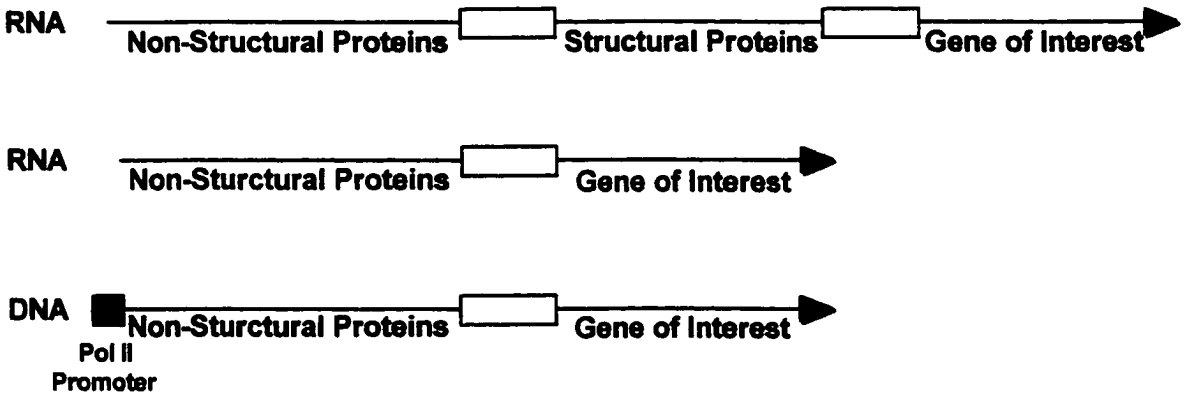

Figure 1.4 Alphavirus vector configurations. A double-subgenomic vector carrying the entire complement of viral protein is replication competent upon transfection of the *in vitro* transcribed RNA molecule (top line). The alphavirus "replicon" vector (middle line) replaces the structural genes with the gene of interest. To produce virus, *in vitro* transcribed RNAs of the replicon and a complementary defective helper (DH) genome, which supply the structural proteins, are made and co-transfected into cells. This vector has been configured into a DNA based virus by incorporating an RNA polymerase II promoter (black box) upstream of the leader sequence of the alphavirus. This allows generation of the RNA vector after transfection of the DNA molecule. Co-transfecting a defective helper genome similarly produces virus. Subgenomic promoter regions (white boxes).

downstream of the structural proteins within the genome (75). While these have utility for many applications they are replication competent and tend to be unstable after several passages. The next class of vectors, "replicons", were created by replacing the structural genes with a gene of interest (for reviews see 22, 144, 192). These RNA transcripts have the ability for self-amplification

and can express very high levels of foreign protein. To produce recombinant viral particles requires co-infection with wild-type virus. Viral stocks produced this way are contaminated with the wild-type virus. To overcome this, defective helper RNAs that could not be packaged into virions were created to supply the structural proteins (21). The replicon and defective helper (DH) plasmids were constructed with a bacteriophage promoter so *in vitro* transcription could be used to produce the RNAs for making virus. Co-transfection of the RNAs into cultured cell lines produces viral particles, which are harvested from the culture supernatant. While these produce moderate levels of virus, the system is cumbersome and the replicon and DH RNAs often can recombine to produce a wild-type genome. Recent advances to the Sindbis viral vector system have addressed this issue by producing inducible packaging cell lines and DNA based expression cassettes to produce the replicon RNA (for review see 65).

Sindbis virus-based systems have been used in a wide array of applications, but are being pursued most intensively for their potential promise as immunotherapeutics or vaccines. There are still a number of problems to overcome in the development of these systems. The structural and non-structural proteins are both toxic to cells in the long term. The virus does not infect all cell types and usually more than a single RNA is needed for expression. Finally, higher titers of viral particles will be needed for the system to be useful.

Adenovirus vectors

The virus responsible for acute respiratory distress was first isolated in 1953 by Rowe and colleagues who named it "adenoid degeneration agent" (162). Independently, Hilleman and Werner isolated immunologically similar viruses, which they named "respiratory illness agents" (85). It was soon realized that the viruses belonged to a new group of viruses, which were named "adenoviruses" and placed in a new viral family *Adenoviridae* (54).

The 50 serotypes of human adenoviruses have been divided into 6 subgroups of which adenovirus type 2 (Ad 2) and adenovirus type 5 (Ad 5), both of subgroup C, are two of the best characterized. The adenovirus particle (approximately 80-90 nm in size) is an icosahedral non-enveloped nucleocapsid within which the 28-43 kb linear double-stranded DNA genome resides. Protruding from the vertices of the nucleocapsid are long fiber proteins, which are attached to the nucleocapsid by the penton base. Viral particles usually gain entry into cells by binding to high affinity receptors on the cell surface followed by internalization via the penton base proteins, which require cellular integrins for uptake (14, 44, 92, 123, 188, 215). Virus can also be directly taken into cells without fiber protein receptors via the penton base interacting with integrins on the cell surface (95). After internalization of the virus, the capsid is actively transported to nuclear pore complexes where the viral genome is released into the cell nucleus (39, 73, 74, 172). The genome is flanked by short (100-140 bp) inverted terminal repeat (ITR) sequences, which each contain an origin of DNA replication. The viral genome is encapsidated using a cis-acting packaging domain contained near the left end ITR of the genome. Adenovirus gene expression can be divided, for the most part, into early (E) genes and late (L) genes, with the onset of DNA replication dividing the two phases (for review see 173). The early region transcription units are early region 1A (E1A), E1B, E2, E3, and E4. The genes encoding protein IX and IVa2 are

expressed late. Most late transcripts initiate from the major late promoter (MLP) and are subsequently processed to generate five families of 3' coterminal transcripts, which each contain the tripartite leader sequence at the 5' end. Other late transcripts include the E2 and VA (see figure 1.5). The host cell RNA polymerase II transcribes almost all of the adenoviral genes, except for the VA genes that are transcribed by RNA polymerase III. The first viral protein expressed is E1A, which is alternatively spliced to produce five proteins involved in transcriptional regulation of the viral genome that are essential for adenovirus replication under normal circumstances. The E1A products also signal the infected cell to enter the S-phase by binding to host cell proteins that normally block cell cycle progression. This event would normally cause the cell to die by apoptosis; however, a second alternatively spliced protein, E1B 19 kD, is produced that blocks E1A-induced apoptosis (212). A second alternatively spliced E1B protein, E1B 55 kD, as well as the E4 34 kD protein, block host mRNA transport, and stimulate

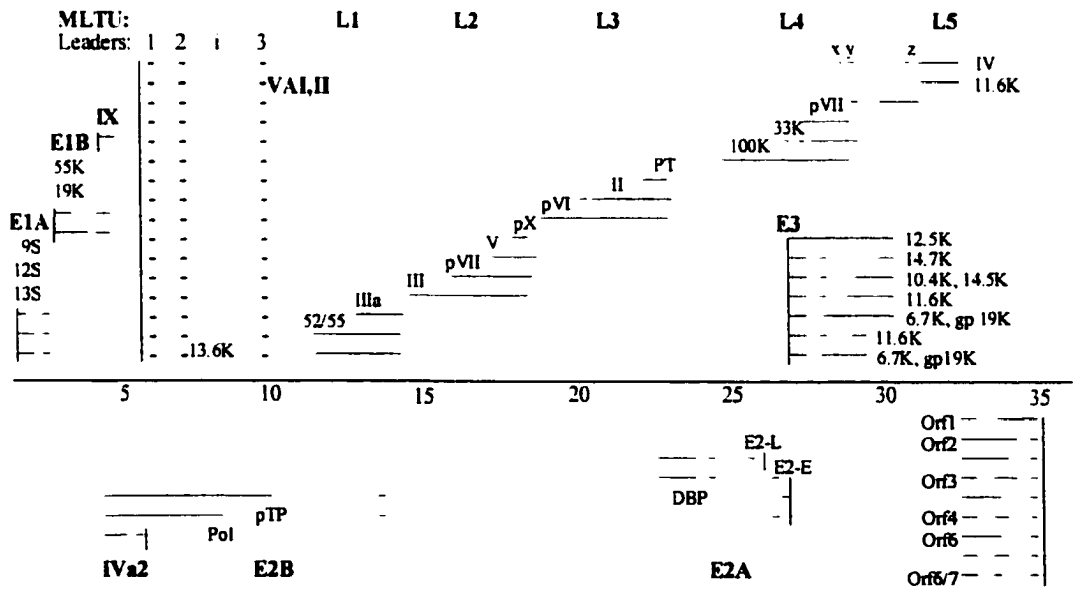

Figure 1.5 The transcription map of the human adenovirus 5 genome. The genome is represented by a line in the center, numbered in kb from the left end, with rightward transcription shown above and leftward transcription shown below.

viral mRNA transport. As E2 gene products accumulate, adenovirus DNA replication begins.

The ITRs serve as replication origins by binding the E2B encoded DNA polymerase. The E2B preterminal protein (pTP) acts as the primer for DNA synthesis after forming a complex with cellular factors NFI and NFII, and covalent linkage to dCMP catalyzed by the adenovirus polymerase (152). Binding with the pTP protects the single-stranded DNA produced by strand displacement replication from nuclease degradation (201). Elongation requires the E2B DNA polymerase and the E2A single strand DNA binding protein as well as cellular factors (for review see 173). Adenovirus late genes begin to be expressed after the onset of viral DNA replication. A single primary transcript is produced, that is spliced into 18 distinct mRNAs (55). At this time E1 and E4 gene products block the accumulation of host cell mRNAs in the cytoplasm (8, 13, 23, 76, 143). These same proteins assist in the accumulation of viral mRNAs in the cytoplasm (226). After the hexon and penton capsomers are formed they accumulate in the nucleus where the viral particle is assembled (93, 193, 204). Empty capsids form and, subsequently, a viral DNA molecule enters the capsid. Packaging of the viral DNA genome is a polar event beginning at the left end and requires several proteins (33, 69). Viral particles continue to be made in the cell for two days producing about 1000 infectious viral particles per cell.

While the E3 gene products are not necessary for viral replication, the ten identified E3 proteins are critical for evading host immune responses against the infected cell (for reviews see 19, 24, 87). In particular the E3 gp 19 kD protein binds to MHC class I molecules, causing their retention in the endoplasmic reticulum. This prevents presentation of viral antigens on the cell membrane to cytotoxic T cells (70). The E3 14.7 kD protein and E3 10.4 kD/14.5 kD complex block cytolysis and inflammation induced by tumor necrosis factor and Fas ligand (110, 181).

Human adenoviral vectors have generally been constructed from the Ad2 or Ad5 serotypes, which have been completely sequenced (30, 156). The first adenoviral vectors appeared in the 1980s and were utilized for delivering genes that could transform cells using the SV40 or polyomavirus T antigens (15, 42, 171, 180, 197, 202, 203). Early vectors generally placed proteins, such as antibiotic selection markers, behind the major late promoter and were shown to drive high levels of gene expression (10-20% of total cellular protein in 293 cells) (5, 15, 42, 101, 189, 220). These studies showed the utility of expressing foreign genes in cells using adenoviral vectors, and more importantly showed that adenoviruses rarely transform cells alone, probably due to the inefficiency of adenoviruses to integrate into the host cell genome (203).

First generation vectors for gene therapy, extended from this earlier work, generally have deletions in the E1 region to render the vector replication-defective, and to prevent virus production and lysis of the target cell. Viral particles are produced by supplying the E1 genes *in trans* with the complementing human embryonic kidney cell-derived 293 line (72). The largest reported deletions extend from nucleotide 342 to 3523, removing most of the E1 sequence (17). To increase the coding capacity of the viral vector, deletions are also made in the non-essential E3 region. Deleting nucleotides 27865 to 30995 removes 3.1 kb of the adenovirus genome and prevents expression of all the E3 genes (17). Combining the E1 and E3 deletions with the ability of adenoviruses to package a genome that is 5% larger than the wild type genome, means these vectors can encode 8.3 kb of foreign DNA.

The most common strategy to construct a recombinant adenovirus employs using a shuttle plasmid constructed from the left end of the virus, deleted of the E1 gene. The foreign gene or cDNA is inserted into the deleted E1 region of the shuttle plasmid, usually with a promoter and polyadenylation signal. The inserted sequence is flanked on both sides by the remaining

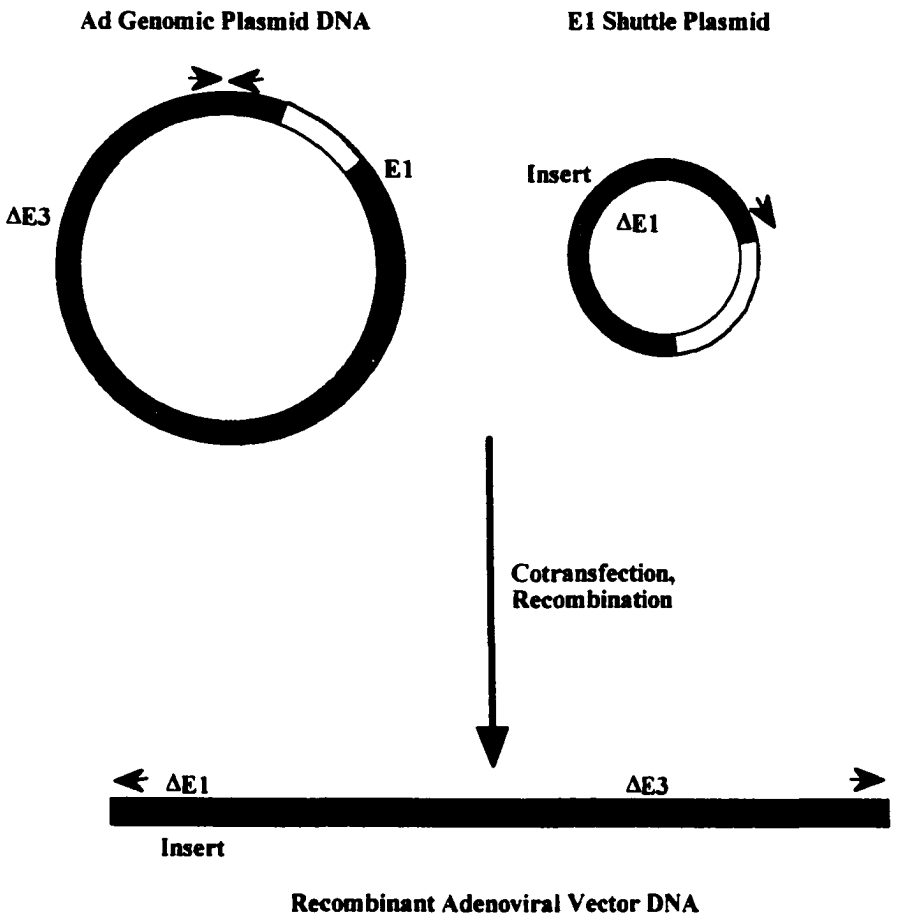

Figure 1.6 Construction of first-generation recombinant adenovirus vectors by homologous recombination. Ad vectors can be rescued following co-transfection of an E1 shuttle plasmid carrying the gene of interest with an Ad genomic plasmid (top). Homologous recombination in 293 cells between the transfected DNAs results in the production of recombinant virus containing the foreign DNA insert (bottom). Adenoviral DNA sequences (shaded bars), plasmid DNA sequences (white bars), insert DNA (speckled bars), ITRs (black arrows).

adenoviral sequence from the left end. Viral particles are produced either by *in vitro* ligation of the shuttle plasmid with the right end of the viral genome followed by transfection of the DNA into the 293 packaging cells, or more usually the shuttle plasmid and a second plasmid carrying the adenoviral genome are co-transfected into the packaging cells (see figure 1.6; 68, 86, 182). Increasing the size of the second plasmid, carrying the adenoviral genome, or removing the

packaging signal from this plasmid, allows for the selection of only recombinant viral particles that have viral genomes of the correct size or genomes carrying the packaging signal (17, 126). A number of other strategies have been employed to increase the efficiency of producing recombinant viruses in this system. While this system has been extremely successful in producing recombinant viruses for gene transfer and expression in mammalian cells, it has been plagued by a host of important problems. At high multiplicities the E1 gene becomes dispensable for replication (103). Even at low multiplicities E1 deleted viruses express low levels of viral gene products, suggesting that cellular factors have E1 like activity (98, 135, 136). These events activate the E2 promoter, which begins replication of adenoviral DNA and expression of other viral gene products that lead to cytopathic effects on the transduced cells and activation of the immune system (25-27, 40, 199, 221-223, 225). New adenoviral vectors have been constructed to try and overcome these problems by removing the E2 genes, the E4 genes, or all of the adenoviral genes that can be expressed in order to eliminate activation of immune responses. The most recent adenoviral vectors are being constructed with the E3 genes intact to attempt to use the immune regulatory functions of these genes (for reviews see 71, 82).

D. Virus vectors for delivery and expression of anti-sense molecules

Anti-sense

The improved understanding of how genes are expressed has led to the promise of many new treatments for diseases. One such concept, anti-sense technology, relies on an understanding of nucleic acid structure and function that was laid down by Watson and Crick. In their model, complementary nucleotides bind to each other to form a stable structure through hydrogen bonds. Both DNA and RNA can form duplexes with complementary sequences. Anti-sense RNA was first recognized in prokaryotes and shown to regulate such processes as replication,

osmoregulation, transposition, and phage reproduction (for review see 200). Anti-sense RNAs to genes in eukaryotes have also been found, such as the mouse dihydrofolate reductase gene, the *Drosophila* dopadecarboxylase gene, and the chicken myosin heavy chain gene, but their regulatory role in gene expression remains undefined (for review see 200).

Anti-sense technology relies on the specificity of nucleotide base pairing to combine with a specific genomic target (see figure1.7). The general approach for targeting a specific gene has

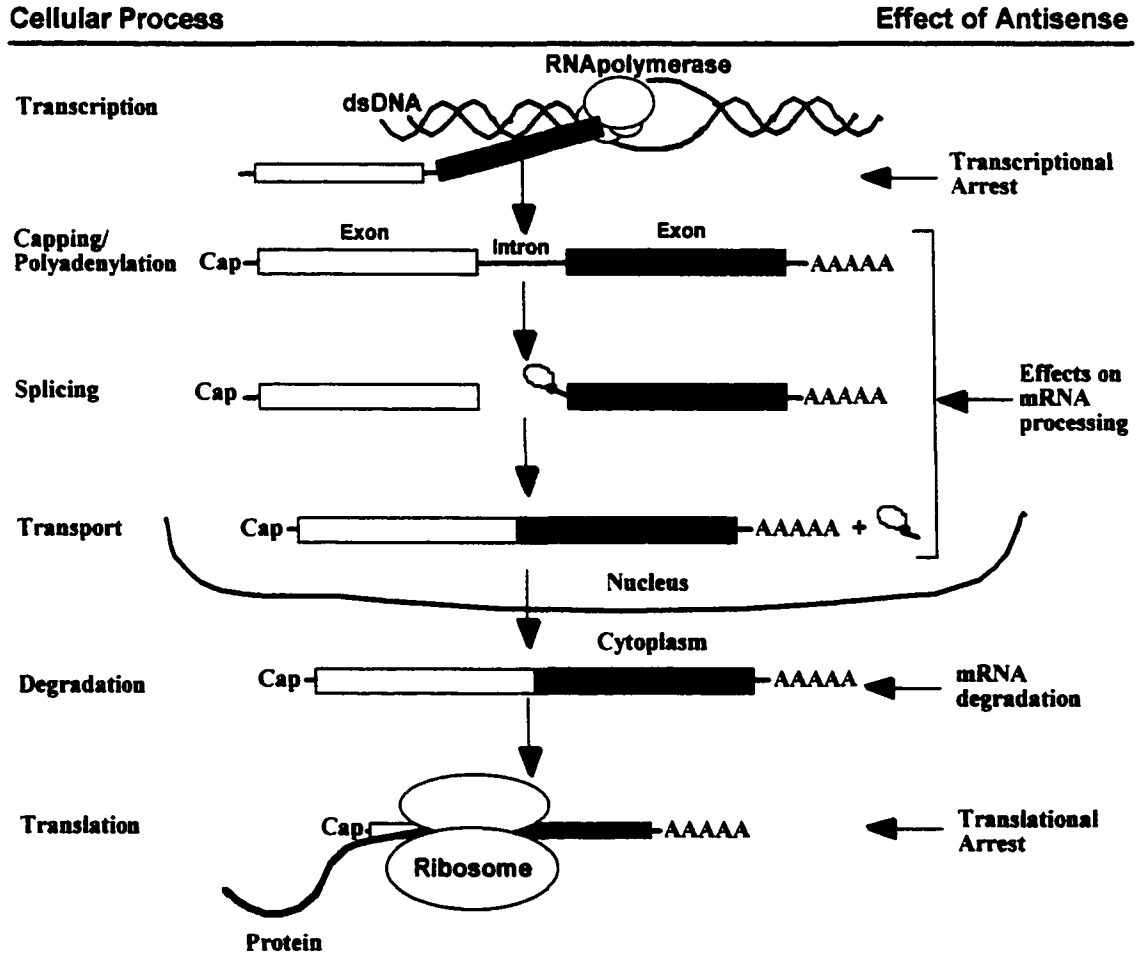

Figure 1.7 RNA processing and possible anti-sense targets. Oligonucleotides or anti-sense RNA can form a duplex with complementary target RNA sequence. This can prevent RNA splicing or transport, or mRNA translation. The duplex can also be a substrate for RNase H. Anti-sense molecules can bind to dsDNA in the major groove forming a triple stranded structure. This can interfere with transcription, replication or protein binding to dsDNA. Boxes (exons), lines attached to boxes (introns or non-translated regions).

been to make synthetic DNA oligonucleotides complementary, or anti-sense, to the cap-site, start codon or splice junctions of the RNA transcribed from this gene (for review see 200). The oligonucleotides are synthesized in ways to accomplish at least three important goals. The first goal is to modify the oligonucleotide to make it more stable and resistant to nuclease degradation. The length of the oligonucleotide also affects its stability, so longer molecules tend to be more stable. Increasing length, however, increases non-specific binding, therefore oligonucleotides generally are made between 12-20 nucleotides long (for review see 200). This maintains specificity to bind the target, but increases the stability of the oligonucleotide. The second goal is to modify the oligonucleotide to make it bind more strongly to the target RNA . One way this has been accomplished has been to conjugate the oligonucleotide with an intercalating agent such as acridine (200). Cells very poorly absorb unmodified oligonucleotides, and the third goal is to make modifications, such as methylation or sulfonation, that render the oligonucleotide more lipophillic and better able to penetrate the cell membrane (36-38, 81, 200).

The mechanisms by which anti-sense oligonucleotides inhibit gene expression are not completely understood. The concept, as it was envisaged, was that the oligonucleotide would bind to the target RNA and block its ability to be spliced or translated, as well as activate host cell RNase H to degrade the RNA-DNA duplex (for reviews see 81, 200). While this does indeed occur with certain oligonucleotides, anti-sense molecules have other specific and non-specific effects.

Another target specific way that anti-sense oligonucleotides have been considered to work is by the formation of a triplex structure with the genomic DNA encoding the gene or binding of the oligonucleotide to the coding strand of the genomic DNA during transcription (for review see 81). Non-specific actions of oligonucleotides include toxicity of the molecules directly to the cells

or binding of the oligonucleotides to cellular proteins, such as DNA polymerase or transcription factors. Synthesis of new oligonucleotides with modifications not yet tried will probably find the balance to protect the oligonucleotide and give specific binding to the target RNA.

The first clear demonstration of anti-sense therapy was through the work of Zamecnik and Stephenson, who used a 13 base oligonucleotide that was complementary to the Rous sarcoma virus genome (184). Anti-sense oligonucleotides have since been applied successfully to prevent expression of a wide array of viral and cellular genes (for review see 200). These methods have evolved to the wide use of synthetic DNA oligonucleotides that base pair with a sense-strand target mRNA (183). Some of the viruses shown to be inhibited by specific anti-sense oligonucleotides include Rous sarcoma virus, human immunodeficiency virus, influenza virus, vesicular stomatitis virus, simian virus 40, human papillomavirus, and herpes simplex virus (for reviews see 2, 18, 81).

Synthetic anti-sense oligonucleotides against HSV-1 have generally been targeted against the immediate early genes of the virus (29, 108, 112-114, 142, 178). These include several publications using oligonucleoside methylphosphonates against the identical splice junction of the pre-mRNAs encoding the ICP22 and ICP47 proteins (29, 108, 112-114, 178). Oligonucleoside methylphosphonates have also been directed against mRNAs for the ICP0 and ICP4 proteins (29, 108, 113). These studies were able to show >90% reduction of virus titer in cells infected with HSV-1 both *in vitro* and *in vivo* (113). Oligonucleoside methylphosphonates, however, form duplexes that are not substrates for RNase H and are thought to work by blocking translation (3). To increase activity oligonucleotides have been synthesized with phosphorothioate modifications (142). Duplexes formed between these types of oligonucleotides and the target RNA are substrates for RNase H (3). Oligonucleotides synthesized against a variety of viral targets

including the mRNA encoding ICP0 with the phosphorothioate modification, however, inhibited HSV-1 replication only to the same extent as the methylphosphonate based oligonucleotides (142). The present methods, while partially successful, have yet to show complete inhibition of HSV-1 replication. It is likely that the difficulties of getting the anti-sense oligonucleotides into specific cell types and the inherent instability of oligonucleotides are interfering with better results.

Virus vectors and anti-sense

An alternative way to overcome many problems inherent to synthetic anti-sense oligonucleotides is to express anti-sense RNA from a virus vector. Virus vectors offer an efficient means to deliver anti-sense RNA into specific cell types with the potential for long-term expression of the anti-sense RNA. This is generally accomplished by expressing the complement of a gene's coding region, or anti-sense RNA. Anti-sense RNA, complementary to the mRNA strand, can then block the expression of its targeted protein by binding to the mRNA, which normally would be translated to produce the functional protein. Originally, this idea was shown by interference with thymidine kinase expression after transfecting cell lines with a plasmid expressing the thymidine kinase gene of chicken or HSV-1 in the anti-sense orientation (99, 100). This was quickly extended to using virus vectors to deliver and express the anti-sense RNA. A variety of viral vectors have been used to produce anti-sense molecules targeted against both cellular genes and genes from infectious agents. Some of the viruses that have been inhibited in this way include human immunodeficiency virus, human T-cell leukemia virus type-I, vesicular stomatitis virus, dengue virus, and LaCrosse virus (67, 137, 147, 195, 205-207). Specific to herpes simplex virus, only one report has appeared using a virus vector to deliver and express anti-sense mRNAs or ribozymes (29).

This report used a vaccinia virus vector to express anti-sense mRNA or ribozymes targeted against the mRNA translation initiation site encoding the ICP4 protein (29). HSV replication was reduced 3-4 logs in Vero cells (29). This study did not examine the use of the vaccinia anti-sense vector to inhibit HSV-1 in neurons. They also selected cell line clones by expressing a neomycin resistance gene from the vaccinia vector. Clones lost the ability to inhibit HSV-1 by passage 20 (29). Anti-sense viral vectors against HSV-1 will have to be used in neurons for efficacy in the cells that harbor the latent virus. Using a similar strategy, we have produced Sindbis and adenoviral vectors, expressing anti-sense RNA targeting the ablation of HSV-1 regulatory proteins (ICP0, ICP4, or ICP27) for studies in neurons.

