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by

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ABSTRACT

CONSTRUCTION OF AN *ESCHERICHIA COLI LAC* REPRESSOR-BASED SYSTEM TO STUDY HUMAN CYTOMEGALOVIRUS GENE EXPRESSION

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An *Escherichia coli lac* repressor-based system was developed to study the roles of human cytomegalovirus (HCMV) genes during viral replication. To this end, a recombinant HCMV expressing the *lac* repressor was generated (RV*lac*), and an HCMV-specific promoter was targeted for conditional expression by inserting the *lac* operator sequence. The promoter of a nonessential gene was chosen in order to be able to assess parameters of repression and derepression of the operator-containing promoter in the endogenous locus, without having virus growth dependent on the specific inducer isopropylthiogalactoside (IPTG). The feasibility of this approach to conditionally express an HCMV promoter was demonstrated by analyzing *lac* operator-containing, HCMV US9 promoters in CAT reporter constructs, using RV*lac* and IPTG in transient assays. This study demonstrates that efficient repression mediated by the *lac* repressor can be achieved, and this repression is efficiently reversed by IPTG. In addition, the operator-containing US9 promoters were inserted into the endogenous locus to investigate the impact of the operator insertion on basal promoter expression in the context of the virus, before these constructs are used to study conditional expression in the viral genome. It was observed that US9 endogenous promoter expression was not affected significantly by the operator insertions. Because attempts to isolate a recombinant virus containing the operator-containing US9 promoter and expressing the

lac repressor were unsuccessful, future studies should target the insertion of the operator-containing US9 promoter and the *lac* repressor gene into two separate recombinant viruses. These viruses could be used in coinfection experiments to address conditional US9 gene expression. Furthermore, alternative sites for insertion of the operator sequence within the US9 promoter should also be evaluated. After demonstrating the feasibility of this approach in the context of the viral genome, the system can then be adapted to target putative essential HCMV genes.

To my parents, my husband, and my children.

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CHAPTER I

INTRODUCTION

Cytomegaloviruses: classification and general characteristics

Cytomegaloviruses (CMV) are animal DNA viruses that are widely distributed in nature. These viruses belong to the *Herpesviridae* family, and were initially defined as salivary gland viruses, due to the central role played by this organ as a target for virus replication. Later, the name cytomegalovirus (CMV) was proposed, to reflect the virus induced cytomegalia, a phenotype characterized by enlargement of the cell, and the distinct cytopathology involving both nuclear and cytoplasmic inclusions in the infected cell. This name also reflects the role of this virus in congenitally acquired cytomegalic inclusion disease (CID) (reviewed by Mocarski, 1996).

Human Herpesvirus 5 (human cytomegalovirus (HCMV)) has been the most studied cytomegalovirus, and is the prototype of the *betaherpesvirinae* subfamily. A significant characteristic of this subfamily is the restricted host range of all of its members. They all present a long replicative cycle, and infection proceeds rather slowly in cells in culture. A typical feature of cytomegalovirus-infected cells is cytomegalia. Like all herpesviruses, CMV persists in the infected host for life (see Viral Pathogenesis section in this chapter) (reviewed by Roizman, 1996).

HCMV is strongly species specific. It will not replicate or cause disease in any animal species other than humans (reviewed by Huang and Kowalik, 1993). The virus is generally acquired early in life, usually causing only mild symptoms upon primary infection. Between 50-80 % of the adult population is seropositive for HCMV, and a significant number of individuals shed the virus in bodily fluids. Symptomatic disease is

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rarely observed in the immunocompetent host. On the other hand, HCMV has become significantly important as a human pathogen in the last two decades because of its involvement in serious morbidity and even mortality in immunocompromised patients. Such patients include those receiving immunosuppressive post transplant therapies, as well as acquired immunodeficiency syndrome (AIDS) patients (see Viral Pathogenesis section).

Human cytomegalovirus: discovery and isolation

Infection of permissive cells with cytomegalovirus results in cell enlargement and the formation of intranuclear inclusions. In the early 1900s, these cytomegalic cells presenting inclusion bodies were thought to result from protozoan or syphillitic infection. Jesionek and Kiolemeoglou described these enlarged cells in several organs of infected fetuses in 1904. Even earlier, Ribbert had observed these cells in kidney sections of a stillborn infant in 1881. These cellular changes were also reported after the examination of numerous postmortem submaxillary glands and other tissues from infants (reviewed by Huang and Kowalik, 1993). All of this evidence led to the term "cytomegalic inclusion disease" (CID) in early studies, when the virus had still not been identified (reviewed by Britt and Alford, 1996).

Lipschutz was the first investigator to propose that CID was caused by a virus. In 1921, he defined the intranuclear inclusions that he observed as being similar to herpetic lesions. The first experimental evidence that CID was caused by a virus was provided by Cole and Kuttner in 1926. These investigators used a guinea pig model, and showed that filtered homogenates of salivary glands bearing intranuclear inclusions were infectious for animals with no previous signs of CID. When histopathologic studies of the salivary

glands of infected animals were compared with the histologic changes observed in the salivary glands of infected humans, the causative agent was proposed as "salivary gland virus".

HCMV was first isolated independently by Smith, and Rowe et al. in 1956, and by Weller et al. in 1957. Rowe and coworkers isolated the currently commonly used laboratory strain AD169. This strain was inadvertently isolated instead of adenovirus from the adenoidal tissue of a child who had undergone tonsil and adenoidectomies (Huang and Kowalik, 1993). Smith and Weller independently isolated the virus from infants with generalized CID. Weller et al. first used the term "cytomegalovirus" to reflect the cytopathology produced by these viruses. Following the isolation of HCMV in the late 1950s, serological studies showed that HCMV causes a common, usually subclinical infection that occurs worldwide. The incidence of HCMV infection is more pronounced in the very young and old, as well as in immunocompromised individuals. Electron microscopic work on the morphology of the virus, as well as the finding that its large genome was composed of DNA, led to the classification of CMV as a member of the *Herpesviridae* family (reviewed by Britt and Alford, 1996).

Structure of the virion

CMV virions present the typical structure of the herpesvirions. The architecture of the virion consists of a core, a capsid, a tegument (also referred to as matrix), and an envelope. The core contains the viral genome (see section below). This is a linear, double-stranded DNA molecule in the form of a torus. The capsid presents icosadeltahedral symmetry, with a diameter of 100 - 110 nm. It is constituted by the major (150 kilodalton

(kD)) and minor (40 kD) capsid proteins. These proteins are analogous to the predominant Herpes Simplex Virus 1 (HSV-1) capsid proteins (Gibson, 1981). The tegument, an amorphous material surrounding the capsid, consists of twenty different proteins, most of which are phosphorylated. This proteinacious structure is believed to be responsible for the anchorage of the envelope to the capsid. Some of the most studied tegument phosphoproteins are pp65 (the most abundant virion protein), pp150, pp71, pp28, and pp64. However, the roles of the tegument proteins, with the exception of the virion transactivator pp71, remain undefined. The virion envelope is derived from the infected cell plasma membrane and contains at least eight viral glycoproteins in the form of spikes, present in four complexes (Pereira et al., 1992). Half of these HCMV glycoproteins, gB, gH, gL, and gM, are homologous to four essential HSV-1 glycoproteins. gB is the major HCMV glycoprotein, and is a predominant target for neutralizing antibodies (Britt et al., 1990). Neutralizing antibodies are also directed to other viral glycoproteins (Pachl et al., 1989), like gH. The viral glycoproteins are involved in virus attachment, penetration and entry into the host cell, as well as in cell-to-cell spread (reviewed by Roizman, 1996).

Virions in the *Herpesviridae* family are pleomorphic; the sizes range from 120 to 300 nm in diameter. This variation is due to differences in the amount of tegument, or to the state of the envelope. Like all herpesviruses, cytomegaloviruses are sensitive to low pH, lipid solvents, and heat (reviewed by Mocarski, 1996).

The viral genome

The herpesvirus DNAs are linear and double-stranded. They vary in molecular weight (120 to 230 kilobase pairs (kbp)) and base composition (31 to 75 G + C mole %). One important characteristic of these viral DNAs is their sequence arrangement. Many herpesvirus DNAs contain repeated sequences both internally and at the termini. This feature allows for the classification of the herpesviruses into six different groups (A, B, C, D, E, and F), based on the six different classes of genomes of the herpesviruses (reviewed by Roizman, 1996).

Cytomegaloviruses have the largest genomes of any of the herpesviruses (180 to 230 -240 kbp) and of any known animal viruses. HCMV DNA is approximately 50 % larger than HSV DNA (reviewed by Gibson, 1993). In addition, the HCMV genome contains a greater density of repeated sequences than that of any other herpesvirus. Particularly, highly repeated regions include the DNA replication origin, *orilyt*, and two transcriptional enhancers. Like HSV, HCMV presents a complex arrangement of unique and inverted repeats that leads to the existence of four genome isomers (genome arrangement of group E). The genome presents a unique long component (UL) and a unique short component (US). Each component is flanked by repeated sequences in an inverted orientation located at the genomic termini and the UL-US junction. Both the UL and US genome components can invert relative to each other to generate four isomeric forms (reviewed by Mocarski, 1996). HCMV virions consist of equimolar concentrations of these four genomic isomers.

The genome of the HCMV AD169 strain has been completely sequenced (European Molecular Biology Laboratory Sequence Database accession number X17403).

It contains 208 predicted open reading frames (ORFs) (Chee et al., 1990), many of which present high degree of DNA sequence homology. These ORFs are believed to originate from gene duplication and have been grouped into gene families. At present, little is known about the function of many ORFs belonging to the various gene families (Mocarski, 1996).

The overall genomic DNA sequence homology among the HCMV strains is approximately 95 %. Restriction mapping analysis of the viral genome has shown that clinical isolates and laboratory-adapted strains are related. However, deletion of genomic sequences occurs after serial undiluted passage of the virus in culture. It is believed that these deletions have led to the loss of some of the host range capabilities of the laboratory-passaged strains AD169 and Towne, when compared to the more limited passaged, more virulent viruses, such as Toledo. At least 19 genes present in clinical isolates are not found in laboratory strains (Cha et al., 1996).

Viral pathogenesis

Pathogenicity

CMV pathogenicity involves a complex balance between acute and persistent infections. The immunocompetency of the infected individual plays a major role in the outcome of viral infection. For HCMV, it is currently unclear if the virus is present in a chronic (with continuous production and release of low levels of infectious virus) or true latent (with no detectable infectious virus) state in persistently infected humans. Macrophages and endothelial cells have been proposed as major sites of HCMV persistence (reviewed by Fish et al., 1995). In contrast, it has recently been demonstrated

that latent murine CMV (MCMV) is harbored in the spleen, the lung, and the kidney of infected mice (Pollock and Virgin, 1995), (Kurz et al., 1997).

Generally, primary infection with HCMV occurs during early childhood. Typically, the virus initially infects ductal epithelial cells within the salivary gland. This is a site of persistent viral replication following primary infection. Transmission occurs via direct or indirect person-to-person contact, by the urine of infants, and the saliva from healthy seropositive individuals (reviewed by Mocarski, 1996). The virus is present in oropharyngeal secretions, urine, tears, feces, semen, cervical and vaginal excretions, breast milk, and blood.

After primary infection, cytomegaloviruses remain persistently in their hosts for life in a quiescent state. These viruses have evolved to coexist in equilibrium with the host. Persistence is achieved by inducing immunosuppression in the host, and by inhibiting immune recognition (Campbell et al., 1989 and 1992), (Campbell and Slater, 1994), (Jones et al., 1995). In humans, reactivation of the virus results in recurrent infections with a variety of clinical manifestations in immunosuppressed individuals, such as after organ transplantation, during pregnancy, and in numerous AIDS and cancer patients.

Studies focusing on the pathology of HCMV infection have demonstrated that when severe disseminated disease occurs (see following section), HCMV can be found in virtually all organs of the infected host (reviewed by Britt and Alford, 1996). The virus replicates in the genitourinary tract and the kidneys. In AIDS patients, the gastrointestinal tract is involved. In newborns with CID, HCMV is present in the bile duct epithelium in the liver. Another site of HCMV replication is the respiratory tract, typically, the mucosa of the upper respiratory tract in immunosuppressed individuals. In the lungs, infected cells

are found in the alveolar and bronchial epithelium. Recently, HCMV infection of adult AIDS patients has been shown to involve the central nervous system (CNS). CNS involvement is also reflected in both generalized and focal structural brain damage when severe fetal HCMV infection occurs (reviewed by Britt and Alford, 1996).

The cell types that are targets for virus replication include epithelial, endothelial and glial cells (reviewed by Mocarski, 1996). The infected cells are typically large, the cytoplasm becomes limited, and the nuclei are prominent. Within the nucleus, the chromatin is marginated, and the characteristic inclusions surrounded by a clear halo are observed. In addition to the cell types mentioned above, HCMV infects monocytes, and this is believed to play a central role in viral pathogenesis (reviewed by Campbell, 1999), (Michelson, 1997).

The murine model has been used extensively to address the role of monocytes/macrophages in CMV infection. This role is dual: MCMV infected- monocytes disseminate the virus within the infected host (Stoddart et al., 1994), and serve as precursors of infected macrophages in the spleen, the lungs and the liver. On the other hand, CMV- infected macrophages trigger the early, non-specific immune response to the virus (see below). Also, as well as allowing productive viral replication, the macrophage has been implicated in MCMV latency (Pollock et al., 1997), (Mitchell et al., 1996). Several antiviral cytokines are produced by CMV-infected macrophages: tumor necrosis factor alpha (TNFα), interleukins IL-8, IL-1β, IL-12, IL-6, and interferon IFNα/β (reviewed by Mocarski, 1996), (Orange and Biron, 1996), (Ruzek et al., 1997) (reviewed by Campbell, 1999). Although these cytokines may control the extent of CMV replication in the macrophage, they fail to completely eradicate the virus. Clearly, the interaction

between CMV and the monocyte/macrophage is intricate, and multiple factors seem to regulate the outcome of infection.

The murine model has been successfully used to study the immune responses to CMV infections (reviewed by Campbell, 1999). In the early stages of MCMV infection, innate non-specific immunity is responsible for controlling virus replication in acutely-infected mice. Natural killer (NK) cells are a significant non-specific defense against CMV. These cells are activated soon after infection, and effectively control early MCMV infection in the target organs. In the early stages of infection, the production of IFNα/β by the infected macrophage also represents a significant non-specific antiviral defense. This defense is effective via direct antiviral activity, as well as the enhancement of NK cell cytotoxicity (reviewed by Campbell, 1999). However, these innate immune responses do not completely eliminate the virus in acutely-infected mice. Cell-mediated immunity involving CD4 and CD8 T lymphocytes is responsible for clearing MCMV infections from target organs. Antibodies, on the other hand, do not participate in clearing a primary infection, but they control the extent of recurrent infections following reactivation of latent MCMV.

In humans, cell-mediated immune responses to CMV are essential for controlling viral infection. pp65 and pp150 tegument phosphoproteins are the principal targets of cytotoxic T lymphocytes (CTLs) (McLaughlin et al., 1994). Other targets of CTLs are the immediate-early protein IE72 and gB (Borysiewicz et al., 1988).

Cytomegalovirus-associated disease

Like other herpesviruses, HCMV is ubiquitous, and the majority of the human population tests positive for CMV infection by adulthood. In most cases, infection of a healthy individual is clinically undetectable. Rarely, however, infection may result in a mononucleosis syndrome clinically identical to the mononucleosis syndrome associated with Epstein-Barr virus (EBV) infection (reviewed by Britt and Alford, 1996).

In contrast, individuals with immature or compromised immune systems may suffer from a variety of disorders associated with HCMV infection. CMV infection is the most common congenital viral infection in humans. In the United States, the incidence is 1-2 % per live births. The majority of these infants present subclinical infections. Transmission *in utero* can result from either primary infection of, or reactivation of latent virus in, the mother. The clinical manifestations include brain damage, mental retardation, deafness, ocular damage, as well as hepatic disease and pneumonia. Severe cases of disseminated disease are fatal. Less severe cases will develop central nervous system abnormalities in the first two years of life. Infants with congenital HCMV infection excrete large quantities of the virus persistently for years after birth. This provides a significant reservoir for virus spread in the population (reviewed by Britt and Alford, 1996), (reviewed by Chang and Lee, 1993).

HCMV is one of the most significant opportunistic pathogens that infects AIDS patients. Syndromes associated with HCMV infection in AIDS patients comprise almost every organ system. Most frequently involved are the CNS (reviewed by Wiley and Nelson, 1993), the gastrointestinal system, and the lung. Diseases like CMV pneumonitis, retinitis, encephalitis, esophagitis, gastritis, hepatitis, and enterocolitis are frequently

observed (reviewed by Fiala et al., 1993), (Jacobson, 1995). Because of the poorer prognosis of CMV-seropositive patients with AIDS, and the observation that HCMV regulatory proteins can regulate expression of the Human Immunodeficiency Virus Long Terminal Repeat (HIV LTR) (Barry et al., 1990), (Biegalke and Geballe, 1990), it has been proposed that HCMV acts as a cofactor in the progression to and development of AIDS. This has been supported by the colocalization of both viruses in brain cells of AIDS patients (Nelson et al., 1988), and by the fact that under certain conditions HCMV has a positive regulatory effect on HIV replication (Lathey et al., 1994). Some reports, however, have revealed that HIV and HCMV do not cooperate, and in fact seem to interfere with replication (Jault et al., 1994), (Koval et al., 1991). To date, the role of HCMV in AIDS progression is still poorly understood, and the data obtained under different experimental conditions are inconclusive. Therefore, the participation of HCMV in AIDS development remains speculative.

In patients receiving allografts, HCMV represents a significant pathogen which can cause severe post transplant complications. The source of HCMV can be reactivated virus in the seropositive recipient, or virus harbored in the transplanted organ from a seropositive donor. Clearly, at greater risk for primary infection are seronegative recipients of organs from seropositive donors. Generally, the severity of HCMV infection and disease correlates with the degree of immunosuppression of the patient. The kidney was the first organ in which allograft transmission of CMV was observed. It was later demonstrated that CMV can be transmitted in transplants of liver, bone marrow, heart, and heart-lung (reviewed by Smyth et al., 1993). Between 60 and 100 % of renal allograft recipients develop HCMV infection. Rates are similar for cardiac and hepatic

transplantation. On the other hand, HCMV infection occurs in 32 to 70 % of bone marrow transplant recipients. The incidence of disease caused by HCMV in the post transplant period is between 20 and 60 %. The clinical manifestations of HCMV infection in allograft transplant recipients include leukopenia, thrombocytopenia, lymphocytosis, elevated hepatic transaminases, and prolonged fever. Life-threatening complications include HCMV infection of the gastrointestinal tract, hepatitis, and pneumonia. HCMV pneumonia is the most significant infectious syndrome in bone marrow transplant patients. If left untreated, this complication can cause the death of 80 % of the patients (reviewed by Britt and Alford, 1996) (reviewed by Winston, 1993).

Antiviral chemotherapy

Several antiviral agents have been used to treat HCMV disease in cases of congenital infection, immunocompetent individuals presenting mononucleosis syndrome, and immunosuppressed cancer and transplant patients with variable severity of clinical symptoms (reviewed by Britt and Alford, 1996). Leukocyte interferon, interferon stimulators such as measles virus and pyran copolymer, nucleoside drugs [iodo-deoxyuridine, fluorodeoxyuridine, cytosine arabinoside, adenine arabinoside (ara-A, vidarabine), and acyclovir (ACV)] have been used individually and in combined therapeutic regimens with little or no clinical benefit. More recently, a nucleoside drug, 9-(1,3- dihydroxy-2-propoxymethyl) guanine (ganciclovir, GCV), and phosphonoformic acid (foscarnet) have been shown to have potent antiviral properties *in vitro* and *in vivo*. Foscarnet is an inhibitor of the viral DNA polymerase. The triphosphate active form of GCV causes nucleic acid chain termination, as well as inhibiting the viral DNA

polymerase. GCV and foscarnet have been used in cases of HCMV pneumonitis, hepatitis, esophagitis, retinitis and colitis in patients with AIDS, as well as to treat CMV infection in allograft recipients. However, after prolonged treatments with either GCV or foscarnet, resistance becomes a common threat. This resistance reflects the appearance of HCMV mutant strains within the infected individual. These resistant strains contain mutations in the DNA polymerase gene (UL54) (Baldanti et al., 1995 and 1996), (Lurain et al., 1996), and/or in the UL97 open reading frame (ORF) (Wolf et al., 1995 and 1995), (Baldanti et al., 1995), (Smith et al., 1996), which encodes a phosphotransferase that is required for phosphorylation of GCV to its active form (Baldanti et al., 1995), (Sullivan et al., 1992), (Littler et al., 1992), (Biron et al., 1986) (see Spontaneous mutants section below). It is presently clear that new antiviral drugs need to be developed in order to efficiently control HCMV infections. Targets in the development of these new drugs include the DNA processivity factor (UL44 ORF), and a protease activity (UL80 ORF) (reviewed by Britt and Alford, 1996).

Vaccines

The first trials using live attenuated vaccines to prevent CMV disease were done in renal transplant patients. The HCMV Towne strain was used. This virus was shown to be immunogenic in these patients, did not cause CMV-associated disease, and failed to reactivate after the patients were subjected to immunosuppressive regimens (reviewed by Marshall and Plotkin, 1993), (reviewed by Adler, 1995).

In the last few years, vaccine development for the prevention of HCMV disease has been focused on subunit vaccines. Candidate HCMV proteins to be produced from

recombinant expression systems include the major envelope glycoprotein gB, the envelope glycoprotein gH, and the pp65 lower matrix phosphoprotein. The most promising candidate for a subunit vaccine is gB (reviewed by Adler, 1995). When gB was cloned into a canarypox (ALVAC) vector, and the recombinant virus (ALVAC-gB) was administered to experimental animals, both humoral and cellular immune responses were elicited (Gonczol et al., 1995). Moreover, human inoculations with isolated gA/gB glycoprotein complex have been reported (Gonczol et al., 1990). CMV specific responses of neutralizing antibodies and lymphocyte proliferation were developed in human volunteers.

DNA vaccination has been tested with expression vectors containing UL83 ORF (encoding the pp65 tegument phosphoprotein). Intramuscular injection of mice with these constructs resulted in the generation of pp65 antibodies in 60% of the injected mice (Pande et al., 1995).

The replicative cycle

General characteristics

HCMV has a highly restricted host range in cell culture. Only cultured primary human cells permit viral replication. The source of these cells has usually been fibroblasts from the skin or the lungs. It is believed that cellular factors involved with differentiation play critical roles in permissiveness for HCMV replication. For example, it has been shown that transcription from the HCMV major immediate early promoter (MIEP) increases with differentiation (Nelson and Groudine, 1986). More recently, Angulo and Ghazal (1995) reported that retinoic acid (an essential regulator of cell differentiation) activated the

MIEP and enhanced the production of virus progeny in human foreskin fibroblasts infected with the HCMV Towne strain. The restriction on host cell range, both in cells in culture and *in vivo*, is revealed by a restrictive viral gene expression, and has been proposed to represent a postpenetration block (LaFemina and Hayward, 1986), (Nelson et al., 1987). Cytomegaloviruses differ from many other herpesviruses in that CMV replication does not result in the shut off of the host cell metabolism; rather, cellular DNA, RNA and protein synthesis are stimulated by CMV. The significance of this event is still not understood (reviewed by Mocarski, 1996). Furthermore, the HCMV envelope is not only involved in virus attachment and entry, but also transduces signals that ultimately result in *fos*, *jun*, and *myc* gene expression. In addition, HCMV infection derepresses cellular enzymes involved in cell proliferation. The virus also induces the expression of genes involved in cellular DNA replication, such as DNA polymerase, topoisomerase II, and ornithine decarboxylase (reviewed by Huang and Kowalik, 1993).

Attachment and Penetration

The initial event of viral replication involves attachment of the virus to the cell surface. Attachment occurs rapidly and efficiently, not only in permissive, but also in nonpermissive cells. This suggests that receptors for CMV are widely distributed on the surface of many cell types (LaFemina and Hayward, 1983 and 1986), (Nelson et al., 1987). Compton et al. (1993) demonstrated that the virus initially attaches to extracellular heparan sulfate proteoglycans. This interaction involves viral envelope glycoproteins, including gB. Following this interaction, binding of the virus to specific cell membrane glycoproteins of 32 and 34 kD occurs. These "viral receptors" are present on the surface

of endothelial, epithelial, fibroblast, and monocytic cells (Nowlin et al., 1991). In addition, CD13 is an aminopeptidase that seems to be involved in stable binding prior to penetration (Soderberg et al., 1993). Penetration occurs after attachment, and it involves the fusion of the viral envelope with the plasma membrane. Specifically, viral glycoproteins gH/gL interact with a cellular protein of 92.5 kD (Keay and Baldwin, 1992) and annexin II (Wright et al., 1994) (reviewed by Compton, 1995). Following penetration, the de-enveloped viral capsids are rapidly delivered to the nuclear pores, and the viral DNA is introduced into the nucleus, where it subsequently circularizes. Once the viral DNA is present in the nucleus, the host cell RNA polymerase II is responsible for transcribing the viral genes.

Immediate-early gene expression

CMV gene expression occurs in a coordinated, sequential fashion. The viral genes are divided into three kinetic classes, on the basis of time of expression. These classes are immediate-early (IE), early (E), and late (L). The genes expressed first during viral replication are the immediate-early genes. These genes are expressed without requiring *de novo* viral protein synthesis. Mapping of the regions expressed at immediate-early times during HCMV infection has revealed several areas on the HCMV genome: UL36-38, UL122-123 (IE1/ IE2) (the major IE locus), TRS1-IRS1, and US3 (Wathen and Stinski, 1982), (Kouzarides et al., 1988), (Colberg-Poley et al., 1992), (Stenberg et al., 1984, 1985 and 1989), (Stasiak and Mocarski, 1992), (Weston, 1988). The most abundantly expressed immediate-early genes are IE1 (UL123) and IE2 (UL122). These transcripts arise via differential splicing, and the protein products share amino acid sequences present

in common exons (reviewed by Stenberg, 1993), (reviewed by Mocarski, 1996).

Expression of the major IE locus is controlled by a strong transcriptional enhancer, the major immediate-early promoter (MIEP). The structure of the MIEP presents a 5' modulator sequence, an enhancer containing repeated elements and transcription factor binding sites, the promoter region including the TATA box, and a leader sequence (reviewed by Ghazal and Nelson, 1993). Both repeated elements and unique sequences are involved in the regulation of IE transcription. A large number of known and/or predicted cellular transcription factor binding sites, including CREB/ATF, AP-1, serum response elements (SRE), SP1, TFIID/TBP, p53, and NFκB, among others, are central to the transcriptional control from the MIEP. Analogous enhancers are present in the immediate-early gene locus of murine CMV and simian CMV (reviewed by Mocarski, 1996). The initial activation of the MIEP is mediated by host cell factors and pp71 (UL82), a virion associated tegument protein. During HCMV infection, expression from the MIEP is first activated, then repressed. Activation differs significantly under permissive and nonpermissive conditions, and this is independent of viral proteins (Nelson et al., 1987), (reviewed by Ghazal and Nelson, 1993).

The protein product from IE1 is a 72 kD phosphoprotein (IE72), originally identified as the major immediate-early protein (Stinski, 1978). The next most abundant immediate-early gene product is encoded by IE2; this is also a nuclear phosphoprotein of 86 kD (IE86). IE1 and IE2 transcripts share the first three exons, and IE72 and IE86 share 85 amino acids at the amino terminal region. These IE proteins regulate the expression of the immediate-early genes, as well as subsequent early gene expression. Other minor proteins are expressed from the major IE locus. The first IE proteins

synthesized from this region following penetration and uncoating are the 72 kD protein, and the 55 kD protein, a positive activator of the MIEP (Baracchini et al., 1992). These proteins appear 1 hr after virus adsorption. At 2.5 hs, the 38 kD, the 86 kD, and 97 kD proteins are produced (reviewed by Stenberg, 1993).

IE72 and IE86 exert both positive and negative regulation on viral gene expression (Depto and Stenberg, 1989), (Klucher et al., 1989), (Klucher and Spector, 1990), (Hermiston et al., 1990), (Pizzorno and Hayward, 1990), (Cherrington et al., 1991). Particularly relevant for the outcome of viral infection is the interaction between IE72 and IE86 to regulate the MIEP (Stenberg et al., 1990). IE72 autostimulates its own transcription by transactivating the MIEP via NFκB sites, whereas IE86 is responsible for the shut off of IE1/ IE2 expression during replication through its ability to repress MIEP function (Lang and Stamminger, 1993). Immediate-early gene expression is reduced (a complete shut off is not observed) during the early phase, but it is resumed late in infection (Stenberg et al., 1989).

IE72 cooperates with IE86 and other transactivators to regulate the subsequent cascade of gene expression. IE72 interacts with SP-1, E2F-1, and CTF-1 (Hayhurst et al., 1995), (Lukac et al., 1994), (Margolis et al., 1995). IE86 is a sequence-specific DNA binding protein thought to be essential for the switch from immediate-early to early gene expression. IE86 has been defined as a promiscuous transactivator. Promiscuous activation of heterologous promoters has been found to be TATA box dependent, and involves direct interaction with TFIID (Hagemeier et al., 1992). IE86 interacts with several components of the cellular transcription machinery, such as TBP (Jupp et al., 1993), TFIIB (Caswell et al., 1993), CREB (Lang et al., 1995), CBP (Schwartz et al.,

1996), and *c-jun* (Scully et al., 1995). The exact mechanism used by IE86 to transactivate is still unknown. The relative contributions of protein-protein interactions with host transcription factors and of direct binding to DNA are still not fully understood. It has been shown that both the amino terminal and carboxy terminal regions of IE86 are involved in transactivation.

Although IE1 and IE2 have been shown to be central to the regulation of viral gene expression, other immediate-early genes whose functions are still undefined are expressed at immediate-early times. These likely play regulatory roles that influence the outcome of infection. Additional immediate-early genes important for the regulation of HCMV gene expression are UL36-UL38 (Colberg-Poley et al., 1992), (Kouzarides et al., 1988), TRS1 and IRS1 (Stasiak and Mocarski, 1992), and US3 (Colberg-Poley et al., 1992), (Weston, 1988). These genes encode products that are believed to function with IE1 and IE2 to regulate transcription during HCMV infection.

Early gene expression

Following the immediate-early phase, the early genes are expressed (reviewed by Mocarski, 1996). These genes are divided into subclasses on the basis of time of expression. Using a temperature -sensitive mutant of HCMV that is impaired in DNA replication (ts66), Stenberg et al. demonstrated that the early mRNAs are differentially regulated relative to the time of DNA synthesis. This gave rise to the classification of the early genes into three subclasses: those genes that are transcribed early and repressed late, those that are expressed at equal levels at early and late times, and those transcripts that

are produced at low levels early, and which are upregulated late (reviewed by Stenberg, 1993).

Studies on several early promoters have shown that both IE1 and IE2 are required for maximal promoter activation (Chang et al., 1989), (Depto and Stenberg, 1989), (Staprans et al., 1988), (Stenberg et al., 1990). Discrete sequences containing direct and inverted repeats within early promoters have been shown to be involved in the regulation of promoter activation (Kerry et al., 1994 and 1996). These repeated sequences vary significantly among different early promoters (reviewed by Stenberg, 1993). In addition to IE1 and IE2, cellular proteins, such as ATF (Kerry et al., 1997), USF (Klucher and Spector, 1990), CREB (Lang et al., 1995), AP-1 (Wade et al., 1992), and E2F (Staprans and Spector, 1986), have been shown to interact with HCMV early promoters to regulate expression. It is believed that several other cellular proteins may act in conjunction with IE1 and IE2 to regulate the expression of early promoters through the diversity of regulatory sequences observed in these promoters (reviewed by Stenberg, 1993).

Many early genes encode viral functions involved in DNA replication. These are the DNA polymerase (UL54), the major early single-stranded DNA binding protein (UL57), the DNA polymerase processivity factor (UL44), and a helicase-primase complex composed of three subunits (UL105, UL102, and UL70). HCMV DNA replication occurs in the same series of steps as for other herpesviruses. After infection, the viral genome circularizes in the nucleus of the infected cell. This occurs approximately 4 hs post infection. Viral DNA synthesis peaks at 18 to 24 hs, and at 60 to 80 hs post infection. These peaks are concomitant with cellular DNA replication (reviewed by Mocarski, 1996). HCMV DNA replication occurs from a sequence of 1.5 kbp, *orilyt*, between

92,200 and 93,700 bp on the genome (Anders et al., 1992). These sequences are rich in clustered and dispersed repeated elements. Several trans-acting factors have been found to be required for *orilyt* replication. These are: UL54, UL57, UL44, UL105, UL102, UL70, IE1 and 2 (UL122-123), UL36/UL37/UL38, TRS1/IRS1, UL112-113, and UL84 (Pari and Anders, 1993), (Pari et al., 1993). Four of these loci (UL36-38, UL112-113, IRS1 and TRS1) cooperate with IE1 and IE2 to activate the expression of replication genes (Iskenderian et al., 1996). DNA replication occurs by a rolling circle mechanism, and results in the formation of concatemers.

Late gene expression

Late genes are expressed after the onset of viral DNA replication These include structural proteins of the capsid, the tegument, and the glycoproteins of the envelope (see section on Structure of the virion in this chapter). To date, very few late genes have been studied in terms of the regulation of their expression. Some of the work reported has involved the true late virion phosphoprotein pp28 (UL99) (Depto and Stenberg, 1992), (Kohler et al., 1994), (Kerry et al., 1997), the true late gene UL94 (Wing et al., 1998), viral glycoproteins (Geballe et al., 1986), and the IE2 late protein p40 (Jenkins et al., 1994). Consequently, late gene expression is currently poorly understood. The reason why these promoters are exclusively expressed at late times in infection, and what viral and/or cellular proteins are involved in the regulation of late gene expression is still undefined.

Virion assembly

During the late phase, cleavage of the replicated DNA into genome length monomers occurs. This newly synthesized DNA is inserted into maturing, pre-formed nucleocapsids. Maturing nucleocapsids bud from the inner nuclear membrane at specific areas of the membrane containing viral glycoproteins. The virions then accumulate in the perinuclear space and subsequently move into cytoplasmic vesicles. Whether the virus follows a single path for envelopment and egress, or deenvelopment and reenvelopment in the cytoplasm occurs, is still not clear (reviewed by Mocarski, 1996).

Identification of viral functions

The current understanding of HCMV gene function has been derived mostly from *in vitro* studies. The roles of IE proteins in the regulation of viral gene expression (reviewed by Stenberg, 1993), as well as the participation of early viral *trans*-acting factors in the regulation of expression of early genes (reviewed by Spector et al., 1990), have been defined in the context of transient expression assays. These studies have provided important information on how viral proteins regulate expression from viral promoters of different kinetic classes. However, they have not answered questions regarding the essential nature of the viral genes or the actual roles of these genes in the context of a natural infection of cells in culture.

To date, very few studies have assessed HCMV gene function during a natural infection of cells in culture. The systems used have relied primarily on random and site-specific mutagenesis to generate mutant viruses (see below).

Random mutagenesis

The classical approach to study gene function in many virus families has depended largely on conditional-lethal mutants. For animal viruses, temperature-sensitive (ts) mutants, generated by UV or chemical random mutagenesis, have been used mostly. Although a complete set of mutants is rarely obtained, ts mutants have been utilized successfully to study viral protein functions of many viruses, including Herpes Simplex virus (HSV) (Subak-Sharpe, 1974) (Schaffer et al., 1970, 1971 and 1973) (Timbury, 1971) (Esparza et al., 1974) (Bone and Courtney, 1974) (Brown et al., 1973) (Marsden et al., 1976), (Preston et al., 1988), (Weir et al., 1989).

In contrast, very few HCMV ts mutants have been isolated and characterized to date (reviewed by Stenberg, 1993), (reviewed by Mocarski, 1996). Yamanishi and Rapp (1977) initially isolated eight ts mutants obtained by mutagenesis with nitrosoguanidine. A total of twenty-one ts mutants had been isolated and reported by 1979 (Yamanishi and Rapp, 1979). Four of these mutants were DNA-negative mutants belonging to different complementation groups. These mutants were used to demonstrate that viral DNA synthesis is not required for the HCMV-induced cellular DNA replication. Attempts to generate mutants with 5-bromodeoxyuridine were unsuccessful. Stenberg isolated an HCMV ts mutant (ts 66) that is impaired in DNA replication. This mutant was useful to study the differential regulation of transcription of several early genes relative to the time of viral DNA synthesis (reviewed by Stenberg, 1993). In contrast with the scarcity of HCMV ts mutants, as many as 24 complementation groups of MCMV ts mutants have been isolated and analyzed (Tonari and Minamishima, 1983), (Sammons and Sweet, 1989), (Akel et al., 1993).

Although ts mutants have traditionally played an important role for defining gene function, there are some problems inherent to them. First, ts mutants produced by UV or chemical random mutagenesis may contain numerous silent nonlethal mutations in several genes. In addition, if a ts mutation is introduced into the DNA sequence encoding a domain shared by more than one viral function, the resulting phenotype cannot be correlated to a particular viral gene. Lastly, in many instances, tight ts mutants are hard to obtain; leakiness is revealed by plating efficiencies with a low permissive to nonpermissive ratio, and this complicates the analysis of the resulting phenotype.

Site-specific mutagenesis

As an alternative to random mutagenesis, site-specific insertion or deletion of genes was first developed for the study of HSV gene function by Post and Roizman in 1981. This protocol is based on the generation of recombinant viruses obtained as a result of the double recombination between intact viral DNA and a DNA fragment containing the mutation (deletion or insertion), and a selectable marker. Using this approach, viable mutants can be obtained as long as the deletions or insertions affect genes that are dispensable for growth of the virus in culture. In order to be able to generate recombinant viruses with mutations in essential genes, the original recombination protocol had to be adapted by generating a host cell line expressing the gene that complements the mutation in the recombinant virus. These complementing cells allow for the propagation of the null mutants.

For HCMV, deletion mutants have been generated by inserting prokaryotic reporter genes, such as β-galactosidase and β-glucuronidase, into the viral genome

(Spaete and Mocarski, 1987), (Jones et al., 1991), (Jones and Muzithras, 1992), (Ripalti and Mocarski, 1991), (Takekoshi et al., 1991), (Browne et al., 1992), (Kaye et al., 1992). Using this approach, Jones et al. demonstrated that the US10 and US11 gene products are nonessential for viral replication in cells in culture. By constructing a deletion mutant using a similar approach, Kaye et al. showed that the UL16 gene encodes a glycoprotein that is nonessential for replication of HCMV in culture. Similarly, Browne et al. demonstrated that the UL18 gene, which encodes the viral homologue of the cellular MHC Class I heavy chain, is dispensable for growth of the virus in human fibroblasts in culture. Thus, these procedures have allowed for the isolation of viruses with nonlethal mutations, namely, viruses with deletions of genes that are dispensable for the replication of HCMV in tissue culture. These deletion mutants have allowed for the definition of 41 ORFs dispensable for growth of HCMV *in vitro*. These ORFs are TRL4 -TRL14, UL1 - UL10, UL16, UL18, UL20, UL33, UL128, IRS1, US1 - US13, and US27. The protein products of most of these genes have still not been defined, but they are believed to play critical roles in viral pathogenesis within the natural host.

Because only primary human cells are permissive for HCMV replication in culture, the identification of essential genes has been hindered due to the lack of permanent cell lines to complement virus null mutants. In the last two years, reports by Mocarski et al. (1996) and Greaves and Mocarski (1998) represent the first attempts to generate recombinant HCMV with mutations in putative essential genes. Using cosmids derived from the Toledo and Towne strains, Mocarski et al. generated an HCMV mutant deleted of the IE1 gene. The protein product of IE1 (IE72) has long been thought to be essential, but direct experimental evidence supporting this notion is lacking. In addition, Greaves

and Mocarski isolated a second IE1 deletion mutant, which lacked exon 4 of the major IE locus. The IE1-negative viruses were propagated in an IE1-expressing human fibroblast cell line (ihfie 1.3). At low multiplicities of infection (MOIs), the deletion mutants failed to replicate in human fibroblasts in culture. However, at MOIs of more than 3 plaque-forming units (PFU) per cell, virus yields were comparable to those of wild type virus. The authors speculated that at high MOIs, the IE1-deficient viruses are capable of growing with wild type kinetics due to compensation for the lack of the IE1 gene product by virion transactivators, which could function in place of IE1 to regulate the initial events of the cascade of HCMV gene expression.

Spontaneous mutants

Spontaneous mutants have also been useful for elucidating the functions of HCMV genes. For example, Zipeto et al. (1993) identified a spontaneous HCMV deletion mutant lacking the pp150 matrix phosphoprotein gene. Together with pp65, pp150 is the main component of the viral matrix. The authors failed to plaque-purify the deletion mutant, therefore they concluded that the variant was growth-defective and dependent on co-infecting wild type virus for replication. This suggested that pp150 is an essential viral function.

Several spontaneous HCMV drug resistant mutants have been isolated from individuals undergoing antiviral treatments with ganciclovir or foscarnet (see Antiviral chemotherapy section in this chapter). These mutants have been very valuable tools for analyzing the efficacy, selectivity and mode of action of these antiviral drugs, as well as for

identifying the viral gene functions that are targets of these drugs: the UL97 and UL54 genes.

The number of HCMV mutants isolated and studied to date is clearly insufficient to unveil the roles of the more than 200 predicted ORFs in the AD169 genome. Because only very few HCMV ts mutants have been generated, and because the lack of complementing cell lines has prevented the generation of HCMV null mutants, a significant number of viral essential gene functions have not been defined yet. In contrast, as mentioned above, deletion mutants have allowed for the definition of 41 ORFs dispensable for growth of HCMV *in vitro*.

At present, our understanding of the HCMV DNA coding capacity is mostly based on comparisons with HSV-1. Approximately 25 % of the ORFs are predicted to code for functions involved in viral DNA replication and metabolism, and the remaining 75 % is believed to code for structural proteins, and proteins involved in virion maturation (reviewed by Mocarski, 1996). Clearly, alternative methods need to be designed to study HCMV essential gene functions in the complex genetic environment of the HCMV-infected cell. These methods should allow for the stringent control of gene expression in order to be able to define the roles of individual viral gene products. Some of these methods are discussed in the following section.

Inducible systems for the study of gene expression

Inducible expression systems in mammalian cells

Inducible expression systems have been used to elucidate the function of many genes in bacteria, yeast, and *Drosophila sp.* Recently, similar strategies have been developed for mammalian cells. Some of these systems consist of promoters that are induced by endogenous control elements, such as promoters responsive to heavy metal ions (Mayo et al., 1982), (Brinster et al., 1982), (Searle et al., 1985). Other systems consist of promoters responsive to heat shock (reviewed by Nouer, 1991) or hormones (Hynes et al., 1981), (Klock et al., 1987), (Israel and Kaufman, 1989). All of these systems have generally suffered from leakiness of the inactive state and, in some cases such as the metallothionein promoter (Mayo et al., 1982), (Brinster et al., 1982), or the mouse mammary tumor virus promoter (Hynes et al., 1981), they show rather modest levels of induction. More importantly, the induction of transcription in these systems results in pleiotropic effects caused by the inducers themselves. For example, elevated temperature or glucocorticoid hormones (Lee et al., 1988) affect the expression of many cellular genes, in addition to the gene under study. This complicates the analysis of the resulting phenotype.

In an attempt to create highly specific regulatory circuits controlled by exogenous effectors, inducible systems that exploit prokaryotic elements have been constructed for the study of gene activity in higher eukaryotic cells. Bacterial regulatory proteins and operator sequences are particularly useful to control transcription in mammalian cells. A distinct advantage is that the recognition DNA sequence (the operator) for the regulatory protein (the repressor) is relatively unique to the gene being studied (Simons et al., 1984).

Thus, monospecific control systems, which are modulated by effectors that are inert to the physiology of the cell, can be established in eukaryotic cells.

The *Escherichia coli lac* operon

Some systems utilized to study inducible gene expression in mammalian cells are based on the *Escherichia coli lac* operon. The operon model (a cluster of genes encoding enzymes of a particular metabollic pathway that are controlled by a single promoter from which a single polycistronic mRNA is transcribed) was proposed by Jacob and Monod. This operon is the classic example of negative regulation of transcription by a repressor protein. The elements in this operon include a regulator gene (*i*) encoding the repressor, which is under the control of a separate promoter that regulates its constitutive expression, an operator site within the operon promoter, and a set of three contiguous structural genes (*z*, *y*, *a*) encoding the inducible enzymes β-galactosidase, galactoside permease, and thiogalactoside transacetylase, respectively. For *Escherichia coli*, the physiologic inducer is allolactose, which is formed from lactose by transacetylation.The *i* gene produces a 37 kD polypeptide that aggregates to form a tetrameric repressor protein. Each subunit consists of 360 amino acids, with a total molecular weight of 154,520. The *lac* operator is a 27 base pair DNA sequence containing a 16-base hyphenated palindrome. It presents 2-fold symmetry that matches the symmetry of the tetrameric repressor. The tetramer binds to the operator very tightly and rapidly when *E.coli* is grown in the presence of glucose or glycerol and in the absence of lactose. This binding results in the block of transcription of the structural genes. As a result, β-galactosidase, an enzyme that is required to hydrolize lactose to galactose and glucose, is

present at very low levels (5 - 10 molecules per cell). However, when lactose, but not glucose, is present in the culture medium, the inducer (allolactose) binds to the repressor with high affinity, and causes an allosteric change in conformation that drastically reduces the affinity of the repressor for the operator. This results in transcription of the structural genes, and the concentration of β-galactosidase increases to several thousand molecules per cell.

The *lac* repressor-operator interaction has been thoroughly characterized (reviewed by Miller and Reznikoff, 1980). The dissociation constant of the repressor-operator complex is 10^{-13} M.The rate constant for association ($\approx 10^{10} M^{-1}s^{-1}$) is very high, which indicates that random association with and dissociation from the DNA is probably not the mechanism for repressor binding to the operator. Rather, it is suggested that facilitated transfer of the repressor to the operator occurs (Fickert et al., 1992). The tetrameric repressor associates to DNA in a nonspecific manner, and this is followed by a looping event that brings the repressor in contact with the operator. This is known as the intersegment transfer mechanism. The *lac* repressor binds $4x10^{6}$ times more strongly to its operator than to other sites on the chromosome. These characteristics of the *lac* repressor-operator interaction are essential for the application of a highly specific regulatory system for the study of gene expression in eukaryotic cells.

The *lac* repressor-operator gene expression system in eukaryotic cells

Brown et al. (1987) was one of the first groups to test the *lac* repressor-operator system in mammalian cells. They used the chloramphenicol acetyl transferase (CAT) reporter gene driven by operator-containing variant SV40 early promoters. Repression

was observed when the reporter constructs were cotransfected with a plasmid encoding the *lac* repressor. Isopropylthiogalactoside (IPTG), a non-metabolizable inducer, was used to relieve repression. The same group described the regulation of a stably integrated CAT gene by the *lac* repressor in monkey cells (Figge et al., 1988).

In 1987, another report on the use of the *lac* repressor-operator system in mammalian cells presented evidence that the *lac* repressor produced in mouse cells can block transcription from an unintegrated CAT gene driven by the Moloney sarcoma virus enhancer containing a *lac* operator insertion. Derepression was observed when cells were exposed to IPTG (Hu and Davidson, 1987). Later, this group proceeded to combine the repressible / inducible *lac* system with induction by glucocorticoids and metal ions in order to achieve higher levels of inducibility. The system presented a high-level-inducible promoter (containing a *lac* operator sequence and a metal-responsive element) that was regulated by both derepression of the *lac* repressor system, and induction by glucocorticoid and Cd^{2+} (Hu and Davidson, 1990).

Other reports have shown the successful application of the *lac* repressor- based gene expression system in mammalian cells. Deuschle et al. (1989) utilized the *lac* repressor protein and the coliphage T3 RNA polymerase to regulate transcription of the firefly luciferase gene downstream of a phage T3 promoter containing a *lac* operator sequence, in rabbit kidney cells. Repression factors comparable to those observed in prokaryotes were obtained. In addition, the *lac* repressor system was used efficiently to regulate expression of genes transcribed by RNA polymerase III in mammalian cells (Syroid et al., 1992).

A different approach for utilizing the *lac* repressor protein to control mammalian gene expression was developed by Labow et al. (1990). They converted the *lac* repressor into a mammalian transcriptional activator by fusing the transcription activation domain from the HSV-1 virion protein 16 (VP16). This fusion protein (LAP) was a potent activator of various promoters containing up to 21 *lac* operator sequences in different positions relative to the transcription start site. The activation was efficiently inhibited by IPTG, although it never completely eliminated activation by LAP.

The versatility of the applications of the *lac* repressor-based system is also illustrated by the adaptation of the system for the regulation of gene expression in plant cells (Wilde et al., 1992), as well as the utility of the system to study the interactions between HCMV and HIV-1 to control viral gene expression in brain cells (Moreno et al., 1997).

Lac-regulated vaccinia virus gene expression

Most of the work published to date reporting the use of the *lac* repressor-operator system for the study of gene expression in eukaryotic cells has been done with vaccinia virus. Rodriguez and Smith (1990) adapted the *lac* repressor-operator system to study inducible gene expression in vaccinia virus. Recombinant viruses containing the *lac i* gene and the *lac* operator sequence were generated, to study the vaccinia virus gene encoding a 14 kD membrane protein. They demonstrated that this viral protein is not essential for the production of intracellular progeny virus, but it is absolutely required for the envelopment of the virus particles in the Golgi apparatus prior to egress from the cell.

Zhang and Moss (1991), (Zhang et al., 1992) constructed recombinant vaccinia viruses constitutively expressing the *lac* repressor, and containing *lac* operator sequences in endogenous promoters, to study the roles of vaccinia virus genes F18R and G8R. From these studies it was concluded that the F18R gene product, an 11 kD virion associated protein, is required for virion assembly. In addition, the G8R gene product was shown to be an essential late transcription factor. Other genes studied by this group include genes involved in virus morphogenesis, such as D13L (Zhang and Moss, 1992), D6R (Hu et al., 1996), and A8L (Hu et al., 1998) genes. Using the *lac* repressor-operator system, all of these genes were shown to encode proteins that are essential for viral assembly.

Recently, a different group of investigators (Klemperer et al., 1997) used the *lac* repressor-operator system to construct an inducible I1 gene in recombinant vaccinia virus. They found that the 35 kD protein product, which is expressed at late times during infection, is essential for the latest stages of viral assembly.

The Tetracycline repressor system

Another inducible system utilizing the *E.coli* Tetracycline (Tet) resistance operon has been developed to control gene expression in mammalian cells. The Tet resistance operon and the *lac* operon regulate gene expression in a similar manner. One of the applications of this system includes the fusion of the tetracycline repressor with the Herpes simplex virion transactivator VP16 to generate a chimeric transactivator that can be regulated by tetracycline (Gossen and Bujard, 1992), (Kim et al., 1995). Kim et al. used the tetracycline repressor fused to VP16 (tTA) to regulate expression of the β-glucuronidase reporter gene driven by tetracycline operator-containing promoters

derived from the HCMV US11 promoter. In transient assays, efficient repression was observed when two or three tetracycline operator sequences were placed adjacent to the TATA box. Repression was also significant in cells constitutively expressing tTA that were infected with recombinant HCMV containing the operator derivative of the US11 promoter driving the expression of the β-glucuronidase gene. This repression was efficiently relieved by tetracycline.

The HCMV US9 gene: a target for testing *lac* repressor-mediated control of HCMV gene expression

In the following chapters, a comprehensive analysis of the expression of *lac* operator-containing, HCMV US9 promoters is presented. This analysis is central to the main objective of this project, which was to test the *lac* repressor-based system using an HCMV specific promoter. Subsequently, because of the nonessential nature of the US9 gene for HCMV replication in human fibroblasts in culture (Jones and Muzithras, 1992) (see below), the endogenous US9 promoter can be targeted for conditional expression using the *lac* repressor system, without jeopardizing the production of virus progeny, or having virus growth dependent on IPTG. The feasibility of this approach to study HCMV gene expression can therefore be assessed. Parameters of repression and IPTG derepression can be tested and optimized, without compromising the production of progeny virus to any of these variables. These experiments will significantly contribute to the long-term goal of the project: to apply the *lac* repressor-based system to study the roles of putative essential HCMV genes in the context of a natural infection of cells in culture.

The US9 gene encodes an envelope glycoprotein whose exact role during HCMV infection is still unknown. This gene is contained within the *Hind* III X region of the HCMV AD169 strain. The genes contained in this region have been defined as the US6 gene family (US6 through US11). The members of this gene family encode proteins of about 180 - 250 amino acids. They present hydrophobic regions at their amino and carboxy termini, and a signal sequence and transmembrane domain have been identified. These proteins also contain N - X - T/S N-linked glycosylation addition sequence. All this evidence has suggested that the US6 gene family encodes related glycoproteins.

The US6 family members have been shown to be dispensable for growth of HCMV in human fibroblasts in culture. This was demonstrated by Jones and Muzithras (1992), who generated a series of recombinant HCMV by inserting the β-glucuronidase gene into the HCMV genome in a manner that resulted in the deletion of one or more of the US6 gene family members. The recombinant viruses grew with similar kinetics to wild type virus. On the other hand, in a study on retinal CMV disease, Pereira et al. (1995) demonstrated that viruses with deletions in US9 and US8 / US9 were not capable of spreading from cell to cell, and infection of polarized retinal pigment epithelial cells with these viruses resulted in the formation of smaller plaques. It was concluded that the US9 glycoprotein is essential for spread of the virus in these cells. More recently, Maidji et al. (1996) used HCMV deletion mutants to demonstrate that the US9 accesory glycoprotein is essential for cell-to-cell transmission of the virus in polarized human retinal pigment epithelial cells. When HCMV mutants deleted in US9 were compared with HSV-1 deletion mutants that are unable to express glycoproteins gE and gI, both HCMV and HSV-1 mutants were impaired in spread across lateral membranes of the infected cells.

In terms of the expression of the US9 gene, Jones and Muzithras (1991) demonstrated that the 1.7kb US9-US8 transcript is expressed with early kinetics. They showed that this mRNA was most abundant at 24 hs post infection, and its abundance was greatly reduced by 48 to 72 hs post infection.

CHAPTER II

SPECIFIC OBJECTIVES

The aim of this study was to construct an *Escherichia coli lac* repressor-based system to control HCMV gene expression. The system includes the *lac* repressor protein (encoded by the *lac i* gene), its cognate operator DNA sequence, and the specific inducer IPTG. In order to test the feasibility of this approach to study HCMV gene expression, a *lac* repressor expressing HCMV (RV*lac*) was generated by homologous recombination resulting in the insertion of the *lac i* gene into a region of the HCMV genome that is dispensable for growth of the virus in culture. RV*lac* was used to assess *lac* repressor protein production in RV*lac*-infected cells in culture, as well as *lac* repressor activity using operator-containing HCMV US9 promoters in transient assays. In these assays, the appropriate number and relative position of the operator sequences could be determined for effective *lac*-mediated repression of the operator-containing US9 promoters, and restoration of promoter activity by IPTG.

The promoter of the US9 gene was chosen for this study because: a) this gene is known to be nonessential for virus replication in tissue culture, and b) the US9 gene is adjacent to the site of insertion of the *lac i* gene, so insertion of both the operator-containing US9 promoter in place of the endogenous promoter and the *lac i* gene could be accomplished in a one step recombination event. Because of the nonessential nature of the US9 gene, future analysis of parameters of repression and derepression of operator-containing promoters can be performed in the endogenous locus, without having virus growth dependent on IPTG. In this way, the feasibility of this approach to control HCMV gene expression in the context of the viral genome could be tested.

This conditional expression system would allow assessment of the functional roles of HCMV putative essential gene products during viral replication. HCMV putative essential genes can be targeted for conditional expression by constructing recombinant viruses containing *lac* operator sequences in their promoters. These promoters can be acted upon by the *lac* repressor produced from the same recombinant virus or a coinfecting recombinant HCMV. In this way, the effect of the loss of expression of the target gene on virus replication can be addressed.

The specific objectives of this study were:

1) To generate recombinant HCMV that expresses the *lac* repressor (RV*lac*). The gene encoding the *lac* repressor (*lac i* gene) was inserted into the HCMV genome by homologous recombination. The strategy involved the replacement of the marker gene β-glucuronidase (β-glu) in the US9-US10 intergenic region of the RV134 (parental recombinant HCMV) genome. RV*lac* was characterized in terms of *lac* repressor protein expression, and repression capabilities of the *lac* repressor produced, using operator-containing heterologous promoters in transient assays.