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"I have yet to see any problem, however complicated

which, when you looked at it in the right way, did not

become still more complicated."

-Poul Anderson

Chapter 2.

COMPARISON OF PROMOTER FUNCTION AND CELL-TYPE SPECIFIC EXPRESSION FROM ADENOVIRAL VECTORS IN NEURONS

The work in part A of this chapter was presented at the annual meeting of the American Society for Virology and has been submitted for publication to the Journal of Virology. The *in vitro* and *in vivo* work on rat brain expression, performed by Drs. Rod Smith and Jerry Clayton, is noted in the figure legends. Dr. Jerry Schaack was responsible for producing the stocks of viruses used specifically for these studies. Dr. Kevin Staley was responsible for electrophysiological characterizations of the rat slice cultures. Dr. Christine Wilcox made the primary rat DRG neuronal cultures. The work in part B will be published upon completion.

References: Traul, D. L., Smith, R. L., Schaack, J. and Wilcox, C. L. (1998) Promoter and neuronal specificity of expression from adenoviral vectors. 17th Annual Meeting of the American Society for Virology, W9-5.

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A. Viral Promoters

1. Abstract

Adenoviral vectors can efficiently transfer and express novel genes in neurons to facilitate their study. The selection of promoters to drive expression of novel genes in neurons remains poorly understood because direct comparisons of function between promoters using the same vector background has not been reported. To examine this issue, six adenoviral vectors were constructed in an identical vector background using three viral promoters, the human cytomegalovirus major immediate early promoter (HCMV-IE), the Rous sarcoma virus long terminal repeat (RSV-LTR), and the adenoviral E1A promoter, to drive the expression of either of two reporter genes, *Escherichia coli lac Z* or green fluorescent protein (EGFP). The intrinsic lack of toxicity and efficiency of the adenoviral vector was demonstrated in primary neuronal cultures. The three promoters produced strikingly different time courses and levels of expression. In acutely prepared hippocampal brain slices significant differences in cell-type specific expression were seen using the different promoters. The RSV-LTR produced expression of the reporter with high efficiency in dentate gyrus and in hilar and pyramidal neurons, while the HCMV-IE promoter produced expression in hilar regions and pyramidal neurons but minimal expression in cells of dentate gyrus. This feature of the HCMV-IE promoter was confirmed *in vivo* following stereotaxic injection. The E1A promoter produced no detectable expression at the times tested, which is consistent with the delayed expression and reduced levels observed in neuronal cultures. These results indicate that markedly different results in vector efficiency reside at the level of the promoter and suggest that promoters used in viral vectors can be matched for specific applications, but require testing of the vectors in specific applications.

2. Introduction

Study of neuronal function has been advanced by the development of viral vector-mediated gene transfer into neurons *in vivo* and *in vitro*. Gene transfer has been achieved in neurons with viral vectors that include recombinant herpes simplex viral vectors (8, 14, 20, 23), herpes simplex virus-based amplicons (15, 16), adenoviral vectors (5, 6, 11, 26), vaccinia virus-based vectors (10, 21), and adeno-associated viral vectors (2, 13, 18, 24). Virtually all of these vectors have provided the opportunity to modify gene expression, although the efficiency of transduction and patterns of gene expression vary considerably between the viral vectors. In addition, some of the viral vectors retain toxicity that is associated with the remaining viral gene products.

Adenoviral vectors have been used extensively for gene transfer into neurons. The human cytomegalovirus immediate early promoter (HCMV-IE), the Rous sarcoma virus long terminal repeat (RSV-LTR), and the adenoviral E1A promoter (E1A) have been successfully used to drive expression of foreign genes in neurons (5, 6, 11, 26, 40). Direct comparison of the efficiency of transduction and promoter function has been complicated by differences in the adenoviral vector background, as well as other experimental conditions. The effects of the immune response in animal studies were also potentially influenced by vector background and limit the ability to directly compare the results from these published studies. Therefore, the extent to which the promoter determines gene expression in the context of the viral vector remains largely unknown.

Marked differences in expression, however, have been observed during studies using the heterologous promoters from HCMV-IE or RSV-LTR (1, 3, 22, 27, 28, 42). The HCMV-IE and RSV-LTR promoters are widely used to provide high level expression in many cell types and significant differences in expression would be unexpected. The promoter from the E1A gene of

adenovirus was also examined, since it has been shown to have special utility for expression of toxic genes that cannot be generated in other viral backgrounds using either conventional or plasmid based recombination in bacteria (25, 40). While these studies suggested the utility of the E1A promoter, they did not characterize the functional properties of the E1A promoter for its general use in adenoviral vectors.

To facilitate comparison of vector-mediated gene expression. adenoviral vectors were constructed in an identical adenoviral vector background (Ad5 *dl*327), which contains deletions in the E1 and E3 regions (36). The *E. coli lacZ* gene or the enhanced green fluorescent protein (*EGFP*) gene was expressed under the control of the HCMV-IE promoter, the RSV-LTR, or the adenoviral E1A promoter. These viral vectors were then utilized to examine the expression in sensory neurons in culture and in acutely prepared slices from the hippocampal region of adult rats, which contain several neuronal types that can be readily identified based on location and distinct cell morphology. The recombinant viruses were also examined after stereotaxic injection into rat brains to confirm the *in vitro* data.

3. Materials and Methods

Construction of adenoviral vectors. Construction of the adenoviral vector encoding *lacZ* under the control of the HCMV-IE promoter was described previously (36). To construct viruses in which *lacZ* or EGFP expression was controlled by the HCMV-IE, adenovirus E1A, or RSV-LTR promoters, bacterial plasmid vectors containing the left end of the adenovirus chromosome through the *Xho*I site at 5788 bp were used. The E1A promoter plasmid (a generous gift of L.-J. Su and I. Maxwell, Univ. of Colorado Health Sci. Center (UCHSC), Denver, Colorado) has the region between bp 502 (the cap site for E1A is at bp 499) and the *Bg*lII site at 3328 bp replaced by a multiple cloning site. The HindIII-BamHI fragment of

pON249 (39) containing the entire *lacZ* coding sequence and eukaryotic translational initiation site was inserted in the sense-orientation 3' to the E1A promoter. To construct the plasmid vector containing the *lacZ* or EGFP genes driven by the RSV LTR, the RSV LTR (a generous gift of R. Mahalingham, UCHSC) was cloned into a plasmid containing the left end of the adenovirus genome with a multiple cloning site replacing the adenovirus fragment from the *Sac*II site at 357 bp to the *Bg*lII site at bp 3328 (a generous gift of L.-J. Su and I. Maxwell, Univ. of Colorado Health Sci. Center). The pON249 fragment containing the *lacZ* gene was then inserted in the sense-orientation 3' to the RSV LTR. The E1A-*lacZ* and RSV-*lacZ* constructs were introduced into adenovirus by overlap recombination (9) in 293 cells (17) using the 2.5-100 map unit fragment of Ad5 *dl*327BB (36) isolated by centrifugation on a sucrose gradient. Plaques were screened for the presence of EGFP using epifluorescence or the presence of β-Galactosidase activity in the presence of the chromogenic substrate X-gal. DNA from purified plaque preparations was further characterized by DNA endonuclease restriction pattern. Positive plaques, which contained the promoter-reporter gene construct replacing the adenovirus E1 region, were replication defective and therefore required growth and titration on the complementing HEK-293 cells. HEK-293 cells were grown in the presence of D-MEM containing high glucose and supplemented with 10% bovine calf serum (Gibco-Life Technologies, Grand Island, New York). The viral vector stocks were prepared, titered, and stored under essentially identical conditions.

Neuronal Cultures. Dorsal root ganglion (DRG) neuronal cultures were prepared and maintained as previously described (37). After two weeks in culture, DRG neurons were infected at a multiplicity of infection of 50 pfu of adenoviral vector per cell for one hour at 35°C. Mock infected cultures were incubated with medium alone. At 1, 3, 7, 14, 28, or 35 days post infection

cultures were examined qualitatively using epifluorescence to detect EGFP in living cultures or quantitatively analyzed for expression of EGFP in protein extracts using a fluorometric assay (Clontech, Palo Alto, CA). β-Galactosidase activity was assessed either by histochemical staining with X-gal (35), or in protein extracts using a β-Galactosidase quantification assay described below.

Neutral Red Assay. Neuronal survival after adenoviral vector infection was assessed using a neutral red assay essentially as described by Greer and Shewen (19). Neutral red must be actively transported across the membrane of living cells. Briefly, medium was removed from the neuron cultures and replaced with 0.5 ml of a 1:10 dilution of neutral red (Stock 3.3 mg/ml, Gibco Life-Technologies, Grand Island, New York) in phosphate buffered saline (PBS, pH 7.4). After one hour at 35°C the neutral red solution was removed and the cultures were washed with 0.5 ml of PBS for 30 minutes at 35°C. After a second wash the cells were lysed with 0.5 ml of lysing solution (0.05 M acetic acid, 0.5% sodium dodecyl sulfate). Cells were lysed overnight at room temperature before reading the absorbance of each sample at 540 nm. Viability of cultures was examined as a function of neutral red uptake by living cells in infected cultures and compared to uninfected, age-matched control cultures. To compare the sensitivity of the neutral red assay to measure cell death with the more traditional method of cell counting, triplicate cultures of neurons were treated with 2'-deoxy-adenosine (0-1000 μM), which kills neurons by apoptosis, for 7 days (43). The neutral red assay or cell counts were performed on the cultures.

β-Galactosidase and EGFP detection and quantification. For histochemical detection of β-galactosidase, neuronal cultures were washed once with PBS, fixed with 4% paraformaldehyde for 5 minutes, washed with PBS and reacted with X-gal as described previously (38). β-Galactosidase activity in neurons was quantified from triplicate samples of cell extracts

using a commercially available assay (Galacton-plus kit, Tropix). Briefly, 1 µl of cell extract from a sample was diluted in 9 µl of lysis buffer containing 0.1 mM dithiothreitol (DTT) and added to 100 µl of reaction buffer containing the galacton substrate. After 1 hour at 37°C the accelerator buffer was added and luminescence was measured in a TD-200 luminometer (Turner, Sunnyvale, CA). Luminescence was standardized to values obtained from reactions performed with known amounts of purified enzyme. Results are expressed as femtograms of β-Galactosidase per mg of protein. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL).

For fluorescence detection of EGFP, neuronal cultures were washed once with 1 X PBS, fixed in 4% parafomaldehyde for at least 30 minutes, and washed with 1 X PBS before microscopic examination using epifluorescence. Fluorescence microscopy was performed using a Nikon Diaphot II equipped with Hoffman-Optics and Epifluorescence (Nikon Inc. Instrument Group, Garden City, NY) to visualize EGFP fluorescence. The filter cubes (Chroma Technology Corp., Brattleboro, VT) included one for fluorescein-isothiocyanate (FITC, Chroma #31648) and one specific for the EGFP (Chroma #41001). EGFP was quantified from duplicate samples using fluorescence spectrophotometry as described by the manufacturer (Clontech, Palo Alto, CA). Briefly, samples were lysed in 0.5 ml GFP sonication buffer (Tris-HCl pH=8.0 [10 mM], NaCl [200 mM], Na_2HPO_4 [50 mM]) and the relative fluorescence was measured in a mini-fluorimeter (Ex 488, Em 540; Shimadzu, Columbia, MD). Fluorescence was standardized to values obtained from reactions performed with known amounts of a purified recombinant GFP (Clontech).

***In vivo* brain slice preparations.** Four hundred µm thick rat brain slices were prepared as described previously for acute experiments (41). The acute brain slice experiments were performed using aseptic conditions and 2 mg/L gentamycin was added to the artificial cerebral

spinal fluid (ACSF) to retard bacterial growth. Slices were maintained in an atmosphere of 95% O_2/ 5% CO_2 at a temperature of 35°C. Infection with virus was performed by gently pipetting 5 µl of viral stock ($1X10^8$ pfu per ml) onto the exposed surface of the slice directly over the dentate/hilar region.

***In vivo* dentate gyrus injections.** Stereotaxic injection of metafane anesthetized rats was performed using standard methods, targeting the dorsal limb of the dentate gyrus with weight normalized coordinates (AP: -3.3, ML: -2.2, DV: -3.0, average weight= 300g) (31, 44). Injection was performed using needles fabricated from glass pipettes filled with 1-1.5 µl of virus vector stock containing 10^5 plaque forming units of virus when grown on the packaging cell line, HEK293. Virus was pressure injected over 10 min and the pipette was left in place for 5 additional minutes to facilitate spread of virus. Acetaminophin (2 mg/ml) was provided *ad libitum* during the first 12 hours of recovery from surgery. Three days after injection animals were anesthetized with pentobarbitol and intracardiac perfusion with 2% paraformaldehyde/PBS was performed. Brains were removed and coronally sectioned into 1 cm thick slices. Thick slices were incubated in X-gal buffer for 30 minutes at 37°C followed by reaction in buffer containing X-gal (1 mg/ml) (31, 35, 44). Tissue blocks were post-fixed in 4% paraformaldehyde/PBS, cryoprotected in 20% sucrose, and cut as 25-40 µm frozen sections.

4. Results

Many of the adenoviral strains utilized for construction of recombinant viral vectors differ, especially in the E3 region of the virus, complicating the direct comparison of viral vector function. The lack of comparison between viruses, differing only in the promoter driving a foreign gene, has resulted in uncertainty as to the contribution of the viral background to differences in adenoviral vector function. To facilitate the direct comparison of promoter

functions, a set of adenoviral vectors was constructed in an identical vector background, Ad5 *dl*327, which contains deletions in the E3 gene (36). These viral vectors were constructed to express either β-Galactosidase or a red shifted, enhanced green fluorescent protein (EGFP) (Clontech) under control of the HCMV-IE, RSV-LTR or the adenoviral E1A promoter. The identical vector background expressing different reporter gene products provided a mechanism to confirm that the observed expression patterns did not reflect cell-type specific differences in mRNA levels, protein stability, or toxicity resulting from expression of the reporter gene product. The examination of expression from two distinct reporter genes, EGFP or *lacZ*, was performed to demonstrate that results were not specific to the expression of a single gene.

Differences of expression in neurons in culture from the promoters transduced using the recombinant adenoviral vectors. Primary neuronal cultures prepared from embryonic rat DRG sensory neurons are efficiently infected with adenoviral vectors, with no evidence of cytotoxicity (40, 45). DRG neuronal cultures were inoculated at a multiplicity of infection (M.O.I.) of 50 pfu per cell, an inoculum that did not result in apparent cell loss as determined in previous studies (40). Expression of β-Galactosidase was examined following infection using either X-gal histochemistry to determine the percentage of neurons expressing the reporter gene in the neuronal cultures (Figure 2.1) or an X-gal protein assay to determine the levels of expression (Figure 2.2).

All the viral vectors demonstrated efficiency in gene delivery. The histochemical detection of expression from the E1A promoter was significantly delayed and never attained the high levels of expression observed from either the HCMV-IE or RSV-LTR promoters. Quantitative measurement of β-Galactosidase activities indicated that the levels of expression from the HCMV, RSV and E1A promoters varied over almost two orders of magnitude, a difference that was

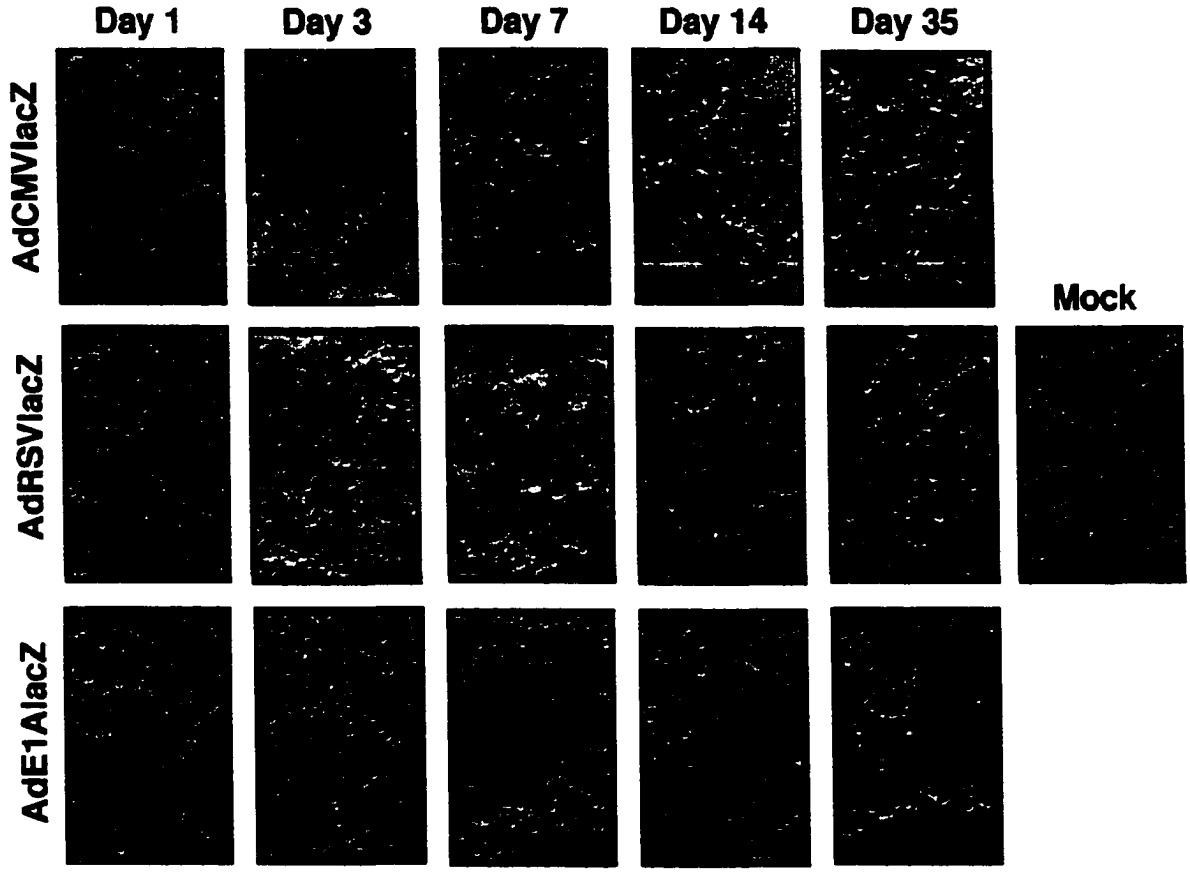

Figure 2.1 Histochemical detection of β-Galactosidase expression from neuronal cultures infected with adenoviral vectors expressed under the control of different viral promoters. DRG neuronal cultures were infected with each of the adenoviral vectors at a M.O.I. of 50 pfu/cell. 1, 3, 7, 14, or 35 days post infection the cultures were examined for β-Galactosidase activity using X-gal histochemistry as described in the methods. Image shows photographs of representative fields observed at each time point using brightfield microscopy (X 100)

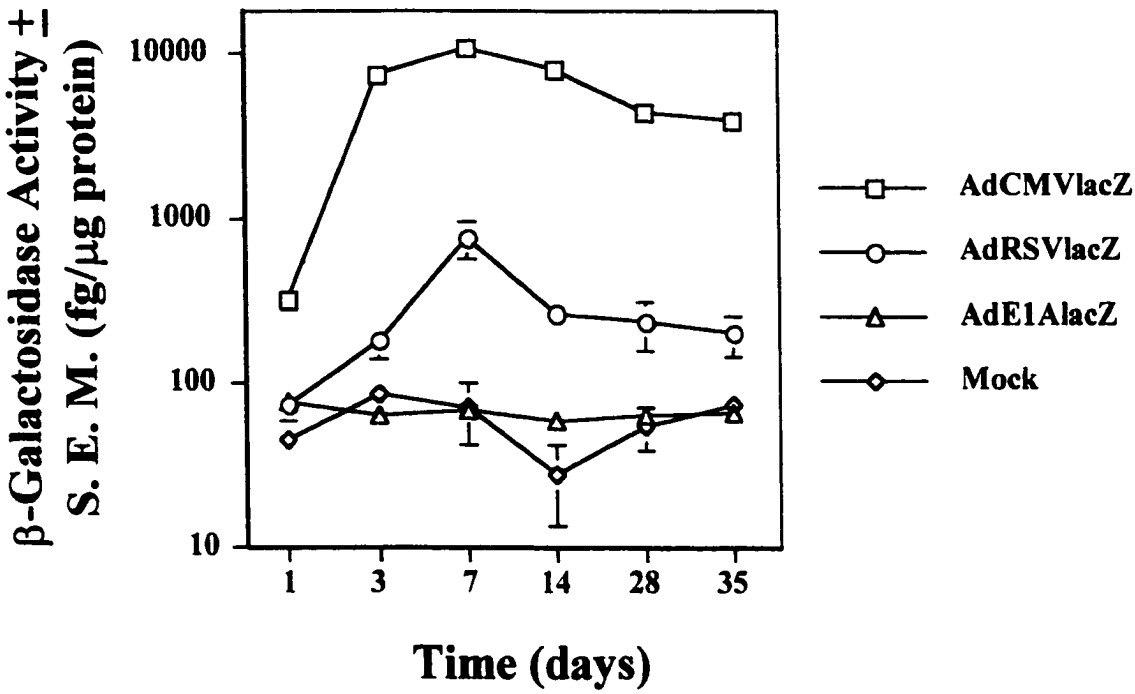

Figure 2.2 Quantitative measurement of β-Galactosidase activity in neuronal cultures at times after infection with adenoviral vectors. β-Galactosidase activity was measured in extracts prepared from neuronal cultures 1, 3, 7, 14, 28, or 35 days post infection with adenoviral vectors as described in figure 2.1. Using the assay for β-Galactosidase as described in the methods, β-Galactosidase activity was quantified using a standard curve and samples were normalized for total protein present in each extract. Data are the mean ± S.E.M. (n=3)

greater than suggested by histochemical methods (Compare figures 2.1 and 2.2). The HCMV-IE promoter produced the highest level of function of the three promoters. While expression from the HCMV-IE peaked rapidly, the RSV-LTR promoter produced levels of β-Galactosidase that continued to increase for many days in culture. In DRG neuronal cultures, the HCMV-IE promoter drove approximately 10-fold higher expression than the RSV-LTR promoter.

These results were confirmed using EGFP as a reporter gene under control of the HCMV-IE, RSV-LTR or E1A promoters (Figures 2.3 & 2.4). The HCMV-IE and RSV-LTR produced fluorescence signal in virtually all of the neuronal profiles, although with different time courses (Figure 2.3). The E1A promoter produced little detectable gene expression at any of the times examined consistent with reduced levels of expression from the *lacZ* gene. Direct measurements of EGFP fluorescence in cell lysates confirmed the pattern of expression as previously determined using β-Galactosidase as the reporter gene (Figure 2.4).

Our previous studies have utilized cell counts after infection as a measure of viral toxicity (40). To extend these analyses, vital staining using neutral red uptake was performed after viral infection of DRG neurons to determine if changes in the level of β-Galactosidase or EGFP expression in the cultures were the result of the vector toxicity and subsequent neuronal cell death (Figure 2.5). No significant changes in neutral red uptake were observed at any time following inoculation of neuronal cultures with the different viral vectors as compared to the uninfected control cultures. As a control for the sensitivity of the neutral red assay, a toxicity dose response curve using 2'-deoxy-adenosine was performed. 2'-deoxy-adenosine causes cell death in neurons by apoptosis (43). After 7 days of incubation with the drug, the neutral red assay and cell counts were performed on the cultures. Comparison of the two assay confirmed that the neutral red assay correlated with neuronal death as determined by cell counts (see figure 2.6).