2) To construct operator-containing US9 promoters. The *lac* operator sequence was positioned at different sites within the US9 promoter. The resulting variant promoters were inserted into the viral genome (specific objectives 3 and 4), and tested for promoter expression (specific objective 6).

3) To generate recombinant HCMV containing operator-containing US9 promoters (RVUS9op). Operator-containing US9 promoters were inserted into the viral genome by homologous recombination, as described in specific objective 1. The resulting recombinant

viruses contained the operator-containing US9 promoter in the endogenous locus. To validate the use of these operator-containing promoters for future analysis of conditional US9 gene expression in the context of the viral genome, US9 gene expression was examined (steady-state levels of US9 mRNA) in RVUS9op-infected cells to control for the impact of the operator insertion in the US9 endogenous locus.

4) To generate recombinant HCMV containing the *lac i* gene and the operator-containing US9 promoter (RV*lac*US9op). The construction and isolation of RV*lac*US9op was pursued in order to obtain a recombinant HCMV that would contain the complete conditional expression system for analysis in the context of the viral genome. The gene encoding the *lac* repressor and the operator-containing US9 promoter were inserted into the viral genome as described in specific objectives 1 and 3.

5) To analyze the growth properties of the recombinant viruses *in vitro*. One step growth curves were performed to determine if the insertion of the *lac i* gene and/or the *lac* operator sequence in the HCMV genome has an impact on the rate of virus replication.

6) To assess expression of operator-containing US9 promoters. The operator-containing US9 promoters were tested in CAT reporter constructs in transient assays, in order to analyze the intrinsic effect of the operator insertion on promoter activation by HCMV immediate-early proteins IE1 and IE2, or wild type AD169 virus. In addition, RV*lac*-mediated repression of these promoters in CAT reporter constructs, and the ability of IPTG to restore promoter activity were assessed.

CHAPTER III

MATERIALS AND METHODS

Cells. Primary human foreskin fibroblasts (HFF) were grown in Minimum Essential Media Eagle (MEM) (Gibco BRL, Rockville, MD), supplemented with 10% heat-inactivated newborn calf serum (Gibco BRL, Rockville, MD), 0.03 % L-glutamine (Sigma, St. Louis, MO), 0.006 % Penicillin (Sigma, St. Louis, MO), and 0.01 % Streptomycin (Sigma, St. Louis, MO). HFF cells were passaged weekly. COS-1 cells (African green monkey, SV40 transformed kidney fibroblasts) were obtained from the American Type Culture Collection (ATCC) (ATCC Number: CRL-1650, Rockville, MD) (Gluzman, 1981), and were propagated in MEM, containing 5% heat-inactivated fetal calf serum (Gibco BRL, St. Louis, MO), 0.03 % L-glutamine, 0.006 % Penicillin, and 0.01 % Streptomycin. All cells were incubated at 37^{0} C, 5% CO_2.

Viruses. The viruses used in this study were the HCMV strain AD169 and its derivative RV134, containing the *Escherichia coli* β-glucuronidase marker gene, under the control of the 2.7 HCMV early promoter, and inserted in the US9-US10 intergenic region of the AD169 genome. RV670, a recombinant HCMV deleted of IRS1, US1 through US5, and the US6 family, was obtained from Dr. T. Jones (Jones and Muzithras, 1992). Viruses were grown in cultured primary human foreskin fibroblasts (HFF) for the preparation of high-titer stocks. The stocks were titered by plaque assay in HFF cells, using standard procedures.

Plasmid constructions. Standard plasmid cloning and plasmid preparation techniques were performed (Maniatis et al., 1987). Restriction endonuclease digestion

protocols were conducted as suggested by the manufacturers (Promega, Madison, WI, New England Biolabs, Beverly, MA).

Plasmid pMIEP*lac i* (shown in Figure 1) was constructed by inserting the 1.6 kilobase (kb) *Escherichia coli lac i* gene in place of the IE1 cDNA downstream of the HCMV major immediate-early promoter (MIEP) in the pIE72kD expression vector. The MIEP consists of a *Hind*III to *Sst*II fragment of 800 nucleotides derived from plasmid p760CAT (Stinski and Roehr, 1985), obtained from Dr. Stinski, and the 1.8 kb IE1 cDNA (encoding the HCMV immediate-early protein IE72) is a *Sst*II to *Hind*III fragment. The *lac i* gene (modified to include the SV40 T antigen nuclear localization signal) was derived from the p3'SS eukaryotic *Lac* Repressor expressing vector in the *Lac* Switch inducible mammalian expression system (Stratagene, La Jolla, CA). A unique *Xba*I site 15 nucleotides upstream of the 5'end of the *lac i* gene was used to linearize p3'SS. The linear DNA molecules were extracted with phenol, and subsequently with chloroform. Following ethanol precipitation, these molecules were treated with DNA Polymerase Large (Klenow) fragment (New England Biolabs, Beverly, MA). Previously kinased *Sst*II linkers were then ligated to the linear vector. The resulting clones were screened for the presence of the *Sst*II linkers. Digestion of pIE72kD with *Sst*II, followed by partial digestion with *Hind*III, and ligation of the *Sst*II to *Hind*III 1.6 kb fragment containing the *lac i* gene, allowed for the generation of pMIEP*lac i*.

Plasmid pRVMIEP*lac i* was constructed by inserting the MIEP-*lac i* gene *Hind*III cassette derived from plasmid pMIEP*lac i*, into a unique *Hind* III site upstream of the US9 promoter in the recombination vector pRV3 (shown in Figure 3). This *Hind*III site was generated by inserting a *BamH*I -*Xba*I -*Hind*III linker into a unique *Apa*I site in the

US9-US10 intergenic region (5'GGGATCCGCTCTAGAGCAAGCTTGGGCC3'). pRV3 contains genes from a nonessential region of the HCMV genome (US8 through US11) that provide the appropriate flanking sequences to allow homologous recombination into the viral genome. The clone in which the orientation of the *lac i* gene conforms to the direction of transcription of the neighboring genes was isolated.

Plasmid pUS9CAT (Figure 11) was constructed by cloning the US9 promoter-leader as a 310 nucleotide (-269 to +41) *Hind*III fragment, from pRV3 (which had been modified with the insertion of a *Hind*III site into the *Sst*II site at +40 in the leader sequence), into a unique *Hind*III site upstream of the chloramphenicol acetyl transferase (CAT) gene in pSVOCAT.

Plasmid pUS9opCAT (shown in Figure 11) was generated by inserting the *lac* operator sequence 5'GAATTGTGAGCGGATAACAATTTC3' flanked by *Sst*II linkers into a unique *Sst*II site at nucleotide position +40 in the leader sequence of the US9 promoter-leader in pUS9CAT.

To generate pUS9op(-16)CAT and pUS9op(-6)CAT (shown in Figure 11), the *lac* operator sequence 5'GAATTGTGAGCGGATAACAATTTC3' was inserted at different alternative sites in proximity to the TATA box within the US9 promoter, by an overlapping PCR mutagenesis strategy (shown in Figure 12) based on modifications of PCR protocols previously described (Zhang and Moss, 1991). Two operator-containing US9 promoters, US9op(-16) and US9op(-6), with the *lac* operator sequence 16 or 6 nucleotides upstream of the transcription start site, respectively, were generated using plasmid pUS9CAT as a template. Two separate PCR reactions were conducted for each operator-containing promoter. For the first reaction, primers pSVO1':

5'GCTCTGATGCCGCATAGTTAAGCC 3', and US92: 5' GCCTCTTTATATCGTCCCGACGTGACGCG 3' were utilized. For the second reaction, the primers used were US93: 5' CGCGTCACGTCGGGACGATATAAAGAGGCAGAATTGTGAGCGGATAACAATT TCCGGTGTTTCGGCTCCCGCAC 3' (underlined sequence represents operator at -16) or US93': 5' CGCGTCACGTCGGGACGATATAAAGAGGCACGGTGTTTCGGAATTGTGAGCG GATAACAATTTCGCTCCCGCAC 3' (underlined sequence represents operator at -6), and CAT4': 5' GCGGGCAAGAATGTGAATAAAGGCCGG 3'. For each operator-containing promoter, these reactions rendered two overlapping products, which were subsequently treated with DNA Polymerase Large (Klenow) fragment (New England Biolabs, Beverly, MA), and then mixed together and PCR amplified using primers pSVO1' (5'-most), and CAT4' (3'-most), to generate the final operator-containing product. The final product was digested with *Hind*III, purified by electrophoresis in 5% polyacrylamide, and ligated into *Hind*III-linearized pSVOCAT, to generate pUS9op(-16)CAT (operator at -16) or pUS9op(-6)CAT (operator at -6). The presence of the operator sequence in these constructs was confirmed by DNA sequencing (Pharmacia LKB.A.L.F. DNA Sequencer, Piscataway, NJ).

Recombination plasmids pRVUS9op-16 and pRVUS9op-6 (Figure 11) were constructed by inserting the operator-containing US9 promoters derived as *Hind*III to *Sst*II fragments from pUS9op(-16)CAT and pUS9op(-6)CAT, respectively, in place of the US9 promoter (removed as a *Hind*III to *Sst*II fragment) in pRV3.

Recombination plasmids pRV*lac*US9op-16 and pRV*lac*US9op-6 (shown in Figure 11) were derived from pRVUS9op-16 and pRVUS9op-6, respectively, by inserting the MIEP-*lac i* gene *Hind*III cassette [derived from plasmid pRVMIEP*lac i* (Figure 3)] into a unique *Hind*III site upstream of the US9 promoter. The resulting clones were screened for orientation of the MIEP-*lac i* gene cassette using *Sst*II. Clones in which transcription of the *lac i* gene proceeds in the same direction as the neighboring genes were isolated.

Plasmids pOp13CAT and pOpRSVICAT (shown in Figure 8A) were obtained from the *Lac* Switch inducible mammalian expression system (Stratagene, La Jolla, CA).

Plasmid pSVH (Depto and Stenberg, 1989) was obtained from Dr. Stenberg. This expression vector contains the genes encoding the HCMV immediate-early proteins IE1 and IE2.

DNA preparations. Infected HFF DNA was isolated using the Genomic Prep Cells and Tissue DNA isolation kit (Pharmacia Biotech, Piscataway, N.J.) following the manufacturer's suggestions. DNA was quantitated on a spectrophotometer (Milton Roy Spectronic 1001 Plus) at 260 nm.

RNA preparations. Total RNA was isolated from infected HFF cells using the Qiagen Rneasy kit (Qiagen, Chatsworth, CA). The protocol was conducted as described by the manufacturer. RNA was quantitated on a spectrophotometer (Milton Roy Spectronic 1001 Plus) at 260 nm.

Construction of recombinant HCMV. Recombinant viruses expressing the *lac* repressor, and/or containing a *lac* operator sequence in the US9 promoter in the endogenous locus were generated by a homologous recombination strategy involving the

replacement of the β-glucuronidase (β-glu) marker gene in the US9-US10 intergenic region of the RV134 (parental recombinant HCMV) genome (see Figure 4). Homologous recombination resulted following cotransfection of infectious RV134 DNA and linearized recombination plasmid DNA into cultured primary human foreskin fibroblasts using a modification of the calcium phosphate precipitation procedure described in Current Protocols in Molecular Biology (1996, Volume 1, Unit 9.1, Supplement 36) (Ausubel et al., 1996). 8 ug of RV134-infected cell DNA and 1-2 ug of CsCl-purified, *Sal*I-linearized recombination plasmid DNA were added to 450 ul of sterile distilled water, followed by the addition of 50 ul of a 2.5 M $CaCl_2$ solution. After gentle mixing, 500 ul of 2X Hepes-buffered saline (38.4 mM Hepes, 274 mM NaCl, 10 mM KCl, 1.6 mM Na_2HPO_4, 0.2% dextrose, pH 7.05) were added dropwise to the DNA-$CaCl_2$ mix, using a Pasteur pipette to mix gently. The DNA precipitate was allowed to form by incubating the mix at room temperature for 15 minutes. The DNA precipitate was then added dropwise onto a monolayer of primary human foreskin fibroblasts in a 100 mm-tissue culture plate, previously washed with 5 ml of 1X Hepes-buffered saline. After an 8-minute incubation at room temperature, 10 ml of HFF culture media were added. This was followed by incubation at 37^0 C for 4 hs. At this time, cells were shocked using 2 ml of a 15% glycerol solution, to increase the cellular uptake of DNA. After 45 seconds, the glycerol was removed, and 5 ml of HFF culture media were quickly added to wash out remaining glycerol. The media was removed 20 seconds later, and 15 ml of fresh culture media were added. Transfected cells were incubated at 37^0 C, with regular changes of fresh media every 4 days. Approximately 10 days after transfection, the first signs of cytopathic effect

(CPE) were visible. When transfected cells reached 100% CPE, primary stocks of putative recombinant virus were harvested and stored at -80^0 C.

Isolation of recombinant HCMV. To identify the primary stocks that contained recombinant virus in addition to parental virus, the stocks harvested as described above were initially screened by PCR. HFF cells were infected with the primary stocks at a multiplicity of infection (MOI) of 2 plaque forming units (PFU) per cell, and at 72 hs post infection, cells were harvested and total infected cell DNA was isolated. For the identification of *lac i*-containing recombinant viruses, *lac i*-specific primers P3: 5'CTCCCACCATGAAACCAGTAACG 3', and M2: 5'GGTATCGTCGTATCCCACTACCG 3' were used. These primers amplified 835 nucleotides within the *lac i* gene. US9 promoter-specific primers US9opTC(scr)1: 5' CCATATAAACGTGGGTTTCGGTGACCACAACC 3', and US9op(scr)3: 5' GACACCAGTGCCAGAAGAAGGAACAGGTGGAC 3', were used for screening for operator-containing recombinant viruses. These primers amplified 318 nucleotides containing the wild type US9 promoter. The presence of the 24- nucleotide *lac* operator sequence resulted in the generation of a PCR product of 342 nucleotides. The screening of primary stocks was also conducted by western blot analysis, for the identification of stocks containing recombinant viruses expressing the *lac* repressor, using an anti-*lac i* polyclonal serum (Stratagene, La Jolla, CA) (see western blot analysis section below). In addition, the screening involved Southern blot analysis using a *lac i*-specific probe (see Southern blot section below). Southern blot analysis was also conducted using an AD169 *Hind*III X probe (spanning the HCMV AD169 genomic

region US7 through US11), for the identification of primary stocks containing recombinant viruses (see Southern blot analysis section below).

Following the identification of primary stocks containing recombinant viruses, these pools of virus were screened by altered plaque phenotype (blue plaques [parental RV134] or white plaques [recombinant]) in the presence of the chromogenic substrate for β-glu: 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-glu) (Biosynth AG). This allowed for the isolation of recombinant viruses. HFF cells in monolayer were infected with primary stocks at an MOI of 10 PFU per plate. After adsorption of the virus at 37^0 C for 2 hs, the inoculum was removed, and 10 ml of fresh culture media were added. 24 hs after infection, the infected cells were overlayed with minimum media (MEM) (Fisher Scientific, Pittsburg, PA) containing 10% heat-inactivated fetal calf serum, and 0.5% agarose. Cells were fed by additional overlays weekly. When plaques became visible macroscopically (approximately 2-3 weeks after infection), plaques were picked as agarose plugs, placed in individual wells of 24 well culture dishes, and incubated until CPE became visible. At that time, the cells were overlayed as described above, with the addition of 75 ug per ml of X-glu. After 3 days, wells with a clear overlay were considered negative for β-glucuronidase activity, suggesting the presence of recombinant virus.

Individual plaques were picked from clear wells as agarose plugs, and the virus was released from the agarose by incubating the plug in 3 ml of culture media for 30 minutes, with frequent pipetting up and down. The resulting virus suspension was divided into 3 equal aliquots, and inoculated into each of 3 100 mm culture plates of HFF monolayers. After virus adsorption at 37^0 C for 2 hs, the inoculum was removed from 2 of the plates, and they were overlayed with fresh culture media until 100% CPE occurred. At

that time, the cells were harvested for 1) the generation of a high-titer stock, and 2) infected cell DNA isolation for Southern blot analysis. When individual plaques became visible in the remaining plate, it was overlayed with MEM containing 10% heat-inactivated fetal calf serum, 0.5% agarose, and X-glu, and incubated at 37^0 C to verify the absence of blue plaque phenotype virus contamination during the selection of the white plaque phenotype virus.

PCR protocols. PCR reactions in this study were performed using Platinum Taq DNA Polymerase (Gibco BRL, Baltimore, MD), following the manufacturer's suggestions. The reactions were conducted in an Ericomp Delta Cycler II System (San Diego, CA). The PCR products were analyzed by electrophoresis in 1.2 % agarose or 5 % polyacrylamide gels, and visualized by staining with ethidium bromide. The gels were photographed with a Polaroid camera (Fotodyne Incorporated). The images presented in chapter IV were generated with a UMAX PowerLook II Scanner with Paintshop Pro version 5.0 Jacs Software.

Transient transfection assays. CAT reporter constructs were used to study the expression of operator-containing US9 promoters in the presence of either HCMV immediate-early proteins IE1 and IE2, using plasmid pSVH (Depto and Stenberg, 1989), or wild type virus (AD169) or recombinant virus expressing the *lac* repressor (RV*lac*). A DEAE-Dextran transfection protocol was performed as previously described (Stenberg et al., 1990). To carry out the aims mentioned above, the general protocol that was conducted was as follows. Plasmid DNA was added to a microfuge tube. 1 ml of sterile Tris buffered saline (30 mM Tris-HCl pH 7.5, 150 mM NaCl) (TBS) was added, and the DNA solution was mixed by vortexing. 0.5 mg DEAE-Dextran (Pharmacia, Uppsala,

Sweden) were then added. This DNA solution was added to HFF cells on 100 mm culture plates, which had been previously washed with TBS. The cells were incubated at 37^0 C for 30 minutes, with frequent rocking. This was followed by the addition of 10 ml of 100uM chloroquine (Sigma, St. Louis, MO) in culture media. After the addition of chloroquine, the cells were incubated at 37^0 C for 3 hs. At that time, the media was replaced with fresh culture media, and the transfected cells were incubated at 37^0 C.

To study IE1 and IE2 activation of operator-containing US9 promoters, 5 ug of pUS9opCAT, pUS9op(-16)CAT or pUS9op(-6)CAT (or pUS9CAT as control) were cotransfected with 5 ug of pSVH into HFF cells. Cells were harvested 48 hs post transfection and assayed for CAT activity. Plasmids pUS9op(-16)CAT and pUS9op(-6)CAT were also tested for activation by AD169 virus. 10 ug of the reporter plasmid were transfected into HFF cells. 24 hs after transfection, the cells were infected with 2 PFU per cell of AD169 virus. After 2 hs adsorption at 37^0 C, the virus inoculum was removed and fresh media was added. Cells were harvested 72 hs post infection and assayed for CAT activity.

To assess *lac* repressor mediated repression of operator-containing promoters (RSV promoters in pOp13CAT and pOpRSVICAT, or US9 promoters in pUS9opCAT, pUS9op(-16)CAT and pUS9op(-6)CAT), 10 ug of either plasmid was transfected into HFF cells. 24 hs after transfection, the cells were mock infected, or infected with 2 PFU per cell of AD169 (as control), or RV*lac*. After adsorption at 37^0 C for 2 hs, the inoculum was removed and fresh media was added onto the infected cells. For IPTG derepression experiments, IPTG (Fisher Scientific, Pittsburg, PA) was added after virus adsorption to final concentrations of 0, 0.2, 0.4, 0.8, or 2 mM. The cells were exposed to IPTG-

containing media until the indicated times of harvest. Cells were harvested at 24, 48, and 72 hs post infection, and assayed for CAT activity.

In order to assess expression of the *lac* repressor, COS-1 cells were transfected with 10 ug of pMIEP*lac i* using the general transfection protocol described above. 48 hs after transfection, cell extracts were prepared for western blot analysis.

Chloramphenicol acetyl transferase (CAT) assay. CAT activity was determined as previously described (Depto and Stenberg, 1989). Transiently transfected cells were washed with cold Tris buffered saline (TBS) twice. Using 1 ml of TBS, cells were scraped into microfuge tubes, pelleted by centrifugation, and subsequently resuspended in 100 ul of 0.25 M Tris-HCl pH 7.8. Three cycles of freeze-thaw were done, and the cell extracts were centrifuged to spin out cell debris. The supernatants were transferred to new microfuge tubes, and heat inactivated at 68^0 C for 10 minutes. For the acetylation reaction, 20 ul of the heat-inactivated extract was added to a reaction mix containing 0.1 uCi ^{14}C-Chloramphenicol (Amersham, Chicago, IL), 70 ul of 0.25 M Tris-HCl pH 7.8, 34 ul of sterile distilled water, and 20 ul acetyl CoA [4 mM] (Boehringer Mannheim, Indianapolis, IN). The reaction was incubated at 37^0 C for 30 minutes. To stop the reaction, 750 ul of ethyl acetate were added, immediately followed by vortexing and centrifugation of the sample for 1 minute. The top aqueous phase was recovered and dried down. The residue was resuspended in 20 ul of ethyl acetate, and spotted onto a thin layer chromatography (TLC) plate (E. Merck, Darmstadt, Germany). Separation of the reaction products was achieved by partition chromatography in a chamber containing 190 ml chloroform and 10 ml methanol, for 1 h. The TLC plate was exposed to X-Ray film (Fuji) with an

intensifying screen at -80^0 C overnight. Percent acetylation was quantitated on the TLC plate by phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA).

Southern blot analysis. The Southern blot protocol was performed as described by Maniatis et al. (1987). 2.5 ug of *Hind*III-digested, infected cell DNA was loaded onto a 0.8% agarose gel, and subjected to electrophoresis at 22 volts overnight. The gel was then stained with ethidium bromide for 45 minutes, and subsequently photographed. Depurination was done by submerging the gel in 0.25 M HCl at room temperature for 10 minutes, followed by denaturation in 0.5 M NaOH, 1.5 M NaCl, at room temperature for 45 minutes. The gel was then soaked in a neutralizing solution (1 M Tris-HCl pH 8.0, 1.5 M NaCl) at room temperature for 45 minutes. After that, the DNA was transferred from the gel onto a positively-charged nylon membrane (Pall Biodyne, Pall Biosupport, East Hills, NY) in 10X SSC (1.5 M NaCl, 150 mM sodium citrate pH 7.0) overnight. The following day, the DNA in the blot was UV cross-linked to the membrane (150 J), and baked at 80^0 C for 1 h. Hybridization was conducted using the Boehringer Mannheim Genius System. The DNA probes used were the 5.2 kb AD169 *Hind*III X fragment, which spans the US7 through US11 region of the HCMV genome, and a *lac i*-specific probe, which was derived by digesting pMIEP*lac i* with *Sst*II and *Hind*III, to render a 1.6 kb fragment containing the *lac i* gene. The probes were generated with Klenow DNA Polymerase by random primed incorporation of digoxigenin (DIG)-labeled deoxyuridine-triphosphate. The blots were exposed to the hybridization solution (containing 7 ng/ml of DIG-labeled DNA probe), at 42^0 C overnight in a Micro Hybridization Incubator, Model 2000 (Robbins Scientific, Sunnyvale, CA). Southern blots were washed 3 times in 2X SSC, 0.1% SDS at room temperature. This was followed by 3 washes in 0.5X SSC, 0.1%

SDS at 65^0 C. Detection of the hybridized DNA fragments was done using an antibody-conjugate [anti-digoxigenin alkaline phosphatase conjugate (anti-DIG-AP)], and a chemiluminescent substrate for alkaline phosphatase (Disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1^{3,7}]decan)-4-yl)phenylphosphate) (CSPD, Tropix, Inc. Bedford, MA).The blots were immediately exposed to X-Ray film (Fuji) with an intensifying screen at room temperature. The images presented in chapter IV were generated with a UMAX PowerLook II Scanner with Paintshop Pro version 5.0 Jacs Software.

Northern blot analysis. HFF cells in 100 mm culture plates were mock infected, or infected with wild type AD169, parental RV134, or recombinant viruses at an MOI of 2 PFU per cell. Virus was allowed to adsorb at 37^0 C for 2 hs. The inoculum was then removed, and fresh culture media was added. Infected cells were harvested 24, 48, and 72 hs post infection, and total RNA was isolated. Northern blot analysis was conducted following the protocol described by Maniatis et al. (1987), with modifications. One tenth of the total RNA recovered was loaded onto a 1 % agarose gel containing 6.6 % formaldehyde. The RNA was subjected to electrophoresis at 70 volts for 4 hs. After electrophoresis, the gel was photographed, and subsequently washed 3 times with sterile distilled water. The RNA was then transferred to a Magna charge nylon membrane (Osmonics Inc. Westborough, MA) in 10X SSC, overnight. The following day, the RNA was fixed to the membrane by UV cross-linking (150 J), and baking at 80^0 C for 2 hs. The membrane was hybridized at 42^0 C overnight, to 10^{7}cpm of a radiolabeled DNA probe to the US9 gene [a *Hind*III-*Eco*RI fragment of 1578 nucleotides derived from pRV3. This fragment comprises the US8 and US9 open reading frames (see Figure 3)]. The

[α^{32}P]dCTP-labeled probe was generated using the Boehringer Mannheim Random Primed DNA labeling kit (specific activity 2 x 10^9cpm/ug). Following hybridization, the blot was subjected to 2 washes in 2X SSC, 0.1% SDS, at room temperature, followed by 2-3 washes in 0.1X SSC, 0.1% SDS at 50^0 C. The blot was exposed to X-Ray film (Fuji) with an intensifying screen, at -80^0 C overnight. The RNA in the blot was quantitated by phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA), and was expressed in arbitrary units. To correct the data for multiplicities of infection, the blot was stripped by boiling in 0.1% SDS for 10 minutes. The RNA in the blot was hybridized to a radiolabeled DNA probe to the UL99 (pp28) gene. This probe was derived from plasmid p28S1 (Depto, 1991) as a 1 kb *Sma*I fragment from *Xba*I-C, comprising the entire pp28 gene sequences. Radiolabeling of the pp28 probe, hybridization, washes, autoradiography, and quantitation were performed as described for the US9 probe.

Western blot analysis. Western blot analysis was conducted to assess *lac* repressor protein expression a) in cells transfected with pMIEP*lac i*, b) in cells infected with primary stocks of putative recombinant virus, to identify those containing *lac i*-positive recombinant viruses, and c) in cells infected with purified *lac i*-positive recombinant viruses. Protein expression was determined at 24, 48, and 72 hs post infection. The protocol was conducted as previously described (Stenberg et al., 1989). Cells were lysed in 30 mM Tris-HCl pH 7.5, 1% SDS, and scraped into a microfuge tube. 60 ul of the lysate were treated with 3 ul of β-mercaptoethanol, and loaded on a 12.5% SDS-polyacrylamide gel. Electrophoresis was performed at 8 milliamps, overnight. The proteins in the gel were then electroblotted onto a Nitropure nitrocellulose membrane (MSI, Westboro, MA), at 100 volts for 2 hs, at 4^0 C. The membrane was subsequently treated

with a protein-blocking solution for 1 h. After that, the blot was incubated with a rabbit anti-*lac i* polyclonal serum (Stratagene, La Jolla, CA), for 1 h at room temperature. This was followed by 3 washes using RIPA-5 buffer (30 mM Tris pH 7.5, 0.5M NaCl, 12 mM deoxycholic acid, 1% NP40, 0.1% SDS) at room temperature. A goat anti-rabbit polyclonal serum conjugated with horseradish peroxidase (Sigma, St. Louis, MO) was used as secondary antibody, and the incubation proceeded at room temperature for 1 h. 3 RIPA-5 washes were then done at room temperature. Chemiluminescent detection was performed by ECL (Amersham Life Science, Buckinghamshire, England), which includes luminol as the luminescent substrate for horseradish peroxidase. The blot was exposed to X-Ray film (Fuji) at room temperature, using an intensifying screen. The images presented in chapter IV were generated using a UMAX PowerLook II Scanner with Paintshop Pro version 5.0 Jacs Software.

Growth curve analysis of recombinant HCMV. HFF cells were seeded onto 25 cm^2 culture flasks. Cells were infected at an MOI of 2 PFU per cell, with either parental virus RV134 or the recombinant viruses. Infections were performed in duplicate. After virus adsorption at 37^0 C for 2 hs, the inoculum was removed, and fresh culture media was added. The infected cells were incubated at 37^0 C. Total virus was harvested daily (day 1 through day 7 post infection), by freezing the infected cells at -80^0 C, followed by thawing at 37^0 C. The infected cells were then scraped into the media, and the resulting virus suspension was transferred to a 50 ml conical tube for sonication (30 seconds). Total infectious virus was quantitated by plaque assay on HFF cells seeded on 6 well culture dishes, using standard procedures. Titers were expressed as $\log_{10}$ PFU/ml.

CHAPTER IV

RESULTS

Construction and characterization of an *E. coli lac* repressor-expressing HCMV

Cloning of the *lac i* gene into an HCMV expression vector

To generate a *lac* repressor expressing virus (RV*lac*) (see specific objectives in chapter II), the 1.6 kb *lac i* gene was first cloned downstream of the HCMV major immediate-early promoter (MIEP) in the pIE72kd expression vector to generate pMIEP*lac i* (Figure 1). The MIEP has been extensively used to express various HCMV genes (reviewed by Stenberg, 1993). Because the MIEP is a very strong promoter that is activated by several viral and cellular transcription factors, and is expressed throughout the course of HCMV infection, it was hypothesized that this promoter would allow for the expression of sufficient amounts of the *lac* repressor protein to efficiently block transcription from operator-containing promoters during all times after infection. Elevated levels of protein are necessary because the *lac* repressor has to kinetically outcompete the binding of transcription factors involved in the formation of an initiation-competent transcription complex at the operator-containing promoter site. Such competition depends on the free concentrations of the transcription factors and the repressor, as well as the rates of complex formation with their respective DNA binding sites. Because little is known about these parameters for different promoters and cell types, the intracellular concentration of *lac* repressor should be high.

Before inserting the MIEP-*lac i* gene cassette into the virus, pMIEP*lac i* (see Figure 1) was first tested for expression of the *lac* repressor by transfecting it into COS cells. COS cells are an established cell line derived from African green monkey kidney

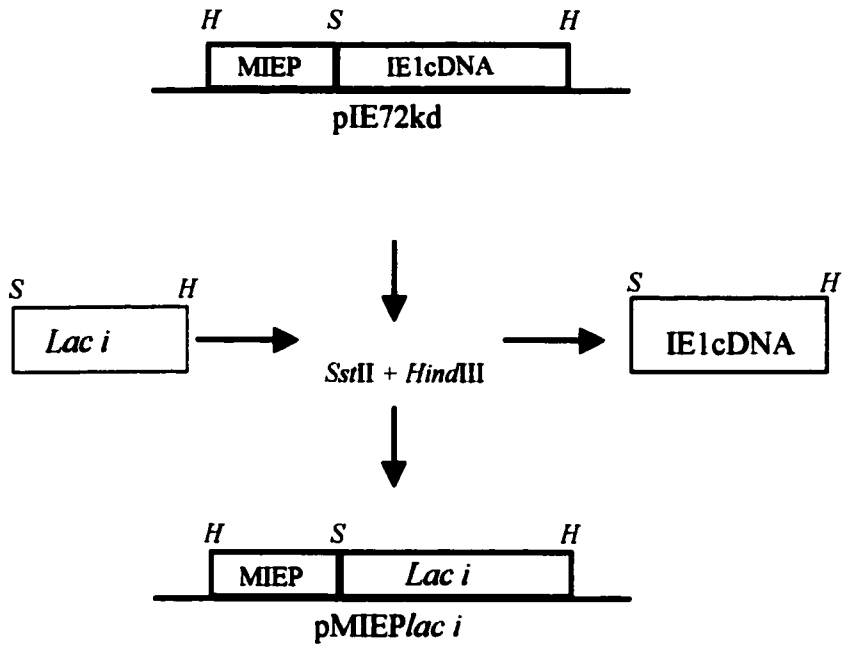


FIG. 1. Cloning of the *lac i* gene into an HCMV expression vector.The1.6 kb *lac i* gene was derived from plasmid p3'SS (*Lac* Switch inducible mammalian expression system, Stratagene, La Jolla, CA). The *lac i* gene was cloned downstream of the HCMV Major Immediate-Early Promoter (MIEP) in place of IE1 cDNA in pIE72kd to generate pMIEP*lac i*. *H*: *Hind*III site, *S*: *Sst*II site.

fibroblasts. These cells have been transformed by an origin-defective mutant of Simian Virus 40 (SV40) that codes for wild type T antigen (Gluzman, 1981). These transformed cells produce T antigen, which makes them a suitable transfection host for expression vectors containing SV40 origin of replication. COS cells were transfected with the expression vector pMIEP*lac i*, or pMIEP*lac i* and pSVH, an expression vector containing the SV40 origin of replication and encoding IE1 and IE2 proteins. These viral transactivators regulate MIEP expression along with cellular transcription factors (see The Replicative Cycle in chapter I). As a negative control for *lac* repressor expression, COS cells were transfected with pSVH alone. Cell extracts were prepared for western blot analysis 48 hs after transfection. The procedure was followed as previously described (Stenberg et al., 1989) (see Materials and Methods). A rabbit anti-*lac i* polyclonal serum was used to detect *lac* repressor protein in the cell extracts. The results are presented in Figure 2. *Lac* repressor protein was undetectable in cells transfected with pSVH alone. On the other hand, significant levels of expression of the *lac* repressor protein were observed in cells transfected with pMIEP*lac i*. Similar levels of *lac* repressor were observed in cells transfected with pMIEP*lac i* and pSVH, suggesting that, at least under the experimental conditions used, expression was mostly affected by cellular transcription factors. The molecular weight of the repressor polypeptide was 37kD as expected. These results demonstrated that the *lac i* gene can be expressed from the MIEP to significant levels, suggesting that insertion of the MIEP-*lac i* gene cassette into the HCMV genome to generate recombinant virus RV*lac* would likely result in the production of elevated levels of the *lac* repressor protein during the course of infection with RV*lac*. However, *lac*

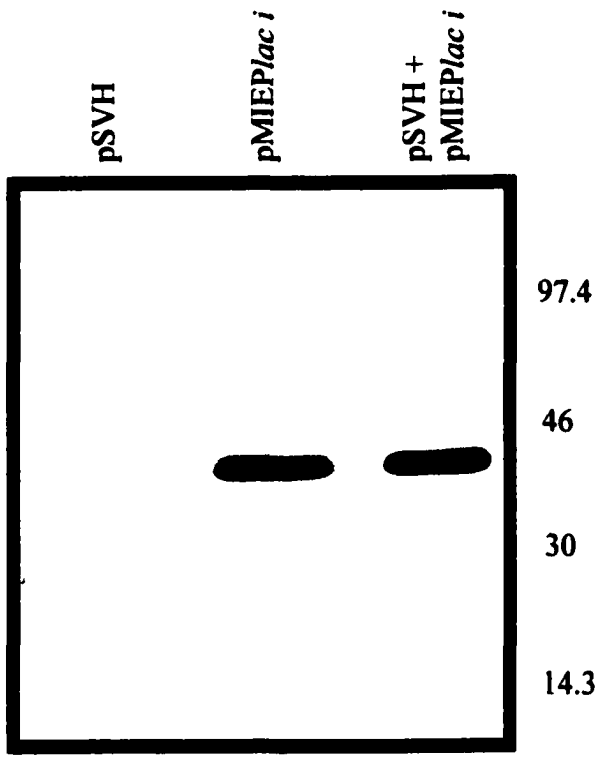


FIG. 2. *Lac* repressor expression from pMIEP*lac i*. COS cells were transfected with either 10 ug of pMIEP*lac i*, or 5 ug of pMIEP*lac i* and 5 ug of pSVH, or 10 ug of pSVH alone as a negative control. 48 hs after transfection, cell extracts were prepared for western blot analysis. A rabbit anti-*lac i* polyclonal serum was used for the detection of the 37 kilodalton-*lac* repressor polypeptide. The numbers indicate molecular weight markers in kilodaltons.

repressor protein expression would have to be measured in RV*lac*-infected cells to confirm these speculations (see corresponding section below).

Insertion of the *lac i* gene into the HCMV genome

The next step was to insert the *lac i* gene (MIEP-*lac i* gene cassette) into the viral genome. The experimental design consisted of a replacement recombination strategy to generate a recombinant HCMV containing the *lac i* gene (RV*lac*) in place of a selectable marker gene. To this end, RV134, a recombinant HCMV containing the β-glucuronidase (β-glu) marker gene located in a nonessential region of the viral genome (between US9 and US10 open reading frames) (Figure 4), was used as parental virus for the homologous recombination leading to the replacement of the β-glu gene with the MIEP-*lac i* gene cassette. Isolation of the resulting recombinant virus was achieved by screening for altered plaque phenotype: blue plaques (parental RV134) or white plaques (recombinant virus) in the presence of the chromogenic substrate for β-glu: 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-glu). The detailed experimental procedure was as follows. To generate RV*lac*, the MIEP-*lac i* gene cassette was first inserted into a unique *Hind*III site between US9 and US10 in the recombination vector pRV3 (Figure 3). The clone in which the orientation of the *lac i* gene conforms to the direction of transcription of the neighboring genes (right to left in the prototype HCMV genome) was isolated. This construct, pRVMIEP*lac i*, was used to insert the MIEP-*lac i* gene cassette into the β-glu locus in RV134 by homologous recombination directed by the appropriate flanking sequences (Figure 4). This was accomplished by cotransfecting infectious RV134 DNA and linearized pRVMIEP*lac i* into primary human foreskin fibroblasts (HFF), using a calcium

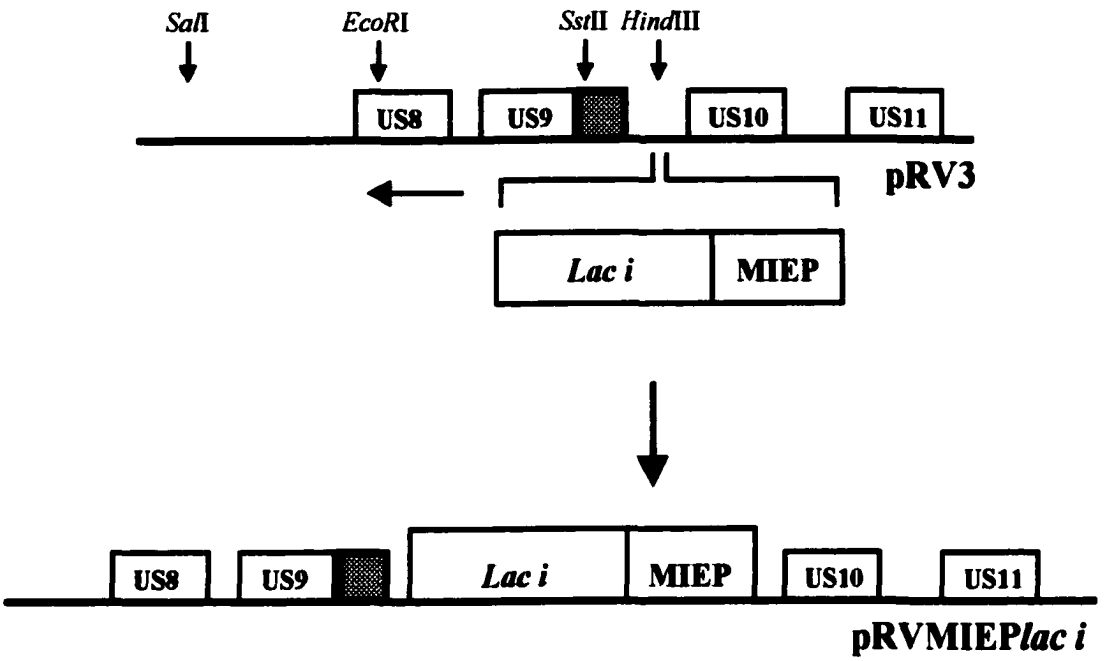
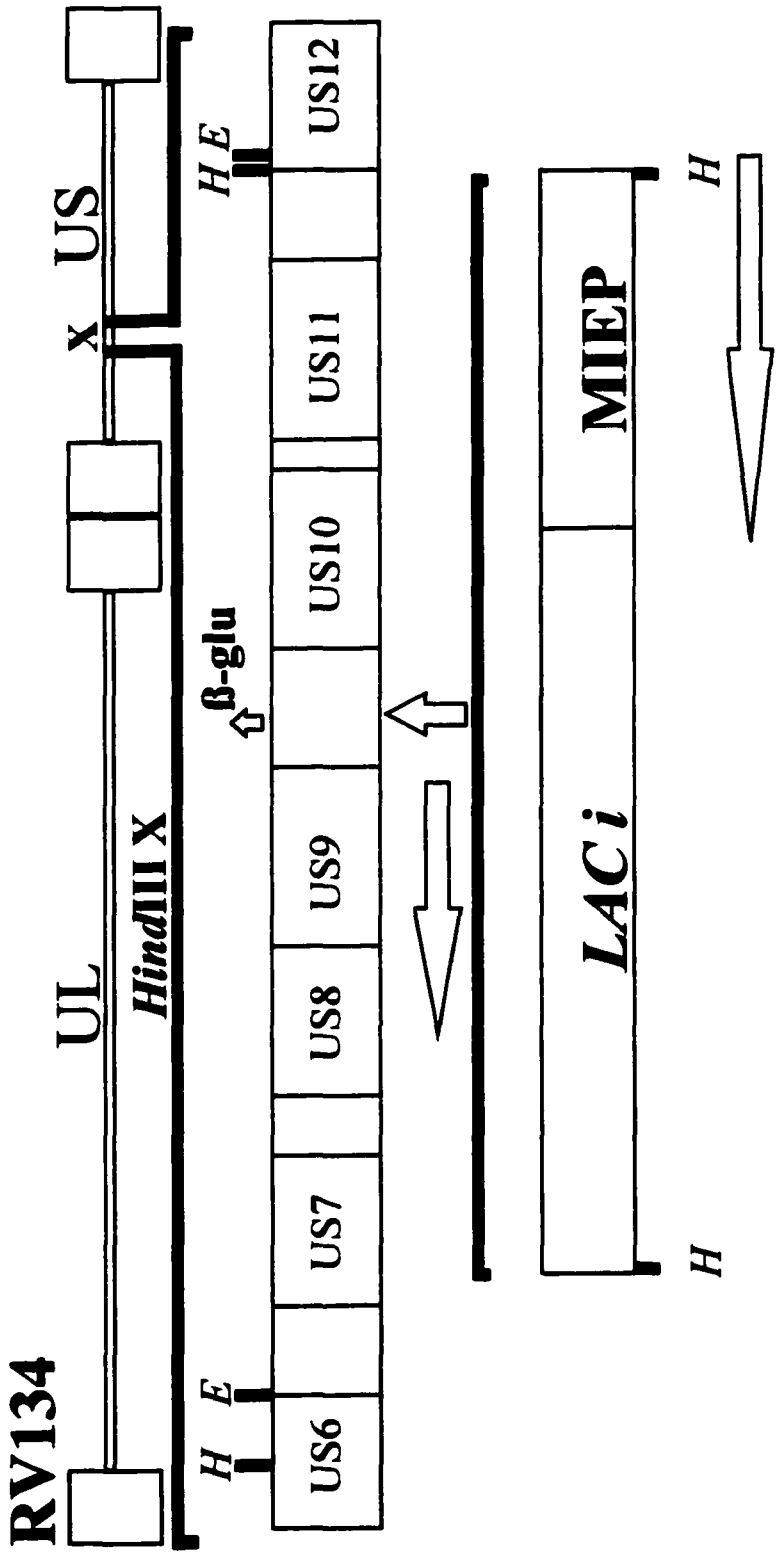


FIG. 3. Diagram of the recombination vector pRV3 and generation of pRVMIEP*lac i*. The MIEP-*lac i* gene expression unit was inserted into a unique *Hind*III site (generated by inserting the linker sequence 5'GGGATCCGCTCTAGAGCAAGCAAGCTTGGGCC3' into an *Apa*I site) between US9 and US10 open reading frames in pRV3. This vector contains genes from a nonessential region of the HCMV genome (US8 through US11) that provide the appropriate flanking sequences to allow recombination into the viral genome. A clone in which transcription of the *lac i* gene proceeds in the same direction as the neighboring genes (right to left in the prototypic HCMV genome, as indicated by the arrow) was isolated and designated pRVMIEP*lac i*.

FIG. 4. Insertion of the *lac i* gene into the HCMV genome. The target region in the viral genome for insertion of the MIEP-*lac i* gene cassette was the *Hind*III X fragment of the AD169 virus strain , which comprises open reading frames US7 through US11. This region is nonessential for viral replication in cells in culture. RV134 is the recombinant HCMV used as parental virus for the homologous recombination leading to insertion of the *lac i* gene into the viral genome. This virus contains the marker gene ß -glucuronidase between US9 and US10. RV134 produces blue plaques in the presence of the substrate for ß -glu: X-glu. Homologous recombination occurs following cotransfection of RV134 DNA and a recombination plasmid containing the MIEP-*lac i* gene cassette flanked by the appropriate flanking sequences (see Fig. 3) into HFF cells. This results in the replacement of the ß-glu gene with the MIEP-*lac i* gene cassette in the US9-US10 intergenic region, leading to the generation of a recombinant virus containing the *lac i* gene. This virus produces white plaques in the presence of X-glu. *UL*: unique long component of the HCMV genome, US: unique short component of the HCMV genome, *H*: *Hind*III, *E: EcoR*I, arrows in the right-left direction indicate direction of transcription of the open reading frames in the *Hind*III X region, and of the MIEP-*lac i* gene expression unit.



phosphate precipitation procedure as described in Materials and Methods. Briefly, 8 ug of RV134 DNA and 2 ug of linearized pRVMIEP*lac i* were transfected into HFF cells. Approximately 10 days after transfection, the first signs of cytopathic effect (CPE) were visible. When transfected cells reached 100% CPE, primary stocks of putative recombinant virus were harvested. In this manner, four primary stocks of virus were obtained.

In order to identify the primary stocks that contained recombinant viruses containing the *lac i* gene, the stocks, containing both wild type and recombinant viruses, were initially screened by PCR, using *lac i*-specific primers (see Materials and Methods). This initial screening involved infecting HFF cells with the primary stocks and harvesting the infected cells at 72 hs post infection. Total cell DNA was extracted, and was subsequently amplified by PCR using forward and reverse primers specific for the *lac i* gene. These primers amplify 835 nucleotides within the *lac i* gene. The PCR products were subjected to electrophoresis in 1.2% agarose, and were visualized by ethidium bromide staining. Figure 5 shows that a PCR product of the correct size (835 base pairs) was obtained for one stock of virus (RV3MIEP*Lac*3 4.26.95), indicating the presence of recombinant virus containing the *lac i* gene.

In order to isolate the recombinant virus, the *lac i*-positive primary stock (RV3MIEP*Lac*3 4.26.95) was screened by altered plaque phenotype. Briefly, HFF cells were infected with the *lac i*-positive stock at 10 PFU per plate. The infected cells were overlayed with minimum essential media (MEM) containing 10% heat-inactivated fetal calf serum and 0.5% agarose. Approximately 550 plaques were picked 2-3 weeks after infection, placed in individual wells of 24 well culture dishes and incubated until CPE

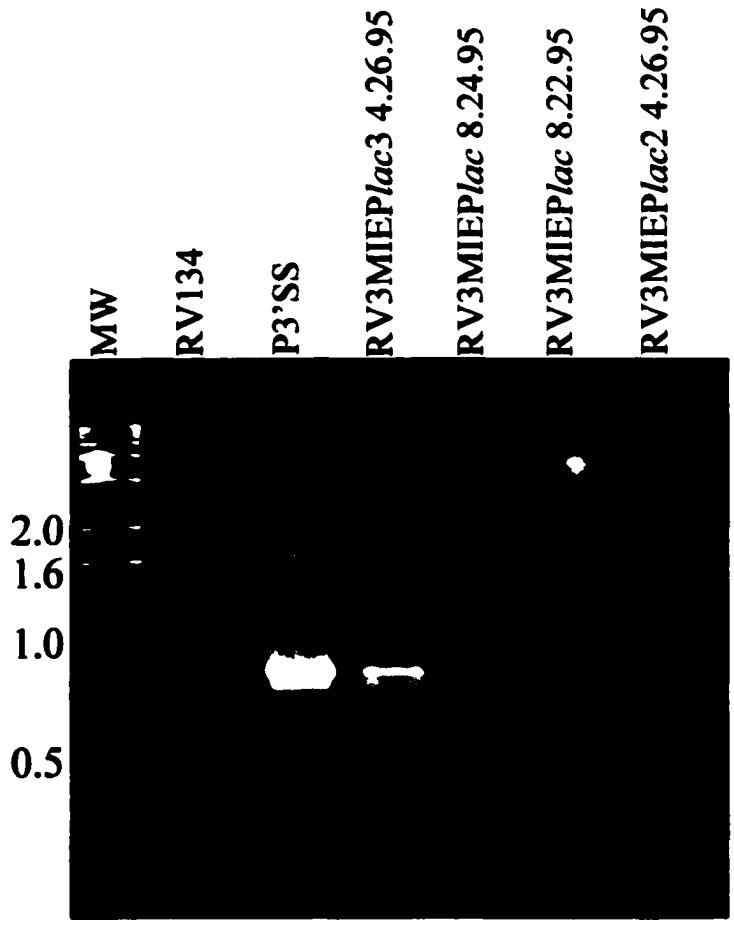


FIG. 5. PCR screening for RV*lac*. Primary stocks resulting from the cotransfection of RV134 DNA and pRVMIEP*lac i* were screened by PCR using *lac i* specific primers (Materials and Methods). HFF cells were infected with the primary stocks. At 72 hs post infection, the infected cells were harvested, and total cell DNA was isolated. The extracted DNA was PCR amplified using the *lac i* specific primers. The PCR products were analyzed by electrophoresis in 1.2 % agarose, and visualized by ethidium bromide staining. The numbers on the left represent molecular weight markers (MW lane) in kilobases. RV134 DNA was used as negative control. Plasmid p3'SS (which contains the *lac i* gene) was used as positive control. Four primary stocks (designated RV3MIEP*lac*, followed by sample number and date of harvest of the stock) were screened.

became visible. At that time, cells were overlayed as described above, with the addition of 75 ug per ml of X-glu. After 3 days, wells with a white or clear overlay were considered negative for β-glucuronidase activity. Four individual clear plaques were isolated, and high titer virus stocks were prepared for the four isolates. These recombinant viruses were designated RV*lac* 1, RV*lac* 2, RV*lac* 3, and RV*lac* 4.

Genetic characterization of recombinant HCMV containing the *lac i* gene (RV*lac*)

To confirm recombination of the *lac i* gene into the viral genome of the white plaque phenotype viruses isolated, as well as *lac i* gene insertion into the appropriate locus, RV*lac* DNA was analyzed by Southern blot. Total cell DNA was prepared from RV*lac*-infected HFF cells (using the four RV*lac* isolates), and was digested with *Hind*III. The resulting DNA fragments were subjected to electrophoresis in 0.8% agarose, transferred to a nylon membrane and hybridized to DIG-labeled probes (see Materials and Methods). The probes used were the HCMV AD169 *Hind*III-X probe (5.2 kb) (which spans the region US7 through US11) (see Figure 4), and a *lac i* probe, a 1.6 kb fragment containing the complete *lac i* coding sequence. Figure 6A shows that *Hind*III digestion of RV*lac* DNA (containing the MIEP-*lac i* gene *Hind*III cassette) resulted in the generation of two fragments (1.8 kb and 3.4 kb) derived from the *Hind*III X region. This contrasts with the single 5.2 kb fragment derived from AD169 DNA digested with *Hind*III, which hybridized to the *Hind*III X probe. The new banding pattern obtained with the recombinant virus suggests the presence of at least one additional *Hind*III site within the *Hind*III X region, suggesting that the MIEP-*lac i* gene *Hind*III cassette was inserted in the appropriate locus (US9-US10 intergenic region). The presence of a 2.4 kb fragment

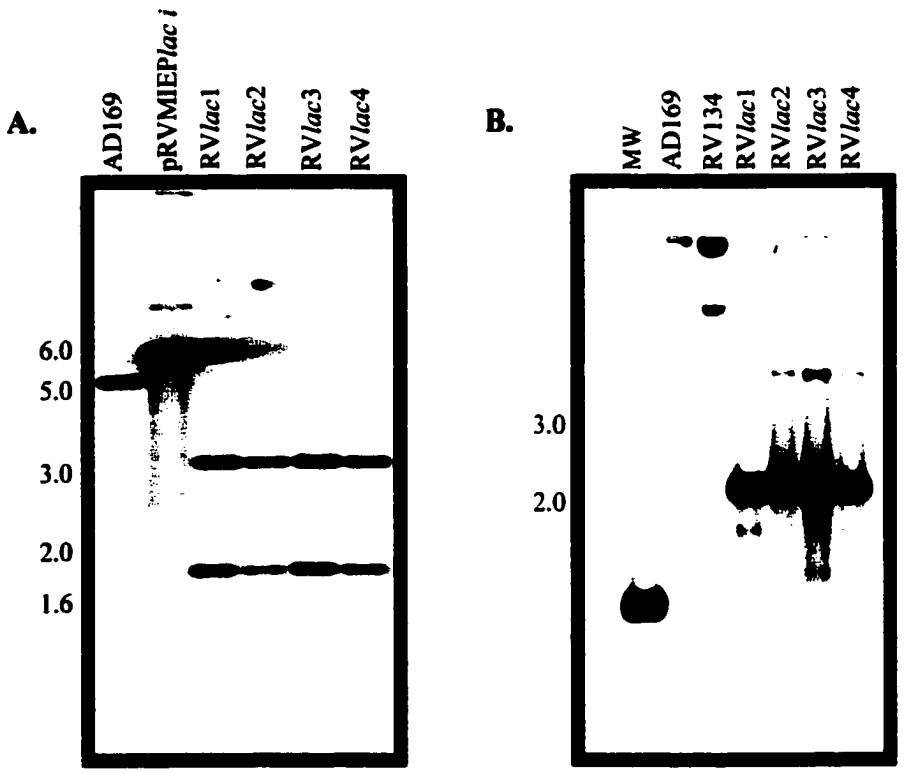


FIG. 6. Genetic characterization of RV*lac* by Southern blot analysis. To confirm the *lac i* gene insertion in place of the ß-glu gene in RV134, total cell DNA was prepared from RV*lac*- or AD169-infected HFF cells, and subsequently digested with *Hind*III. The resulting DNA fragments were subjected to electrophoresis in 0.8 % agarose, transferred to a nylon membrane, and fixed. The DNA in the blot was hybridized to an AD169 *Hind*III X DIG-labeled probe (Materials and Methods). This 5.2 kilobase (kb) probe spans the US7 through US11 region of the AD169 genome, and was used in the Southern blot analysis depicted in panel A. Panel B shows the results of Southern blot analysis using a 1.6 kb *lac i* gene DIG-labeled probe (comprising the entire coding sequence of the *lac i* gene). The numbers on the left of the blots represent molecular weight markers (expressed in kb). AD169 and RV134 DNAs were used as negative controls for *lac i* gene-specific hybridization. MW lane in panel B: molecular weight markers.

hybridizing to the *lac i* probe (shown in Figure 6B) confirms the presence of the *lac i* gene in the viral genome. The results obtained using both probes were identical for the four isolates of RV*lac*.

Lac repressor expression in RV*lac*-infected cells

To determine if the *lac* repressor protein was expressed in RV*lac*-infected cells, western blot analysis was conducted as described in Materials and Methods, using a rabbit anti-*lac i* polyclonal serum. HFF cells were infected with the four RV*lac* isolates at an MOI of 2. As a negative control for *lac* repressor expression, cells were infected with RV134 at an equivalent MOI. Infected cells were harvested at 24 hs after infection, and cell extracts were prepared for western blot analysis. The results obtained (Figure 7A) using the four RV*lac* isolates indicated that these viruses are capable of expressing significant amounts of the 37 kD *lac* repressor polypeptide. This figure illustrates *lac* repressor expression at 24 hs post infection. In order to address expression throughout the course of infection, an experiment was conducted in which HFF cells were infected with RV*lac* 1, and were harvested at 24, 48, and 72 hs after infection, for western blot analysis. As negative controls, mock and RV134 infections were performed similarly. Figure 7B shows that the repressor is expressed throughout the course of infection, with levels increasing at 48 and 72 hs. At those times after infection, low levels of two smaller bands were observed. These polypeptides increased at later times of infection, and likely represent degradation products of the 37 kD repressor polypeptide. RV*lac* 1 was used in all the subsequent experiments in this study, and is referred to as RV*lac*.