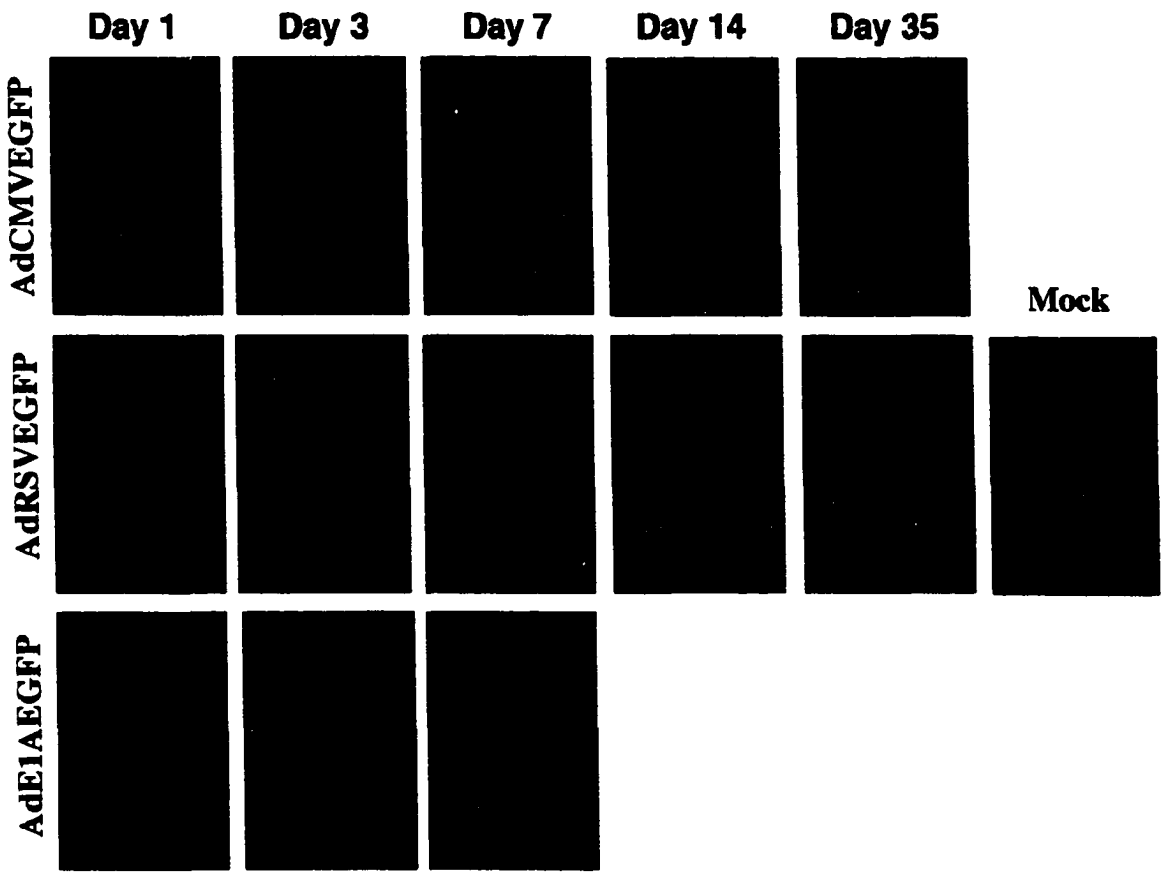

Figure 2.3 Fluorimetric detection of EGFP expression from neuronal cultures infected with adenoviral vectors expressed under the control of different viral promoters. DRG neuronal cultures were infected with each of the adenoviral vectors at a M.O.I. of 50 pfu/cell. 1, 3, 7, 14, or 35 days post infection the cultures were examined for EGFP expression using fluorescence microscopy. Image shows photographs of representative fields observed at each time point using epifluorescence microscopy (X 100)

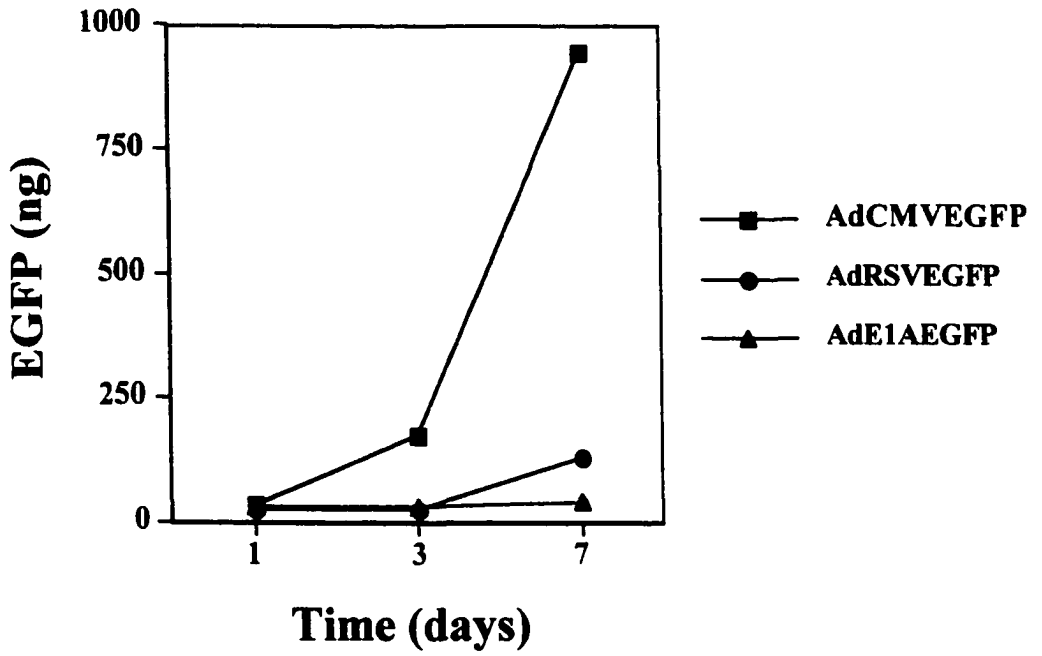

Figure 2.4 Quantitative measurement of EGFP fluorescence in neuronal cultures at times after infection with adenoviral vectors. EGFP expression was measured in extracts prepared from neuronal cultures at 1, 3, or 7 days after infection with adenoviral vectors as described in figure 2.3. EGFP fluorescence was quantified using a standard curve. Data are the average relative fluorescence (n=2)

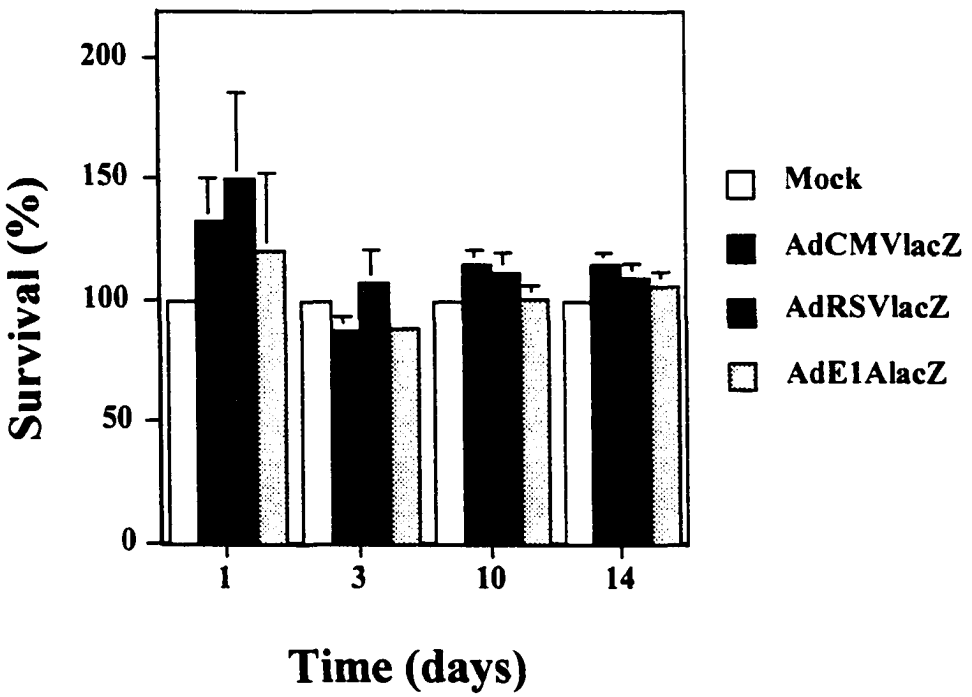

Figure 2.5 Evidence of cell death is not detected following infection of neuronal cultures with the adenoviral vectors. Neuronal cultures were infected with the adenoviral vectors at a M.O.I. of 50 pfu/cell. 1, 3, 10, or 14 days post infection, the neuronal cultures were harvested and percent survival was assessed using the neutral red viability assay as described in the methods. The data are the mean ± S.E.M. of triplicate wells of one experiment (n=3)

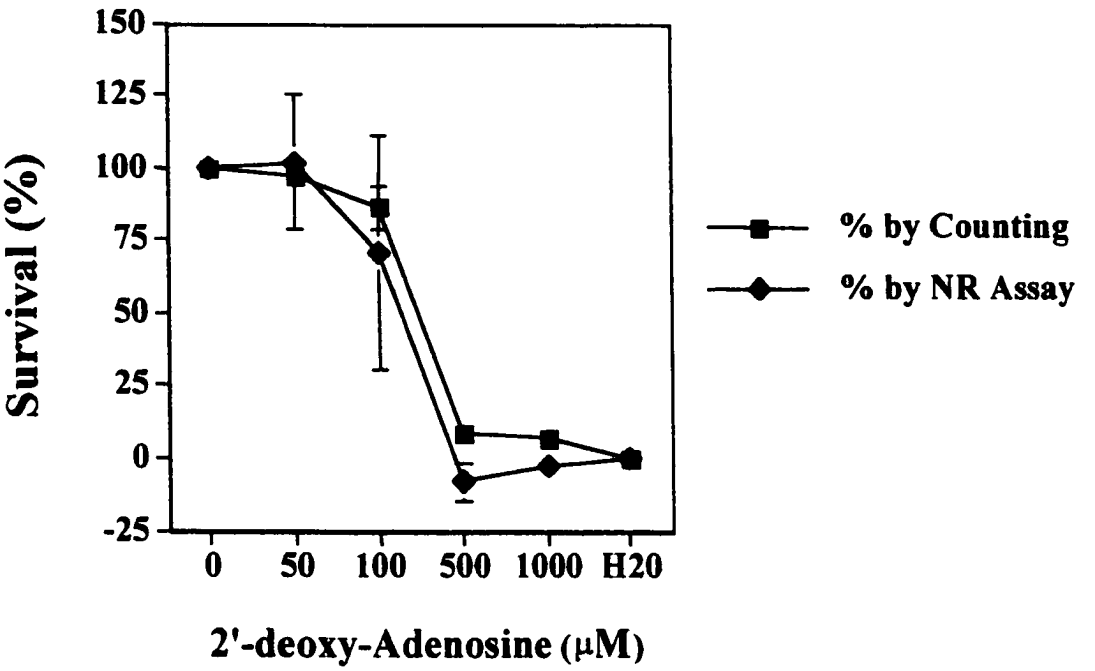

Figure 2.6. Neuronal cell death compared using the neutral red assay or cell counting. Neuronal cultures were treated with 0-1000 μM 2'-deoxy-adenosine for 7 days. The neuronal cultures were then assayed for percent survival using the neutral red viability assay as described in the methods or by counting viable cells. The data are the mean ± S.D. of one experiment performed in triplicate.

indicated that significant neuronal cell death did not occur as a result of the viral vector infection or reporter gene product toxicity, even in neurons expressing very high levels of the reporter gene over relatively long periods of time.

Hippocampal slice infection shows cell-type specific expression dependent upon the promoter used to drive expression of β-galactosidase. The DRG neuronal cultures described above are peripheral neurons obtained from embryonic sources and may not reflect characteristics of mature neurons. Hippocampal slices acutely prepared from rat brain can be maintained for 24 hours or longer in culture with maintenance of metabolic and electrophysiologic properties (12, 30, 34). Hippocampal slice preparations have been used to examine viral vector function with several types of viral vectors (7, 29, 32, 33). The ability to maintain brain slices from mature animals for a period of greater than 24 hours allowed the functions of the viral vectors to be examined in mature neurons that maintain many of their *in vivo* connections and functions. Control studies were performed to demonstrate preservation of neuronal function indicated by stability of excitatory postsynaptic potentials and population spikes recorded by stimulation of the lateral perforant path (data not shown).

The hippocampal formation contains multiple neuronal cell types, which can be identified based on location and cell morphology. This provides a preparation for examining the properties of vector promoter function in different classes of adult neurons. Viral inoculations were performed after the slice preparations had stabilized in the incubation chamber for an hour. Adenoviral vectors were introduced in a volume of 5 μl (2 $X10^7$ pfu) to the surface of the individual slices, directly over the hippocampal formation, and allowed to diffuse into the slice passively. Twelve or 24 hours after inoculation, slices were processed for detection of β-

Galactosidase using X-gal histochemistry. HCMV-IE-mediated expression was detected as early as 12 hours after inoculation in a limited population of cells in the hilar region of hippocampus (data not shown). By 24 hours after infection with the HCMV-IE-lacZ vector, intense β-Galactosidase expression in the hilus obscured identification of individual cells and included large regions of pyramidal cells extending to CA1 (Figure 2.7 B&E). However, expression in the granular cell layer was minimal. In contrast, the RSV-LTR-lacZ vector produced significant levels of β-Galactosidase staining 24 hours after inoculation in the granular cell layer, which extended into the molecular layer. A more restricted staining in the hilar region was observed and included many cell profiles (Figure 2.7 A & D). Expression of β-Galactosidase under the control of the E1A promoter was not detected over the time course of these experiments, consistent with the reduced levels of expression observed in cultured DRG neurons (Figure 2.7 C & F).

The RSV-LTR Ad β-Galactosidase -vector (Ad5 *dl*327 RSV-*LacZ*) demonstrated a pattern of infection in hippocampal slices *in vivo* that was consistent with published *in vivo* findings, utilizing a similar vector (26). Specifically, expression within both the granule cell layer and hilar region was quite extensive when virus was injected into the hilar region. In contrast, the restricted expression by HCMV-IE in the granular cell layer would not be predicted from published studies; however, the very high levels of expression from the HCMV-IE could complicate analysis and may make detection of regions of low expression within the dentate gyrus difficult (11). To address this issue, small volume inoculations of the dorsal limb of the dentate gyrus were performed using 1-1.5 μl containing 10^5 pfu of adenoviral vector. Rat brains were examined histochemically for β-Galactosidase 72 hours after inoculation. Representative results are shown in figure 2.8. Similar to *in vivo* hippocampal slice studies, a small subpopulation of

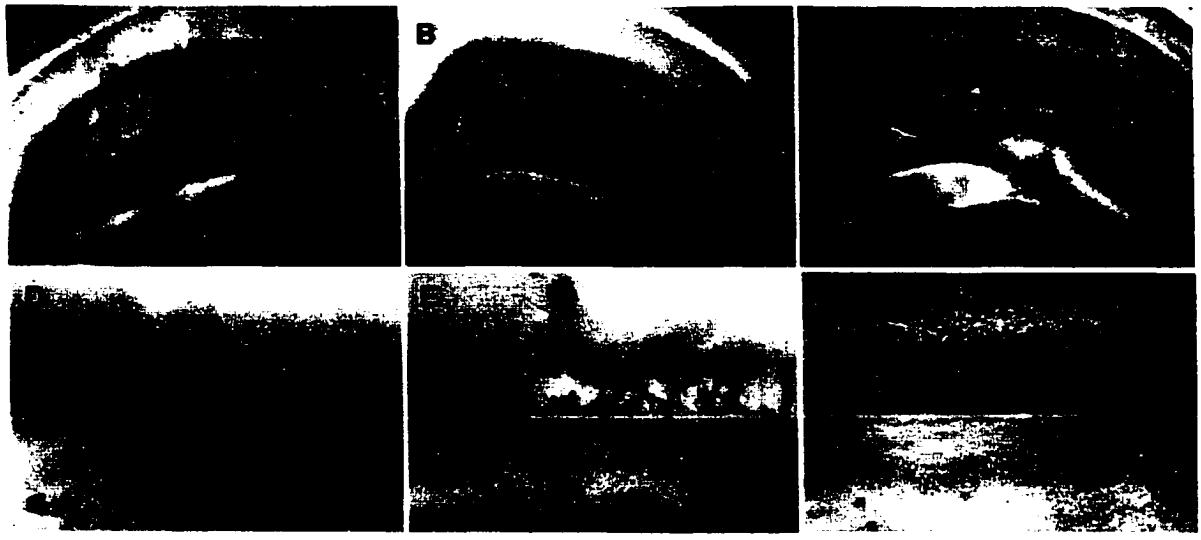

Figure 2.7 Adenoviral vector mediated β-Galactosidase expression in rat hippocampal brain slices *in vitro*. Hippocampal slices were prepared from adult rat brain and infected with adenoviral vectors as described in the methods. Twenty-four hours after infection with 5 X 10^7 pfu of each viral vector, hippocampal slices were processed for histochemical detection of β-galactosidase, seen as positive blue-staining. Representative results of infection with Ad-RSV-lacZ (A & D) Ad HCMV-IE-lacZ (B &E), or Ad-E1A-lacZ (C & F). Low power views of hippocampus (A-C) and higher power magnification of views of the dorsal blade of the dentate gyrus and hilar regions (D-F) are shown (These experiments were performed by Drs. Rod Smith and Jerry Clayton).

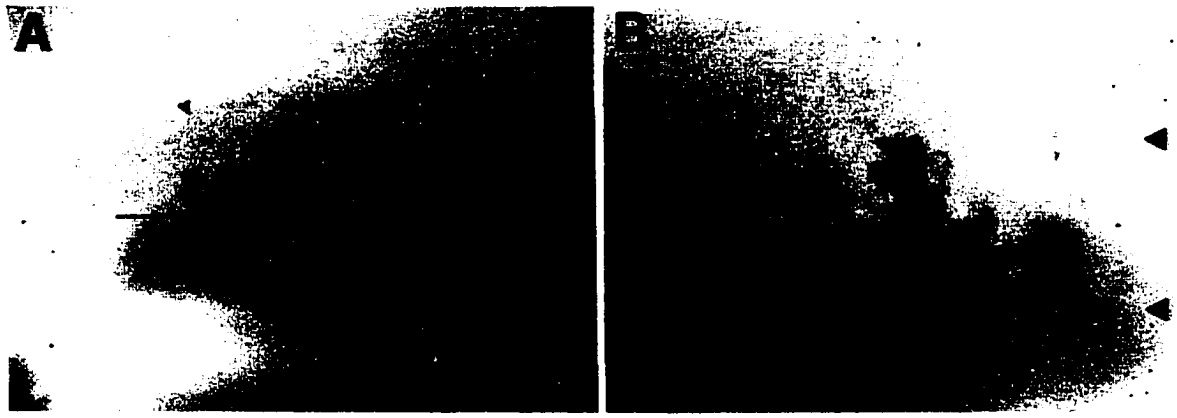

Figure 2.8 Adenoviral vector mediated β-Galactosidase expression in rat dentate gyrus *in vivo*. An adenoviral vector constructed to express β-Galactosidase under the control of the HCMV-IE promoter (Ad HCMV-IE-lacZ) was injected *in vivo* into the dorsal blade of the dentate gyrus using stereotaxic methods. Representative views of spread of virus in the dentate gyrus are shown at low (A) and high magnification (B). In panel (A) the position of the hippocampal fissue, the dorsal blade of the dentate gyrus, and the hilar region is indicated. In panel (B) arrows indicate the extent of the granular cell layer (These experiments were performed by Drs. Rod Smith and Jerry Clayton).

cells localized to the subgranular zone of the dentate gyrus expressed β-Galactosidase, although scattered cells within the granular cell layer were also labeled, confirming that the viral inoculum spread throughout dentate gyrus (Figure 2.8). The expression of β-Galactosidase in a subpopulation of cells with the evidence of viral spread to other dentate regions clearly demonstrates that the HCMV-IE promoter has very limited function in the majority of cells in the dentate gyrus. The distinctly different functional properties of the HCMV-IE and RSV promoters in the context of the adenoviral vectors have important implications for the design of virus vectors for gene delivery to neuronal populations of the hippocampus and, potentially, other cell-types.

5. Discussion

Despite marked technical improvements, development of an adenoviral vector can present technical challenges with the requirements for the appropriate expression of functional genes (25, 40). Selection of promoters is an important determinant for the feasibility of generating viral vectors that express active proteins and is one of the determinants of the utility of the vector. As demonstrated here, promoter selection has important implications for gene expression. The striking differences observed would not be predicted based on the high activity of the respective promoters in cell lines (Schaack, personal communication). This strongly indicates the need for empirical examination of promoter function.

The function of the E1A promoter in neurons is an important issue, since intrinsic regulatory properties of this promoter have allowed construction of viruses to express toxic genes, which can not be obtained with other promoters (25, 40). The utility of the E1A promoter is based on the ability of the E1A gene product to down-regulate the promoter, which allows packaging cell lines that supply E1A in trans to avoid potentially toxic effects of genes and

efficiently package virus (Schaack, personal communication). E1A mediated gene expression measured in HeLa cells is 50% of that seen with the HCMV-IE promoter (Schaack et al., submitted for publication). In contrast, the E1A promoter demonstrated a nearly 100-fold lower level of expression in sensory neurons compared to the HCMV-IE promoter and was not detectable in hippocampal brain slices. While the level of E1A expression is significantly reduced compared to other promoters it has been demonstrated to be sufficient to allow functional gene expression (25, 40). In many cases the E1A promoter allows levels of protein expression that are more consistent with those that occur *in vivo*.

The hippocampal slice provides a useful preparation for comparison of differences in gene transfer among cell types and classes of neurons. The hippocampal slice preparation is widely used as a model system for examination of neuronal physiology. Rat hippocampal slices can be maintained for 24 hours or longer allowing viral vector mediated gene transfer and expression (7, 29, 32, 33). The experiments shown here demonstrate that significant expression of genes can be achieved within the time of viability of tissue slices with use of the HCMV-IE or RSV promoters, although the cell type specificity of expression differs considerably.

The differences in function of the HCMV-IE and RSV-LTR promoters would not be expected based on the efficiency of these promoters in non-neuronal cell types. The use of identical adenoviral vector constructs allows demonstration that the differences are at the level of the gene promoter and are not related to differences in vector background. The restricted expression of the HCMV-IE promoter in dentate gyrus was also demonstrated by injection of virus *in vivo*. Therefore the restricted expression of the HCMV-IE promoter is not restricted to conditions of the *in vivo* experiments and will be an important consideration for design of viral vectors to target expression in these cell types. These promoter-specific effects are likely to

be relevant to other vector systems, including adeno-associated virus, where a HCMV-IE promoter driven construct did not express significant levels of β-Galactosidase in granular neurons compared to hilar regions (4). The data presented demonstrate the marked differences in promoter function that can occur when promoters are used in neuronal cell types. Ultimately differential promoter function may prove to be a useful tool for selective gene expression in specific nervous tissues.

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B. Neuron Specific Promoters

1. Abstract

An important aspect of developing viral vectors is the appropriate and regulated expression of the gene of interest in the target cells. Most commonly, viral promoters (such as the human cytomegalovirus immediate early promoter or the Rous sarcoma virus long terminal repeat) have been used to drive gene expression. We have reported that viral promoters, in the context of an adenoviral vector, can express genes in different populations of neurons, with varying levels and kinetics of expression. This implies that neuronal factors can affect these promoters and this can alter the cell types and the patterns of expression obtained using them. A major limitation of using non-neuronal promoters for neuron specific expression, however, is that the promoter probably will not be appropriately regulated. To address this issue we are developing adenoviral vectors with neuron specific promoters. We have constructed, in an identical vector background, six adenoviral vectors to drive expression of either the green fluorescent or β-Galactosidase marker proteins. The promoters we have chosen include the neuron-specific enolase (NSE) promoter, the *N*-methyl-D-aspartate (NMDA) promoter, the Tα1 tubulin promoter, the tyrosine hydroxylase (TH) promoter, the GAP43 promoter, and the HSV-1 LAT promoter. Analysis of reporter gene expression from the recombinant adenoviruses constructed with these promoters is in progress. Recombinant adenoviruses have been made with all promoters driving EGFP except NMDA, and all but the NSE and NMDA promoters driving *lacZ*. The NSE and NMDA promoters when combined with the *lacZ* and SV40 polyadenylation signal are above the packaging limit of the viral genome. So far only the Tα1 and the LAT promoters have shown activity in dorsal root ganglia neurons in culture. The eventual completion

of this study will include studies of the recombinant viruses on acute hippocampal slice cultures and stereotaxic injection of the viruses into rat brains.

2. Introduction

Viral vectors have become important tools to deliver and express genes in cells of the nervous system (for review see 12). While many viral vectors are being considered as gene delivery vehicles to the nervous system, our studies have focused on adenovirus-based vectors. Adenoviral vectors have been very successful for this application, as they are readily taken up by neuronal cells (1, 4, 6, 14). Viral vectors have generally been constructed by placing the gene of interest between a viral promoter to drive gene expression and a viral polyadenylation signal for proper processing of the mRNA transcript. While this produces functional proteins, the gene is usually overexpressed and not regulated in a controlled physiologic manner. By selecting known neuron-specific promoters we potentially can achieve regulated cell type specific expression.

Most of our understanding of the regulation of gene expression in the nervous system has come from studies on embryonic development. Through these studies a host of neuron specific genes have been found that are expressed in specific types of neurons and at specific times during development (for review see 16). Promoters that regulate genes during development have generally been studied using transgenic mice that contain the promoter linked to a reporter gene (such as *lacZ*). Neuron-specific enolase (NSE) is a distinct glycolytic enzyme expressed in most terminally differentiated neurons and neuroendocrine cells (24). Expression is linked to synaptogenesis and increases with the accumulation of mature CNS neurons, becoming one of the most abundant brain-specific proteins in the adult (17, 18, 21, 23, 28). The promoter regulating this gene is a 4.0 kb region upstream of the NSE coding region (7). The NMDA promoter controls the regulation of two genes, the N-Methyl-D-aspartate receptor (NMDAR) 1 gene and

the NMDAR2 gene. These receptors are members of the glutamate family of ligand-gated ion channels and are important in neurotrophic and neurotoxic mechanisms. NMDAR1 is expressed widely in the CNS with prevalent expression in the hippocampus, cerebral cortex and olfactory bulb. Expression increases until cortical neuronal migration ceases in the developing mammalian CNS. The corresponding full-length promoter region is 3.0 kb in length (2, 3). Tα1 tubulin is a major cytoskeletal component of growing neurites and is expressed at high levels in developing neurons during morphological growth. The 5' regulatory region is about 1.0 kb in size (10). GAP-43 is an abundant protein in growth cones. This protein is expressed predominantly in immature neurons, but can be upregulated after neuronal injury. The promoter region is approximately 1.0 kb in size (20). Tyrosine hydroxylase (TH) converts tyrosine to dihydroxyphenylalanine (DOPA), which is the rate-limiting step in the catecholamine pathway generating the neurotransmitters dopamine, norepinephrine, and epinephrine. TH is expressed in the adrenal medulla, sympathetic ganglia and certain defined nuclei of the brain. There is increased expression of TH during development and the 1.0 kb promoter is affected by glucocorticoids, nerve growth factor and trans-synaptic activity (15).

It has also been shown that many neuron specific-promoters, like the sodium channel promoter, contain a regulatory DNA sequence called the neuron restrictive silencer element (NRSE) (19). This element regulates neuron specific expression through binding to a protein called the neuron restrictive silencing factor (NRSF) (25). The NRSF is expressed in non-neuronal cells, but not neuronal cells, and binds to the neuron specific silencing element blocking transactivation (25). A promoter that has recently been suggested to have a NRSE like element is the 1.0 kb Lat promoter region from HSV-1 (8, 9). This promoter has been used in HSV based viral vectors to drive stable and long term expression in the nervous system (13).

We have inserted each of these promoter regions into an identical adenoviral vector so direct comparisons on the patterns of expression can be made. Potentially, this will lead to adenoviral vectors that give regulated, neuron specific expression of the foreign gene they carry.

3. Materials and Methods

Cells and Neuronal Cultures. The Vero cells (ATCC, Rockville, MD) HeLa cells (ATCC, Rockville, MD), and 293 cells (generously provided by Jerry Schaack, (11) were cultured in Dulbecco's modified eagle medium (D-MEM) plus 10% fetal bovine serum (FBS). Dorsal root ganglion (DRG) neuronal cultures were prepared and maintained as previously described (26). After two weeks in culture, DRG neurons were infected at a multiplicity of infection of 50 pfu of adenoviral vector per cell for one hour at 35°C. Mock-infected cultures were incubated with medium alone. At 1, 3, 7, 14, 28, or 35 days post infection cultures were examined qualitatively using epifluorescence microscopy to detect EGFP in living cultures or quantitatively analyzed for expression of EGFP in protein extracts using a fluorometric assay (Clontech, Palo Alto, CA). β-Galactosidase activity was assessed either by histochemical staining with X-gal (22), or in protein extracts using a β-Galactosidase quantification assay described below. All culture reagents were from Life Technologies (Grand Island, NY, USA) unless otherwise noted.

Production of recombinant adenoviruses. Recombinant adenoviruses were constructed by sub-cloning defined promoter regions into a promoter-less adenovirus left end plasmid (pXC15 #7 or #18) (obtained from L.-J. Su and Ian Maxwell, UCHSC). These left end plasmids are deleted between 358 and 3318 bp, which deletes the E1A promoter, and replaced with a polylinker containing a multi-cloning site.

To produce recombinant adenovirus the left end plasmid containing the promoter region with the marker protein were co-transfected with the pJM17 plasmid into the 293 packaging cell

line using the calcium phosphate transfection method as described by Graham (for review see 5). Briefly, 1 X 10^6 293 cells were cultured in a 25 cm^2 vented flask 24 hours prior to transfection. The next day the medium was removed and 4 ml of fresh medium was added 1-4 hours prior to transfection. Into a sterile tube 5 μg of the left end plasmid and 5 μg of the pJM17 plasmid were added together with 31.25 μl of 2 M $CaCl_2$ (final concentration 125 mM) and water (total volume 250 μl total). The DNA mixture was then added dropwise, while bubbling with a pipette, into 250 μl 2X HEPES-buffered salt solution (final concentration 1X). After 10' at room temperature the mixture was added dropwise to the cells, while gently rocking the flask. The cells were then incubated at 37°C. The next day the medium was changed and the cells were maintained until recombinant viruses formed and the cell monolayer showed complete cytopathic effects. The cells and supernatant were harvested and virus was plaque purified at least two times before expanding and titrating. Titrations of recombinant adenoviruses were performed using 293 cells, and lack of wild type recombination was confirmed using HeLa cells.

β-Galactosidase and EGFP detection. For histochemical detection of β-Galactosidase, neuronal cultures were washed once with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 5 minutes, washed with PBS and reacted with X-gal as described previously (27). For fluorescence detection of EGFP, neuronal cultures were washed once with 1 X PBS, fixed in 4% parafomaldehyde for at least 30 minutes, and washed with 1 X PBS before microscopic examination using epifluorescence microscopy.

Microscopy. Fluorescence microscopy was performed using a Nikon Diaphot II equipped with Hoffman-Optics and Epifluorescence (Nikon Inc. Instrument Group, Garden City, NY). The filter cubes (Chroma Technology Corp., Brattleboro, VT) included one for fluorescein-

isothiocyanate (FITC, Chroma #31648), one specific for the humanized green fluorescent protein (EGFP, Chroma #41001), and one for DAPI (U.V., Chroma # 11000).

4. Results

Larger promoters cause inefficient virus production. Recombinant adenoviruses expressing β-Galactosidase have been made with the LAT, Tα1-tubulin, GAP43, and TH promoters. The NMDA and NSE promoter constructs with the β-Galactosidase reporter did not produce virus when co-transfected into 293 cells. The combination of these larger promoters (3.0 or 4.0 kb) with the β-Galactosidase gene and SV40 polyadenylation signal were greater than the coding capacity of the vector we used (Table 2.1). The adenoviral vector system should accommodate 5.5 kb of DNA insert; however, as one gets close to the packaging limit it becomes more difficult to generate a recombinant genome, which can be packaged into viral particles.

Promoter	Approximate Size (kb)	Virus Produced		Expressed in DRG	
		lacZ	EGFP	lacZ	EGFP
CMV	1.0	yes	yes	yes	yes
RSV	1.0	yes	yes	yes	yes
E1A	0.5	yes	yes	yes	yes
Lat	1.0	yes	yes	yes	yes
Tα1	1.0	yes	yes	yes	yes
GAP43	1.0	yes	N/A	no	N/A
TH	1.0	yes	N/A	no	N/A
NMDA	3.0	no	N/A	N/A	N/A
NSE	4.0	no	yes	N/A	no

Table 2.1 Promoters used in parts A and B of this chapter to construct recombinant adenoviruses to express the *lacZ* or EGFP reporter genes. The table shows the approximate size or each promoter and whether virus was produced after co-transfecting the shuttle plasmid and pJM17 plasmid into the 293 packaging cell line as described in the methods. The table also indicates whether expression of the reporter gene was seen in DRG neurons infected individually with a M.O.I. of 50 pfu/cell with each recombinant virus. N/A, not applicable, as virus was not produced.

Expression patterns in 293 cells and Vero Cells. One of the aims of this research is to find promoters that do not express in the 293 packaging cell line or non-neuronal cells. Recombinant viruses could then be made with neuronal genes that are potentially toxic to other cell types. So far, the LAT and Tα1-tubulin recombinant viruses expressing the β-Galactosidase or EGFP marker proteins have shown expression in 293 cells and Vero cells (Table 2.1). The GAP43 and TH viruses have not shown expression in either 293 cells or Vero cells. The NSE recombinant virus expressing EGFP has also not shown any expression in the two cell lines.

Expression patterns in neuronal cells. The LAT and Tα1-tubulin promoter containing viruses showed expression of EGFP or β-Galactosidase in DRG neurons (Table 2.1). None of the other viruses showed expression of either marker protein. This was expected for GAP43 and TH, as they should express in other neuronal cell types such as cortical neurons or sympathetic neurons, respectively. We did expect the NSE promoter to drive good levels of expression in the DRG neurons. Further analysis of the recombinant viruses using Southern blot analysis needs to be done before we can make any conclusions.

5. Discussion

The development of adenoviral vectors with promoters to drive specific types of expression is important for gene delivery in a system as complex as the central nervous system. We are examining six different neuron-specific promoters, specifically, the NSE promoter, the NMDA promoter, the TH promoter, the GAP43 promoter, the Tα1-tubulin promoter and the Lat promoter. These promoter regions were chosen for their characterized expression patterns in transgenic animals. Recombinant viruses were produced for the TH, GAP43, Tα1-tubulin, or Lat promoters expressing either β-Galactosidase or EGFP marker proteins. The NMDA produced virus when the EGFP marker protein was used, but neither the NSE nor the NMDA promoter

produced virus when the *lacZ* marker protein was used. Expression has been observed in cell lines or DRG neurons using the LAT or the Tα1-tubulin promoter. The NSE, TH or GAP43 did not show expression of EGFP in cell lines or DRG, cortical or sympathetic neurons. This may reflect that these promoters are developmentally regulated and not expressed in cell culture. It may also mean the expression of EGFP was less than can be detected by fluorescence [<28 nM]. Potentially, when the recombinant viral genome was produced, during homologous recombination, a rearrangement occurred in the promoter or reporter sequence. If this occurred there could be loss of reporter gene expression. While this work is far from complete, it shows the importance of analyzing promoters for function in the context of the viral vector in which they are to be used. Adenoviral vectors, using the system described in the methods, are designed with a minimal promoter region to give cell type specific expression, with consideration of the size of the gene of interest. Future work for completion of these studies will include analysis of expression patterns in acute hippocampal slice or rat brains after infection with the recombinant viruses.

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"The important thing in science is not so much to obtain

new facts as to discover new ways of thinking about them."

-Sir William Lawrence Bragg

Chapter 3.

RECOMBINANT ADENOVIRUSES EXPRESSING HSV-1 IMMEDIATE EARLY PROTEINS ICP0, ICP4, AND ICP27 FUSED TO THE GREEN FLUORESCENT PROTEIN

Part of the work in this chapter was presented at the annual meeting of the American Society for Virology, and will be submitted to the Journal of Virology for publication. The western blot analyses performed by Sarah Richart, a co-author on the paper, are noted in their respective figure legends. Joe Morroni and Joe Gogain were responsible for construction of the pCMVEGFP-1535 and pCMVEGFP-ICP4, respectively.

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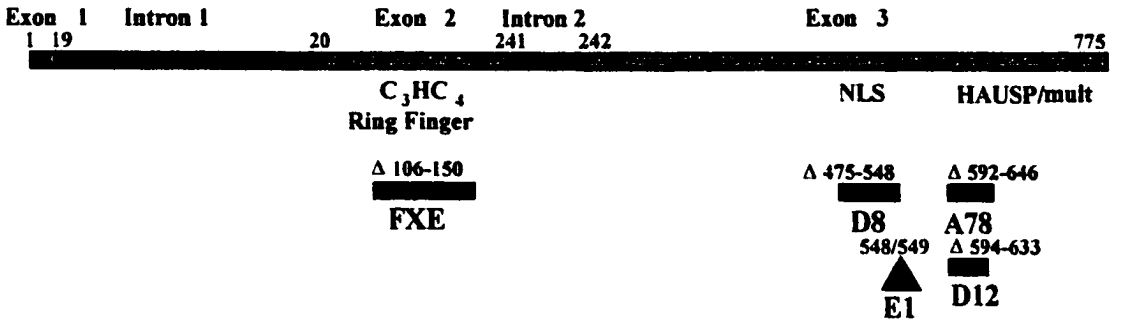
1. Abstract

The herpes simplex virus type-1 (HSV-1) immediate early infected cell proteins (ICP) ICP0, ICP4 and ICP27 are transcription factors that modify HSV-1 gene expression in cultured cell lines; however, their activities during HSV-1 gene expression in neurons are not as well characterized. We have constructed recombinant adenoviruses expressing ICP0 (or ICP0 mutants), ICP4 or ICP27 fused to the humanized green fluorescent protein (EGFP-ICP4, EGFP-ICP0 or EGFP-ICP27, respectively) to allow observation of the expression of these proteins in live neurons. Co-transfection of HeLa cells with plasmids expressing the EGFP-ICP0 or EGFP-ICP4, or EGFP-ICP27 fusion proteins and an HSV-1 glycoprotein D (gD) promoter or thymidine kinase (tk) promoter renilla-luciferase reporter plasmid showed that the fusion proteins were transcriptionally active. The recombinant adenoviruses expressing the fusion proteins were able to complement HSV-1 mutants deleted of the respective immediate early proteins in Vero cells. In Vero cells, expression of the EGFP-ICP0, or EGFP-ICP4, or EGFP-ICP27 fusion proteins from expression plasmids or recombinant adenoviruses showed the characteristic nuclear localization of EGFP-ICP4 and EGFP-ICP27, and punctate nuclear localization of EGFP-ICP0 that has been described for the non-fused proteins. Infection of primary dorsal root ganglion neurons with the recombinant adenoviruses showed that the EGFP-ICP4 or EGFP-ICP27 localized to the nucleus, while the EGFP-ICP0 gave little or no expression. These studies demonstrate that HSV-1 immediate early proteins fused to the humanized green fluorescent protein are functional and localize to their correct sub-cellular compartments in cell lines. Importantly, the recombinant adenoviruses were able to infect neurons, allowing the expression patterns and cellular localization of the proteins to be analyzed.

2. Introduction

The immediate early (IE) proteins of herpes simplex virus type 1 (HSV-1) control the cascade of viral gene expression during a productive infection in permissive cells (45). Several studies have demonstrated that the immediate early proteins ICP0, ICP4, and ICP27 are nuclear phosphoproteins that can activate and repress transcription of many HSV and cellular promoters in cultured cell lines (37, 45, 49, 58). The expression and functions of these IE proteins in primary sensory neurons are poorly understood. It has been suggested, however, that neuron-specific factors may silence expression or change the function of the proteins (31, 41, 53, 56).

ICP0 mutants, with deletions and insertions in the coding region, have been used to characterize the functional domains of ICP0 important during the productive HSV-1 infection (15-18, 20, 23). Through the construction and expression of these types of mutants in cell lines, both from plasmids as well as in recombinant herpesviruses, many functions of the domains of ICP0 have been elucidated (see figure below). Exon one is encoded from amino acids 1-19, exon



two is encoded from amino acids 20-241, and exon three is encoded from amino acids 242-775 (44). The amino terminus, residues 1-103, contains an acidic region that activates transcription when tethered to a DNA binding domain (35). Exon two contains a cysteine-histidine (C_3HC_4) zinc binding motif, or RING finger, from amino acids 115-156 (18, 23, 35). There are two

centrally located proline-rich domains and a serine-rich tract, which may be the site of phosphorylation (5, 58). Regions both on the amino and carboxyl ends have been demonstrated to be important for synergy with ICP4 (18). The carboxyl end contains the amino acid sequence from residues 501-506, Arg-Pro-Arg-Lys-Arg-Arg, which is similar to other highly basic nuclear localization signals (15). Nuclear localization, however, requires at least amino acids 475-548 to be functional. ICP0 has been shown to have important consequences on infected cells including disruption of nuclear domains, association with the ubiquitin-proteosome pathway, and destruction of kinetochores essential to cell division (22, 24, 26, 38). Amino acids 680-767 are necessary for targeting ICP0 to nuclear domains, known as ND10 domains. The RING finger is required for dispersal of proteins contained in these ND10 domains, such as the promyelocytic leukemia protein PML or ND10 protein Sp100 (24, 38). IPC0 can form multimers when a region including amino acids 617-711 is present (6). The region important in interactions with the ubiquitin-proteosome pathway has been mapped to amino acids 594-663 (39, 40). Finally, ICP0 has been shown to associate with cellular proteins cyclin D3 and translation elongation factor eEF1-Bα (30). The functions of these domains in neurons have not been addressed. Because of the post-mitotic nature of neurons, several of these functions may be altered, unnecessary, or domains may have functions not yet defined when HSV-1 infects neurons. If there is a loss of certain functions in neurons this may push the balance of the virus lifecycle in favor of the latent state over the lytic state.

Common to all three of these proteins is the ability to regulate transcription. ICP4 is a strong transcriptional activator of HSV-1 promoters, such as the glycoprotein D (gD) or thymidine kinase (tk) promoter (for review see 45), and a repressor of gene expression, including its own promoter (for review see 37). ICP0 also is a transcriptional activator alone, but more

importantly it greatly enhances the activity of ICP4 when both are present together (20). ICP27 has been shown to transactivate viral promoters and to repress the transactivation of both ICP4 and ICP0 (49). A crucial aspect of these proteins is the ability to interact with each other, which not only regulates the genes they can transactivate, but alters their location within the cell. This could have important effects on protein interactions and on potential functions that the proteins have at different times in the virus lifecycle.

An inherent difficulty in studying transcription factors in neurons is that neurons are recalcitrant to conventional transfection methods using lipofectamine or calcium phosphate. Furthermore, neurons are difficult to manipulate for immune labeling techniques. In order to overcome these obstacles, we have constructed recombinant adenoviruses expressing the immediate early proteins ICP0 (or ICP0 mutants), ICP4, or ICP27 fused to the humanized form of the green fluorescent protein (EGFP). It has previously been shown that adenoviruses can efficiently infect neurons both *in vivo* and *in vitro* (1, 2, 7, 33). Furthermore, an adenovirus lacking the E1A gene can infect HSV-1 latently infected neuronal cultures without causing reactivation (Wilcox, unpublished). Adenoviruses expressing the ICP0 or ICP4 have been constructed (51, 60). The ICP4 recombinant adenovirus was constructed with a temperature sensitive mutation of the ICP4 under the control of its own promoter (51). These studies were undertaken to determine if the strong transcriptional activity of ICP4 could substitute for the deleted E1A protein (51). The ICP0 expressing adenovirus placed the entire ICP0 coding region into the E1A region of the adenovirus genome under the control of the adenoviral major late promoter or its own native promoter (60). These ICP0 adenoviruses were also used to determine if ICP0 could replace the functions of the E1A deleted protein (60). Other reports using the ICP0 expressing adenoviruses showed that the expression of ICP0 can reactivate HSV-2 in the human

fetal lung model (46, 57). ICP0 functions were also shown to be necessary for the efficient establishment of latency in the DRG model of HSV latency *in vitro* (57). While these reports have begun to address the effects of these proteins on latency, the expression patterns of the proteins in neurons during the phases of the HSV-1 infection have not been examined.

The green fluorescent protein (GFP), or mutated derivatives of GFP, has been fused to many proteins and become a prominent tool to study the interactions and expression patterns of the fused proteins in live cells (for review see 54). An important advantage of fusing proteins with the very small green fluorescent protein is that the fusion protein usually retains its normal functions (4, 54). Reports of fusing the green fluorescent protein to HSV proteins include the viral capsid protein VP26, the tegument protein VP22, and the thymidine kinase protein (9, 11, 13, 14, 36). Another report used the ICP27 nuclear export signal to show that it could translocate a nuclear protein fused to GFP from the nucleus into the cytoplasm. Importantly, all of these fusion proteins appeared to retain their normal functions. Fusions of the full-length immediate early proteins to the green fluorescent protein have not been reported. We have fused the EGFP to the immediate early proteins and expressed them from an adenoviral vector to enable us to examine the expression patterns of these proteins in live neurons. Using this combined strategy of adenoviral vectors and fusion proteins, we were able to deliver and examine the expression patterns of ICP0 (and ICP0 mutants), ICP4, and ICP27 in live neurons.

3. Materials and Methods

Cells and viruses. The Vero cells (ATCC, Rockville, MD), HeLa cells (ATCC, Rockville, MD), 2-2 cells (generously provided by Rozanne Sandri-Goldin, (50), E5 cells (generously provided by Neal DeLuca, (10), and 293 cells (generously provided by Jerry Schaack, (29) were cultured in Dulbecco's modified eagle medium (D-MEM) plus 10% fetal bovine serum

(FBS). 2-2 cells also were supplemented with 750 μg/ml gentamycin to maintain the transgene. Primary dorsal root ganglion neurons were prepared as previously described (56). All tissue culture reagents were from Life Technologies (Grand Island, NY, USA) unless otherwise noted.

Viruses included: 17^{+} (wild type HSV-1), D30EBA (17^{+} mutant lacking both copies of the ICP4 coding region,(43), and 27lacZ (KOS mutant lacking the single copy of ICP27, (50).

Expression plasmids. The humanized green fluorescent protein (EGFP) was fused in frame to the N-terminus of the immediate early proteins ICP0 (or ICP0 mutants) or ICP4 (17^{+} strain of the HSV-1). pCMVEGFP-ICP0 was constructed in two steps. First the *Sst*I to *Hpa*I fragment from pJR3 containing the wild type ICP0 coding region (generously provided by Roger Everett, (20) was subcloned into the pEGFP-C1 (Clontech, Palo Alto, CA) expression plasmid from *Sst*I to *Sma*I. To create the in-frame fusion with the EGFP coding region, the plasmid was cut with *Bgl*II and *Nco*I, filled in with Klenow DNA polymerase and dNTPs, and religated with T4 DNA ligase. We constructed EGFP fusions to a series of ICP0 deletion mutants that lack several functional regions of ICP0 (see figure above). Plasmids p110FXE, p110E1, p110A78, p110D8, and p110D12 (generously provided by Roger Everett, (15, 17, 18, 23) were restriction digested with *Bam* HI and *Sal* I. The fragments, which contained the different mutations, were then subcloned into the original pCMVEGFP-ICP0 expression plasmid using the *Bam* HI and *Sal* I restriction sites. These plasmids will be used directly for transfection assays.

The pCMVEGFP-ICP4 was also made in two steps. The *Xba*I-*Pst*I fragment containing the mutant ICP4 coding region lacking the last 7 amino acids from pXK966 (generously provided by Kent Wilcox, (59) was subcloned into the pEGFP-C1 expression plasmid from *Xho*I to *Pst*I to produce EGFP-1535. To accomplish this in the correct reading frame, the plasmids were restriction digested with *Xba*I or *Xho*I and filled in with Klenow DNA polymerase and dNTPs.

The plasmids were then restriction digested with *Pst*I, and the appropriate fragments were ligated with T4 DNA ligase. The last 7 amino acids and stop codon of the wild-type ICP4 were replaced using an *Nru*I-*Bam*HI fragment from pXK350, which contains the entire wild type ICP4 coding region under the control of the CMV promoter (generously provided by Kent Wilcox, (59). This fragment was subcloned into the EGFP-1535 construct to produce pCMVEGFP-ICP4 containing the full length ICP4 fused on the N-terminus with EGFP.

The EGFP was also fused in frame to the N-terminus of the ICP27 immediate early protein (KOS strain of HSV-1). pCMVEGFP-ICP27 was constructed by subcloning the *Age*I to *Eco*RI fragment from pGEM-2 (generously provided by Rozanne Sandri-Goldin, (49), which contains the entire ICP27 coding region into the *Age*I to *Eco*RI sites of the pEGFP-C1 expression plasmid. This plasmid was cut with *Eco47*III and *Eco*RI and subcloned into pBS(KS) (Stratagene, LaJolla, California) from *Hinc*II to *Eco*RI. This plasmid was then cut with *Kpn*I and *Eco*RI and subcloned into the pEGFP-C1 plasmid to create the in-frame fusion of the EGFP and ICP27 coding regions.

Plasmids used to express non-fused proteins and controls included: the pEGFP-C1 plasmids expressing EGFP, pXK350 expressing the wild type ICP4 protein, pCMV-ICP0 expressing the wild type ICP0, and pCMV-ICP27 expressing the wild type ICP27 protein. The pCMV-ICP0 plasmid was constructed by restriction digesting the pCMVEGFP-ICP0 plasmid with *Eco47*III and *Bsp*EI to remove the EGFP coding region, filling in with the Klenow enzyme and dNTPs, and religating with T4 DNA ligase. pCMV-ICP27 was constructed by restriction digesting pCMVEGFP-ICP27 with *Age*I to remove the EGFP coding region and religating with T4 DNA ligase. All of the plasmids described above used the humanized cytomegalovirus immedate early promoter (HCMV IE) for expression in mammalian cells. Plasmid DNAs for

transfections were extracted from bacteria and purified on Qiagen columns as described by the manufacturer.

Production of recombinant adenoviruses. Recombinant adenoviruses were constructed by subcloning the plasmids encoding the fusion proteins, described above, into an adenovirus left end plasmid (pXC15 #9 or #12) under the control of the E1A promoter (generously provided by L.-J. Su and Ian Maxwell, Univ. of Colorado Health Sci. Center, Denver, Colorado (UCHSC). To produce recombinant adenovirus the left end plasmid containing the fusion protein was co-transfected with the pJM17 plasmid into the 293 packaging cell line using the calcium phosphate transfection method as described by Graham (for review see 3). Briefly, 293 cells (1 X 10^6) were cultured in a 25 cm^2 vented flask 24 hours prior to transfection. The next day the medium was removed and 4 ml of fresh medium was added 1-4 hours prior to transfection. Into a sterile tube 5 μg of the left end plasmid and 5 μg of the pJM17 plasmid were added together with 31.25 μl of 2 M $CaCl_2$ (final concentration 125 mM) and water (total volume 250 μl total). The DNA mixture was then added dropwise, while bubbling with a pipette, into 250 μl 2X HEPES-buffered salt solution (final concentration 1X). After 10' at room temperature the mixture was added dropwise to the cells, while gently rocking the flask. The cells were then incubated at 37°C. The next day the medium was changed and the cells were maintained until recombinant viruses formed and the cell monolayer showed complete cytopathic effects. The cells and supernatant were harvested and virus was plaque purified at least two times before expanding and titrating. Titrations of recombinant adenoviruses were performed using 293 cells, and lack of wild type recombination was confirmed using HeLa cells.

Transfection and dual-luciferase assays. To assess the function of the EGFP fusion proteins as transcriptional regulators, a dual-luciferase assay was performed. Reporter plasmids

included a gD-renilla luciferase (pRL-gD) or TK-renilla luciferase plasmid (pRL-TK) and an internal control plasmid, pminLUC (based on pminCAT; 27), driving firefly luciferase to assess transfection efficiency. The two types of luciferase, renilla and firefly use different substrates. This allows two reporter plasmids to be analyzed in a single sample. The pRL-gD reporter plasmid was constructed by restriction digest of a gD-luciferase (LUC) plasmid pXK352 (generously provided by Kent Wilcox) with *Hind*III and *Bam*HI to remove the luciferase coding region. Into this site a *Hind*III to *Bam*HI fragment containing the renilla coding region from pRL-TK (Clontech, Palo Alto, CA) was inserted by ligation with T4 DNA ligase. HeLa cells (2×10^5) were plated into each well of a 6 well dish 24 hours prior to transfection. The next day the medium was removed and replaced with 1 ml of Opti-MEM one hour before transfecting the cells (Life Technologies, Grand Island, New York). The gD-renilla or TK-renilla reporter plasmid (300 ng) and pminLUC (300 ng) control plasmid along with varying concentrations of the expression plasmids and pBS(KS) plasmid were transfected into the HeLa cells. A total of 2.6 μg of DNA and 10 μl of Lipofectamine in 100 μl total volume of Opti-MEM was used for each transfection. 24 hours post-transfection, the lipid containing Opti-MEM was removed and replaced with DMEM plus 10% FBS. Cells were harvested 48 hours later in passive cell lysis buffer and 5 μl of each sample was assayed for renilla and luciferase activity as described for the dual-luciferase assay (Promega, Madison, WI) with a TD-200 luminometer (Turner Research, Sunnyvale, CA). Experiments were performed in triplicate and are reported as the average luminescence from triplicate wells for each treatment.

Infections. Recombinant adenoviruses expressing the fused or non-fused proteins were infected at a multiplicity of infection (M.O.I.) of 50 pfu/cell into Vero cells or primary dorsal root

ganglion neurons. At various times post infection the cultures were analyzed for protein expression using fluorescence microscopy, western blot analysis, or luciferase assays.

Complementation of mutant viruses lacking ICP4 (D30EBA) or ICP27 (27-lacZ) were performed by co-infecting Vero cells with the complementing recombinant adenovirus and the mutant HSV-1. Cells and supernatant were collected and plaque assays were performed with E5 cells (expressing ICP4) or 2-2 cells (expressing ICP27).

Western Blot Analysis. Western blot analysis was performed on 293 cells infected with the recombinant adenoviruses or HSV expressing the immediate early wild type or EGFP-fusion proteins. Briefly, cells (1 X 10^6) were plated per 60 mm dish for each virus 24 hours before infection. The cells were infected at a M.O.I of 10 pfu/cell and harvested at times post infection in RIPA buffer as previously described and frozen at -20°C until analyzed by SDS-PAGE (47). Proteins were quantified with the bicinchoninic acid (BCA) protein assay kit based on the biuret reaction developed by Lowry (Pierce Immunochemicals, Rockford, Illinois). Each sample or standard (10 μl) was added to 200 μl of the BCA working solution and the plate was incubated at room temperature for 2 hours. Each protein sample (30 μg) was separated on a 7.5% SDS-PAGE denaturing gel and transferred to nitrocellulose (Pro-bind, Amersham-Pharmacia) for western blot analysis. The nitrocellulose blots were blocked with phosphate buffered saline (PBS, pH = 7.4) plus 0.1% Tween-20 and 1.0 X Uniblock (Analytical Genetic Testing Center, Inc., Denver, CO) overnight at 4°C before antibodies were added. Primary antibodies were diluted 1:250 in PBS with 0.1% Tween 20 and 1.0 X Uniblock and incubated with the blots. Primary antibodies used were mouse monoclonals H1101 anti-ICP0, H1114 anti-ICP4 (Goodwin Institute for Cancer Research, Plantation, FL), H1112, anti-ICP27 (generously provided by Rozanne Sandri-Goldin), and anti-EGFP (Clontech, Palo Alto, CA). After washing, an anti-mouse

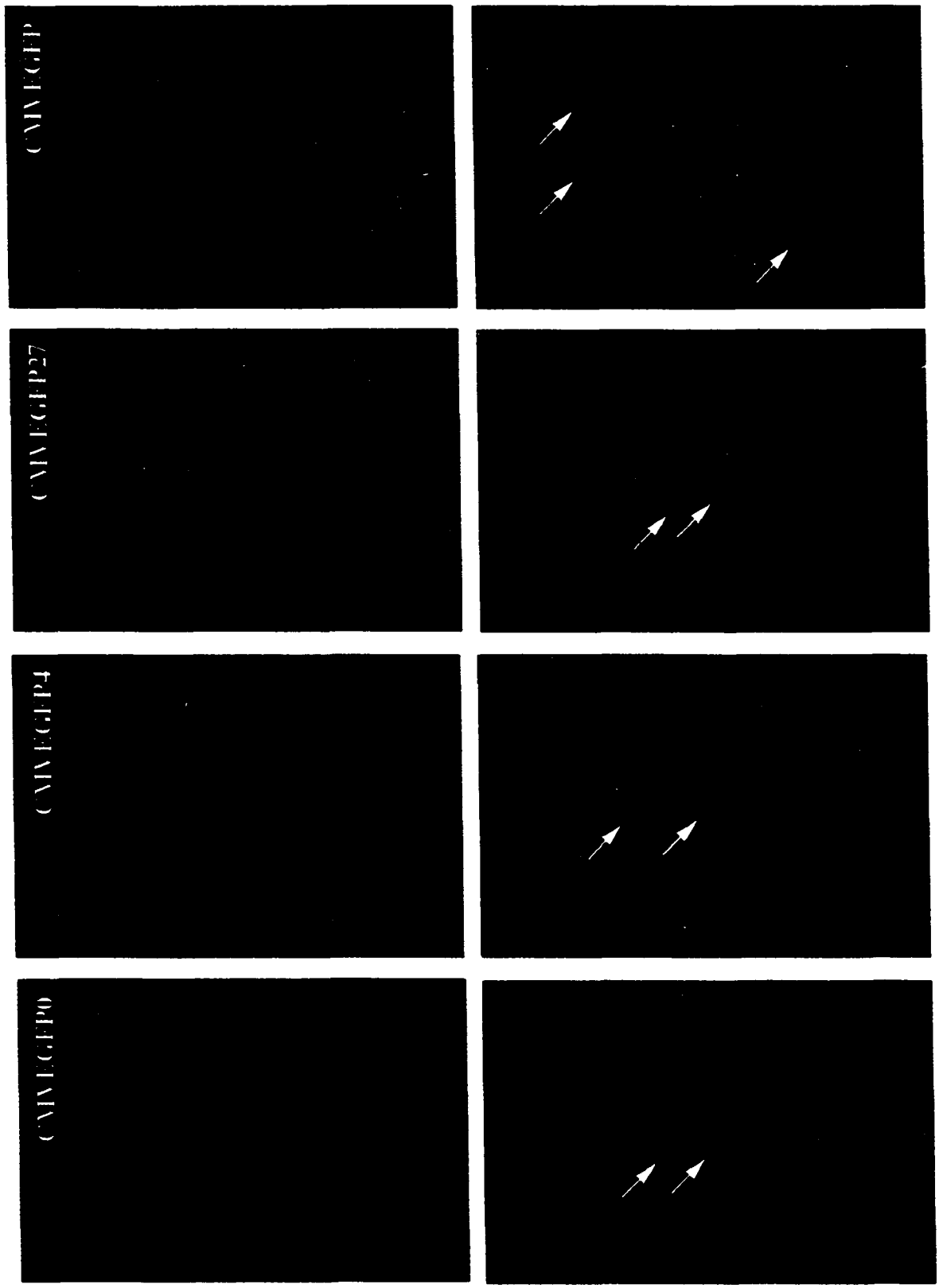
secondary antibody conjugated to horseradish peroxidase (Vector Labs) was diluted 1:750 in PBS with 0.1% Tween 20 and 1.0 X Uniblock and incubated with the blot. Blots were developed using a NEN chemiluminescent detection kit and Kodak Biomax film.