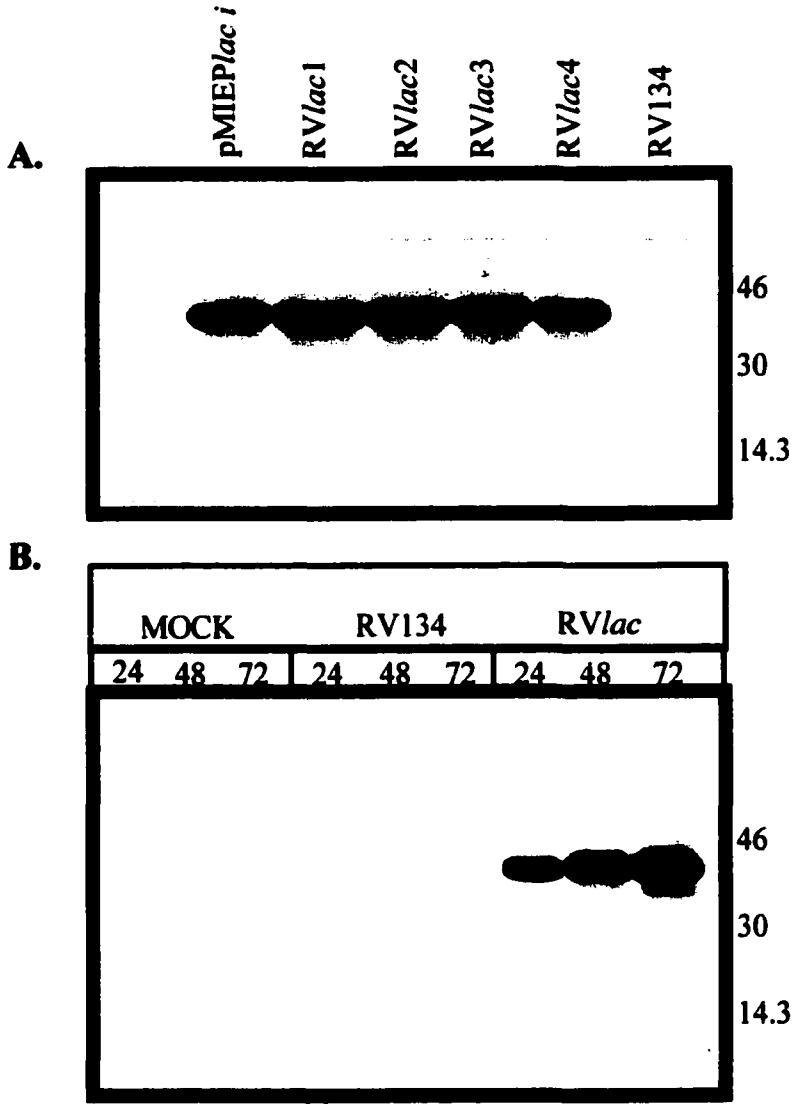


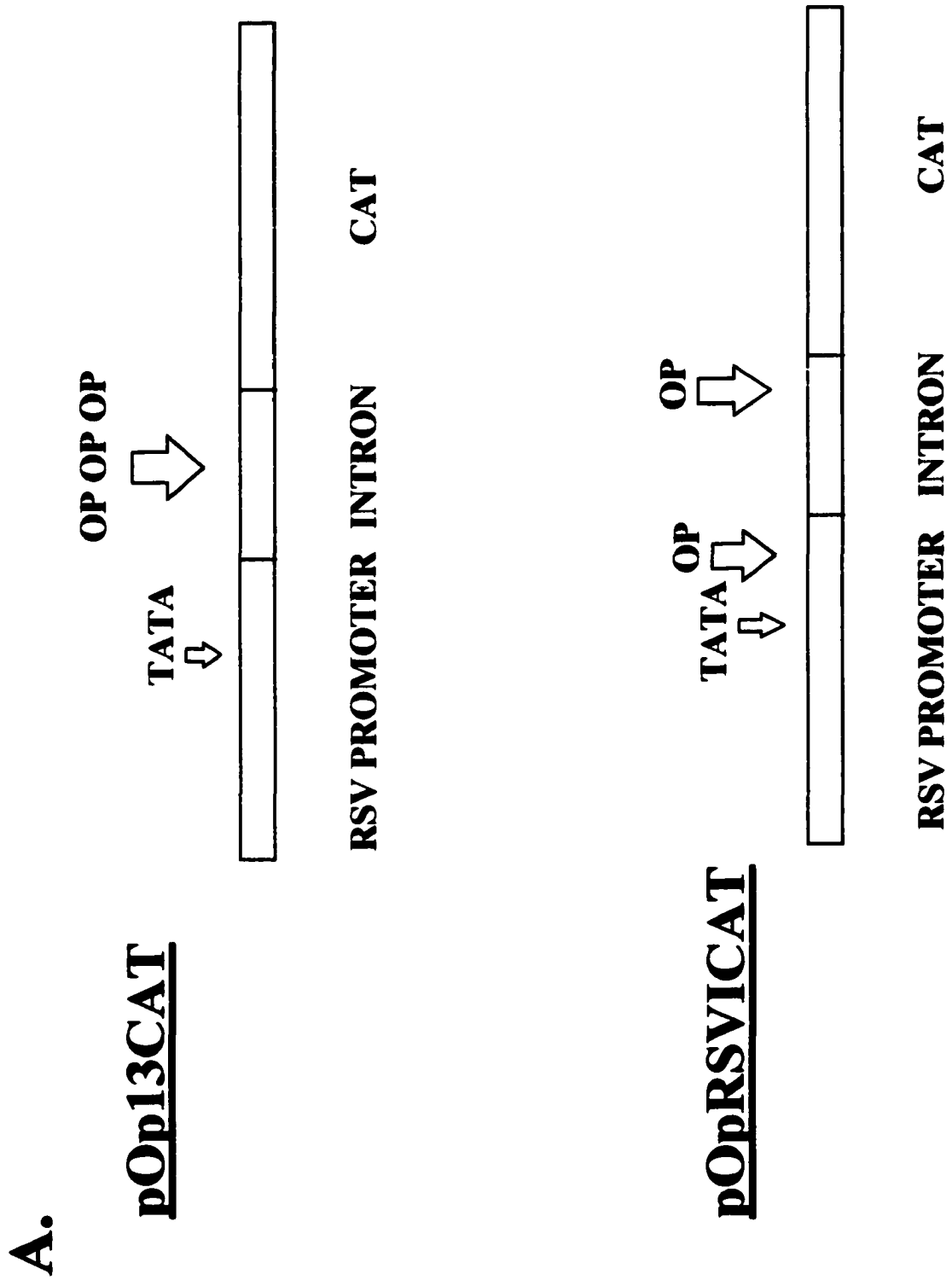
FIG. 7. Expression of the *lac i* gene product in RV*lac*-infected cells. (A) HFF cells were infected with four isolates of RV*lac* at 2 PFU per cell. Cells were harvested at 24 hs, and cell extracts were prepared for western blot analysis. As a positive control, *lac i* gene expression in COS cells transfected with pMIEP*lac i* was included in the analysis. A RV134-infected cell extract was used as negative control. (B) *Lac i* gene expression was measured in cells infected with RV*lac*1 at 24, 48, and 72 hs post infection. Cells that were mock infected, or infected with RV134 and harvested at the indicated times were used as negative controls. The numbers on the right of the blots represent molecular weight markers in kilodaltons.

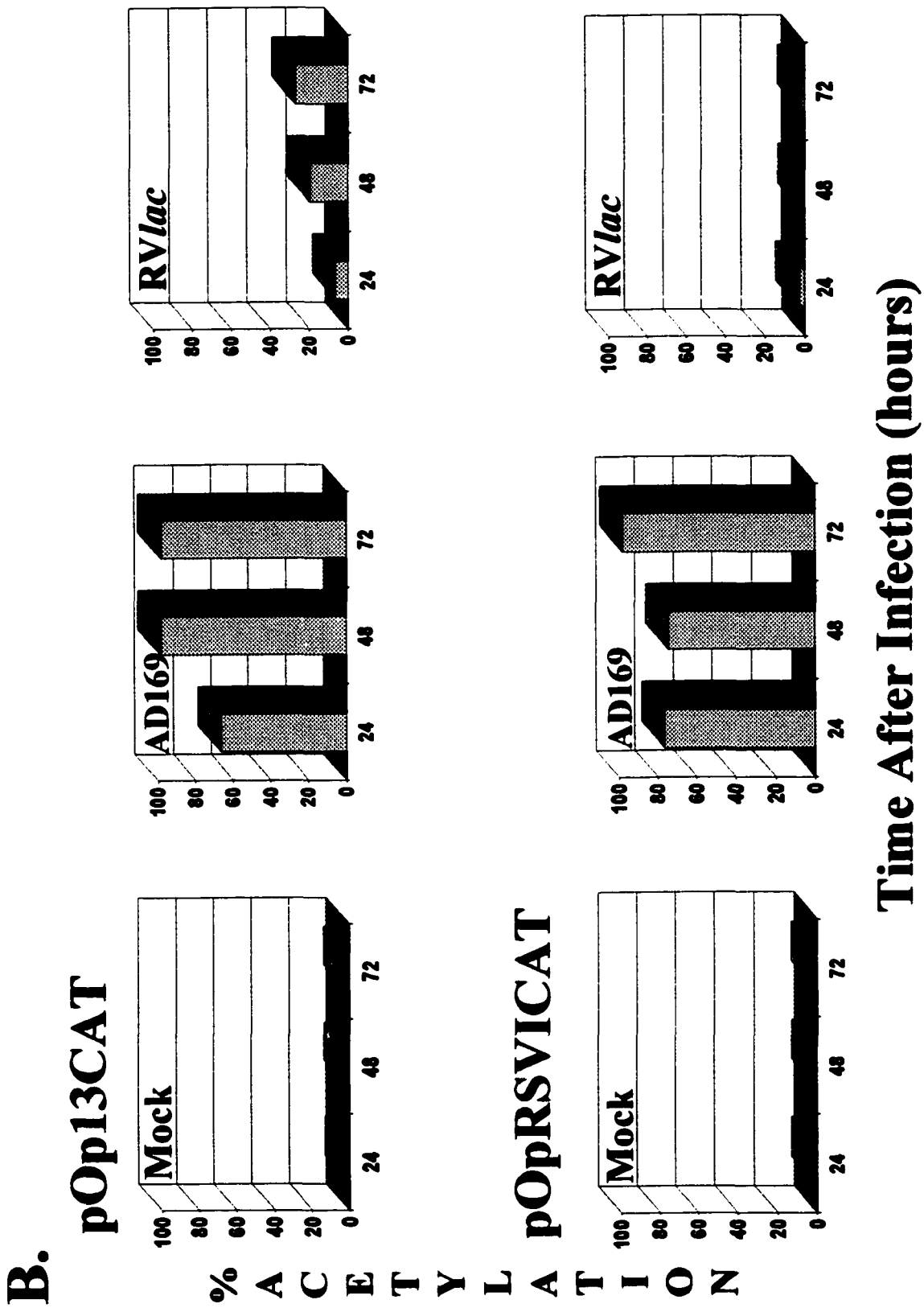
Biological function of the *lac* repressor expressed from RV*lac*

Before addressing the response of HCMV-specific, operator-containing promoters to repression by RV*lac* (see specific objectives in Chapter II), the *lac* repressor protein produced in RV*lac*-infected cells was tested for its repression capabilities, using operator-containing promoters that differ in the number and relative positions of the operator sequences (pOp13CAT and pOpRSVICAT, shown in Figure 8A). These reporter plasmids were obtained along with the plasmid encoding the *lac i* gene (p3'SS) as part of the *Lac* Switch inducible mammalian expression system (Stratagene, La Jolla, CA) (see Materials and Methods).

Briefly, 10 ug of a CAT reporter plasmid containing *lac* operator sequences in the RSV promoter (either pOp13CAT or pOpRSVICAT) (see Figure 8A) were transfected into HFF cells using the DEAE-Dextran transfection protocol described in Materials and Methods. At 24 hs after transfection, cells were infected with RV*lac* at 2 PFU per cell. As controls, mock infection or infection with wild type virus AD169 at an equivalent MOI were performed. At 24, 48 and 72 hs post infection, cells were harvested and assayed for CAT activity as previously described (Depto and Stenberg, 1989) (see Materials and Methods). Figure 8B shows that infection of transfected cells with RV*lac* resulted in a highly significant reduction in CAT activity (80% for pOp13CAT and 100% for pOpRSVICAT), as compared with CAT activity in cells infected with wild type virus AD169. It is worth noting that a more effective repression of promoter activity resulted when the operator sequence was adjacent to the TATA box (in pOpRSVICAT). These results suggest that sufficient amounts of functional *lac* repressor were present in the infected cells to significantly repress expression of the operator-containing promoters.

FIG. 8. (A) Schematic diagram of reporter constructs pOp13CAT and pOpRSVICAT. *Lac* operator-containing reporter constructs pOp13CAT and pOpRSVICAT contain the Rous sarcoma virus (RSV)-Long Terminal Repeat (LTR) promoter, the *lac* operator sequence 5'TGTGGAATTGTGAGCGCTCACAATTCCACAGTC3' inserted at various positions, and the chloramphenicol acetyl transferase reporter gene (from Stratagene *Lac* Switch inducible mammalian expression system). (B) Analysis of the biological function of the *lac* repressor produced from RV*lac*. A representative experiment is shown. HFF cells were transfected with 10 ug of pOp13CAT or pOpRSVICAT. At 24 hs after transfection, cells were mock infected, or infected with AD169 or RV*lac* at 2 PFU per cell. Infected cells were harvested at 24, 48, and 72 hs post infection, and cell extracts were assayed for CAT activity. Percent acetylation was quantitated by phosphorimager analysis.





To determine if IPTG is capable of reversing the observed repression, and to establish the optimal concentration and time of exposure to IPTG for a maximal effect, an IPTG titration curve was performed in which CAT activity was expressed as a function of IPTG concentration. HFF cells were transfected with 10 ug of the reporter plasmid pOpRSVICAT (see Figure 8A), using the DEAE-Dextran transfection protocol described in Materials and Methods. At 24 hs after transfection, cells were infected with RV*lac* or AD169, at 2 PFU per cell, and 18 hs after infection, IPTG was added to the media at 0, 10, 25, 50 and 100 uM. 7 hs after the addition of IPTG, cells were harvested and assayed for CAT activity as described previously. The results of these experiments are shown in Figure 9. Consistent with the experiments shown in Figure 8, repression in RV*lac* infected cells in the absence of IPTG was highly significant (6.51% acetylation) when compared with the level of promoter activation in AD169-infected cells at all concentrations of IPTG tested (98% acetylation, data not shown). A 4-fold increase in CAT activity was observed in cells infected with RV*lac* in the presence of 100 uM IPTG, as compared to the activity in cells infected with RV*lac* in the absence of IPTG. The level of IPTG mediated derepression observed (about 40%), although significant, is probably insufficient to accurately assess conditional expression of an HCMV promoter in the context of the viral genome. Therefore, in order to obtain a more effective derepression, higher concentrations of IPTG, as well as different experimental conditions, such as time of exposure to IPTG, were also tested in subsequent experiments in this study (see corresponding section below).

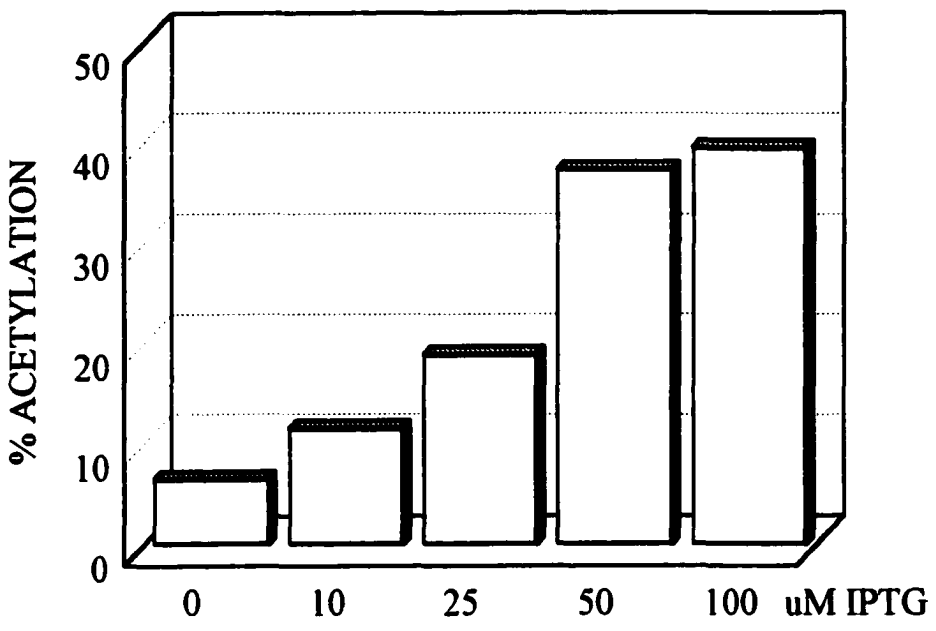


FIG. 9. IPTG induction of reporter CAT activity in RV*lac*-infected cells. HFF cells were transfected with 10 ug of reporter plasmid pOpRSVICAT, and at 24 hs after transfection, cells were infected with AD169 (data not shown), or RV*lac*, at 2 PFU per cell. 18 hs after infection, IPTG was added to the media at 0, 10, 25, 50, and 100 uM. 7 hs after the addition of IPTG, cells were harvested and assayed for CAT activity. Percent acetylation was quantitated by phosphorimager analysis. In AD169-infected cells, 98% acetylation was observed for all the IPTG concentrations tested (data not shown). A representative experiment is shown.

Insertion of a *lac* operator sequence into an HCMV nonessential gene promoter

Central to the objectives of this project was to test the conditional expression of an HCMV promoter utilizing the *lac* repressor-based system. The main aim of this study was to address all the fundamental aspects of the system using an operator-containing HCMV promoter and RV*lac* in transient assays, before assessing the feasibility of this approach in the context of the HCMV genome. To this end, an operator-containing HCMV promoter was constructed and characterized. The operator-containing promoter(s) was cloned into a CAT reporter construct for analysis of expression in transient assays (see corresponding section below). In addition, this promoter(s) was inserted into the HCMV genome in the endogenous locus, with or without the *lac i* gene adjacent to it, for future analysis of conditional expression in the context of the virus. The promoter of a nonessential gene was chosen because it would be suitable to test conditional gene expression in the context of the virus without affecting the production of progeny virus. In other words, the lack of expression of the operator-containing promoter in the repressed state would not affect the ability of the virus to replicate in culture. In this way, propagation of this recombinant virus would not be dependent on IPTG.

The US9 gene was chosen as a target for insertion of a *lac* operator sequence into its promoter because the US9 gene product is known to be dispensable for growth of the virus in tissue culture (Jones and Muzithras, 1992). Thus, a recombinant virus susceptible to conditional expression of the US9 gene offers the advantage to test a *lac*-regulated HCMV promoter in the context of a natural infection, and to perform experiments to

establish optimal conditions for IPTG derepression. In addition, the US9 gene is located immediately adjacent to the site of the *lac i* gene insertion (see Figure 4). This permits the insertion of the *lac i* gene and the operator-containing US9 promoter into the virus in a single recombination event, and the blue-white plaque selection method can be used for the screening of the double insertion.

Several reports have addressed the insertion of single or multiple *lac* operator sequences into various target promoters (Hu and Davidson, 1987), (Rodriguez and Smith, 1990). The obvious advantage of using multiple copies of the operator sequence is that higher levels of repression can be obtained (Rodriguez and Smith, 1990). However, it has also been observed that this is accompanied by a reduced ability of the inducer (IPTG) to restore promoter activity to wild type levels. Thus, the insertion of a single, 24-base *lac* operator sequence (5'GAATTGTGAGCGGAT AACAATTTC 3') was favored in this study.

The approach for generating an operator-containing US9 promoter initially involved the insertion of one *lac* operator sequence into the leader of the US9 gene at a unique *Sst*II site at +40 (see Figure 10). This first step allowed for a rapid and convenient cloning of the operator sequence. An oligonucleotide containing the *lac* operator sequence was inserted into the US9 leader in pUS9CAT (Figure 11) (see Materials and Methods).

The position of the operator sequence has been shown to be critical for effective repression mediated by the *lac* repressor (Rodriguez and Smith, 1990), (Hu and Davidson, 1987). Operator positions closer to the TATA box seem to result in higher

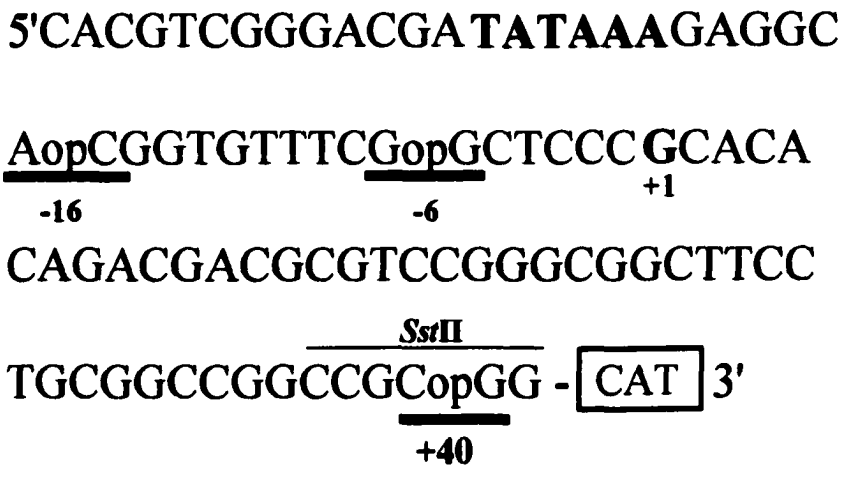
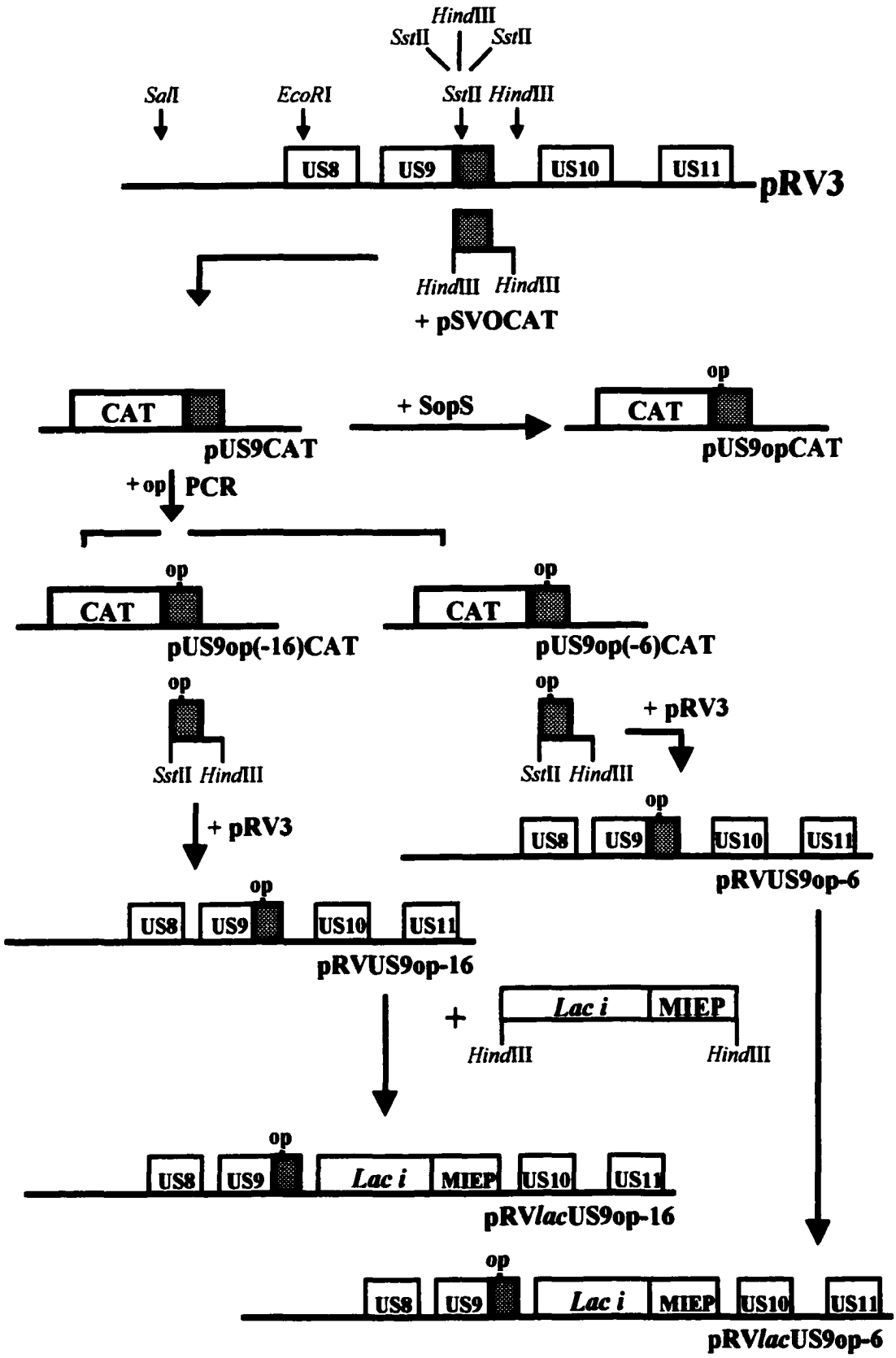


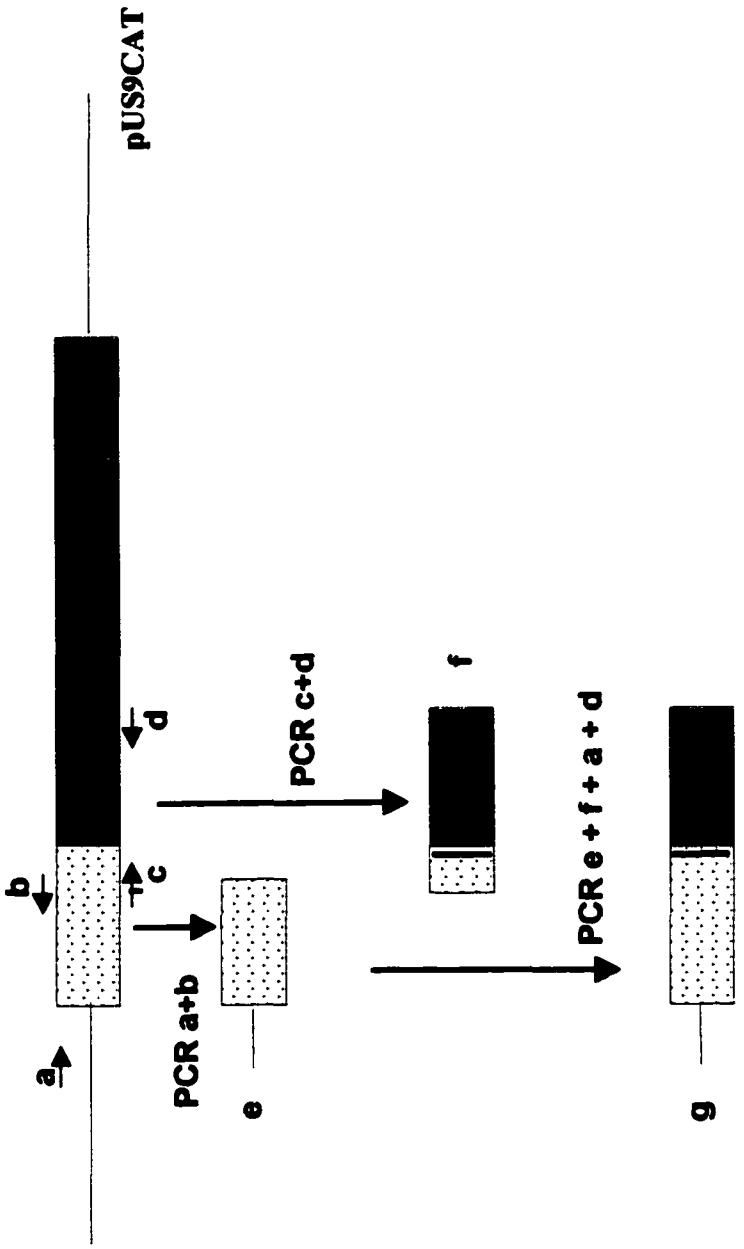
FIG. 10. Insertion of *lac* operator sequences into the HCMV US9 promoter. Partial sequence of the US9 promoter-leader upstream of the CAT gene in the reporter construct pUS9CAT. Op represents the *lac* operator sequence 5'GAATTGTGAGCGGATAACAATTTC3', cloned into a unique *Sst*II site at +40 in the leader sequence to generate pUS9opCAT, or inserted by an overlapping PCR insertional mutagenesis protocol at the positions indicated -16 and -6, to generate pUS9op(-16)CAT, and pUS9op(-6)CAT, respectively. The TATA box and the CAP site (G at +1) are indicated in bold. The CAT reporter plasmids containing these US9 variant promoters are represented in Fig. 11.