Microscopy. Fluorescence microscopy was performed using a Nikon Diaphot II equipped with Hoffman-Optics and Epifluorescence (Nikon Inc. Instrument Group, Garden City, NY). The filter cubes (Chroma Technology Corp., Brattleboro, VT) included one for fluorescein-isothiocyanate (FITC, Chroma #31648), one specific for the humanized green fluorescent protein (EGFP, Chroma #41001), and one for 4',6-diamidine-2-phenylindole-dihydrochloride (DAPI, U.V., Chroma # 11000).

4. Results

Expression of EGFP-fusion proteins in Vero cells using transfected plasmids or recombinant adenoviruses. Analysis of EGFP fluorescence and localization of the fusion proteins was performed after transfecting different expression plasmids into Vero cells. For these experiments, the HCMV-IE promoter was used to express the EGFP fused genes. Fluorescence microscopy revealed that the EGFP was fluorescent when fused to each of the immediate early proteins. Importantly, the fusion proteins gave the predicted characteristic patterns of expression identified using immune labeling methods (8, 42, 61). The EGFP-ICP4 produced nuclear expression 24 hours after transfection (Figure 3.1). The EGFP-ICP0 gave punctate nuclear staining 24 hours after transfection (Figure 3.1). The EGFP-ICP0 mutants also gave the expected patterns of expression after transfection into Vero cells (data not shown). The EGFP-ICP27 gave predominantly nuclear expression, but there was also cytoplasmic expression (Figure 3.1). All patterns were compared to the EGFP protein, which showed expression throughout the cells

Figure 3.1 Transfection of fusion-protein expression plasmids into Vero cells. Expression of EGFP-ICP0, EGFP-ICP4 EGFP-ICP27, or EGFP 24 hours after transfection of Vero cells (5 X 10^4) with 5 μg of the plasmid DNA containing the fusion constructs under the control of the CMV promoter (Upper). Cells were fixed with 4% paraformaldehyde and counterstained with DAPI tovisualize the nucleus of each cell (Lower). Fluorescence microscopy using a Nikon Diaphot II with Hoffman-Optics and GFP (Upper) or U.V. (Lower) filters (Oil X 600). Arrows indicate the nucleus of cells in the upper image as visualized with DAPI.



(Figure 3.1). Cells were counterstained with the DNA fluorophore DAPI, to locate the nucleus of each cell.The fusion-proteins were also expressed after infection of Vero cells with the recombinant adenoviruses containing the immediate early fusion proteins under the control of the adenovirus E1A promoter (Figure 3.2). EGFP-ICP4 and EGFP-ICP27 showed the same patterns of nuclear and cytoplasmic staining observed in cells transfected with the expression plasmids. The EGFP-ICP0, however, showed punctate staining that was not nuclear (Figure 3.2). Unlike the EGFP-ICP0 result, the EGFP-ICP0 mutants showed the same patterns of expression from the adenoviral vector as was seen with the transfected plasmids (Figure 3.3).

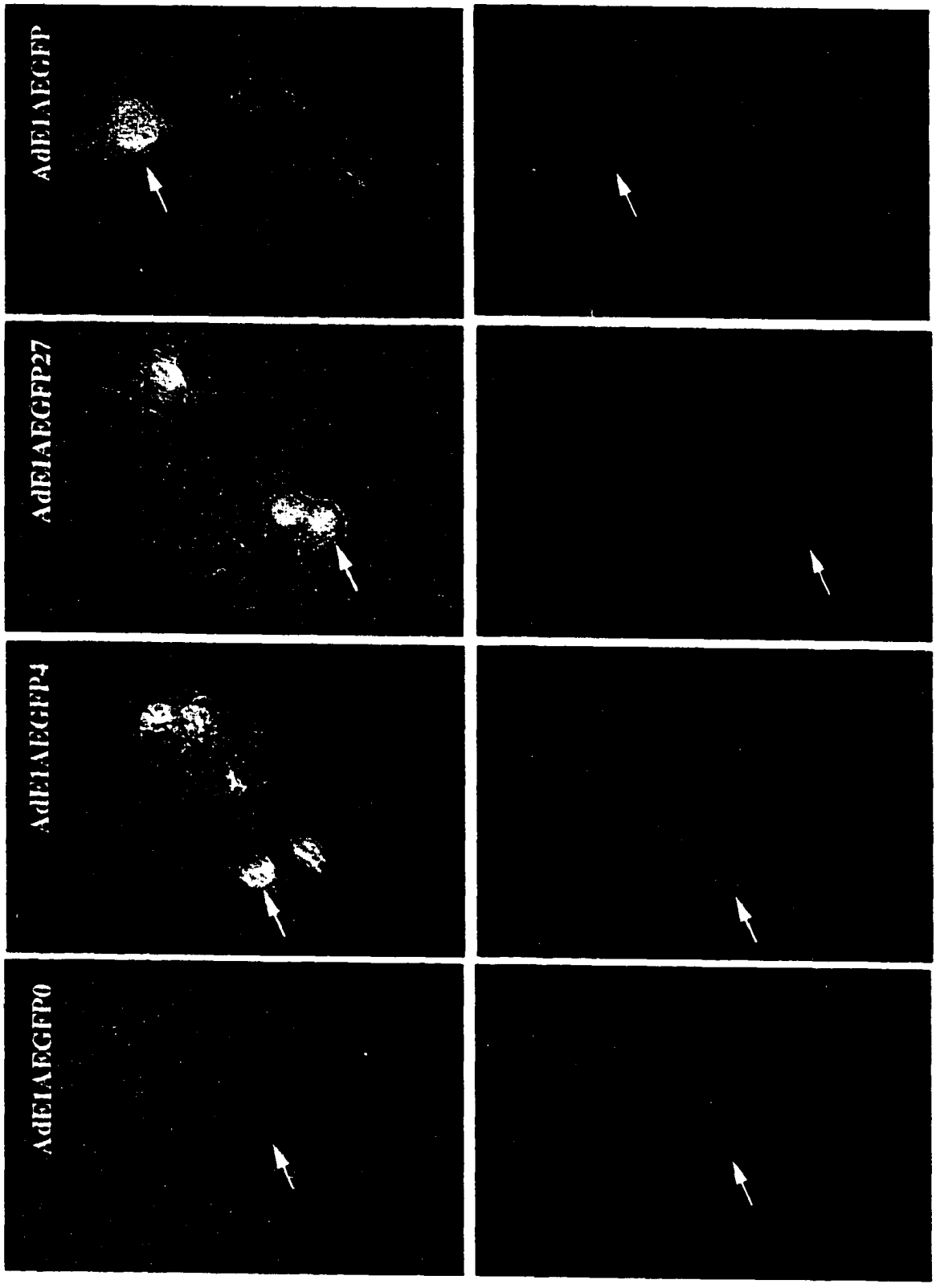
Differences in expression of the EGFP-fusion proteins in primary neurons infected with the recombinant adenoviruses. Primary dorsal root ganglion neurons were established in cell culture for two weeks before infecting with the recombinant adenoviruses. The pattern of expression for EGFP-ICP4 was nuclear and comparable to the patterns of expression in cultured cell lines (Figure 3.3). EGFP-ICP27 showed nuclear and cytoplasmic staining, also as observed in cultured cell lines. The EGFP-ICP0, however, showed little if any expression in the primary neuron cultures, even though it was clearly expressed in cell lines (Figure 3.4). This result extended to the EGFP-ICP0 mutants, which also did not show any expression in the primary neuron cultures (data not shown).

Transactivation of the gD-renilla or TK-renilla reporter plasmid with the EGFP-fusion proteins. One potentially important role of ICP0 and ICP27 is to regulate the activities of ICP4 (37). Fusion of EGFP to the amino terminus of the three proteins could potentially affect how the proteins interact and change how they regulate each other. To compare the ability of the fusion-proteins to transactivate viral promoters in a similar manner to the non-fused proteins,

Figure 3.2 Infection of recombinant adenoviruses expressing fusion-proteins in Vero cells. Expression of EGFP-ICP0, EGFP-ICP4, EGFP-ICP27, or EGFP after infection of Vero cells with a M.O.I. of 50 pfu/cell with the different the recombinant adenoviruses containing the fusion constructs under the control of the E1A promoter. Cells were fixed with 4% para-formaldehyde and counterstained with DAPI to visualize the nucleus of each cell (Lower). Fluorescence microscopy using a Nikon Diaphot II with Hoffman-Optics and GFP (Upper) or U.V. (Lower) filters (Oil X 600). Arrows indicate the corresponding cells between the upper and lower images.

Figure 3.3 Infection of Vero cells with recombinant adenoviruses expressing fusion-proteins. Expression of EGFP-ICP0, EGFP-FXE, EGFP-E1, or EGFP-A78, EGFP-D8, or EGFP-D12 24 hours after infection of Vero cells with a M.O.I. of 50 pfu/celll with the different recombinant adenovirus. Cells were fixed with 4% paraformaldehyde and counterstained with DAPI to visualize the nucleus of each cell (Lower). Fluorescence microscopy using a Nikon Diaphot II with Hoffman-Optics and GFP (Upper) or U.V. (Lower) filters (Oil X 600). Arrows indicate the corresponding cells between the upper and lower images.

Figure 3.4 Infection of DRG neurons with fusion-protein recombinant adenoviruses. Expression of EGFP-ICP0, EGFP-ICP4, EGFP-ICP27, or EGFP after infection of DRG neurons with a M.O.I. of 50 with the recombinant adenoviruses containing the fusion constructs under the control of the E1A promoter. Cells were fixed with 4% paraformaldehyde and counterstained with DAPI to visualize the nucleus of each cell (Lower). Fluorescence microscopy using a Nikon Diaphot II with Hoffman-Optics and GFP (Upper) or U.V. (Lower) filters (Oil X 600). Arrows indicate the corresponding cells between the upper and lower images.



combinations of the fused or non-fused proteins were transfected into Vero cells or HeLa cells and transactivation experiments were performed with a dual-luciferase assay. The maximum transactivation of the tk-promoter with the ICP4 and EGFP-ICP4 was four-fold in Vero cells (Figure 3.5). The EGFP-ICP0 or ICP0 showed similar activity with the EGFP-ICP4 or ICP4, to transactivate the gD-promoter in HeLa cells (Figure 3.6). The EGFP-ICP27 or ICP27 inhibited the ability of EGFP-ICP4 or ICP4. to transactivate the gD-promoter in HeLa cells (Figure 3.7).

The pminLUC control plasmid gave relatively uniform luminescence throughout all experiments on all days (data not shown) with an average expression of $0.072 \pm .047$ [range = -0.028 to 0.160]. This suggested that the cells uniformly took up different combinations of the plasmids during transfection and that the plasmids or proteins expressed were not toxic to the cells.

Complementation of viral mutants. Another test to indicate that the fusion-proteins function similarly to the wild-type proteins was to show that the fusion proteins could rescue deletion mutants of HSV-1 lacking ICP4 or ICP27. As these proteins are absolutely essential for virus replication, no virus will be produced from the deletion mutant unless the protein is supplied in *trans*. Vero cells were infected with either the 17^{+} deletion mutant D30EBA, lacking both copies of ICP4, or the KOS deletion mutant 27-lacZ, lacking the single copy of ICP27. The mutant viruses were rescued by co-infecting the cells with the respective recombinant adenovirus expressing the EGFP-fusion protein. Results in Table 3.1 show that recombinant adenoviruses expressing EGFP-ICP4 or EGFP-ICP27 were able to rescue the deletion mutants. Control adenoviruses expressing no protein or expressing EGFP alone produced no virus when co-infected with either deletion mutant (Table 3.1).

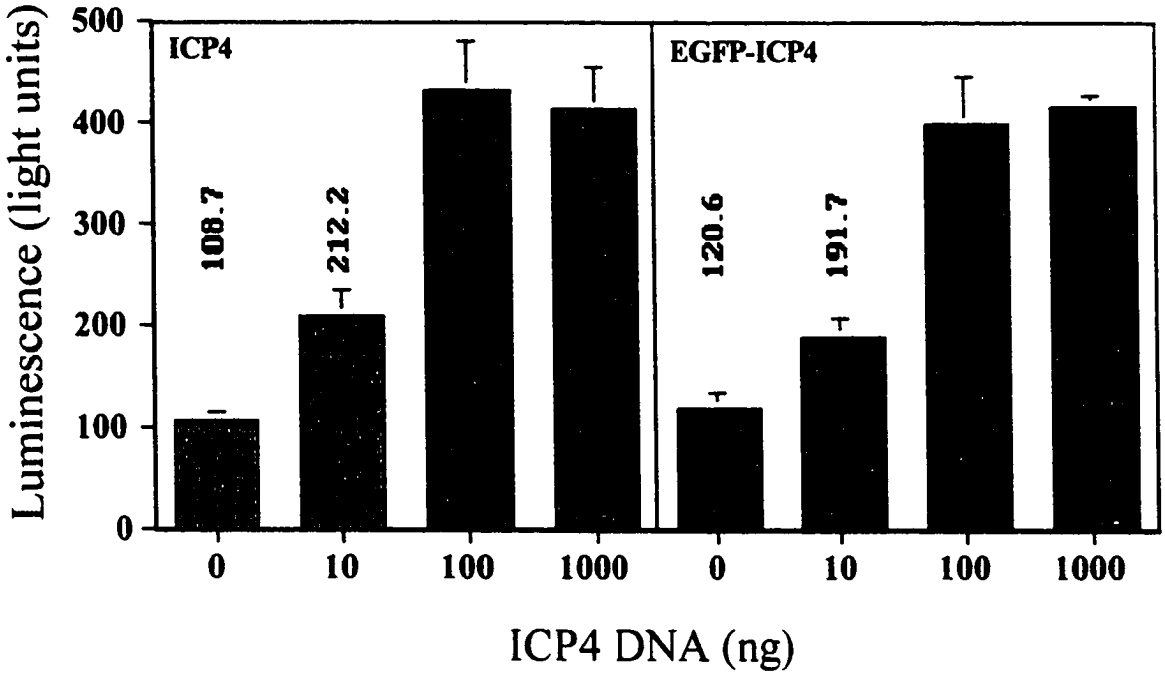


Figure 3.5 Renilla luciferase activity of cell extracts 48 hours after transfection of Vero cells with 300 ng of the TK-pRL (and 300 ng of the pminLUC control reporter plasmid, data not shown) reporter plasmid and 2 μg total of control and expression plasmids carrying the fused or non-fused protein constructs under the control of the CMV promoter. The data represent the mean ± the S.D. from one of two independent experiments done in triplicate.

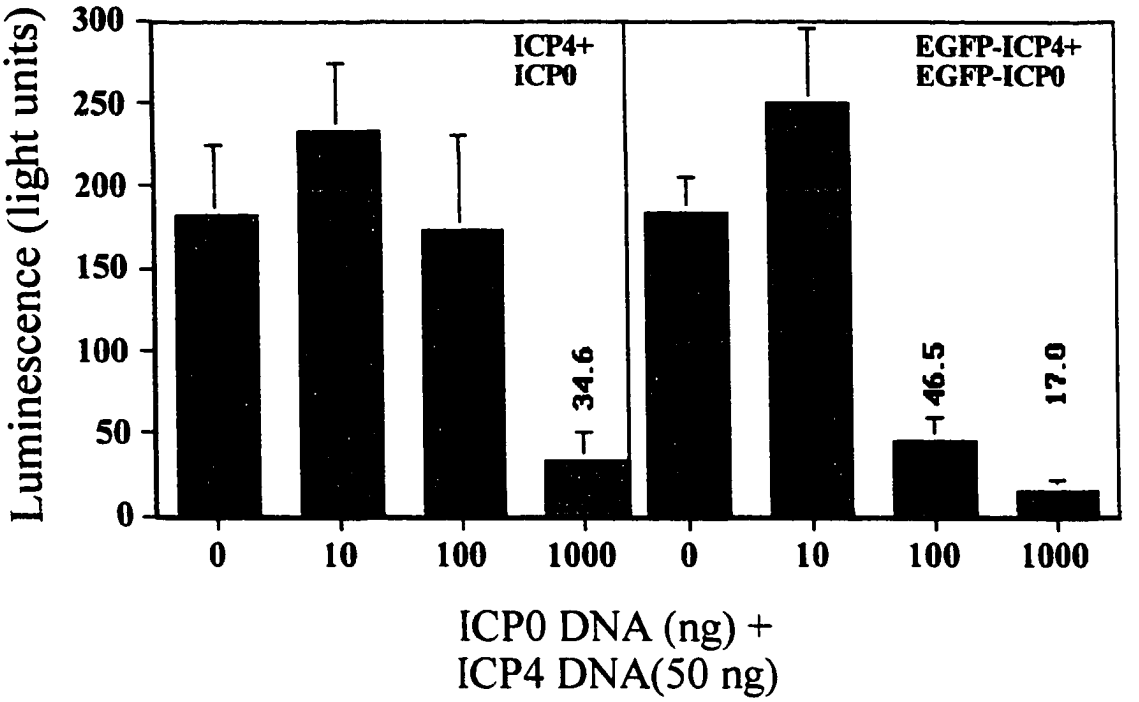


Figure 3.6 Renilla luciferase activity of cell extracts 48 hours after transfection of HeLa cells with 300 ng of the gD-pRL (and 300 ng of the pminLUC control reporter plasmid, data not shown) reporter plasmid and 2 μg total of different combinations of control or expression plasmids carrying the fusion-protein constructs under the control of the CMV promoter. The data represent the mean ± the S.D. from one of two independent experiments done in triplicate.

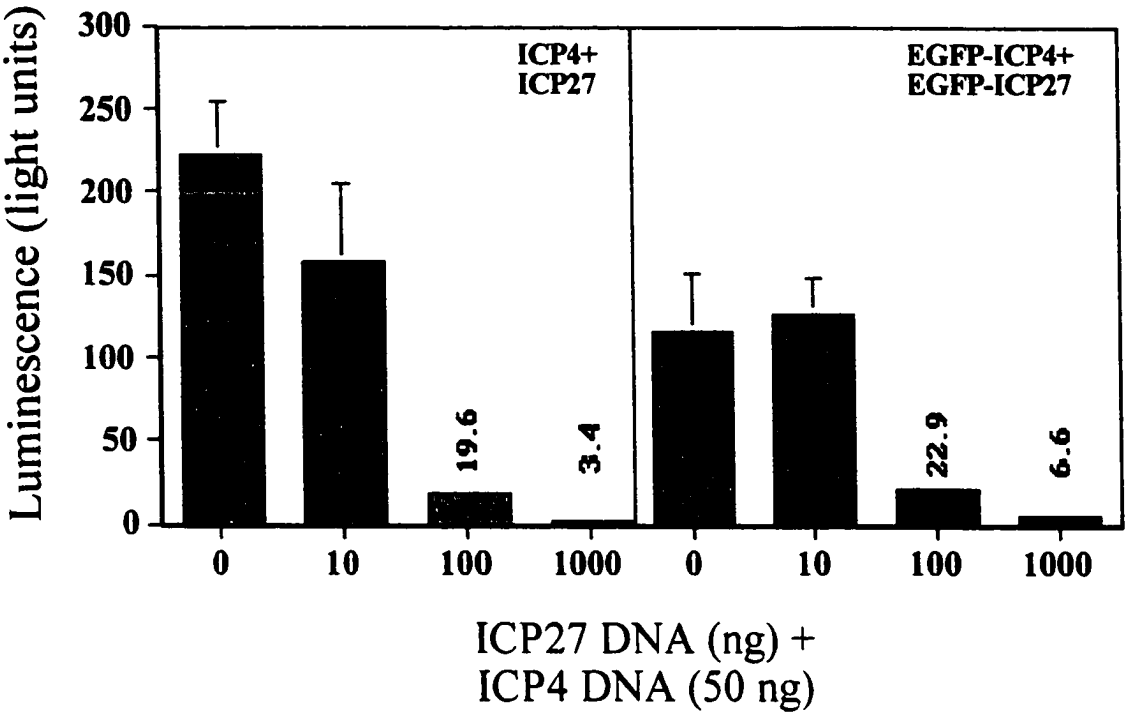


Figure 3.7 Renilla luciferase activity of cell extracts 48 hours after transfection of HeLa cells with 300 ng of gD-pRL (and 300 ng of the pminLUC control reporter plasmid, data not shown) reporter plasmid and 2 μg total of different combinations of control or expression plasmids carrying the fusion-protein constructs under the control of the CMV promoter. The data represent the mean ± the S. D. from one of two independent experiments done in triplicate.

Mutant HSV[a]	Complementing Adenovirus[a]	Titer (pfu/well)
Mock[b,c]	-	0
pACpLpA[b,c]	-	0
pACpLpA[b,c]	D30EBA(Δ4)[b]	0
pACpLpA[b,c]	Δ27-lacZ[c]	0
AdE1AEGFP[b,c]	-	0
AdE1AEGFP[b,c]	D30EBA(Δ4)[b]	0
AdE1AEGFP[b,c]	Δ27-lacZ[c]	0
-	D30EBA(Δ4)[b]	0
AdEGFP-ICP4[b]	-	0
AdEGFP-ICP4[b]	D30EBA(Δ4)[b]	4.0×10^5
-	Δ27-lacZ[c]	0
AdEGFP-ICP27[c]	-	0
AdEGFP-ICP27[c]	Δ27-lacZ[c]	4.4×10^6
17^{+}[b,c]	-	2.4×10^7

Table 3.1. Complementation of HSV-1 deletion mutants with recombinant adenoviruses co-infected into Vero Cells.
[a]Vero cells were infected with a M.O.I. of 50 with each recombinant adenovirus and a M.O.I. of 10 with each herpes virus.
[b,c]Titer (assayed in E5 cells[b], ICP4; or 2-2 cells[c], ICP27) per well of one representative experiment (n=3).

Western blot analysis of proteins expressed from recombinant adenoviruses.

Western blot analysis of cells infected with the recombinant adenoviruses expressing the fused or wild-type proteins, or cells infected with HSV-1 (strain 17^{+}), confirmed that the viral proteins were expressed from the adenoviral vectors under the control of the E1A promoter (Figure 3.8). The blot showed bands that correlated with the expected size of each protein fused to EGFP (EGFP-ICP4, 202 kD; EGFP-ICP0 137 kD; EGFP-ICP27, 90 kD), although other isoforms were also observed potentially as the result of post-translational modifications or degradation of the proteins.