FIG. 11. Schematic diagram of operator-containing plasmid constructions. A unique *Hind*III site upstream of the US9 promoter (gray box) in the recombination vector pRV3 was generated by inserting a *BamH*I-*Xba*I-*Hind*III linker (restriction enzyme recognition sequences are represented in bold in the linker sequence below) into a unique *Apa*I site in the US9-US10 intergenic region (5'**GGGATCCGCTCTAGAGCAAGCTT**GGGCC3'). A unique *Sst*II site at +40 in the US9 leader sequence was modified with the insertion of a *Hind*III site. Plasmid pUS9CAT was generated by cloning the US9 promoter-leader (-269 to +41) *Hind*III fragment obtained from pRV3, into a unique *Hind*III site upstream of the chloramphenicol acetyl transferase (CAT) gene in pSVOCAT. Plasmid pUS9opCAT was generated by inserting the *lac* operator sequence 5'GAATTGTGAGCGGATAACAATTTC3' (op) flanked by *Sst*II linkers (SopS) into a unique *Sst*II site at +40 in the leader of the US9 promoter-leader sequence in pUS9CAT. Using an overlapping PCR mutagenesis strategy, the *lac* operator sequence was inserted at -16 or -6 in the US9 promoter using pUS9CAT as template, to generate pUS9op(-16)CAT or pUS9op(-6)CAT, respectively. The US9 promoters containing the operator at -16 or -6 were cloned as *Hind*III-*Sst*II fragments into pRV3 to replace the wild type promoter in pRV3, to generate the recombination vectors pRVUS9op-16 and pRVUS9op-6, respectively. The MIEP-*lac i* gene expression unit was cloned as a *Hind*III fragment (derived from pRVMIEP*lac i*), into the unique *Hind*III site in the US9-US10 intergenic region in pRVUS9op-16 and pRVUS9op-6, to generate pRV*lac*US9op-16 and pRV*lac*US9op-6, respectively. Transcription from all the constructs in this figure proceeds from right to left. The empty boxes represent HCMV open reading frames of the unique short component of the viral genome US8 through US11, the CAT gene, the HCMV major immediate-early promoter (MIEP), and the *lac i* gene.



levels of repression, both for the *lac* repressor-based system (Brown et al., 1987), and the tetracycline repressor-based system (Kim et al., 1995). Therefore, strategies were developed for the insertion of the operator sequence at different sites in proximity to the TATA box within the US9 promoter. These strategies were based on modifications of PCR protocols previously described (Zhang and Moss, 1991). A DNA fragment containing the US9 promoter-*lac* operator-CAT gene was assembled from PCR products (see Figure 12). As mentioned previously, insertion of the operator sequence in a US9 promoter-CAT construct would allow for assessing the expression of the operator-containing promoters in transient assays, before analyzing these variant promoters in the context of the virus. Two PCR reactions were performed to amplify the 5'half or the 3'half of the initial US9 promoter-CAT gene DNA fragment (obtained from pUS9CAT), utilising two different primer pairs specifically designed to target the operator sequence between the TATA box and the CAP site. This strategy was conducted in order to insert the operator sequence at two alternative positions in the US9 promoter [-16 or -6, relative to the transcription start site (+1)] (see Figure 10). For each operator location, the PCR reactions rendered two overlapping products, which were then mixed together and PCR amplified using the 5'-most and 3'-most primers (see Figure 12). Because a *lac* operator sequence was present in one of the internal primers, the resulting PCR product contained the *lac* operator in the US9 promoter upstream of the CAT gene. The final PCR product was digested with *Hind*III, purified by electrophoresis in 5% polyacrylamide, and ligated back into a unique *Hind*III site upstream of the CAT gene in pUS9CAT, to generate pUS9op(-16)CAT (operator at -16), and pUS9op(-6)CAT (operator at -6) (Figure 11).

FIG. 12. Overlapping PCR mutagenesis strategy. The *lac* operator sequence 5'GAATTGTGAGCGGATAACAATTTC3' was inserted between the TATA box and the CAP site in the HCMV US9 promoter (see Fig. 10). This was done by PCR amplifying a DNA fragment containing the US9 promoter (dotted box) and the CAT gene (stripped box), using pUS9CAT as template (see Fig. 11). Two separate PCR reactions were conducted utilizing primers a+ b, or c+ d, to generate two overlapping PCR products (e or f, respectively) (see Materials and Methods for actual primers used). e and f were mixed and PCR amplified using primers a+ d, to generate the final mutagenized product g (containing the *lac* operator sequence). The vertical bar in primer c, and in the DNA fragments f and g, represents the *lac* operator. Two alternative insertion sites were developed: -16 or -6, relative to the transcription start site.



The presence of the *lac* operator sequence in these constructs was confirmed by DNA sequencing.

Generation of recombinant viruses using the *lac* repressor-operator system

Insertion of the *lac i* gene and the *lac* operator- containing US9 promoter into the HCMV genome

In order to utilise the *lac* repressor and the operator-containing US9 promoter to analyse conditional expression of the US9 gene in the context of the HCMV genome, the *lac i* gene and the operator-containing US9 promoter needed to be inserted into the viral genome in a manner that resulted in 1) adequate levels of *lac* repressor expression, 2) appropriate operator location, and 3) a measurable operator-repressor interaction in the context of viral infection. The *lac* operator sequence and the *lac i* gene can be inserted into a single recombinant HCMV, or into two recombinant HCMV that can be used in coinfection experiments. In this study, the complete expression system (the *lac i* gene, and the operator-containing US9 promoter) was inserted into a single recombinant HCMV. Because the US9 gene is located next to the site of insertion of the MIEP-*lac i* gene cassette (see Figure 4), the introduction of both the operator-containing US9 promoter in place of the endogenous promoter, and the *lac i* gene could be accomplished in a one step recombination event. Thus, the blue-white plaque phenotype screening method could be utilized to identify HCMV recombinants with the double insertion. Because the operator insertion may affect sequences that are relevant for promoter expression in the context of the viral genome, [these observations have been reported

with other promoters (Kohler et al., 1994)], a control recombinant virus in which only the operator-containing US9 promoter was inserted upstream of the endogenous US9 gene in RV134 was generated. Both the operator at -16 and the operator at -6 were utilized for this purpose.

Recombinant HCMV containing an operator sequence at -16 or -6 in the US9 promoter, with or without the *lac i* gene (MIEP-*lac i* gene cassette), were constructed by the replacement recombination strategy described in previous sections for the generation of RV*lac*. The strategy involved the insertion of the *lac i* gene in place of the β-glu gene in the RV134 genome (see Figure 4), as well as the replacement of the endogenous US9 promoter with the operator-containing US9 promoters. The recombination plasmids used were pRVUS9op-16 or pRVUS9op-6 (Figure 11), which were derived from pRV3 by replacing the US9 promoter with the operator-containing promoters from pUS9op(-16)CAT and pUS9op(-6)CAT, and pRV*lac*US9op-16 or pRV*lac*US9op-6 (Figure 11), which resulted from the insertion of the MIEP-*lac i* gene cassette into pRVUS9op-16 and pRVUS9op-6, respectively (see Materials and Methods).

In addition, a control recombinant virus (RV0) was generated. This virus was constructed by recombination between RV134 DNA and the recombination vector pRV3, which contains no operator or *lac i* gene insertions. The purpose for constructing this recombinant virus was to control for the impact of the linker sequences between US9 and US10 (see *Hind*III site in Figure 3) on US9 gene expression.

The recombination plasmids were linearized with *Sal*I, and subsequently cotransfected with RV134 DNA into HFF cells, using the calcium phosphate precipitation protocol described. 8 ug of RV134 DNA and 1-2 ug of linearized recombination vector

were used. When transfected cells reached 100% CPE, primary stocks of recombinant virus were harvested.

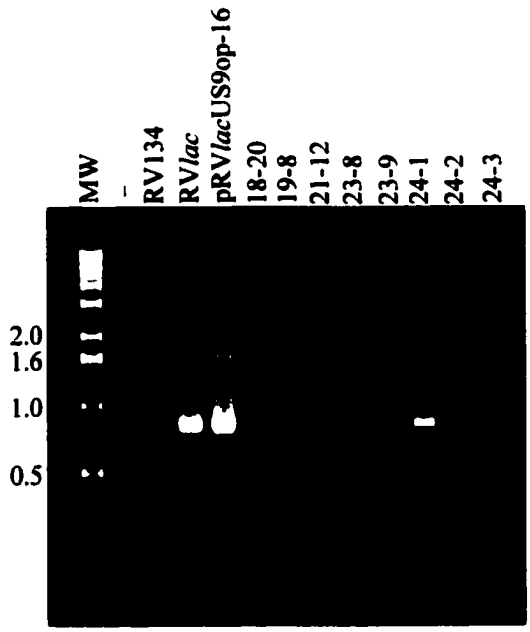
Screening for HCMV recombinants

In order to identify primary stocks that contained recombinant viruses, the screening was done by PCR, using *lac i*-specific and US9 promoter-specific primers (see Materials and Methods). Briefly, HFF cells were infected with the primary stocks, and infected cell lysates were obtained 72 hs. post infection for total cell DNA extraction. Subsequently, the DNA was amplified by PCR, using forward and reverse primers specific for the *lac i* gene or the US9 promoter. The PCR products were subjected to electrophoresis in agarose or polyacrylamide gels, and visualized by staining with ethidium bromide. Figure 13 shows the PCR products obtained using the *lac i*-specific primers to screen 18 primary stocks of virus derived from the cotransfection of RV134 and pRV*lac*US9op-16 or pRV*lac*US9op-6. A band of 835 nucleotides could be detected as the PCR amplification product using the DNA from cells infected with several of the stocks, suggesting the presence of recombinant virus containing the *lac i* gene. Because some degree of nonspecific DNA amplification was observed in these experiments, Southern and western blot analyses were conducted to confirm these results (see below).

US9 promoter-specific primers were utilized to screen for operator-containing recombinant virus in the stocks derived from the cotransfection of RV134 with pRVUS9op-16, or pRVUS9op-6. These primers amplify 318 nucleotides within the US9 promoter. The presence of the 24 base-operator sequence resulted in the amplification of a larger DNA fragment (342 nucleotides) relative to the wild type US9 promoter.

FIG. 13. PCR screening for RV*lac*US9op-16 and RV*lac*US9op-6. Primary stocks resulting from the cotransfection of RV134 DNA and pRV*lac*US9op-16 or pRV*lac*US9op-6 (see Fig. 11) were screened by PCR using *lac i* specific primers (Materials and Methods). HFF cells were infected with the primary stocks, and at 72 hs after infection, total cell DNA was prepared from the infected cells. The DNA was PCR amplified using the *lac i* specific primers. The PCR products were subjected to electrophoresis in 1.2 % agarose, and visualized by ethidium bromide staining. The numbers on the left represent molecular weight markers (in lanes MW) in kilobases. No DNA or RV134 DNA were used in PCR reactions as negative controls. RV*lac* DNA and the recombination plasmid pRV*lac*US9op-16 were used as positive controls. 18 primary stocks were screened. The nomenclature represents transfection experiment number, followed by sample number. Panel C contains repeats of the PCR screening reactions for stocks 29-1 and 24-1, which served as controls.

A.



B.

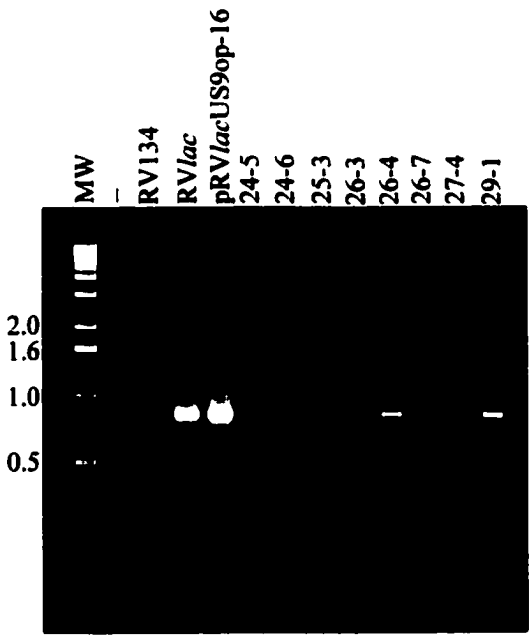


Figure 13. Continued

C.

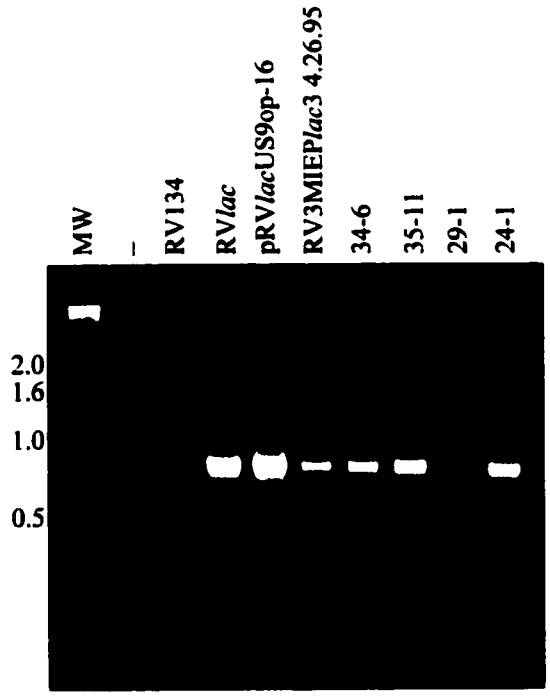
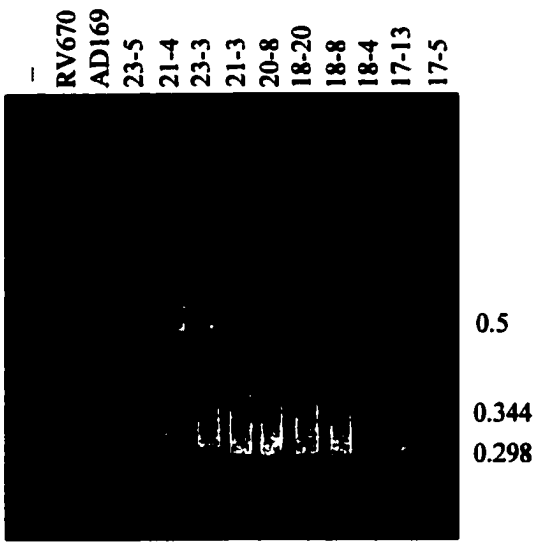


FIG. 14. PCR screening for RVUS9op-6 (panel A) and RVUS9op-16 (panel B). Primary stocks resulting from the cotransfection of RV134 DNA and pRVUS9op-6 or pRVUS9op-16 (see Fig. 11) were screened by PCR using US9 promoter specific primers (Materials and Methods). HFF cells were infected with the primary stocks, and 72 hs after infection, cells were harvested and total DNA was isolated. The DNA was PCR amplified using the US9 promoter specific primers. The resulting products were analyzed by electrophoresis in 5 % polyacrylamide or 1.2 % agarose, and visualized by ethidium bromide staining.The numbers on the right in panel A and on the left in panel B represent molecular weight markers in kilobases (MW lane in panel B). No DNA or RV670 (a recombinant HCMV deleted of the US9 gene) DNA were used as negative controls. AD169 DNA was used as positive control for the wild type US9 promoter. In panel B, RV134 and RV*lac* DNAs were also used as positive controls for the wild type US9 promoter. The positive control for operator-containing US9 promoter 14B3 in panel B is a recombinant virus isolated from stock 21-4 (panel A) which contains the operator sequence at -6 in the US9 promoter. The nomenclature for the primary stocks screened includes transfection experiment number followed by sample number. A band indicating the presence of the operator sequence in the US9 promoter was detected for stocks 21-4 (panel A), 34-3 and 34-4 (panel B).

A.



B.

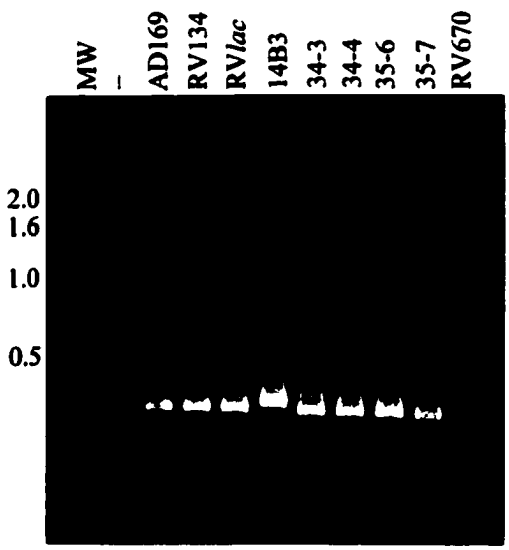
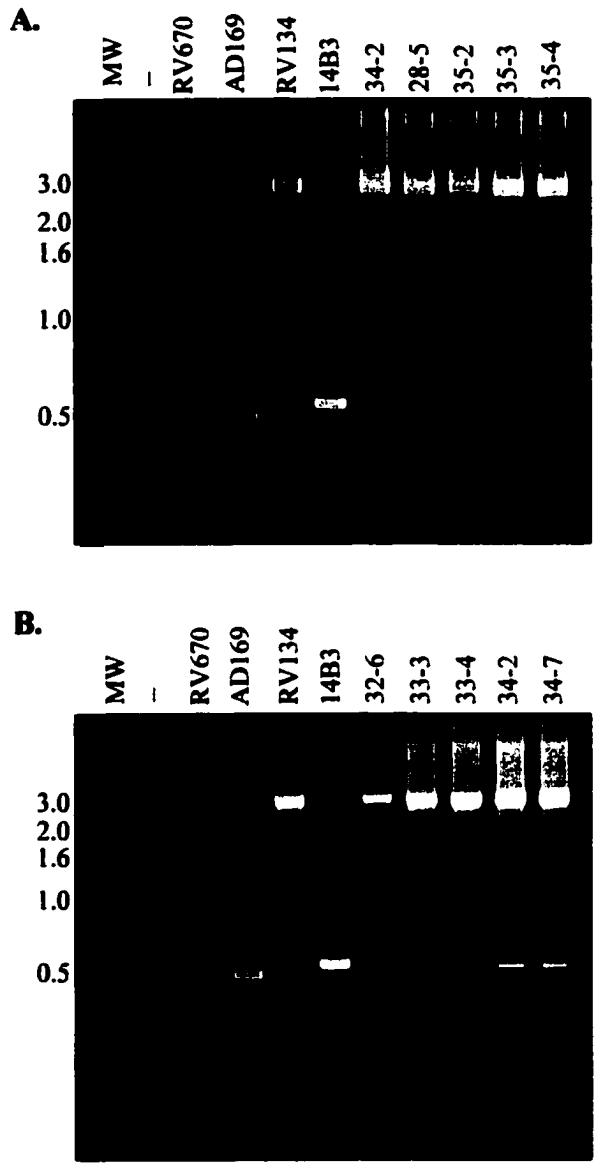


Figure 14A-B shows that three stocks were identified to contain operator-containing recombinant virus (21-4, 34-3, and 34-4), as evidenced by the presence of a smaller band (318 nucleotides) corresponding to the parental virus present in the stock, and a larger band (342 nucleotides) indicating the presence of operator-containing virus. The nomenclature of the primary stocks reflects transfection experiment number followed by sample number. Stock 21-4 was derived from the cotransfection of RV134 DNA with pRVUS9op-6. Several recombinant viruses were isolated from this stock (see corresponding section below). One of these viruses, 14B3, was used as a positive control for the PCR screening shown in panel B. Stocks 34-3 and 34-4 resulted from cotransfecting RV134 DNA with pRVUS9op-16.

In order to screen for the control virus constructed by cotransfecting RV134 and pRV3 (RV0), another pair of US9 promoter-specific primers were used. These primers (US9 1777: 5'CGATCCCTCCTCCTGATATG3', and US9 2280: 5' CTCGAGAGTACACTTACTGC3') amplified 503 nucleotides upstream of the US9 open reading frame in the wild type virus AD169, and in RV0, but the presence of the β-glu gene in the parental virus RV134 resulted in the amplification of 3,000 nucleotides. Figure 15A-B shows the PCR products obtained when screening primary stocks derived from the cotransfection of RV134 and pRV3. The presence of a band of about 500 nucleotides indicates the presence of recombinant virus (RV0) in stocks 34-2, 28-5, 35-2, 33-3, and 34-7.

To confirm the PCR results, Southern blot analysis was performed using an AD169 *Hind*III X probe, in order to screen primary stocks for RV0, and for *lac* operator-containing viruses, and using a *lac i*-specific probe to identify *lac i*-positive stocks. HFF

FIG. 15. PCR screening for RV0. Primary stocks resulting from the cotransfection of RV134 DNA and pRV3 (see Fig. 11) were screened by PCR using the primers described in Chapter IV. HFF cells were infected with the primary stocks, and 72 hs after infection, cells were harvested and total cell DNA was isolated. The DNA was subsequently PCR amplified. The resulting PCR amplified products were analyzed by electrophoresis in 1.2 % agarose, and visualized by ethidium bromide staining. The numbers on the left represent molecular weight markers in kilobases (MW lanes). No DNA or RV670 DNA were used as templates for PCR reactions that served as negative controls. AD169, RV134 and 14B3 (RVUS9op-6) DNAs were used as positive controls. The nomenclature of the primary stocks was as described in Figs. 13 and 14.



cells were infected with the primary stocks. Infected cell DNA was prepared at 72 hs post infection, subsequently digested with *Hind*III, subjected to electrophoresis in agarose gels, blotted onto nylon membranes, and hybridized to DIG-labeled probes (see Materials and Methods). Figure 16A-B shows that *Hind*III digestion of the DNA derived from the primary stocks identified by PCR to contain *lac* operator-containing recombinant viruses (21-4, 34-3, and 34-4) (Figure 14A-B) resulted in the generation of two fragments derived from the *Hind*III X region (1.8 kb and 3.4 kb), corresponding to recombinant virus DNA, as well as the 7.7 kb fragment corresponding to the *Hind*III X region containing the β-glu gene in the parental virus RV134. These fragments were detected using the *Hind*III X probe. For RV0, Southern blot analysis using the *Hind*III X probe failed to detect the bands corresponding to recombinant virus in the primary stocks. Only the 7.7 kb band, representing parental RV134 DNA was detected (Figure 16C). Possibly, the amount of recombinant virus in the stocks was too low to be detected using this technology, while these same stocks were shown to contain recombinant virus by PCR analysis (Figure 15). On the other hand, the use of the *lac i* probe resulted in the detection of one primary stock (34-6), derived from the cotransfection of RV134 DNA and the recombination plasmid pRV*lac*US9op-16, containing the *lac i* gene (see Figure 17A). This contrasts with the PCR data using the *lac i*-specific primers, where PCR amplification of the DNA derived from several primary stocks showed the presence of a *lac i*-specific product.

In order to identify primary stocks containing *lac* repressor-expressing virus, western blot analysis was conducted. HFF cells were infected with the stocks that had

FIG. 16. Southern blot screening for RVUS9op-6 (panel A), RVUS9op-16 (panel B), and RV0 (panel C). Total cell DNA was prepared from cells infected with RV134, RV*lac*, or the primary stocks previously screened by PCR. The DNA was digested with *Hind*III, and was subjected to electrophoresis in 0.8 % agarose. The DNA fragments were then transferred to a nylon membrane, fixed, and hybridized to the AD169 *Hind*III X probe described in Materials and Methods. The numbers on the right of the blots represent molecular weight markers in kilobases. The expected 1.8 kb and 3.4 kb fragments (which indicate the presence of recombinant virus in the stock) were observed in stocks 21-4, 34-3, and 34-4.