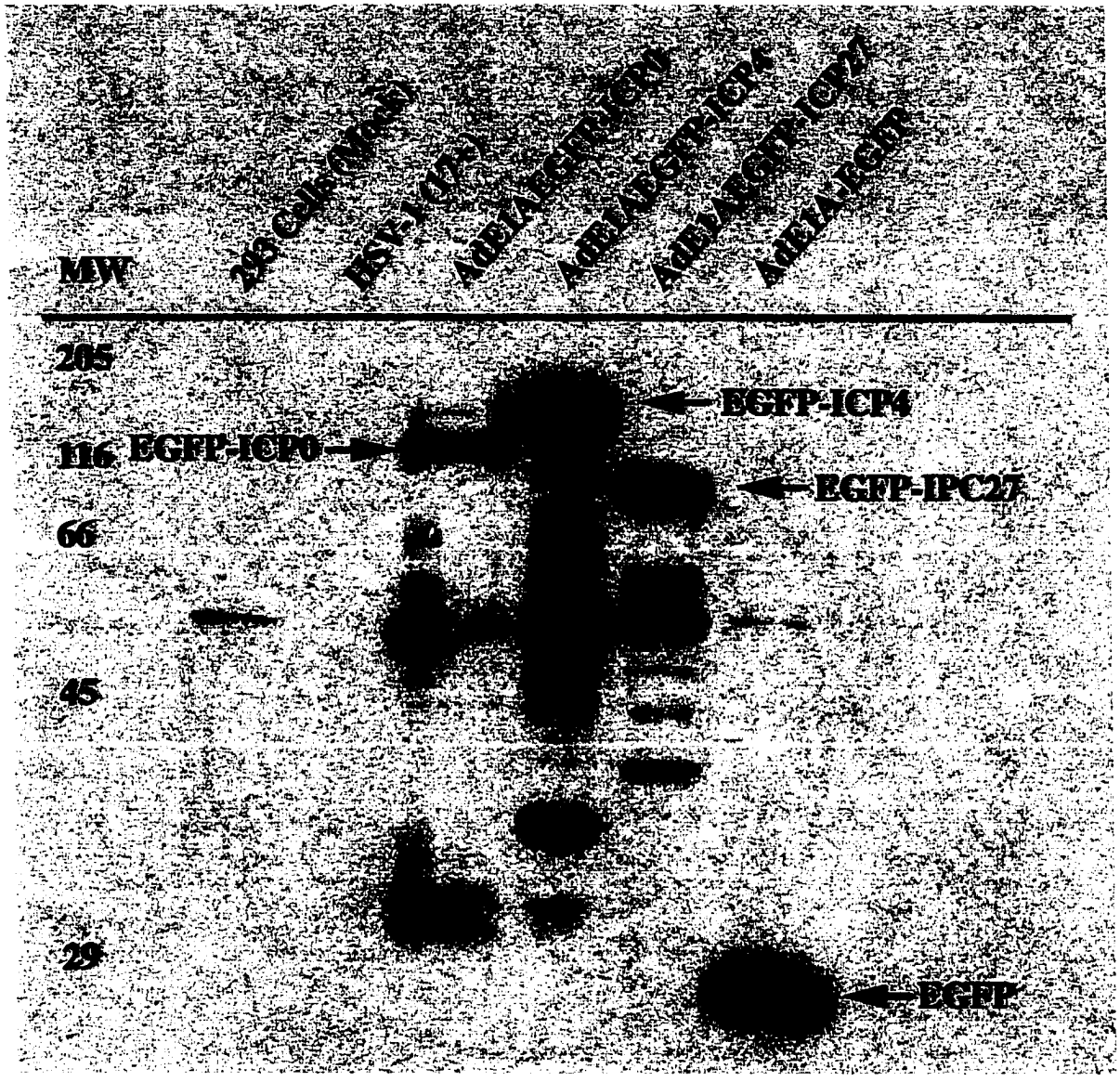


Figure 3.8. Western blot analysis of 293 cells infected with recombinant adenoviruses expressing the immediate early fusion proteins. Briefly, 293 cells were infected with a M.O.I. of 10 pfu/cell with the different recombinant adenoviruses expressing EGFP, EGFP-ICP0, EGFP-ICP4, or EGFP-ICP27. Controls included infection of 293 cells with mock or HSV-1 17+ strain. The cells were harvested 24 hours post infection and analyzed by western blot analysis using a primary monoclonal antibody specific for the EGFP. The molecular weights of the wild type proteins are ICP0, 110 kD; ICP4 175 kD; ICP27 63 kD; and EGFP, 27 kD. The blot shows bands of the correct size increase for the fusion proteins EGFP-ICP0, 137 kD; EGFP-ICP4, 202 kD; and EGFP-ICP27, 90 kD. Bands for the correct size immediate early fusion-protein are indicated with arrows. This western blot was performed by Sarah Richart.

5. Discussion

The roles the HSV-1 immediate early transcription factors ICP0, ICP4 and ICP27 in neurons during the establishment, maintenance, or reactivation from latency remain unknown. This is primarily because of the unique challenges of studying proteins in neurons, including difficulties using antibody staining to localize the proteins or transfection to introduce and express the proteins. To address this first obstacle, we have used recombinant adenoviruses, which can efficiently deliver and express foreign genes in neurons. To overcome the second obstacle, we have constructed fusions of the humanized form of the green fluorescent protein to each of the HSV-1 immediate early proteins ICP0, ICP4, and ICP27.

Localization and expression of the EGFP-ICP0, EGFP-ICP4 or EGFP-ICP27 fusion proteins from expression plasmids under the control of the HCMV IE promoter transfected into Vero cells showed the characteristic patterns of expression that have been reported using immunohistochemistry for the wild-type proteins (8, 42, 61). Transactivation studies in Vero cells using expression plasmids also gave similar results of transactivation for EGFP-ICP4 or transrepression for EGFP-ICP27, as has been reported previously for the wild-type proteins (19, 49). We did not see enhanced transactivation for EGFP-ICP0 or ICP0 with the fused or non-fused ICP4 as has been described; however, both the fused or non-fused ICP0 showed similar levels of activity when co-transfected with the EGFP-ICP4 or ICP4 (19, 49). The pminLUC internal control plasmid did not appear to be affected by the transcription factors we were expressing, during optimization experiments for the luciferase experiments (data not shown). This reporter plasmid contains the TATA-box element connected to the luciferase reporter gene. As expected it gave very low values and was not transactivated above a baseline value in these experiments. While a better internal luciferase reporter control would have been one that shows

transactivation in cell lines at a constant level throughout all of the experiments, we did not have one available. In preliminary experiments, the luciferase reporter constructs we had available (including the SV40, tk, CRE, gD, and VP5 promoters) were affected by the immediate early transcription factors (data not shown).

The recombinant adenoviruses were constructed using the native adenoviral E1A promoter to drive expression of the fusion proteins. This promoter was chosen for two main reasons. Firstly, the E1A protein expressed in the wild type virus and the 293 packaging cell line downregulates the E1A promoter and genes expressed using this promoter during adenovirus production are repressed (32, 48, 52). This was potentially important, as ICP0, ICP4 and ICP27 are strong transcriptional regulators, which could have deleterious effects on cells over-expressing these proteins. Secondly, the E1A promoter is a weak promoter in neurons and low levels of proteins are expressed after infection (32, 48, 52). Recombinant adenoviruses expressed the EGFP fusion proteins in Vero cells at levels that could be visualized using fluorescence microscopy for EGFP.

An important indication that the EGFP immediate early fusion proteins expressed from the adenoviral vectors were still functional was their ability to rescue deletion mutants of HSV-1 lacking ICP4 or ICP27. Vero cells infected with HSV-1 deletion mutants lacking, ICP4 or ICP27, produced virus when co-infected with adenovirus expressing the respective fusion-protein.

While expression patterns for the EGFP-ICP4 and EGFP-ICP27 were the same as those seen in transfection studies, EGFP-ICP0 appeared to be absent from the nucleus after viral infection compared to being seen in the nucleus after transfection of Vero cells. Interestingly, the EGFP-ICP0 mutant expression plasmids or recombinant adenoviruses did show the same patterns of expression after transfection or infection of Vero cells. In neurons, EGFP-ICP4 and EGFP-

ICP27 showed strong expression in the nucleus, and some cytoplasmic staining for the EGFP-ICP27. The EGFP-ICP0 and EGFP-ICP0 mutants, however, showed no specific EGFP expression in neurons even one week after infection with the recombinant adenoviruses. This was unexpected and could mean several things. Firstly, because the E1A promoter is a weak promoter, it is possible that only low levels of EGFP-ICP0 were expressed that were below the detection limit for visualizing EGFP by epifluorescence microscopy (a minimum intracellular concentration of 28 nM has been reported to be observable above cellular autofluorescence, 4). This seems unlikely as both the ICP4 and ICP27 fusion proteins were expressed and readily visible. Second, the ICPO fusion was readily detectable in Vero cells, albeit with an unusual staining pattern (punctate but non-nuclear). It may be that in neurons ICP0 has the ability to downregulate the E1A promoter, whereas ICP4 or ICP27 do not. Another possibility is that an adenoviral protein or the adenoviral genome competes for the same nuclear domains where ICP0 is stably expressed in cell lines. Reports have shown that adenoviral proteins interact with nuclear domains and adenoviral replication is associated with nuclear domains (12, 34). An alternative hypothesis is that the ICP0 fusion is expressed and a neuron-specific function rapidly degrades the protein or changes the expression pattern so that it is below the limit of EGFP detection. Nuclear domains, believed to be sites of transcriptional activity, potentially could be absent or different in post-mitotic neurons, so that ICP0 remains diffuse in the nucleus and is rapidly degraded. A neuron-specific function that could have effects on IPC0 is neuron-specific splice recognition sites (28). Alternatively spliced forms of ICP0 have been shown during HSV-1 infection of cell lines (16). One splice variant is a protein from the first and second exons, and a glycine tail from the second intron (16, 21, 55). The alternately spliced ICP0R is 262 amino acids long and represses transactivation of promoters that ICP0 usually upregulates (21, 25, 55). It has also been found

that the dominant negative ICP0R can inhibit viral replication in cell lines (55). It is not known which is the predominant form in neurons, and different splice variants may have different half-lives in neurons.

For ICP4 and ICP27, the results show that the green fluorescent protein can be used to generate fusion-proteins that retain the ability to localize appropriately and function as transcriptional activators, even when both proteins are fused. These viral vectors allow observation of protein expression and localization in live neurons. The results also show the importance of analyzing the fusion proteins for normal function in several different assays and cell types. It was not expected that the ICP0 or ICP0 mutant fusion proteins would not be seen in neurons when they were seen in Vero cells. The results suggest that neurons process ICP0 differently than Vero cells.

Acknowledgements

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"It's the virus, stupid"

-David Ho

Chapter 4.

VIRUS VECTORS EXPRESSING ANTI-SENSE RNA IN NEURONS

The two parts in this chapter consider RNA or DNA based viral vectors to express anti-sense RNA targeted against the HSV-1 immediate early proteins to ablate gene expression and interfere with viral replication. While the work presented in this chapter has not been published, it has lead to several other publications and abstracts that were presented at meetings.

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A. Sindbis viruses Expressing Anti-sense RNAs

1. Abstract

Neurotropic herpesviruses remain significant pathogens in animals and humans. Viral infections of the nervous system are usually serious and very difficult to treat because the nervous system is an immunologically and drug restricted site. In addition, viral infections in the brain usually have very serious consequences. Because of the problems inherent in treating infections of the nervous system, novel antiviral approaches are necessary. Even with the development of several successful antiviral drugs for the treatment of HSV infections, there are increasing reports of HSV isolates that are drug resistant. The ultimate goal of this research is to develop a novel, specific antiviral therapy against neurotropic herpesviruses.

Replication-defective virus vectors, designed to interfere with transcription of the infecting agent, have great potential to specifically inhibit pathogenic viral infections, particularly within the nervous system. A promising replication-defective virus vector is the Sindbis replicon system. Our preliminary studies indicated that a recombinant Sindbis replicon successfully infected neurons and expressed a foreign gene with no apparent pathogenic effects. The goal of this research is the development of Sindbis virus vectors to express an essential HSV-1 transcript in anti-sense orientation to interfere with HSV-1 replication and thereby reduce or eliminate the pathogenic virus infection.

2. Introduction

Recombinant Sindbis viral vectors have been developed to introduce and express foreign genes in mammalian and insect cells (4, 20, 24, 38). The success of Sindbis virus to express foreign genes is based on its genomic organization and replication strategy (4). When infectious virus enters a cell the single-stranded, positive-sense RNA, which is capped and polyadenylated,

can act as a mRNA template for replication to begin. The 5' two-thirds of this RNA encodes the non-structural proteins, while structural proteins are encoded in the 3' one-third of this RNA. Only non-structural proteins involved in RNA replication are translated from this genomic RNA. Structural proteins are translated from a subgenomic mRNA from an internal promotor on the genome complementary negative strand RNA. The non-structural and structural genes can be independently expressed from separate mRNAs (25). To produce Sindbis replicons, large deletions in the structural genes are made and replaced with a heterologous gene. A defective "helper" RNA expressing the structural proteins is able to package the Sindbis replicon without being packaged itself, thus creating viral particles which are able to infect cells efficiently but are incapable of forming progeny virions (see figure 4.1) (3). Preliminary studies in our laboratory showed that Sindbis replicons successfully introduced and expressed foreign genes (the *E. coli LacZ* gene or GFP) in neurons with long-term expression and minimal toxic effects (6, 17). These findings led us to propose the introduction of a specific anti-sense herpes simplex virus type-1 (HSV-1) sequence into this replicon expression system to act as an anti-viral agent.

A variety of viral vectors have been used to produce anti-sense molecules targeted against genes from both cells and infectious agents. Some of the viruses that have been inhibited in this way include human immunodeficiency virus, human T-cell leukemia virus type-I, vesicular stomatitis virus, dengue virus, and LaCrosse virus (11, 20, 24, 30, 32-34). Specific to HSV, only one report has appeared using a virus vector to deliver and express anti-sense mRNAs or ribozymes (5). This report used a vaccinia virus vector to express anti-sense mRNA or ribozymes targeted against the translation initiation site of the mRNA encoding the ICP4 protein (5). HSV replication was reduced 3-4 logs in Vero cells (5). This study did not examine the use of the vaccinia anti-sense vector to inhibit HSV-1 in neurons. They also selected for anti-sense

expressing cell line clones by including a neomycin resistance gene in the vaccinia virus vector. Clones lost the ability to inhibit HSV-1 by passage 20 (5). Anti-sense viral vectors against HSV-1 will need to be used in neurons, the cells that harbor the latent virus, for potential efficacy in treating all phases of the virus infection.

ICP4 is an immediate early gene product that is produced earliest during the productive infection and is absolutely essential for HSV-1 virus replication (19, 26). ICP4 controls the expression of early and some late viral genes. HSV-1 mutants lacking ICP4 express only the immediate early genes, and can only be propagated in cells transformed to express ICP4 (26). The HSV-1 ICP4 gene product provides an excellent target to test the ability of expression of anti-sense RNA by the Sindbis replicon system to inhibit viral replication.

3. Materials and Methods

Construction of Sindbis replicon containing the HSV-1 anti-sense ICP4. The Sindbis replicon plasmid (SINrep5, generously provided by Charles Rice, Washington University, St. Louis, Missouri; 3), was used to produce the anti-sense and sense producing vectors (see figure 4.1). The methods of construction have been described (for reviews see 3, 18, 22). Control viruses included one expressing the green fluorescent protein (GFP) and one expressing a chloride channel cDNA in the anti-sense orientation. To produce the Sindbis virus vector expressing the anti-sense ICP4 transcript, a 1.84 kb *Bam*HI fragment from pGX58 (containing the full length HSV-1 ICP4 coding region, generously provided by Kent Wilcox, Medical College of Wisconsin, Milwaukee, Wisconsin) was first subcloned into the *Bam*HI site of pBS(KS). The new plasmid was then cut with *Xba*I and *Hinc*II to remove a 1.68 kb fragment, which was subcloned in the anti-sense orientation into the SINrep5 plasmid using the *Xba*I and *Stu*I restriction sites. We also constructed a double subgenomic replicon vector, in which mRNA from a second cDNA could be

expressed downstream. The rationale for this was to be able to express a marker protein as well as an anti-sense RNA (see figure 4.1)

Generation of recombinant Sindbis virus replicons. Virus particles were generated essentially as described (3, 6). The plasmid for transcription of defective helper (DH) RNAs has the same 5' terminus as the Sindbis virus genome RNA (3) and was used to transcribe RNAs for co-transfection into BHK cells to produce transducing viral particles (7). All of the Sindbis virus cDNA plasmids are positioned downstream from the SP6 DNA-dependent RNA polymerase promotor. Plasmids were digested with XhoI to generate linear DNA templates for run-off transcription with a commercially available kit (Message machine, Ambion) using SP6 RNA polymerase and the 5' cap analog 7^{m}G5'ppp5'G (Ambion). Transcription reaction products were aliquoted and stored at -80°C. Liposome mediated transfection of RNA into cells was performed

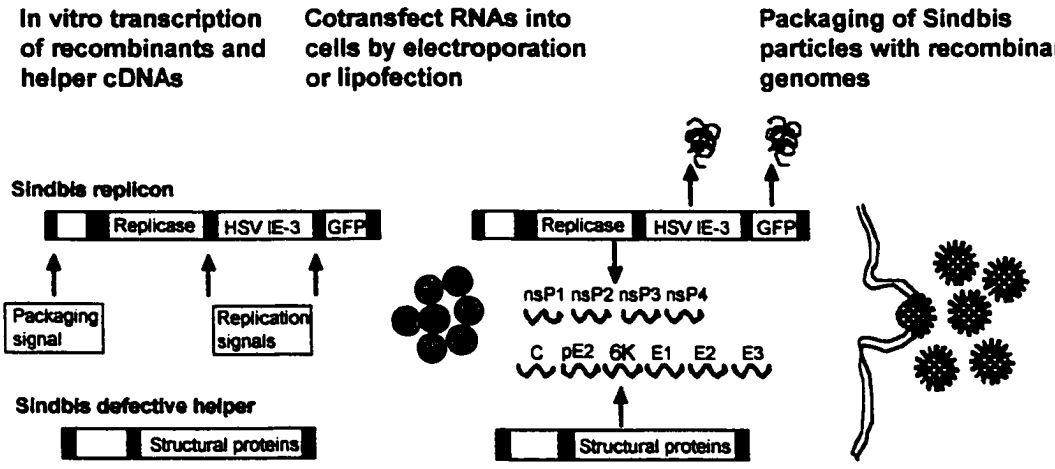

Figure 4.1 The scheme for production of recombinant Sindbis virus particles using the "replicon" system. The Sindbis replicon contains the non-structural genes necessary to produce the RNA dependent RNA polymerase. This plasmid replaces the structural genes with the gene of interest. In this figure two sub-genomic promoters have been inserted upstream of the anti-ICP4 and GFP cDNAs. This gives expression of two mRNA species, one for the GFP and one with the anti-sense and GFP joined. To produce virus a second "defective helper" plasmid is used to supply the structural genes. RNA from both plasmids is produced by *in vitro* transcription and co-transfected into BHK cells. When complete cytopathic effects are seen the cells and supernatant are harvested.

using 25 μl RNA and 25 μl lipofectin as previously described (21). Virus particles were harvested from transfected monolayers at the time when the cells showed evidence of cytopathic effects. Viral stocks were analyzed for wild-type recombination by plaque assay on BHK cells. *LacZ* transducing particles were titered using X-gal staining of infected cells.

Cell lines and neuronal cultures. BHK and Vero cell lines were maintained in Dulbecco's modified eagle medium (D-MEM) plus 10% fetal bovine serum. Sensory neuron cultures were prepared from dorsal root ganglia (DRG) of embryonic day 15 rats as described (37). Based on cell counts from the representative cultures after anti-mitotic treatment, cultures were plated to provide 1-5 X 10^3 neurons/12.5 mm culture well for analysis of HSV-1 replication, and 1-5 X 10^5 neurons/24.0 mm culture well for RNA analysis.

Analysis of virus-specific anti-sense RNA. Northern blot analysis was used to analyze mRNA expression as previously described (29). Briefly, acid guanidinium thiocyanate phenol/chloroform extraction was used to isolate total RNA from cell lines or neuronal cultures after different treatments. Denaturing formaldehyde agarose electrophoresis was used to separate RNA species, after which the RNA was transferred and crosslinked to a nitrocellulose membrane . ^{32}P-labeled RNA probes were added to pre-hybridized filters and hybridized at 50°C for 12-14 hr. Filters were then washed under high stringency conditions at 65°C and exposed to a phosphoimager screen (Molecular Dynamics) to detect labeled probe signals.

Infection of cell lines and neurons to determine the functional consequences of anti-sense RNA expression on ICP4. BHK cells or DRG neurons were infected with the Sindbis replicon expressing the anti-sense ICP4 RNAs or with control Sindbis replicons expressing GFP or a non-specific anti-sense RNA. The cultures were co-infected 24 hours later with 0.2 ml of ten-fold serial dilutions (10^3-10^7 pfu/ml) of HSV-1 (17^+). The plates were frozen 24 hours after

co-infection at -70°C until analyzing virus growth by plaque assay on Vero cells. The percentage of cells surviving infection was examined using a neutral red assay (14).

4. Results

Analysis of RNA expression. Neurons or BHK cells infected with the Sindbis-anti ICP4 transducing particles expressed specific anti-sense RNAs as analyzed by Northern blot analysis using riboprobes (Figure 4.2). A time course of RNA expression in DRG neurons in culture showed that high levels of RNA were detected by 24 hours post infection, but decreased to undetectable levels 2 weeks post infection with the Sindbis-lacZ transducing particles (Figure 4.3). Controls to look at a normal cellular message (such as β-actin or substance P) were not performed and the results may suggest uneven loading of samples or degradation of the RNA.

Toxicity of the Sindbis virus particles. Loss of RNA detection suggested the possibility that cytotoxicity was occurring with the amount of virus used for the RNA experiments. A multiplicity of infection (M.O.I.) of 2000 (equal to $1x10^6$ transducing particles/ml) was used, which would infect a large percentage of neurons in each culture. A neutral red vital stain assay was used to measure cell viability 24 hours, 1 or 2 weeks after infection with SINrep5-lacZ/26 transducing particles at M.O.I.s of 2000, 200, or 20, respectively. At a M.O.I. of 2000, approximately 25% of neurons were non-viable two weeks post infection (Figure 4.4), whereas with a M.O.I. of 200 or 20 there was less toxicity. Values greater than 100% at day one post infection can be attributed to more neutral red being taken up by the virally infected cells. Viruses alter membrane polarization and these results may reflect this.

Using three different defective helper constructs (DH-SH, DH-26, or DH-BB), transducing particles were produced with Sindbis-GFP or Sindbis-*lacZ* replicons (see table 4.1).

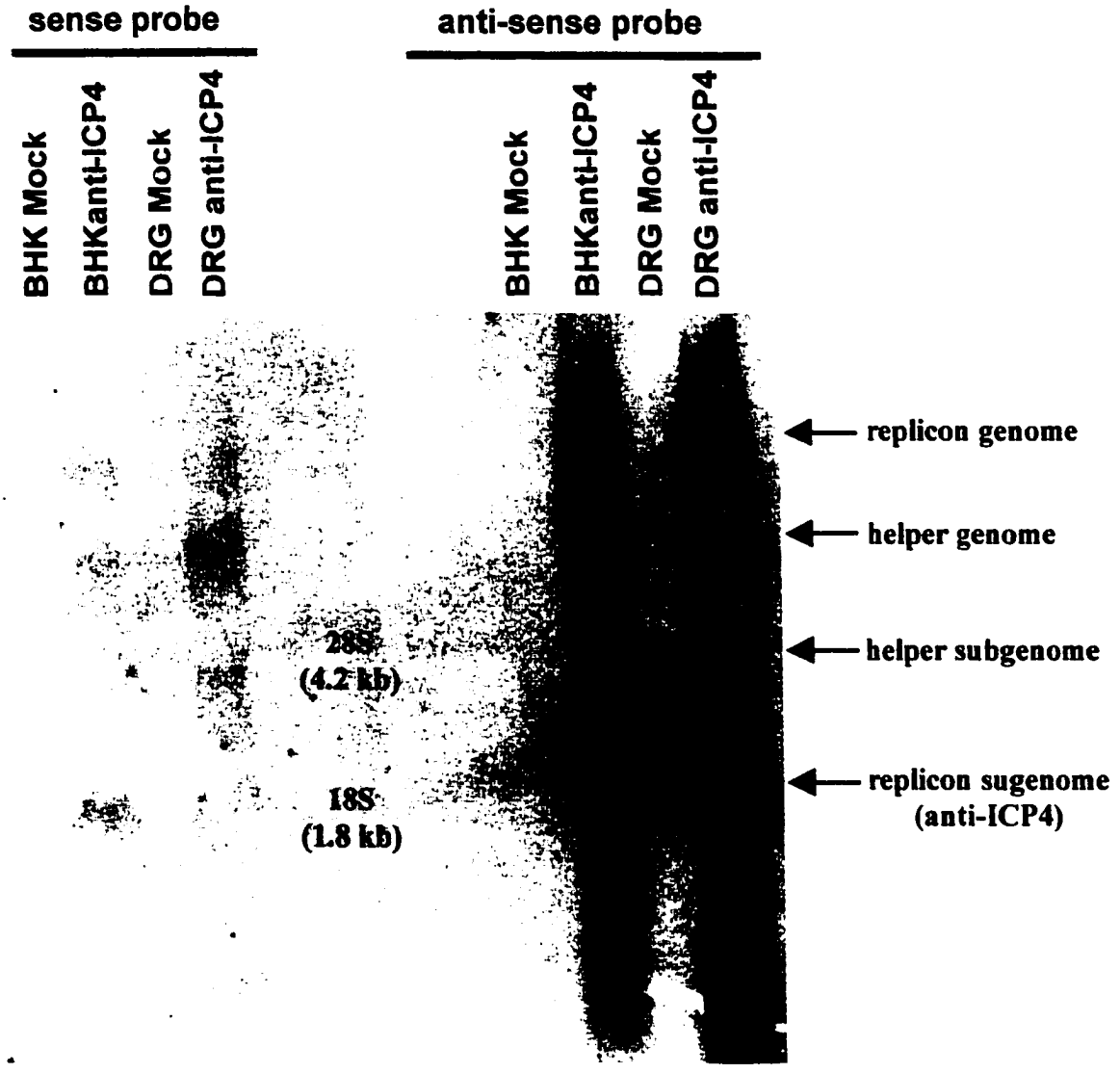

Figure 4.2 Expression of anti-sense RNA. Northern blot analysis of total RNA extracted from BHK or DRG neurons 24 hours after infection with the SINrep 5 anti-IPC4 viral particles at a M.O.I. of 2000. Equal amounts (10 μg) of RNA were separated on two agarose gels and transferred to nitrocellulose. A sense or anti-sense riboprobe specific for the first 390 bp of ICP4 was then used to probe the blots. The anti-sense blot shows several RNA species specific for the anti-ICP4 RNA including the Sindbis vector genomic and helper genomic RNAs as well as the subgenomic anti-ICP4 RNA.

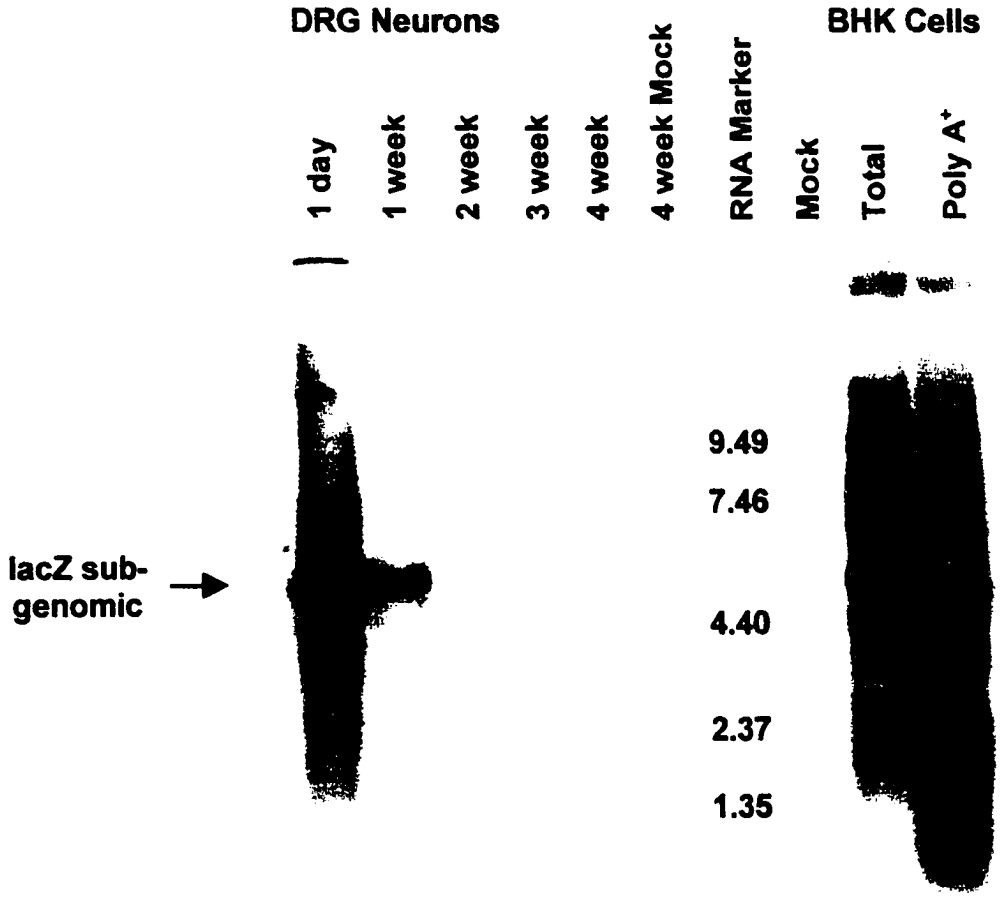

Figure 4.3 Time course of RNA expression. Northern blot analysis of *lacZ* mRNA expression in BHK or DRG neurons infected with the SINrep5 *lacZ* viral particles at a M.O.I. of 2000. Total RNA was harvested from cells at 1, 7, 14, 21, or 28 days after infection. RNA (10 μg) at each timepoint was separated by denaturing gel-electrophoresis. The blot was probed with a sense strand specific riboprobe for the *lacZ* transcript.

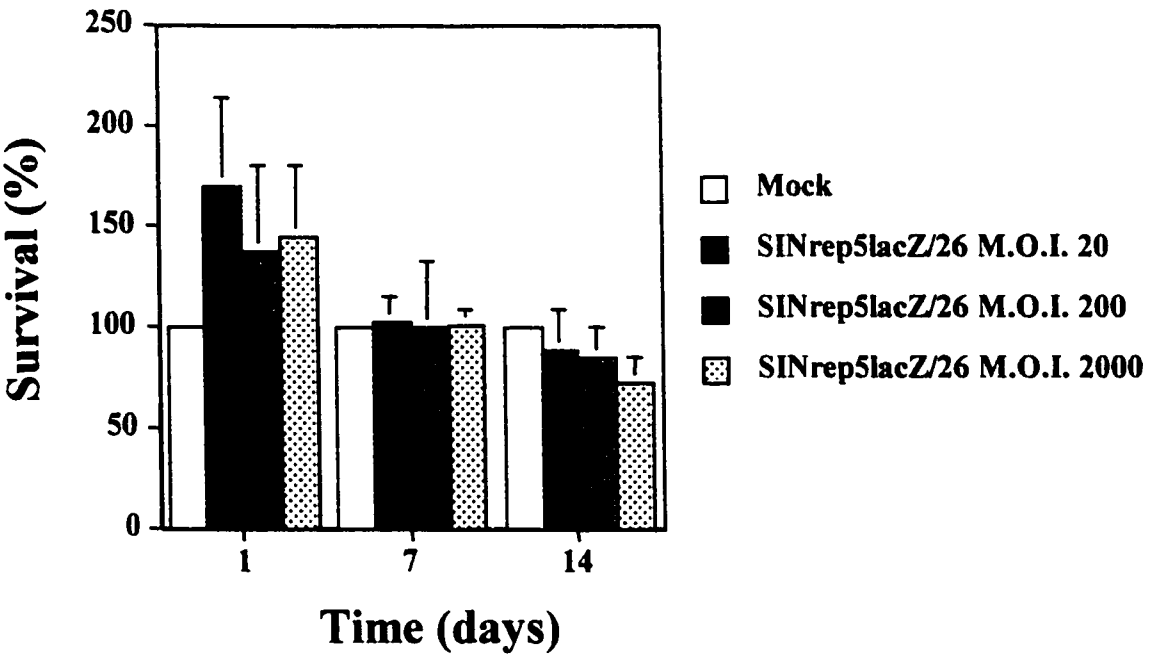

Figure 4.4. Neutral red assay of cytotoxicity in DRG neurons mock infected or infected with three different M.O.I. of the SINrep 5 *lacZ* viral particles. Cells were analyzed 1, 7, or 14 days after infection and the percent survival was calculated as compared to the mock infected cultures at each time point. Each bar represents the average percent survival of n = 3 wells +/- the standard deviation from one of two independent experiments.

Sindbis Virus	Titer of β-galactosidase positive cells (blue positive cells/ml)	Titer (pfu/ml)
BHK (ATCC)		
SINrep5lacZ/SH	**1.5×10^6**	**2.5×10^7**
SINrep5lacZ/26	**6.0×10^6**	**1.2×10^8**
BHK (Joe)		
SINrep5lacZ/SH	**4.8×10^6**	**4.3×10^4**
SINrep5lacZ/26	**3.0×10^7**	**1.9×10^4**
SINrep5lacZ-GFP/26	**9.3×10^6**	**3.45×10^4**
SINrep5Sind/-/26	**N.A.**	**1.5×10^4**
SINrep5anti-ICP4/26	**N.A.**	**1.8×10^3**
SINrep5anti-CLC2-GFP/26	**N.A.**	**2.8×10^3**

Table 4.1 Analysis of transducing particle titers and wild-type contamination. Sindbis recombinant viruses were produced by transfection of BHK cell lines from ATCC or one used by Joe Corsini with *in vitro* transcribed RNA from replicons containing genes of interest and defective helper plasmids DH-SH or DH-26. The cells and supernatants were harvested for virus 48 hours post transfection. Titers were performed for β-galactosidase activity using the X-gal substrate (blue positive cells/ml), and for the ability to form plaques (pfu/ml). N. A. not applicable as these viruses do not contain the lacZ gene.

Of the three helpers, DH-26 helper produced the highest titers of transducing particles (see table 4.1). More importantly, the DH-26 helper produced transducing particles that were more neurotropic and infected neurons preferentially over non-neuronal cells in the cultures. The DH-SH and DH-BB produced only moderate titers and infected non-neuronal cells preferentially over neurons in the cultures (6). Recombination between the replicon and helper RNA has been reported to occur at high frequency between the subgenomic promoter regions of the two RNA species (35). To determine if wild type or recombinant virus was produced during virus production, viral stocks were analyzed for plaque forming ability on BHK cells. All three helper RNAs generated infectious virus particles (see table 4.1, 10^3-10^4 plaque forming units/ml in the original BHK cell line). Using a BHK cell line obtained from ATCC produced even higher amounts of wild-type virus (see table 4.1, $>10^7$ pfu/ml). It is unknown what difference there is between the two BHK cell lines that caused these results. These results, however, show the importance of checking viral stocks for wild-type contamination compared to particles expressing the gene of interest.

Interference of HSV-1 replication with the recombinant viruses. The HSV-1 ICP4 gene is essential for viral replication. Infection of neuronal cultures or BHK cells with the Sindbis ICP4 anti-sense transducing particles and co-infecting with HSV-1 showed that HSV-1 replication could be inhibited by one log; but control Sindbis transducing particles, without a subgenomic message or expressing GFP or another anti-sense message, also inhibited HSV-1 replication (Table 4.2). To address the non-specific interactions, Sindbis replicon RNA, without a subgenomic message or expressing the anti-ICP4 message or another non-specific anti-sense message, was transfected into BHK cells and then infected with HSV-1. HSV-1 was inhibited by one log by each of the replicon RNAs (Table 4.3).

Sindbis Virus	BHK Titer of HSV-1 (pfu/ml)	Neurons Titer of HSV-1 (pfu/ml)
Mock	**$2.6 \times 10^6 \pm 1.2 \times 10^6$**	**$4.6 \times 10^5 \pm 1.8 \times 10^5$**
SINrep5anti-ICP4/26	**$3.2 \times 10^5 \pm 1.5 \times 10^5$**	**$4.6 \times 10^5 \pm 6.7 \times 10^4$**
SINrep5anti-CLC3/26	**$7.4 \times 10^5 \pm 2.7 \times 10^5$**	**$7.7 \times 10^4 \pm 5.5 \times 10^4$**
SINrep5GFP/26	**$3.4 \times 10^5 \pm 2.0 \times 10^5$**	**$2.6 \times 10^5 \pm 5.9 \times 10^4$**

Table 4.2. BHK or DRG neurons were infected with recombinant Sindbis viruses. The cells were co-infected 24 hours post infection with varying amounts of HSV-1. The cultures were frozen at -70°C 24 hours post co-infection until analysis for infectious HSV-1 by plaque assay on Vero cells. The data are from one experiment ($n = 6 \pm$ the standard deviation)

Replicon	HSV-1 Titer (pfu/ml)
Mock	**$1.80 \times 10^6 \pm 1.27 \times 10^6$**
SINrep5	**$1.35 \times 10^5 \pm 1.35 \times 10^5$**
SINrep5antisense-ICP4	**$2.07 \times 10^5 \pm 6.70 \times 10^4$**

Table 4.3. Co-infection of HSV-1 into BHK cells either mock transfected or transfected with 5 μg of the SINrep 5 replicon or the SINrep 5 replicon expressing the anti-ICP4 gene. 24 hours post infection cultures were analyzed for virus production as in table 4.2. The data are from one experiment ($n = 3 \pm$ the standard deviation).

5. Discussion

We have developed Sindbis viral vectors for gene transfer into neurons to test their ability to express anti-sense RNA. Neurons infected with Sindbis-anti ICP4 expressed large amounts of the specific anti-sense RNA by 24 hours post infection. RNA expression was undetectable by two weeks post infection and cytotoxicity was evident by neutral red assay using a high M.O.I. needed to infect a large percentage of the neurons in each culture. At high multiplicities, wild-type contamination was evident and this was one source of toxicity. Even at high multiplicities, using more neurotropic helper plasmids, not all of the neurons in a culture were infected, as seen in studies using marker proteins (6). HSV-1 replication was inhibited by at least one log, but non-specifically. This non-specific inhibition appears to be caused by the Sindbis replicon itself, which alone interferes with HSV-1 replication. The reason we do not see greater inhibition of HSV-1 replication with the anti-ICP4 expression may reflect differences in where these two viruses replicate. The DNA virus, HSV-1, replicates in the nucleus and the RNA virus, Sindbis, replicates in the cytoplasm (26, 27). It is possible that to produce interference with a DNA virus nuclear expression of the anti-sense RNA will be necessary. Our results may also reflect the interactions of the non-structural proteins of the Sindbis virus to inhibit host cell macromolecular synthesis (22).

To improve Sindbis replicons for neurons will require eliminating plaque-forming virus and toxicity of the non-structural proteins. In this way higher titers of virus could be used to infect more of the neurons in a culture. As of this writing several new Sindbis viral vectors have been reported and selection of non-cytotoxic mutants have been found (9, 10, 16, 23). To reduce wild-type contamination or recombinants between the defective helper and the replicon, replicon vectors have been made using DNA-based expression of the RNA viral genomes or producing

inducible packaging cell lines for the structural or non-structural proteins (9, 16, 23). To limit cytotoxicity will require finding Sindbis viruses that are not pathogenic to cells. Mutations of the viral glycoproteins E1 and E2 alter neurovirulence in mice, and genetic mapping studies have located some of the regions that cause cytopathic effects (15). Construction of new defective helper plasmids or packaging cell lines using these mutations will likely reduce toxicity. A second problem is that the non-structural proteins are also toxic in cell lines. After PCR mutation of the nsP2 gene, Rice's group has selected replicons that are non-cytopathic in mammalian cells (10). Combining these changes should produce a non-toxic replicon that contains no wild type contamination. The ability of Sindbis virus to express large amounts of RNA quickly after infection, combined with new technology, has significant potential for greater success for anti-sense interference.

B. Adenoviruses Expressing Anti-sense RNAs

1. Abstract

While the Sindbis replicon viral particles can infect and express high levels of RNA in neurons, they are limited by wild-type contamination and toxicity. These problems interfered with our ability to use this viral vector to produce specific anti-sense inhibition of HSV-1 in neurons. To overcome these obstacles we have made anti-sense expressing recombinant viruses using an adenoviral vector. Adenoviruses have shown great utility in the delivery and expression of genes in neurons (see chapter 1 for review of adenovirus vectors). Adenoviral vectors also offer several other advantages over the Sindbis viral vector system, including no wild-type contamination, higher titers of virus particles (10^8), and no apparent toxicity. Recombinant viruses were constructed to express anti-sense RNA targeted to the ICP0, ICP4 and ICP27 genes of HSV-1. ICP4 and ICP27 are essential genes required for viral replication. ICP0 is not an essential gene, but plays essential roles in the efficiency of virus replication and the different stages of the virus lifecycle *in vivo*. Analysis of cells infected by two anti-ICP4 adenoviruses by northern blot hybridization showed high levels of RNA expression. Recombinant adenovirus co-infected with HSV-1 did not block HSV-1 viral replication in a specific manner. Interestingly, however, control adenoviruses were able to block 4-5 logs of HSV-1 growth. The mechanism by which adenoviruses block HSV-1 replication is not known, but potentially the two DNA viruses can compete for replication compartments they both use in the nucleus.

2. Introduction

The success of adenoviruses for gene therapy lies in the ability to produce recombinant viruses expressing foreign genes without any wild type contamination (for review see 2). Advantages of adenovirus vectors are a capacity to express large inserts of foreign DNA (up to 8

kb in size), the ability to produce high-titer viral stocks (10^8-10^{12} particles/ml), a broad range of infectivity of mammalian cells, and the availability of vectors with many different promoters (for review see 2). Adenovirus is a non-enveloped DNA virus with a genome of approximately 36 kb. During the lytic infection genes expressed before viral DNA replication include the E1 regions E1A and E1B, the E2 regions E2A and E2B, E3 and E4. After viral DNA replication, the major late promoter is activated and transcribes most of the alternatively spliced mRNAs used to produce the structural proteins. The strategy used to generate recombinant viruses is to delete regions of the adenovirus genome and insert foreign genes of interest in their place. Deleting the essential E1 gene, renders recombinant viruses replication defective unless the E1 gene is provided in trans (for review see 2). Foreign genes are cloned into plasmids containing the first 5,000 bp of the genome (pACCMVpLpA) with the E1 gene deleted. A second plasmid (pJM17), with deletions in the E3 genes and carrying the rest of the adenovirus genome, is used to generate a recombinant virus after co-transfection into cells where homologous recombination can occur. To produce viral particles the plasmids are co-transfected into the 293 packaging cell line, which provides the E1 gene functions in trans (Figure 4.5) (for review see 2). Recombinant viruses are selected for because the second plasmid pJM17, also carrying the ampicillin and tetracycline resistance genes and the bacterial origin of replication, is too large to be packaged into viral particles. Initial studies in our lab have shown that several different promoters can be used to produce adenoviruses that express β-galactosidase or the green fluorescent protein in neurons without any toxic affects on the cells (8). The promoters allow long term expression, high titers of virus are produced (>10^8 particles/ml), and greater than 90% of the neurons in a single culture can be infected (8). Using the HCMV IE promoter high levels of foreign genes can be expressed in neurons. Using the green fluorescent protein (GFP), production of viruses can be monitored,

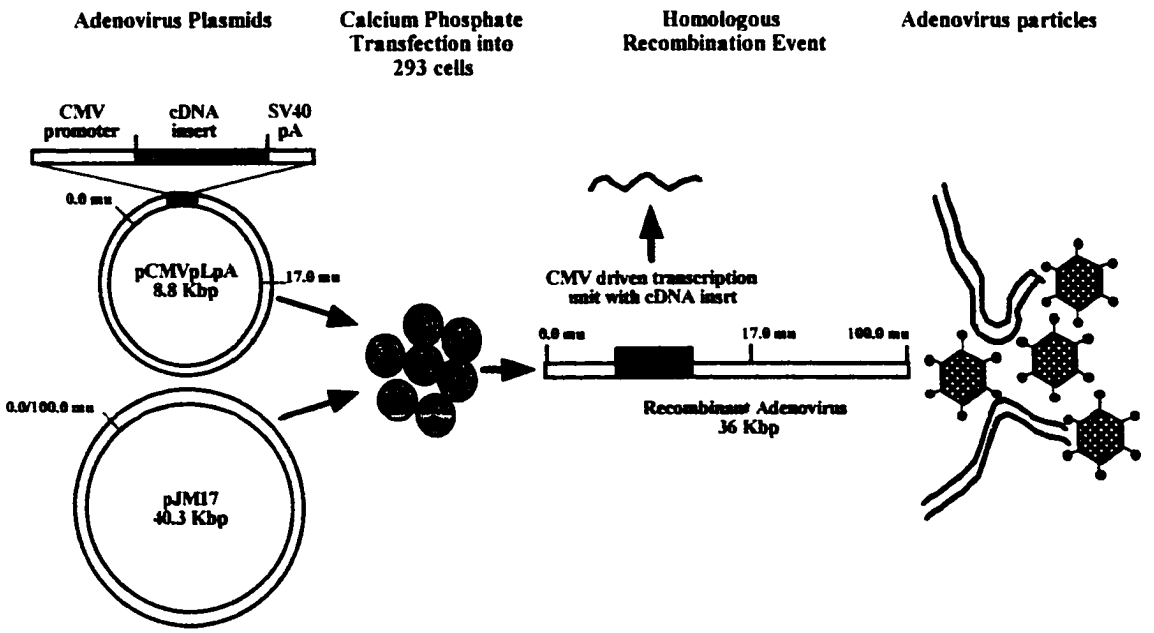

Figure 4.5 Scheme for producing recombinant adenoviruses. An adenovirus left end shuttle plasmid (pACCMVpLpA) is used to insert the gene of interest between the HCMV IE promoter and a polyadenylation signal. To produce virus the left end plasmid and a second plasmid (pJM17) are co-transfected into the 293 packaging cell line which supply the E1 proteins necessary for efficient replication. The two plasmids can recombine to produce a viral genome of the correct size to be packaged into viral particles.

and infected neurons can be identified. These findings led us to propose the development of adenovirus vectors as an anti-sense therapeutic agent to treat alphaherpesvirus infections.

ICP4 and ICP27 are immediate early HSV-1 gene products that are produced earliest during the productive infection and are absolutely essential for HSV-1 replication (for review see 26). ICP4 and ICP27 control the expression of early and some late viral genes. HSV-1 mutants lacking ICP4 and ICP27 express only the immediate early genes, and are propagated only on cells transformed to express ICP4 or ICP27 (for review see 26). The HSV-1 ICP4 and ICP27 gene products provide excellent targets for the adenovirus anti-sense system to test the potential for the inhibition of HSV-1 replication. ICP0 is not an essential gene for viral replication, but appears to

be important for the ability of HSV-1 to establish a latent infection in neurons (36). Targeting the ICP0 gene product will test whether the adenovirus anti-sense system can prevent the establishment of a latent infection in neurons.

3. Materials and Methods

Cell lines and neuronal cultures. Human embryonic kidney cells transformed with Ad 5 viral DNA, 293 cells(13) , generously provided by Jerry Schaack, Univ. of Colorado Health Sci. Center (UCHSC), and African green monkey kidney cells (Vero cells, ATCC, Rockville, MD) were maintained in Dulbecco's modified eagle medium (D-MEM) plus 10% fetal bovine serum. Sensory neuron cultures were prepared from dorsal root ganglia (DRG) of embryonic day 15 rats as described (37). Based on cell counts from the representative cultures after anti-mitotic treatment, cultures are plated to provide 1-5 X 10^3 neurons/12.5 mm culture well for analysis of HSV-1 replication, and 1-5 X 10^5 neurons/24.0 mm culture well for RNA analysis.

Adenovirus left end plasmids for constructing anti-sense expression vectors. Genomic DNAs containing the HSV-1 ICP4, ICP27, or ICP0 genes were used to produce the anti-sense adenovirus constructs (see figure 4.6). Each anti-sense DNA was subcloned into an adenovirus plasmid containing 5 kb of the left end of the virus (pACCMVpLpA, (12). Anti-sense constructs were placed between the HCMV IE promoter and SV40 polyadenylation signal to control expression of the anti-sense DNA.

Generation of recombinant adenovirus. Recombinant viruses were generated essentially as described (for review see 2). Briefly, the left end plasmid containing the anti-sense construct was linearized with *XhoI*, and 5 μg of the plasmid DNA were cotransfected along with 5 μg of the pJM17 shuttle plasmid DNA containing the Ad 5 genome into 293 cells. Recombination occured between the two plasmids while in the 293 cells. The recombinant virus

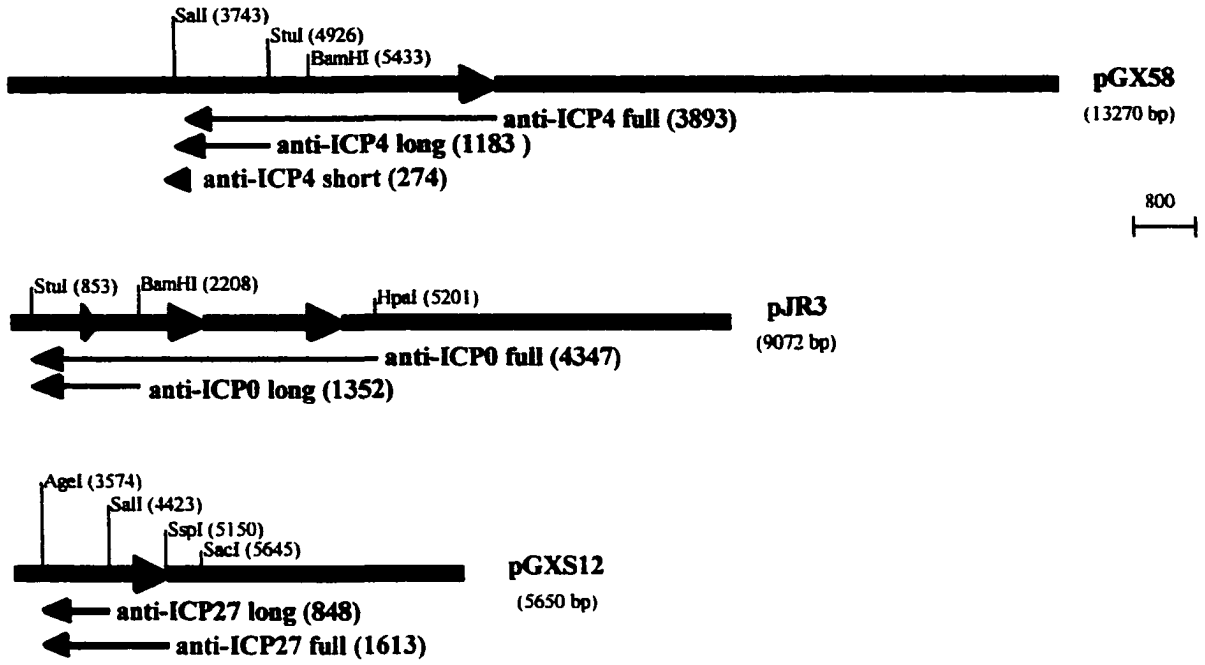

Figure 4.6 Diagram of the ICP4 (top), ICP0 (middle), and ICP27 (bottom) containing plasmids used for obtaining anti-sense fragments. The coding regions of each protein are shown as solid black boxes with rightward arrows in the plasmid box. Anti-sense fragments used to produce recombinant adenoviruses are show as thin lines below the plasmid box with leftward arrows. Restriction sites are shown. Numbers in parenthesis are sizes of fragment (bp).

lacks the E1A and E1B genes essential for efficient viral replication, and can only readily produce virus in 293 cells which constituitively express the E1A and E1B proteins. Virus was harvested from the cells and supernatant by several freeze-thaw cycles and virus was plaque purified. Lack of wild-type contamination was confirmed by no plaque production on HeLa cells. Anti-ICP4 viruses were also analyzed by restriction digest and Southern blot analysis to confirm that the recombinant viruses contained the DNA insert (data not shown).

Analysis of virus-specific anti-sense RNA.

Northern blot analysis. Northern blot analysis was used to analyze mRNA expression as previously described (29). Briefly, acid guanidinium thiocyanate phenol/chloroform extraction was used to isolate total RNA from Vero cells after adenovirus infection. Denaturing

formaldehyde agarose electrophoresis was used to separate RNA species, after which they were transferred to a nitrocellulose membrane and crosslinked. ^{32}P-labeled RNA probes, produced by *in vitro* transcription of linearized plasmid DNA with T3 or T7 RNA polymerase, were added to prehybridized filters and hybridization at 50°C was continued for 12-14 hr. Filters were then washed under high stringency conditions at 65°C and exposed to a phosphoimager screen (Molecular Dynamics) to detect labeled probe signals.

Infections of cell lines and neurons with adenovirus to determine the functional consequences of anti-sense RNA expression. 5 X 10^4 Vero or 293 cells were infected with adenovirus expressing the anti-sense mRNA, or control adenoviruses expressing EGFP or no transcript with 0.2 ml of 1 X 10^7 pfu/ml for 24 hours. To determine if anti-sense expression could effectively block HSV-1 replication, Vero or 293 cells were co-infected with 0.2 ml of 1 X 10^5-1 X 10^2 pfu/ml of HSV-1, and virus replication was determined 24 hours later by plaque assays of cell cultures and supernatants after freeze-thawing.

4. Results

Analysis of RNA expression. To determine if the recombinant viruses had recombined correctly and were able to express anti-sense RNA northern blot analysis was used on total RNA extracted from cells. Vero cells infected with two different recombinant anti-ICP4 adenoviruses expressed high levels of the anti-sense RNAs 24 hours post infection as shown by northern blot analysis using riboprobes (Figure 4.7). The 1.690 kb coding region containing adenovirus expressed a single band of the correct size. The 390 bp coding region containing adenovirus expressed the correct size band and a larger and smaller band. The other ICP27 or ICP0 anti-sense adenoviruses have not been analyzed for RNA expression.

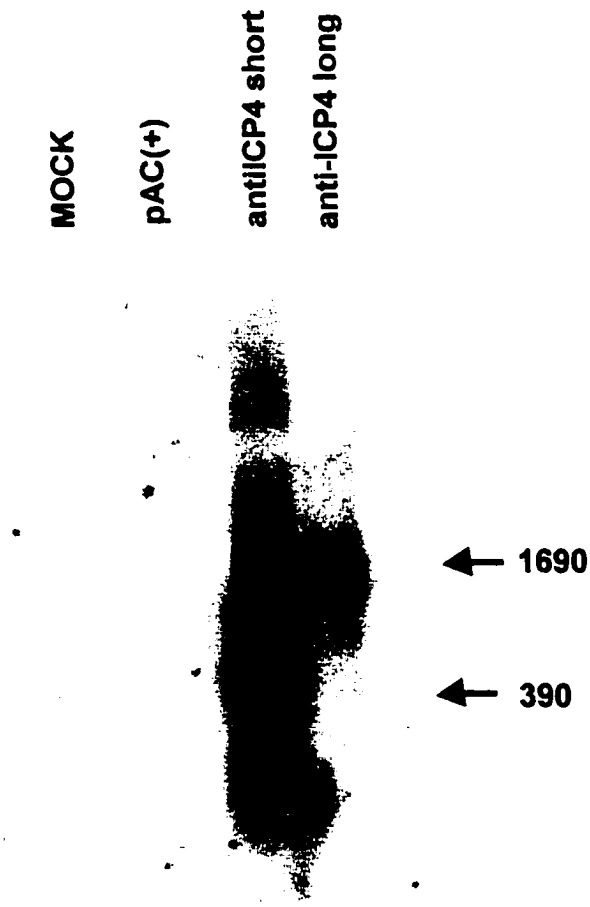

Figure 4.7 Northern blot analysis of total RNA from Vero cells infected with a M.O.I. of 50 with different adenoviruses (pAC, Ad anti-ICP4 Start, or Ad anti-ICP4 Long). Cells were harvested 24 hours after infection and 10 μg of each RNA was separated by gel electrophoresis and analyzed using an anti-sense specific riboprobe for the first 390 bp of the ICP4 coding region.