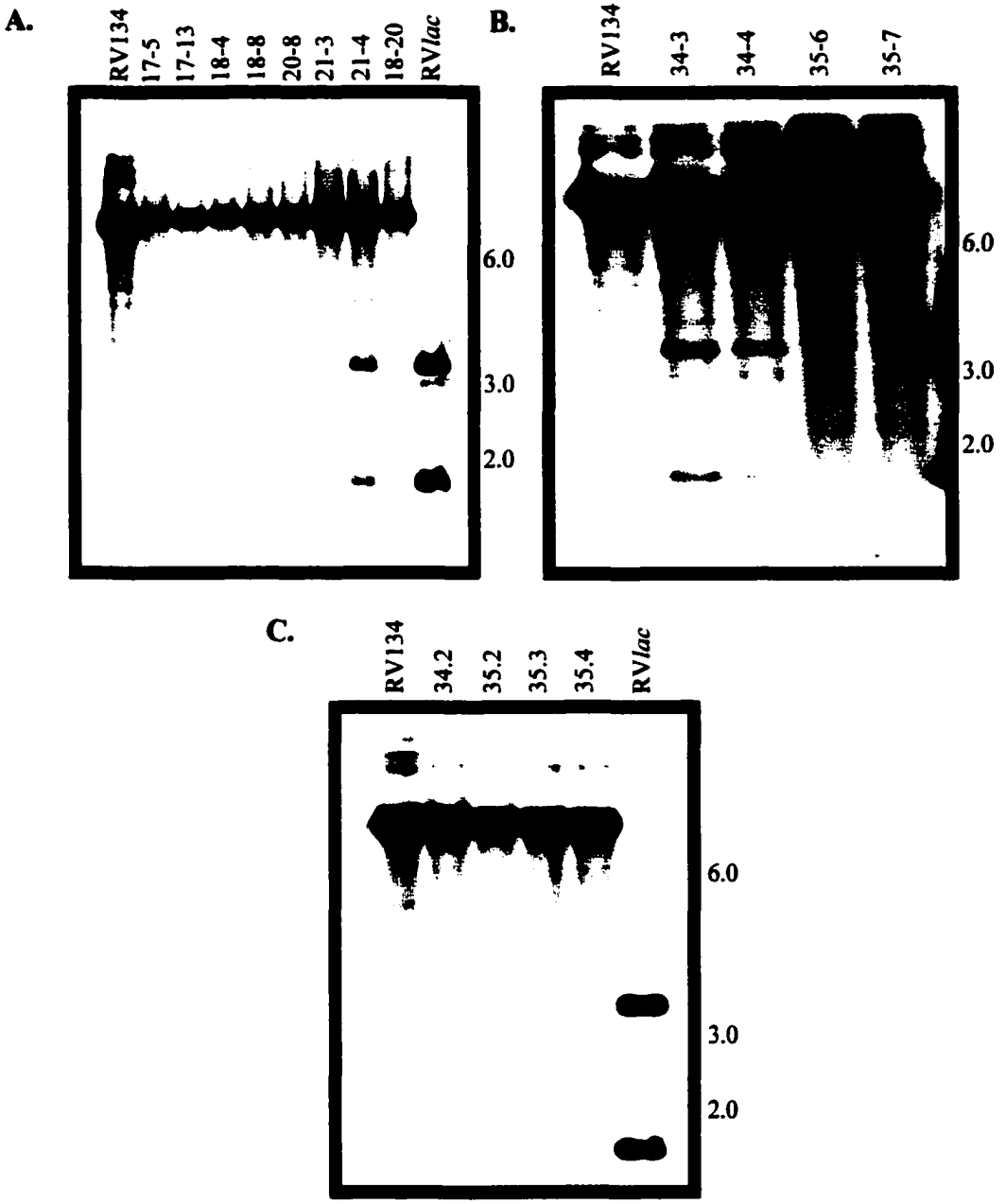
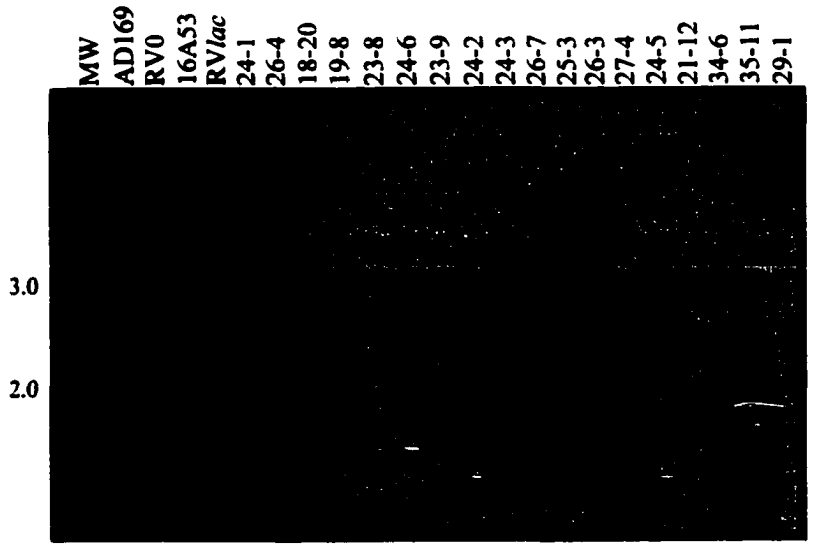
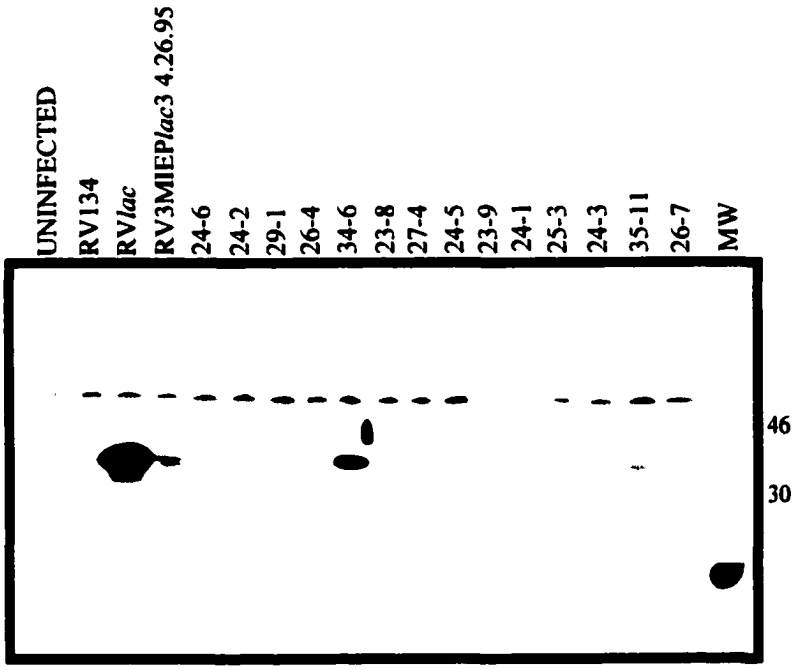


FIG. 17. Southern (panel A) and western (panel B) blot screening for RV*lac*US9op-16. (A) Total cell DNA was prepared from cells infected with AD169, 16A53 (RVUS9op-6, isolated from primary stock 21-4), RV0 (352 3B2) (negative controls), or RV*lac* (positive control), or the primary stocks previously screened by PCR. The DNA was digested with *Hind*III, and was subjected to electrophoresis in 0.8 % agarose. The DNA was then transferred to a nylon membrane, fixed, and hybridized to the *lac i* gene specific, DIG-labeled probe described in Materials and Methods. The numbers on the left of the blot represent molecular weight markers (MW lane) in kilobases. A band of 2.4 kb was detected with the *lac i* probe for stock 34-6, indicating the presence of recombinant virus containing the *lac i* gene in the stock. (B) HFF cell extracts were prepared from uninfected cells, or 24 hs after infection with RV134 (negative control), RV*lac* (positive control), RV3MIEP*lac*3 4.26.95 (see Fig. 5) (positive control), or the primary stocks previously screened by PCR. The cell lysates were subjected to electrophoresis in SDS-polyacrylamide, electroblotted onto a nitrocellulose membrane, and reacted with a rabbit anti-*lac i* polyclonal serum. MW: molecular weight markers, expressed in kilodaltons on the right of the blot. Stock 34-6 contains recombinant virus expressing the *lac* repressor. The nomenclature of the primary stocks in both panels was as described in previous figures.

A.



B.



tested positive for the *lac i* gene by PCR analysis, and 24 hs post infection, cells were lysed and harvested. The cell lysates were subjected to electrophoresis in SDS-polyacrylamide gels, electroblotted onto a nitrocellulose membrane, and reacted with a rabbit anti-*lac i* polyclonal serum. Figure 17B shows that one stock (34-6, which had been identified by Southern blot analysis using the *lac i* probe) contains recombinant virus expressing the *lac* repressor.

These procedures allowed for the identification of primary stocks of virus that contained 1) operator-positive, *lac i*-negative recombinant virus (RVUS9op(-16) [stocks 34-3 and 34-4] and RVUS9op(-6) [stock 21-4]), 2) operator-positive, *lac i*-positive recombinant virus (RV*lac*US9op(-16) [stock 34-6]), and 3) operator-negative, *lac* i-negative recombinant virus (RV0 [stocks 34-2, 28-5, 35-2, 33-3, and 34-7]).

Isolation of HCMV recombinants

Altered plaque phenotype screening was conducted on the primary stocks identified as described above, in order to isolate the recombinant viruses. This screening was performed as described for the isolation of RV*lac*. The recombinant viruses (white plaque phenotype viruses) isolated using these procedures were: 13 isolates of RVUS9 op-6, 6 isolates of RVUS9op-16, and 1 isolate of RV0. In spite of numerous attempts to isolate the double insertion recombinant virus RV*lac*US9op-16 (more than 4,000 individual plaques were picked and screened for the presence of the virus), this virus could not be isolated. Although several white plaques were obtained, these were found to contain false positive recombinant viruses, as determined by Southern and western

blot analyses of 33 isolates. These viruses likely represent deletion mutants involving the β-glu locus as a result of abnormal recombination events. The reason(s) for failing to isolate the double insertion recombinant virus is unknown at this point. One possible explanation is that the presence of both the *lac i* gene and *lac* operator sequence in the recombination vector could result in expression of the *lac* repressor, followed by binding of the *lac* repressor produced to the operator sequence and thereby obstructing or preventing recombination. Thus, only very rarely would appropriate homologous recombination occur to generate the double insertion recombinant virus. The extremely small number of viruses resulting from this rare recombination event would make the isolation of such viruses highly unlikely.

Genetic characterization of the white plaque phenotype viruses

To verify that the white plaque phenotype viruses isolated as described in the previous section are recombinant viruses where homologous recombination had occurred in the US9-US10 intergenic region in the viral genome, Southern blot analysis of recombinant virus infected cell DNAs was conducted as previously described (see Materials and Methods). The AD169 *Hind*III X DIG-labeled probe (5.2 kb) (spanning the region US7 through US11 in the HCMV genome), was used. Figure 18A shows that *Hind*III digestion of AD169 DNA resulted in the generation of a 5.2 kb fragment hybridizing to the *Hind*III X probe. On the other hand, *Hind*III digestion of recombinant virus DNA resulted in the generation of two fragments derived from the *Hind*III X region (1.8 kb and 3.4 kb). Hybridization to the *Hind*III X probe resulted in the detection of these two bands when the DNAs of 13 isolates of RVUS9op-6 (Figure 18A), 4 isolates of

FIG. 18. Genetic characterization of white plaque phenotype viruses. Southern blot analysis of recombinant virus infected cell DNA was conducted, along with AD169- and RV*lac*- infected cell DNAs as controls. The probe used to hybridize to *Hind*III-digested DNAs was the *Hind*III X probe described in Materials and Methods. (A) Isolates from stock 21-4 (RVUS9op-6). (B) Isolates from stock 34-3 (RVUS9op-16). (C) Isolate from stock 35-2 (RV0). The numbers on the right of the blots represent molecular weight markers in kilobases. The expected 1.8kb and 3.4 kb fragments hybridizing to the *Hind*III X probe were observed for the white plaque phenotype viruses isolated, except 15D5 and 7D1 (panel B).

RVUS9op-16 (Figure 18B), and 1 isolate of RV0 (Figure 18C) were analyzed. For RVUS9op-16, 4 of 6 isolated recombinant viruses presented the appropriate *Hind*III digestion pattern (20D6, 4A6, 14C3, and 13B3).

Analysis of the growth properties of recombinant viruses

To analyze the growth rate of recombinant viruses RV0, RV*lac*, RVUS9op-16, and RVUS9op-6, and to compare them with the growth of parental virus RV134, one step growth curves were performed as described (Jones et al., 1991), (Kerry et al., 1997). In these experiments, the objective was to determine if the insertion of the *lac i* gene and the *lac* operator sequence has an effect on the rate of virus replication. HFF cells were infected with the indicated viruses at a multiplicity of infection (MOI) of 2. Total virus was harvested daily, and virus yields were determined by plaque assay on HFF cells, using standard procedures. Figure 19 shows the results of these experiments. No significant differences in the growth rates of the recombinant viruses relative to the parental virus RV134 were observed. This suggests that the insertions of the *lac i* gene and the *lac* operator sequence in the viral genome, and the phenotypes derived from these insertions, do not affect the rate of production of virus progeny. In addition, RV0 also replicated at similar rates, which indicates that the presence of the linker sequences in the US9-US10 intergenic region does not have an impact on virus replication. These results have an important implication. They suggest that the components of the *lac* repressor-based system can be integrated into the HCMV genome for the assessment of conditional expression of an individual viral gene, without impacting on the capacity of the virus to produce progeny. Thus, the system is viable in the context of the viral genome. It is worth

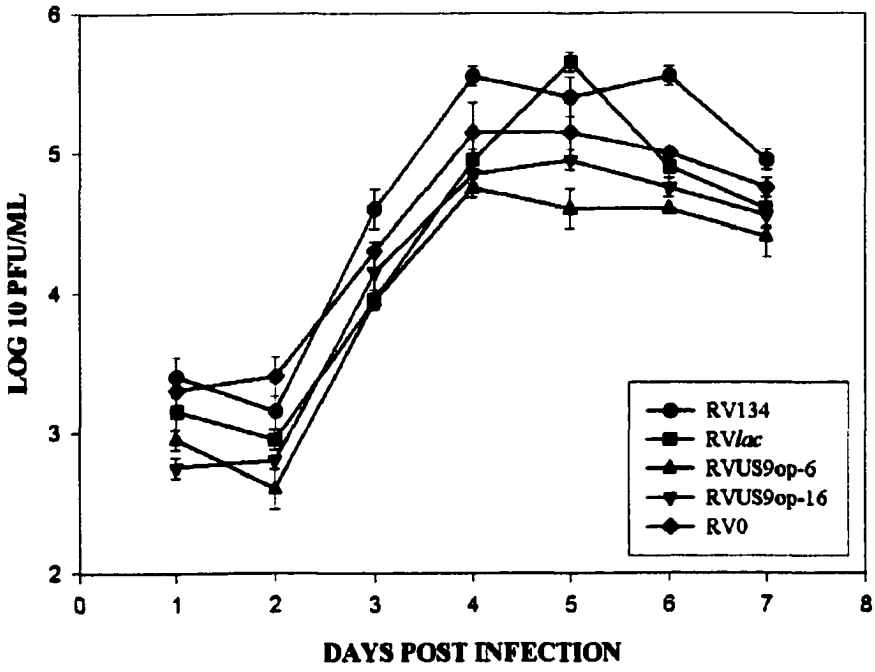


FIG. 19. One step growth curve analysis of recombinant viruses. HFF cells were infected at an MOI of 2 PFU per cell, with either parental virus RV134, or the recombinant viruses RV*lac*, RVUS9op-6, RVUS9op-16, or RV0. Total virus was harvested daily, from day 1 to day 7 after infection. Infectious virus was quantitated by standard plaque assay on HFF cells. Titers were expressed as $\log_{10}$ PFU/ml.

noting, however, that these observations with the US9 promoter should not be generalized to the study of conditional expression of all other HCMV genes, for which the use of the *lac* repressor-based system has to be optimized and tested.

Analysis of expression of operator-containing US9 promoters

Of vital importance to accomplish the objectives of this project was to demonstrate that the operator-containing US9 promoters can be efficiently repressed by the *lac* repressor, and that this can be relieved by IPTG. In addition, it was critical to demonstrate that the operator insertion has a minimal intrinsic effect on basal promoter expression, in order to validate the use of these promoters in this study.

US9 promoter expression in pUS9opCAT

The operator-containing US9 promoter-leader in pUS9opCAT was tested for the impact of the operator insertion on promoter expression, as well as its susceptibility to repression and derepression. To ensure that the insertion of the operator *per se* did not affect expression of the US9 promoter, transient transfection assays were conducted as described in previous sections. Briefly, pUS9opCAT, or pUS9CAT as control, was cotransfected with pSVH into HFF cells. The US9 promoter belongs to the early kinetics class, and is known to be activated by IE proteins (reviewed by Stenberg, 1993), (Spector et al., 1990). A CAT reporter construct containing the HCMV DNA polymerase promoter (ppolCAT) was used as a control of transfection. Cells were harvested 48 hs post transfection, and assayed for CAT activity as described in Materials and Methods. Figure 20 shows a complete abrogation of CAT activity in cells cotransfected with pUS9opCAT

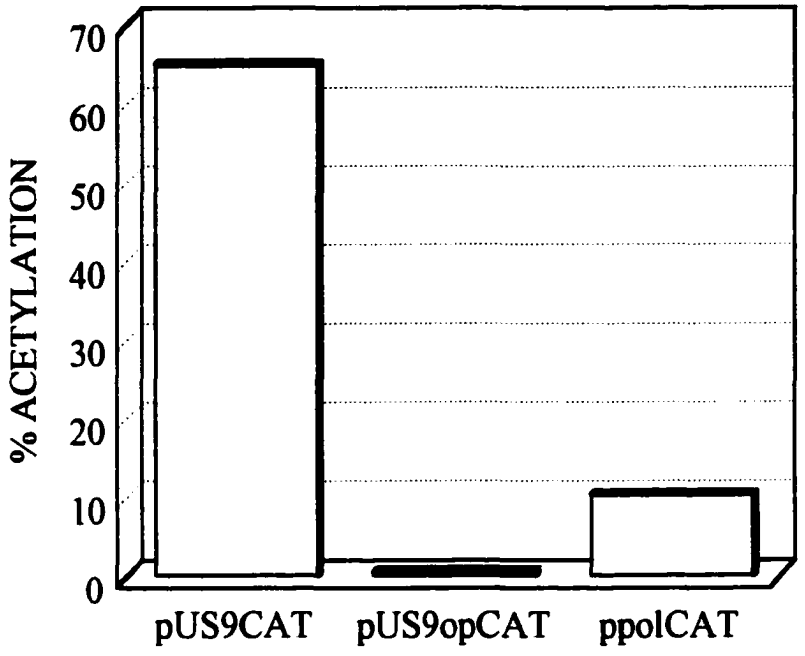
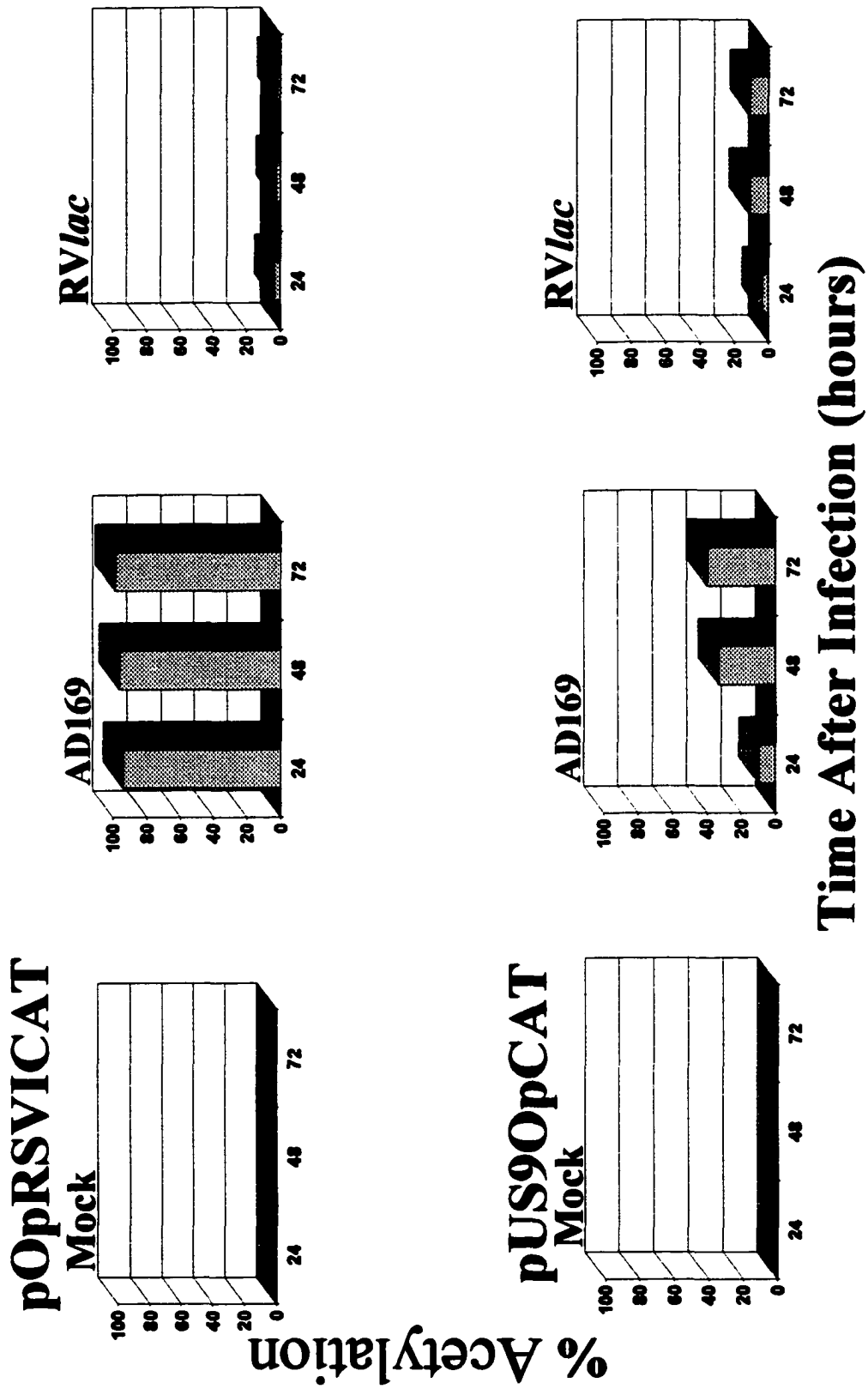


FIG. 20. US9 promoter activation by HCMV immediate-early proteins IE1 and IE2. HFF cells were cotransfected with 5 ug of pSVH (expressing HCMV IE1 and IE2), and 5 ug of pUS9CAT (containing the wild type US9 promoter), or 5 ug of pUS9opCAT (containing the operator sequence at +40). ppolCAT (containing the HCMV DNA polymerase promoter) was used as control of transfection, by cotransfecting 5 ug of ppolCAT with 5 ug of pSVH. Cells were harvested 48 hs after transfection, and cell extracts were assayed for CAT activity. Percent acetylation was determined by phosphorimager analysis. A representative experiment is shown.

and pSVH, suggesting that the operator insertion at +40 has a negative impact on expression. Possibly, the presence of the operator sequence in the leader adversely affected the translation of the CAT mRNA (see Discussion).

pUS9opCAT was also tested for repression by the *lac* repressor in a transfection-RV*lac* infection experiment, which also included the reporter plasmid pOpRSVICAT for parallel analysis. Briefly, pUS9opCAT was transfected into HFF cells, and the transfected cells were infected with RV*lac* (at an MOI of 2) at 24 hs post transfection. As controls, mock infection or infection with wild type virus AD169 at an equivalent MOI were performed. Cells were harvested at 24, 48, and 72 hs post infection and assayed for CAT activity. The results of this experiment are shown in Figure 21. The data obtained with pOpRSVICAT is in accordance with the experiment presented in Figure 8B. On the other hand, very low levels of CAT activity were obtained from pUS9opCAT upon infection with wild type virus AD169, in accordance with the results obtained with IE1 and IE2 (Figure 20), supporting the conclusion that the operator insertion in pUS9opCAT adversely affects promoter expression. Some degree of repression was observed (about 50%) in cells infected with RV*lac*. Collectively, the results regarding promoter expression in pUS9opCAT indicated that alternative sites for operator insertions needed to be tested, in order to obtain higher levels of promoter expression in the absence of the repressor. To this end, the US9 promoters containing the *lac* operator sequence at -16 and -6 in the CAT reporter constructs pUS9op(-16)CAT and pUS9op(-6)CAT (Figure 11) were analyzed. The results of these experiments are described in the following sections.

FIG. 21. Repression of the US9 promoter in pUS9opCAT. HFF cells were transfected with 10 ug of pOpRSVICAT (control) or pUS9opCAT. 24 hs after transfection, cells were mock infected, or infected with AD169 or RV*lac* at 2 PFU per cell. Infected cells were harvested at 24, 48, and 72 hs post infection, and cell extracts were assayed for CAT activity. Percent acetylation was determined by phosphorimager analysis. A representative experiment is shown.



Impact of the *lac* operator insertion at -16 or -6 on US9 promoter expression

It was of critical importance to first determine if the operator insertion *per se* had an impact on promoter expression in pUS9op(-16)CAT and pUS9op(-6)CAT. Ideally, the operator insertion would have minimal intrinsic effect on US9 promoter activation. Thus, such a promoter would be suitable to test conditional expression mediated by the *lac* repressor-based system both in transient assays and in the context of the viral genome. To address this question, transient transfection experiments were conducted to assess activation of these variant promoters by HCMV IE proteins IE1 and IE2. The experiments were performed as described for pUS9opCAT, and they demonstrated that the *lac* operator sequence can be inserted into an HCMV promoter without significantly affecting its basal level of expression in transient assays. Figure 22 shows the results of those experiments. pUS9op(-16)CAT was activated to about 70% of wild type pUS9CAT, whereas pUS9op(-6)CAT was activated to about 20%. Thus, the operator insertion at -16 (closer to the TATA box) affected promoter activation by IE1 and IE2 to a lesser extent, suggesting that in this promoter the intrinsic impact of the operator sequence was minor. As a result, promoter activation did not differ significantly from wild type promoter activation by IE1 and IE2. However, because promoter expression in the endogenous locus could render different results for these promoters, given the higher level of complexity of regulation of promoter activity in the viral genome, both constructs were included for the generation of recombinant viruses in this study (see corresponding section in this chapter).

To further analyze the impact of the operator insertion on promoter expression in pUS9op(-16)CAT and pUS9op(-6)CAT, the effect of the presence of all viral proteins on

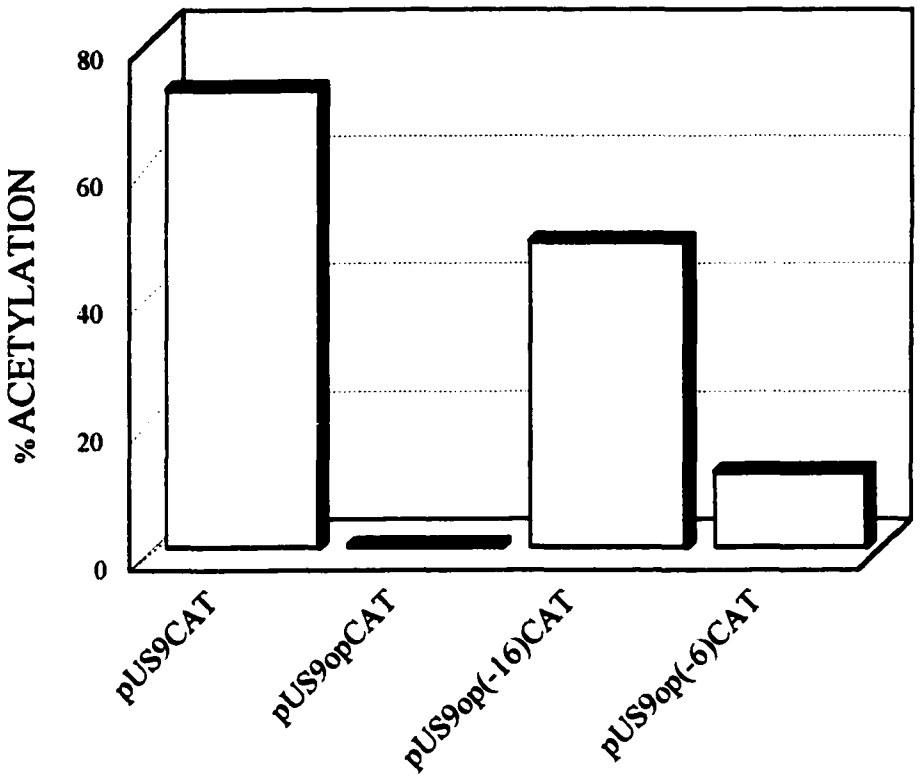


FIG. 22. Impact of the operator insertion on US9 promoter activation by HCMV immediate-early proteins IE1 and IE2. HFF cells were cotransfected with 5 ug of pSVH (expressing IE1 and IE2), and 5 ug of either pUS9CAT (wild type US9 promoter), pUS9opCAT (operator at +40), pUS9op(-16)CAT (operator at -16), or pUS9op(-6)CAT (operator at -6). Cells were harvested 48 hs after transfection, and cell extracts were prepared for CAT activity assay. Percent acetylation was quantitated by phosphorimager analysis. A representative experiment is shown.

activation of the operator-containing US9 promoters in the CAT reporter constructs was addressed. Transfection of these constructs into HFF cells was followed by infection with AD169 at 2 PFU per cell, 24 hs after transfection. Cells were harvested 72 hs post infection, and cell extracts were prepared and assayed for CAT activity. The results of these experiments are shown in Figure 23. In the presence of whole virus, activation of pUS9op(-16)CAT was approximately 2-fold that of the wild type promoter in pUS9CAT, and for pUS9op(-6)CAT, the level of activation was 6-fold relative to pUS9CAT. These data suggest that the presence of the operator sequence at positions -16 and -6 (relative to the transcription start site) in the US9 promoter results in the disruption of sequences that participate in the regulation of expression of the US9 promoter in the presence of all viral proteins. The analysis of these sequences within the US9 promoter and of the phenotype(s) associated is beyond the scope of this study.

Supporting the observations regarding IE1 and IE2-mediated activation of the operator-containing US9 promoters, expression of the promoter in pUS9op(-16)CAT in the presence of AD169 virus was affected by the operator sequence to a lesser extent. Overall, it was concluded that the operator sequence inserted at -16 has a lesser intrinsic effect on transient US9 promoter expression than does the operator insertion at -6. Thus, the US9 promoter with the operator insertion at -16 was considered more suitable for the analysis of *lac*-mediated repression in transient assays.