Interference of HSV-1 replication with the recombinant viruses. The most direct way to show that the anti-sense viruses were functional was to determine whether they could inhibit HSV-1 viral growth. To do this, Vero cells were infected with control adenoviruses or adenoviruses expressing the anti-sense RNAs and 24 hours later the cultures were co-infected with HSV-1. Figure 4.8 shows that HSV-1 growth was inhibited in Vero cells infected with adenoviruses expressing β-galactosidase or two different anti-ICP4 viruses. Surprisingly, the control virus inhibited HSV-1 growth more than either virus expressing anti-sense RNA (Figure 4.8). Because of the dramatic decrease in HSV-1 growth (3-4 logs), a second set of experiments was performed using control viruses alone (see figure 4.9). The pAC control adenovirus has no insert in the expression cassette, and the MLP0 adenovirus expresses the ICP0 immediate early gene and should enhance viral growth. In Vero cells pAC inhibited HSV-1 viral growth as before; MLP0, however, allowed growth similar to mock infected cultures at two concentrations of HSV-1 and inhibited the lowest concentration of HSV-1 (see figure 4.9). When this was repeated in 293 cells, where the defective viruses should be able to replicate, both of the control viruses inhibited growth (4-5 logs) at all concentrations of HSV-1 used (see figure 4.9). Furthermore, there was even greater inhibition in the 293 cells compared to the experiments performed in Vero cells. To date the other ICP0 or ICP27 adenoviruses have not been carefully analyzed for inhibition of HSV-1 growth.

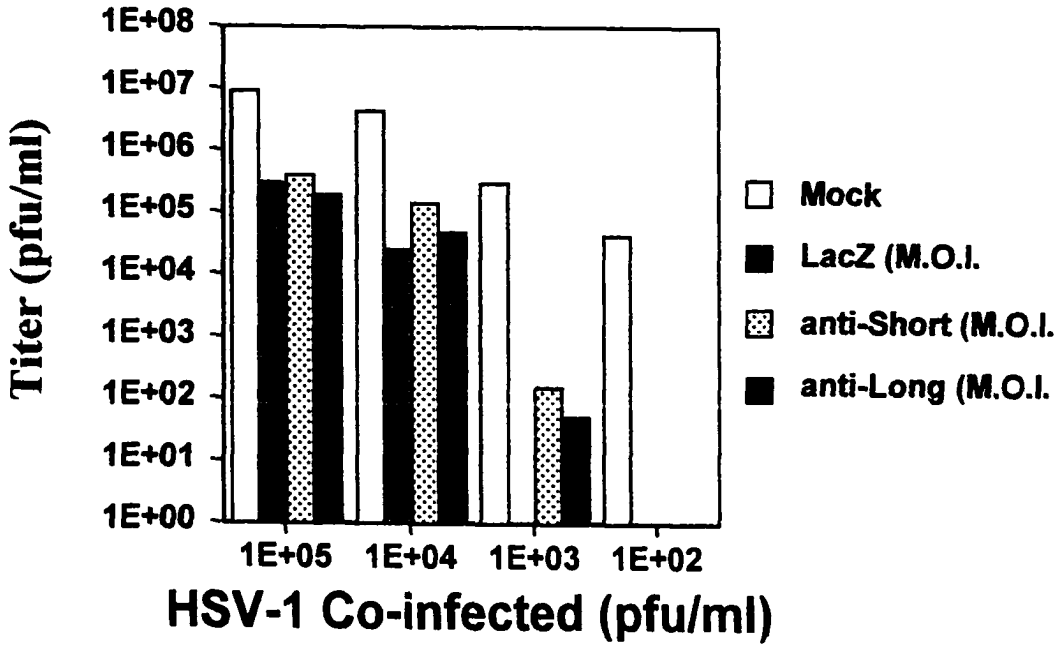

Figure 4.8 Vero cells were infected with 0.2 ml of 1 X 10^7 pfu/ml of different adenoviruses. The cells were co-infected 24 hours later with 0.2 ml of 1 X 10^2-1 X 10^5 pfu/ml of HSV-1. The cultures were harvested 24 hours later and titers for infectious HSV-1 were determined by plaque assay on Vero cells. The data represent one of two experiments done in triplicate.

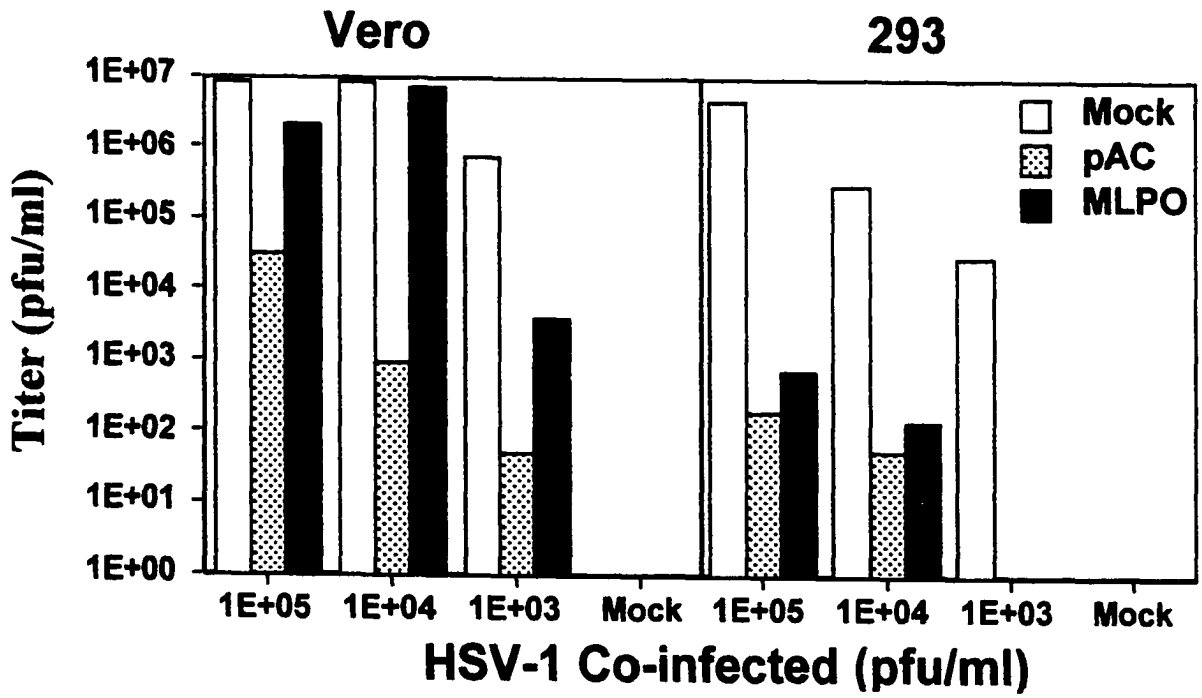

Figure 4.9 Vero cells were infected with 0.2 ml of 1 X 10^7 pfu/ml of different adenoviruses. The cells were co-infected 24 hours later with 0.2 ml of 1 X 10^3-1 X 10^5 pfu/ml of HSV-1. The cultures were harvested 24 hours later and titers for infectious HSV-1 were determined by plaque assay on Vero cells. The data represent one of two experiments done in triplicate.

5. Discussion

This study attempted to overcome the limitations of the Sindbis anti-viral study and examine the use of a DNA viral vector as a means to express anti-sense RNA to interfere with HSV-1 viral replication in cells. Adenoviral vectors can infect neurons more efficiently than the Sindbis viral particles. They also do not have the same types of problems with wild-type contamination of viral stocks. High titer stocks, that make manipulations of the virus much easier, can be generated. Finally, adenoviral vectors can accommodate a larger coding capacity for foreign genes.

Several adenoviral vectors were constructed to express anti-sense RNA targeted against the immediate early proteins ICP0, ICP4 and ICP27. Analysis, to date, has been done using the anti-ICP4 recombinant adenoviruses. The adenoviruses were able to express the anti-sense messages, but specific inhibition of HSV-1 replication was not observed. Unexpectedly, at the titers of virus used, control adenoviruses and anti-sense viruses were able to block HSV-1 growth by 3-4 logs in Vero cells and 4-5 logs in 293 cells. While we have not determined how the adenoviruses are blocking HSV-1 replication, there are many possibilities. Both of these DNA viruses are known to associate with nuclear domains inside the infected cell (for reviews see 26, 28). These domains are thought to be areas where replication and transcriptional activity occur for the viruses. It is possible that adenoviruses can block or compete with HSV-1 for these domains. Another possibility is that the adenovirus blocks co-infection with HSV-1 or competes for the macromolecules necessary for virus production. This is unlikely as experiments in Chapter 3 demonstrate that adenoviruses can rescue HSV-1 deletion mutants that cannot replicate on their own. These experiments, however, used a very low M.O.I. (50 pfu/cell) compared to the experiments performed in this chapter where HSV-1 viral inhibition was seen. A ratio of 10,000:1

(adenovirus particles to HSV-1 particles) showed the maximum inhibitory effect of HSV-1. Future experiments include trying to determine which domains of the adenovirus are responsible for this effect.

While the use of viral vectors to specifically block HSV-1 replication was not successful, it led to a potentially interesting finding that adenoviruses can block HSV-1 replication. Reasons for the failure of the anti-sense expression to block HSV-1 replication, other than vector interference, are not clear. Anti-sense RNA is usually directed against the 5' cap site of the targeted gene, although splice junctions have also been used as targets (for reviews see 1, 31). Anti-sense viral vectors constructed against cellular genes or genes from pathogen have been made with truncated or entire coding regions of the targeted genes (for reviews see 1, 31). A longer RNA is more likely to form secondary structures that can block binding to the target mRNA; however, longer RNA also provides more potential sites for interaction between the anti-sense RNA and the target mRNA. Another concern has been that the capping and polyadenylation sites using expression cassettes will interfere with the anti-sense effect. This does not appear to happen, but using tRNA or snRNA promoters may be a way to produce stable anti-sense RNAs that do not have these extra elements.

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"It is a capital mistake to theorize before one has data.

Insensibly one begins to twist facts to suit theories

instead of theories to suit facts."

-Sherlock Holmes

SUMMARY

This dissertation is divided into two major themes, viral vectors and herpes simplex virus. In the first part we compared different promoters for their ability to express genes in neurons. This led to the work in the later parts, where viral vectors were used to express or interfere with expression of HSV-1 genes in neurons.

Viral vectors.

The success of viral vectors lies in their ability to efficiently deliver and express genes in cells that cannot readily be studied using conventional approaches. Neurons are cells that pose unique challenges for expression of foreign genes because of their post-mitotic state and unique structural architecture. While viral vectors have become powerful tools to study genes in the nervous system, there are still many problems with viral vectors that need to be resolved. These include lack of regulated expression of the foreign gene from the viral vector and immune responses against the virally infected cells.

In developing viral vectors for use in the nervous system, we have focused on characterizing different viral and cellular promoters to express marker proteins in rat neurons, both *in vitro* and *in vivo*. The HCMV-IE, RSV LTR, and E1A viral promoters were compared directly by constructing recombinant adenoviruses with these promoters driving *lacZ* or EGFP marker protein expression using the same viral vector backbone. Our results showed that these viral promoters drove different levels and kinetics of expression in DRG neurons *in vitro*. The HCMV-IE promoter expressed the highest amounts and earliest detection of protein after infection of neurons with the recombinant virus. The RSV promoter drove an intermediate level of protein expression that took several days to reach. The E1A promoter gave the lowest, usually

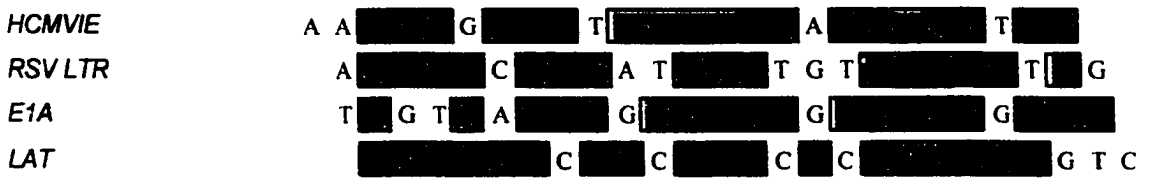
undetectable, levels of expression. Infection of the recombinant adenoviruses onto acute hippocampul rat brain slices or injected into rat brains *in vivo*, drove different levels of expression, and expressed the β-galactosidase marker protein in different regions and cells of the rat brain in a promoter specific manner. This is an important finding in that one would not have expected cell-type specific expression using these viral promoters. It also shows the importance of analyzing promoter functions for gene expression and recombinant viral vectors in the context in which they are to be used (such as the brain).

While viral promoters showed different levels, kinetics, and cell-type specific gene expression, they will not give regulated expression of genes (even with inducible promoter systems based on tetracyline or steroids). This will not matter for uses such as cancer treatment or vaccines, as high levels of expression are the goal. However, gene replacement therapies, such as L-DOPA replacement in Parkinson patients, will probably be most successful using regulated expression of the gene by placing it under the control of its native promoter. Another rationale for using neuron-specific promoters is to allow neuronal genes that are potentially toxic to non-neuronal cells to be expressed only in neurons. This includes eliminating expression in the 293 packaging cell line so viruses can be produced with genes (either neuronal genes or genes for cancer treatment) that could be toxic to the packaging cells as well. To develop recombinant adenoviruses with promoters that are regulated by neurons, we constructed adenoviral vectors with promoters from neuronal genes as well as a viral HSV-1 promoter that has neuron-specificity. Adenoviral vectors were constructed with the NSE, NMDA, TH, Tα1, GAP43 or LAT promoters driving marker protein expression as above. Preliminary results have shown that the Tα1 and LAT promoters drove expression of lacZ or EGFP in DRG neurons.

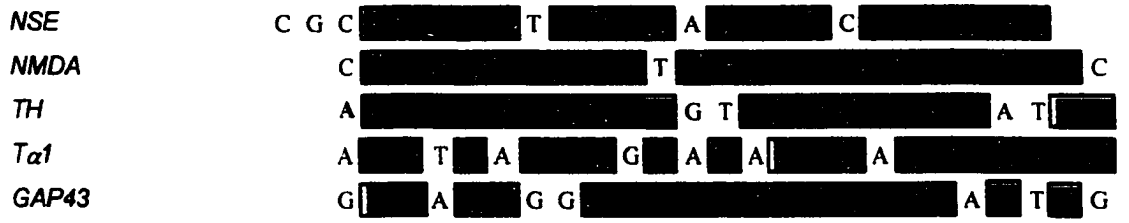
To address why these promoter regions might give cell-type specific expression, subsequence, pairwise and multiple alignment analyses were performed (MacVector, Oxford Inc.) on the promoter regions used to construct the recombinant adenoviruses. Subsequence analysis showed that the promoters contained many common DNA elements where cellular transcription factors could interact. Pairwise and multiple analysis of promoter regions with known neurons restrictive silencing elements (NRSE) showed that the HCMV-IE, RSV LTR and E1A viral promoters (as well as the neuronal promoters) contained regions that showed identity (68-88%, see figure on p. 152) with known neuron restrictive silencing elements (NRSE). These elements, found in the promoters of neuronal genes, suppress gene expression in non-neuronal cells. While the viral promoters we used were active in non-neuronal cells, the NRSE-like regions suggest that cell-type specific promoter elements may be contained in viral promoters. Subsequence analysis also revealed that all of the promoters contained regions that are found in adenoviral promoters (see figure on p. 153). While it is unknown if either of these DNA elements are functional in the promoters we used, the results are potentially important in explaining the observed cell-type specific expression. They are also important for finding promoters that will not be functional in the 293 packaging cells.

While we did not address immune responses against viral vectors, transient expression of genes due to immune responses against the infected cells or the expressed gene is a significant problem limiting gene therapy applications. Overcoming the pre-existing or induced host immune response against the virally infected cells is a daunting task. While viruses have evolved elaborate mechanisms to downregulate the immune response against them, the host still seems to clear most

Viral Promoters



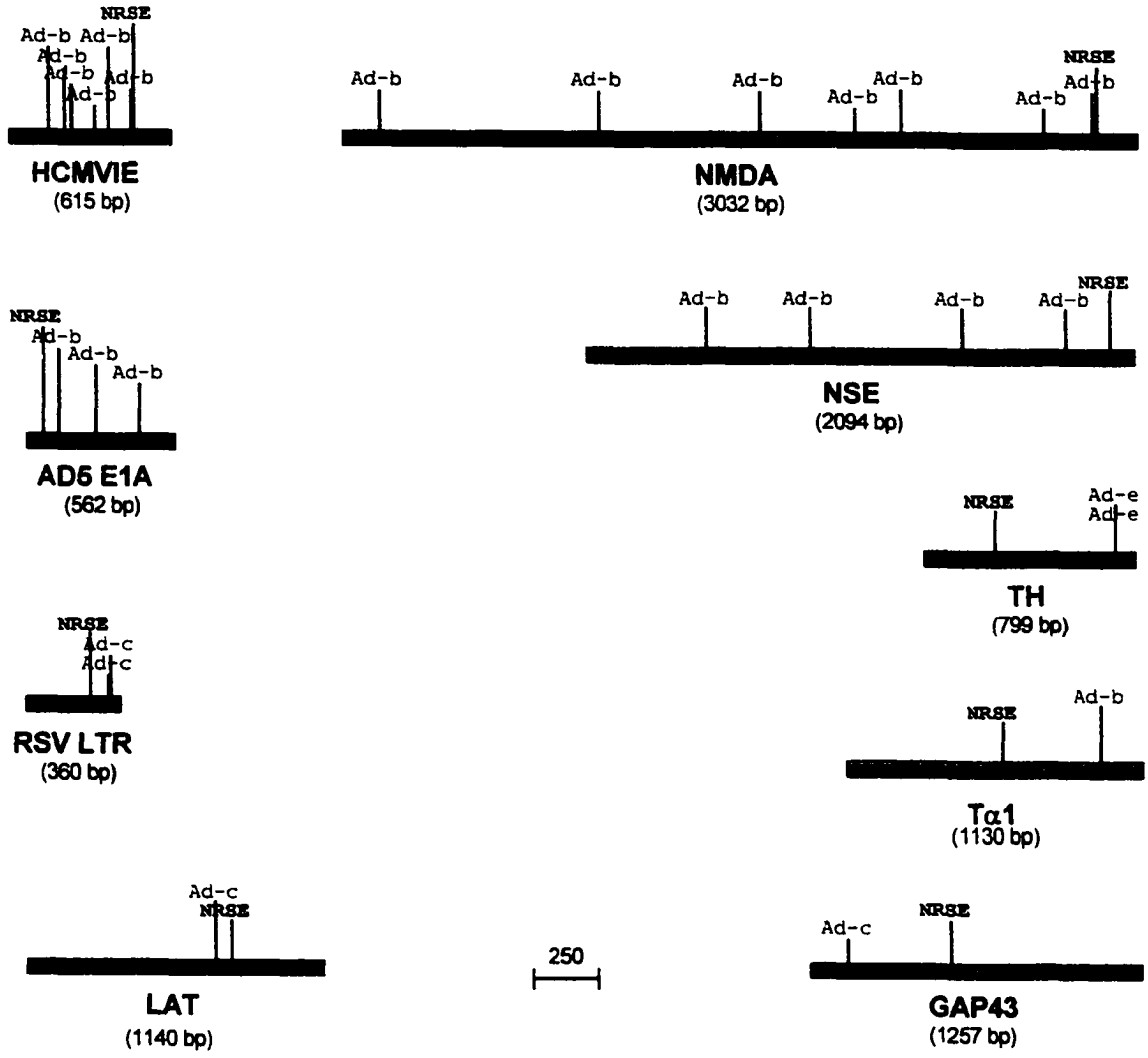
Neuronal Promoters



Neuron Restrictive Silencing Elements

NRSEsyn2	T T T A G T A C C G C G G A C A G A G C C
NRSENg-Cam3	G G C G T T G T C C G T G G T A T C G C A A
NRSENg-Cam2	G G C G C T G T C C G C G G T G C T G A A
NRSEsyn	T T C A G C A C C G C G G A C A G T G C C
NRSE1	T T C A G C A C C A C G G A C A G C G C T C
NRSE2	G C C A G C A C C A C G G A C A G T T C C
NRSE3	G G A G C T G T C C G A G G T G C T G A A
NRSEcons	T T C A G C A C C A C G G A C A G T G C C
NRSENa	T T C A G A A C C A C G G A C A G C A C C
NRSENg-Cam	G G C G C T G T C C G C G G T G C T G A A
NRSESCG10	T T C A G C A C C A C G G A G A G T G C C

Viral and Neuronal Promoters with NRSE Motifs. The diagram shows results from multiple ClustalW alignment analysis of the viral (human cytomegalovirus immediate early, HCMVIE; Rous sarcoma virus long terminal repeat RSV LTR; adenoviral early region 1A, E1A; and HSV-1 latency associated antigen, LAT) and neuronal (neuron specific enolase, NSE; N-methyl-D-aspartate, NMDA; tyrosine hydroxylase, TH; Tα1 alpha tubulin Tα1, and GAP 43) promoter regions used to construct recombinant adenoviruses. Shown below the viral and neuronal promoters are the known neuron restrictive silencing element (NRSE) subsequences. The 25 bp region of each promoter that had similarity with known NRSE found in neuron-specific genes is shown. Bases in promoters that matched with any of the NRSE bases are boxed and shaded in gray.



Neuron restrictive silencing elements (NRSE) and Adenoviral Promoter Elements. Diagram shows where the NRSE (shown in blue) and some of the adenoviral subsequences (Ad-b, GCCAA; Ad-c, AATGA; or Ad-e, TGACGT) are in each promoter used for constructing the recombinant adenoviruses expressing marker proteins. The promoters are shown from 5' to 3' (left to right) ending at the start (ATG) of translation for each gene.

viral infections without any apparent pathologic effects. The successful viral vector will likely be one that does not elicit any immune response. The two basic structures of viruses that make them useful as vectors are their outer envelope or capsid, that binds to specific moieties on a cell and allows for the viral particle to fuse or be taken into the cell, and the genome that can persist in the cell and express a gene of interest. If minimal genomes could be packaged into non-immunogenic viral particles, the current limitations of viral vectors could be overcome still allowing the benefits of efficient delivery and expression unique to viral vectors. Minimal or "gutless" vectors have been made to express genes; however, viral particles that do not elicit an immune response have not been as well developed.

Herpes simplex virus.

HSV has evolved with humans over eons to form a relationship whereby the virus can remain in the host despite a strong immune response. The key to the success of HSV as a pathogen lies in its ability to form a latent infection in the neurons of the host from which reactivation and transmission of the virus can occur throughout the lifetime of the host. This latent or "quiescent" state is best characterized as the complete absence of viral antigens and the expression of only one major RNA species (LAT). The virus-host interactions during latency continue to be intensely studied to ascertain the mechanisms the virus uses to establish, maintain and reactivate from the latent state in neurons. Only through a complete understanding of latency in neurons will we be more successful in reducing transmission of HSV-1.

The immediate early proteins are critical for regulating viral gene expression during a productive infection in permissive cells. Characterization of these proteins in neurons, where latency occurs, has not been done. It is likely that the immediate early proteins have novel functions during the establishment, maintenance or reactivation from latency in neurons. We

constructed adenoviral vectors expressing fusion proteins of the immediate early proteins ICP0 (or mutants or ICP0), ICP4 and ICP27 fused to EGFP. We showed that these recombinant adenoviral vectors expressed the fusion proteins and that the fusion proteins were functional in cell lines. Infection of neurons with ICP4 or ICP27 adenoviral vectors showed that the fusion proteins were expressed and localized to the nucleus as was seen in cell lines. However the fusion proteins were not detected in neurons infected with ICP0 or ICP0 mutant adenoviral vectors. These results suggest that there might be differences in the way HSV-1 regulatory proteins are expressed in neurons as compared to permissive cells. The results underscore the importance of determining what individual HSV-1 proteins do in the context of the cells in which latency occurs. It also suggests that adenoviral vectors are a good way to deliver and express HSV-1 proteins in neurons.

The ultimate goal of HSV-1 research is to "cure" an HSV-1 infected individual, thereby breaking the transmission cycle of the virus to non-infected individuals during an episode of reactivation. Using adenoviral vectors we attempted to ablate the expression of HSV-1 immediate early genes ICP4 and ICP27, which are essential for a productive infection. Using either the Sindbis viral vector or adenoviral vector, we showed that anti-sense RNA targeted against the 5' start of translation for ICP4 was expressed in cell lines or primary neurons. Anti-sense viral vectors, however, did not show specific inhibition of HSV-1 growth in cells. The Sindbis viral vector controls inhibited one log of HSV-1 and, surprisingly, the adenoviral vector controls inhibited greater than four logs of HSV-1. The mechanism of this adenoviral inhibition is not known. One hypothesis is that the recombinant adenoviruses competed for the same nuclear domains where both of these DNA viruses replicate. Alternatively, the adenoviral vectors may also have induced interferon responses in the cells, which blocked HSV-1 growth. These

inhibitory effects were multiplicity dependent, as we showed replication incompetent HSV-1 mutants with deletions of the ICP4 or ICP27 genes could be rescued using low multiplicities of adenoviral vectors expressing the deleted genes. These differences may give insight into how adenovirus can inhibit HSV-1 at high multiplicities. Adenoviral inhibition of HSV-1 was an unexpected result that could potentially lead to new understandings about HSV-1 replication and novel therapies against HSV-1. These results also suggest that anti-sense viral vectors against DNA viruses may not work in the same way that has been shown to be successful against RNA viruses.

Understanding the molecular mechanisms during the establishment and reactivation from HSV-1 latency will do much for preventing new individuals from becoming infected. A more difficult problem is to understand how the viral genome is maintained during latency. Unless we can discover a way to eliminate the viral genome from latently infected individuals, a reservoir will remain in the population that can transmit the virus. Potentially, this could be accomplished by eliminating latently infected neurons or eliminating viral genomes within these cells.

It is generally believed that a small percentage of neurons in any one ganglion are actually infected with the latent virus. It is thought that these neurons die during episodes of reactivation. The lack of overt neuropathologic effects in individuals suffering from lifelong episodes of HSV-1 reactivation lends support to the idea that only small numbers of neurons are infected. Thus, if one could deliver an agent or toxic gene that could be activated specifically in HSV latently infected neurons, one could potentially eliminate infected neurons. Eliminating the viral genome without killing the infected neuron seems more technically improbable. This is probably due to an initial lack of understanding about how the viral genome is maintained in neurons. Viral or cellular DNA binding proteins (e.g. cellular histone proteins) probably protect the genome from

cellular nucleases. If a drug or agent could be found to compete with the protective proteins maybe the nucleases would be able to degrade the foreign genome.

Large strides have been made in deciphering the lifecycle and molecular make-up of HSV over the past 70 years. And, while we have not eliminated HSV infections, we are much closer to finding better ways to treat infected individuals and protect individuals who have yet to suffer from an HSV infection.