However, a more complex level of regulation of promoter expression occurs in the viral genome. First, viral transactivators cooperate with cellular transcription factors to control viral promoter expression within the viral genome. Second, promoter

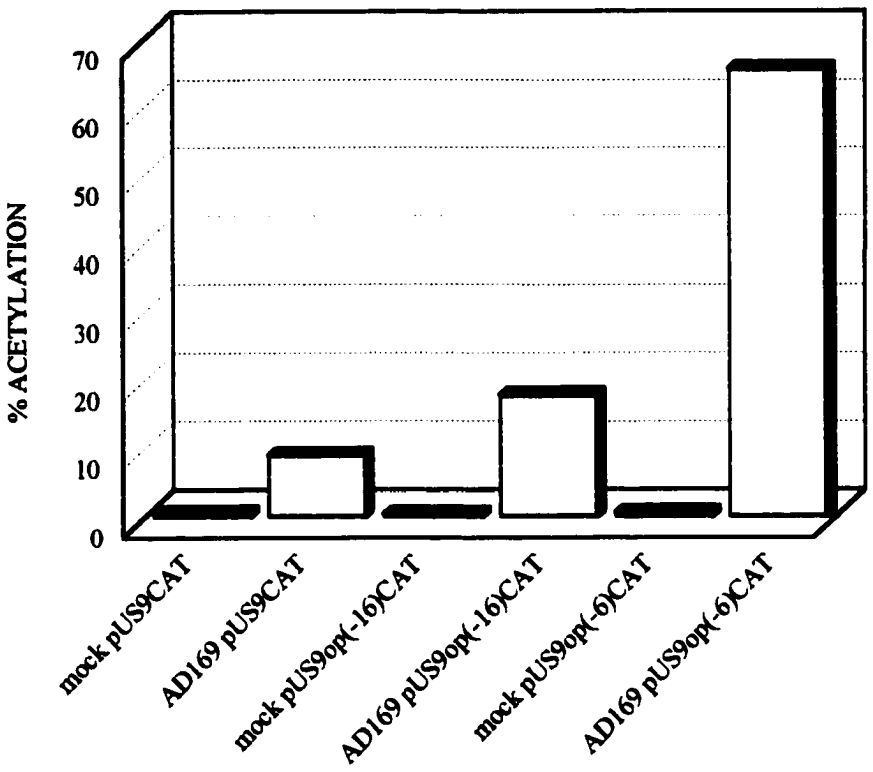


FIG. 23. Impact of the operator insertion on US9 promoter activation by viral proteins. HFF cells were transfected with 10 ug of pUS9CAT (wild type US9 promoter), pUS9op(-16)CAT (operator at -16), or pUS9op(-6)CAT (operator at -6). 24 hs after transfection, cells were mock infected, or infected with AD169 at 2 PFU per cell. Infected cells were harvested 24, 48, and 72 hs post infection, and cell extracts were prepared for CAT activity assay. Percent acetylation was quantitated by phosphorimager analysis. A representative experiment is shown.

expression in the endogenous locus is also affected by distant *cis* acting elements present in the viral genome. Thus, the activity of the operator-containing US9 promoters in the endogenous locus could vary significantly from that observed in the CAT reporter constructs. To address this question, both variant promoters were constructed into the recombination vector for insertion into the virus as described in this chapter. In this way, US9 promoter expression could be assessed in the endogenous locus to investigate the impact of the operator insertions in the context of the viral genome (see following section).

Impact of the *lac* repressor-based system on US9 gene expression in the context of the viral genome

A key control for validating future experiments on conditional expression of the US9 gene in the context of the viral genome was to analyse US9 mRNA levels in RVUS9op(-16)- and RVUS9op(-6)- infected cells, to determine if the operator insertion *per se* has a significant impact on US9 gene expression. In addition, US9 expression was measured in RV*lac*-infected cells, to control for the impact of the MIEP-*lac i* gene cassette insertion adjacent to the endogenous US9 promoter. Moreover, US9 mRNA levels were also assessed in RV0- infected cells, in order to determine if the linker insertion *per se* affects US9 promoter activity in the context of the viral genome. In order to perform these controls, northern blot analysis was conducted on RNA isolated from *lac i*-negative viruses RVUS9op(-16)-, RVUS9op(-6)- or RV0- infected HFF cells, and from RV*lac*-infected HFF cells. The results were compared to parental RV134 virus and wild type virus AD169. Briefly, total RNA was isolated from HFF cells infected with

2 PFU per cell of either wild type virus, parental RV134 virus, RV0, RVUS9op(-16), RVUS9op(-6), or RV*lac*, at 24, 48, and 72 hs post infection. RNA samples were subjected to northern blot analysis using a radiolabeled US9 specific probe as described in Materials and Methods. The results are presented in Figure 24.

Several observations can be made from the results obtained. First, in contrast with previously reported observations (Jones and Muzithras, 1991), US9 mRNA levels in cells infected with AD169 did not differ significantly throughout the course of infection. The highest levels were observed at 24 hs, but these were not significantly reduced at 48 and 72 hs post infection. Second, it was interesting to observe that for all the recombinant viruses, the kinetics of US9 gene expression was altered. All the recombinant viruses presented lower levels of US9 mRNA at 24 hs post infection. These levels increased at 48 hs, and even more at 72 hs after infection. Thus, the US9 gene was expressed with early-late kinetics from all the recombinant viruses. Third, in addition to a change in the kinetics of expression, the highest US9 mRNA levels measured in cells infected with RV0, RVUS9op-16 and RVUS9op-6 (at 72 hs post infection) were approximately 2-fold those in cells infected with wild type AD169 virus (at 24 hs post infection), or parental RV134 (at 72 hs post infection). For RV134 and RV*lac*, the highest US9 mRNA levels achieved (at 72 hs post infection) were approximately equal to those in cells infected with AD169 at 24 hs.

Overall, these results showed that the insertions done within and in proximity to the US9 promoter in the endogenous locus affected promoter expression in terms of time of expression, leading to a change in kinetics of expression of the US9 gene.

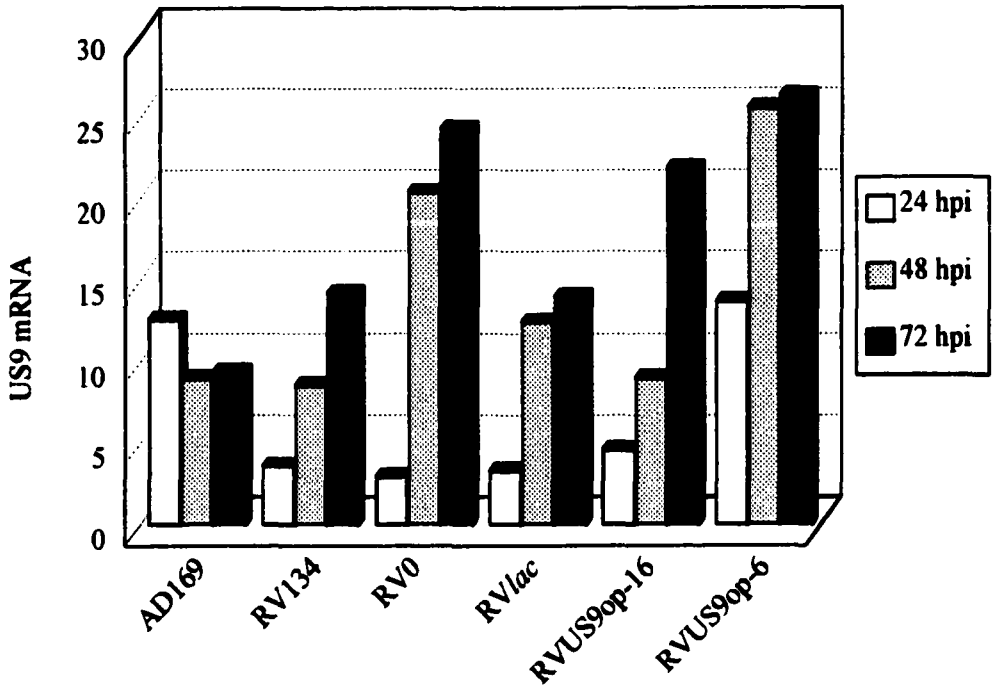


FIG. 24. Impact of the *lac* repressor-based system on US9 gene expression in the context of the viral genome. Northern blot analysis was conducted on total RNA isolated from cells infected with AD169, RV134, RV*lac*, RV0, RVUS9op-16, or RVUS9op-6, at 2 PFU per cell, at 24, 48, and 72 hs post infection (hpi). The RNA was subjected to electrophoresis in 1 % agarose, transferred to a nylon membrane, fixed, and hybridized to a radiolabeled US9 specific probe (a DNA fragment comprising the entire US9 and the majority of US8) (Materials and Methods). The RNA was quantitated by phosphorimager analysis, and is expressed in arbitrary units. A UL99 (pp28) specific radiolabeled probe was used to correct for multiplicities of infection. The results presented are average of the results from two replicate experiments.

Furthermore, it led to a 2-fold increase in mRNA levels when the insertions were done at -6 and -16 in the US9 promoter (in RVUS9op-16 and RVUS9op-6), and when the linker sequences were inserted immediately upstream of the US9 promoter (in RV0). The mechanism(s) underlying these phenotypic changes are unknown at this point. They likely include disruption of sequences important for the regulation of promoter activation, which in turn influence the interactions of these sequences with viral and cellular proteins that participate in the control of US9 promoter expression. This led to a change in kinetics, and in the levels of mRNA produced. However, the magnitude of these changes is probably not significant in terms of the utility of the operator-containing US9 promoters in the endogenous locus for the application of the *lac* repressor-based system to assess conditional viral gene expression. The implications of these results are presented in chapter V.

RV*lac*-mediated repression of operator-containing US9 promoter

The aim of the experiments described in this section was to assess *lac*-mediated repression of operator-containing US9 promoter expression in transient assays. In previous sections it was hypothesized that pUS9op(-16)CAT would be a more suitable construct to test *lac*-mediated repression in transient assays, since activation of this variant promoter in the presence of viral proteins occurs at levels comparable to those of the wild type US9 promoter. To this end, HFF cells were transfected with 10 ug of pUS9op(-16)CAT. At 24 hs after transfection, transfected cells were either mock infected, or infected with wild type AD169 or RV*lac* at 2 PFU per cell. Cells were

harvested at 24, 48, and 72 hs post infection, and cell extracts were prepared for CAT activity assay. The results are presented in Figure 25. RV*lac* infection of cells transfected with pUS9op(-16)CAT resulted in a 90 % reduction in CAT activity when compared with wild type virus infection, indicating that the operator-containing US9 promoter in pUS9op(-16)CAT can be efficiently repressed by the *lac* repressor. This significant level of repression was maintained throughout the course of infection. These findings were of vital importance for this study: an operator-containing, HCMV-specific promoter that can be efficiently acted upon by the *lac* repressor produced from RV*lac* demonstrates the feasibility of this approach to study HCMV gene expression.

Derepression of operator-containing US9 promoter by IPTG

Experiments utilizing IPTG were performed in order to assess derepression of the operator-containing US9 promoter in pUS9op(-16)CAT. Several reports on the use of the *lac* repressor based system in mammalian cells have shown that the IPTG concentrations used to achieve maximal derepression could be varied through a low dose (1-500 uM), a medium dose (0.5-15 mM), and a high dose (5-50 mM). Toxicity has been observed at concentrations higher than 50 mM (Figge et al., 1988). The times of exposure to IPTG, using operator-containing promoters upstream of the CAT reporter gene (Brown et al., 1987), (Hu and Davidson, 1987) (Figge et al., 1988), (Labow et al., 1990), or the luciferase gene (Rodriguez and Smith, 1990), have varied in different studies, from 7-12 hs, to up to 3 days.

Initially, an experiment to test toxicity was performed by exposing HFF cells to 0, 5, 10, 25, and 50 mM IPTG for 72 hs. Figure 26 shows HFF monolayers that had been

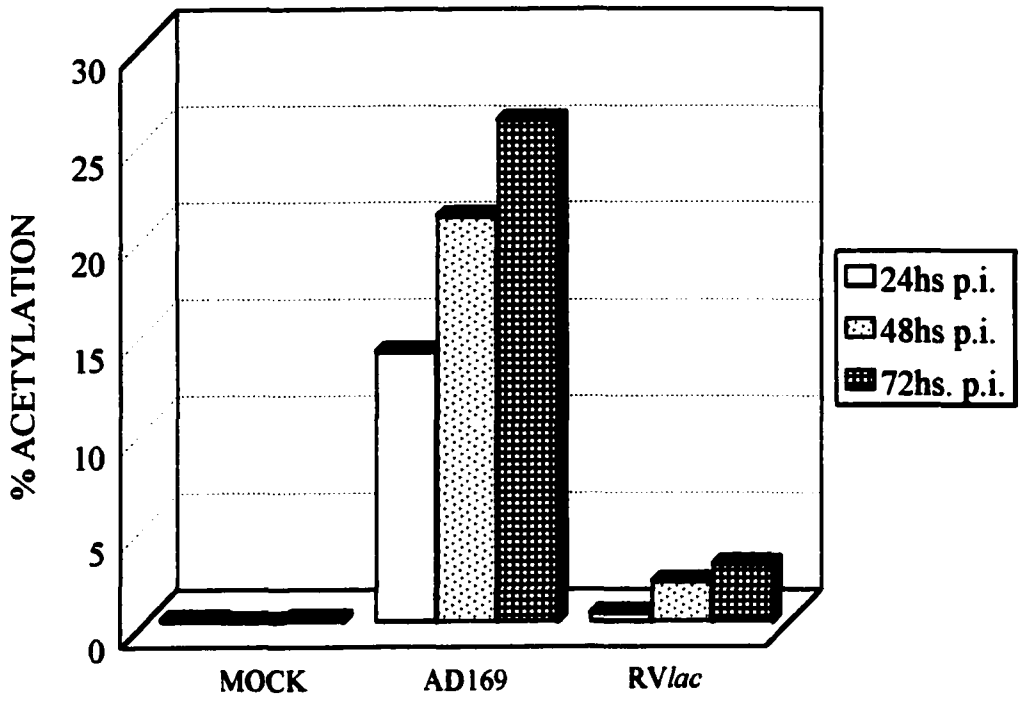


FIG. 25. Repression of the US9 promoter in pUS9op(-16)CAT. HFF cells were transfected with 10 ug of pUS9op(-16)CAT. At 24 hs after transfection, cells were either mock infected, or infected with AD169 or RV*lac* at 2 PFU per cell. 24, 48, and 72 hs post infection, cells were harvested, and cell extracts were assayed for CAT activity. Percent acetylation was quantitated by phosphorimager analysis. A representative experiment is shown.

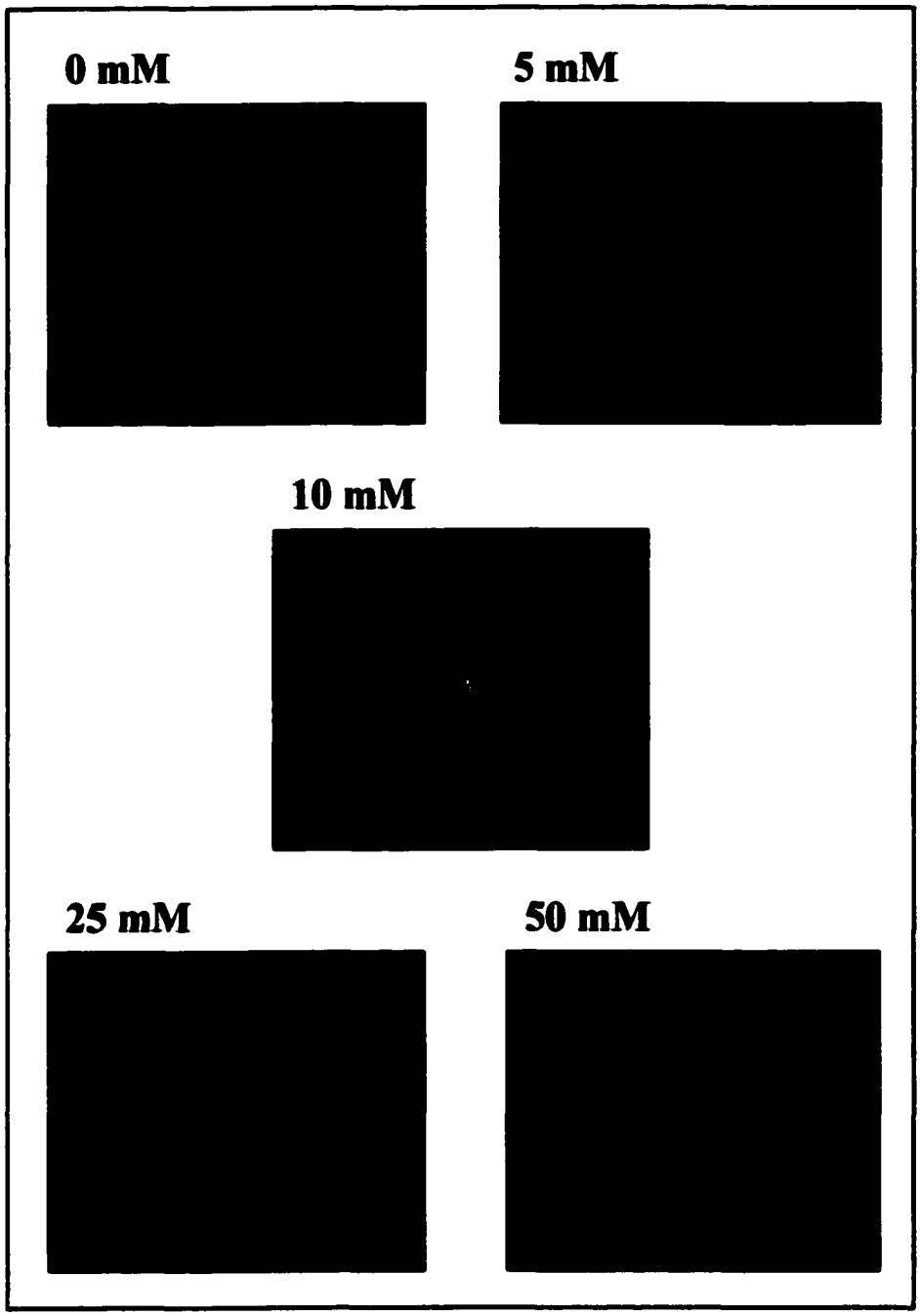


FIG. 26. Exposure of HFF cells to increasing concentrations of IPTG. To test IPTG toxicity, HFF cells were exposed to 0, 5, 10, 25, and 50 mM IPTG for 72hs. At that time, cells were photographed using a Photo Zoom inverted microscope (Bausch & Lomb), and a Pentax K1000 camera. The images presented were generated with a UMAX PowerLook II Scanner with Paintshop Pro version 5.0 Jacs Software.

exposed to the indicated concentrations of IPTG for 72 hs. Morphology and cell viability did not seem to be affected by exposure to IPTG. Therefore, all the concentrations tested were considered to be nontoxic for HFF cells, thus suitable for use in the derepression experiments.

In this study, the procedure to assess IPTG derepression involved transfecting HFF cells with 10 ug of pUS9op(-16)CAT, and 24 hs after transfection, the cells were infected with RV*lac* at an MOI of 2. After adsorption of the virus for 2 hs, the virus inoculum was removed, and fresh culture media containing 0, 0.2, 0.4, 0.8 or 2 mM IPTG was added. A lower medium dose range was initially tested in order to find the minimum concentration of IPTG that was necessary and sufficient to efficiently cause derepression. Cells were harvested 72 hs post infection, and assayed for CAT activity as previously described (see Materials and Methods). Figure 27 shows that 0.8 mM IPTG was capable of derepressing pUS9op(-16)CAT expression to levels comparable to wild type levels. This is a significant finding, since it demonstrates that the system functions efficiently to reverse *lac*-mediated repression of an HCMV promoter. Similar results were obtained when cells were exposed to 2 mM IPTG. The data suggest that 0.8-2 mM IPTG could be the optimal levels of inducer to utilise in the analysis of the operator-containing US9 promoter in the viral genome. However, the conditions for IPTG induction, in terms of IPTG concentration and time of exposure to IPTG, will have to be optimized for studying conditional expression of the US9 promoter in the context of the viral genome.

The observations in the last two sections demonstrate that *lac*-mediated repression (using a recombinant HCMV expressing the *lac* repressor), and IPTG derepression can be readily achieved using an HCMV-specific promoter in transient assays. This validated

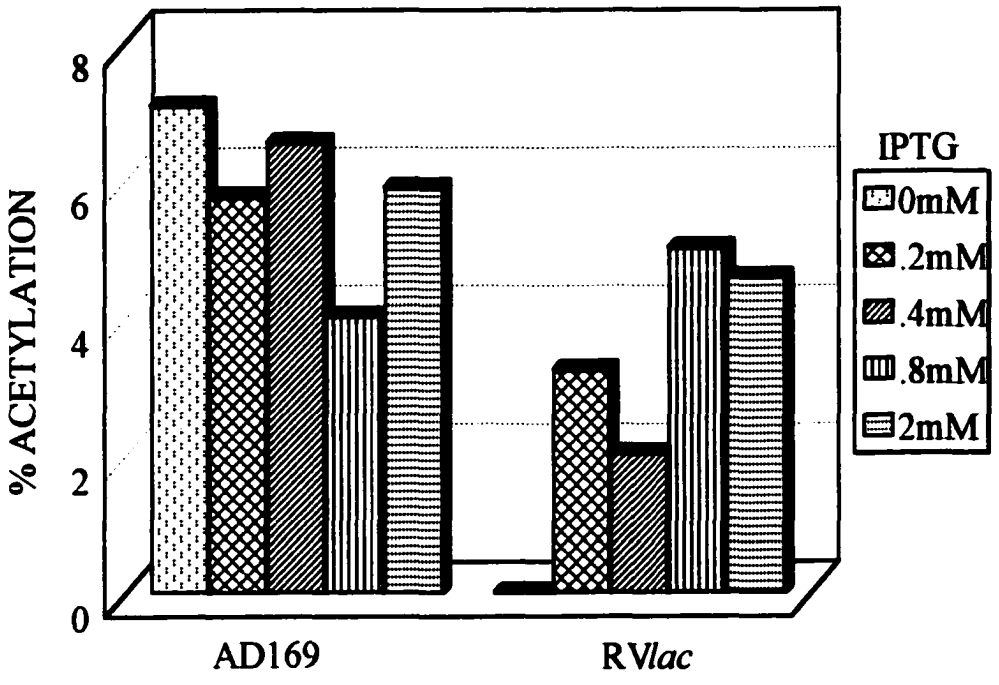


FIG. 27. Titration of IPTG for induction of pUS9op(-16)CAT in cells infected with RV*lac*. HFF cells were transfected with 10 ug of pUS9op(-16)CAT. At 24 hs after transfection, cells were infected with either AD169 or RV*lac* at 2 PFU per cell. After virus adsorption for 2 hs, IPTG was added at 0, 0.2, 0.4, 0.8, or 2 mM. Cells were harvested 72 hs post infection, and cell extracts were assayed for CAT activity. Percent acetylation was quantitated by phosphorimager analysis. Results shown correspond to a representative experiment.

efforts to construct this system into the virus for future analysis of conditional gene expression during the course of HCMV infection of cells in culture.

CHAPTER V

DISCUSSION

HCMV essential genes encode protein functions that are absolutely required for replication of the virus in cell culture. In other words, deletion or mutation of such genes is lethal. Thus, these genes are believed to play central roles in the replicative cycle, leading to the production of virus progeny. To date, these genes have not been directly tested for their roles in virus replication. Hence, their essential nature remains speculative, and it is mostly based on homologies with HSV-1 essential genes. The unavailability of a significant number of HCMV conditional-lethal mutants, and the lack of cell lines to complement virus null mutants, have presented an obstacle for addressing the role of individual essential viral gene products in the context of a natural infection (reviewed by Stenberg and Kerry, 1995). Therefore, the need for the development of new methodologies to address gene expression in the context of the HCMV genome, as well as for the propagation of viruses with mutations/deletions in genes likely to be essential, is presently clear.

In this study, a conditional expression system was developed to study the functional roles of HCMV gene products during viral replication. This approach is based on the *Escherichia coli lac* repressor protein, the *lac* operator DNA sequence to which the repressor binds with high affinity and sequence specificity, and the specific inducer IPTG. By using this system, HCMV putative essential genes can be targeted for conditional expression by constructing recombinant viruses containing *lac* operator sequences in target promoters. These operator sequences within the target promoters can be acted upon by the *lac* repressor produced from the same recombinant virus or a

coinfecting recombinant HCMV. The effect of the loss of expression of the target gene on virus replication can then be assessed.

Several questions were addressed in this study. Can the *lac* repressor-operator system be inserted into the HCMV genome to study viral gene expression? Specifically, can the *lac* repressor be expressed from recombinant HCMV? Will the *lac* repressor produced recognize the *lac* operator sequence in an HCMV promoter, in a CAT reporter construct to first test the system in transient expression assays, as well as in the endogenous locus in the viral genome? Will this interaction block promoter activation? What levels of repression and IPTG-mediated derepression can be achieved? These objectives were successfully accomplished by generating a *lac* repressor expressing recombinant HCMV and a *lac* operator-containing, HCMV-specific promoter, and analyzing the *lac* operator-repressor-IPTG interactions in the context of such a promoter. The approaches used and the relevant findings in this study are discussed in this chapter.

The *lac* repressor-operator system has been shown to regulate the expression of transfected and integrated reporter genes in mammalian cells (Brown et al., 1987), (Hu and Davidson, 1987), (Deuschle et al., 1989), (Figge et al., 1988), as well as in recombinant vaccinia virus-infected cells (Rodriguez and Smith, 1990 and 1990), (Fuerst et al., 1989), (Zhang and Moss, 1991). As a novel transcriptional regulatory system in mammalian cells, the *lac* repressor-operator interaction offers significant advantages for use as a conditional genetic switch. Firstly, the use of these prokaryotic elements, which are inert to the physiology of the eukaryotic cell, can potentially be applied for the stringent regulation of expression of any chosen HCMV gene. In this manner, the target

gene, and therefore viral replication, can be externally controlled. Secondly, the repressor-operator interaction is highly specific. The *lac* operator sequence is present extremely infrequently in the genomes of mammalian cells. Therefore, only the gene under study is susceptible to regulation by the *lac* repressor. This strikingly contrasts with other gene expression systems, like induction mediated by hormones, heavy metals, or heat shock, where the inducers can affect the expression of several genes simultaneously. Thirdly, the system can be allosterically regulated by IPTG, allowing for the reversion of the repressed phenotype. This is an important aspect for the application of the system in this study, due to the absolute requirement of IPTG for the propagation of recombinant viruses with repressible promoters of essential viral genes.

For use in an eukaryotic environment, the gene encoding the *lac* repressor (*lac i* gene) contained a modified initiation codon (GTG to ATG) for efficient translation in an eukaryotic cell. Also, the gene was modified to include the SV40 T antigen nuclear localization signal, in order for the protein product to localize to the nucleus. Transport into the nucleus was thought to be of critical importance if the repressor was to be used to regulate transcription of HCMV genes, which occurs in the nucleus and depends on the cellular RNA polymerase II. However, in one of the earliest reports using the *lac* repressor in mammalian cells, Hu and Davidson (1987) showed that expressing a *lac* repressor lacking a nuclear localization signal resulted in 10% of the total repressor localized to the nucleus, and 90% to the cytoplasm. Although the majority of the repressor molecules were present in the cytoplasm, those present in the nucleus were biologically active and repressed operator-containing promoters by 90%. Nonetheless, in this study, the presence of a nuclear localization signal was considered to be important;

since larger amounts of the repressor protein will be localized in the nucleus, lower levels of expression of the protein will be needed to effect regulation.

The initial goal was to construct a recombinant HCMV that expresses the *lac* repressor from the HCMV major immediate-early promoter (MIEP) (RV*lac*) (Specific objective 1). This recombinant virus would allow for the assessment of *lac* repressor protein expression in the infected cells throughout the course of infection. In addition, RV*lac* could be used to test operator-containing HCMV promoters in transient assays. These assays would determine the most appropriate number and relative position of the operator sequences for effective *lac*-mediated repression, to validate the insertion of such promoters into the virus for analysis in the context of the viral genome. The MIEP-*lac i* gene expression unit was inserted between open reading frames US9 and US10, a transcriptionally barren region in the HCMV genome. Expression driven by the MIEP allowed for the *lac* repressor to be produced immediately after infection, and throughout the course of viral replication. As presented in Figure 7, significant levels of the 37 kD repressor polypeptide were observed at 24, 48, and 72 hs post infection. *A priori*, the amounts of the repressor polypeptide present in the RV*lac*-infected cells were presumed to be sufficient to form enough repressor tetramers to efficiently block transcription from operator-containing promoters, although there were no previous data to support that assumption. The fact that significant levels of the repressor were observed during the complete replicative cycle has an important implication: expression of the *lac* repressor gene under the control of the MIEP would allow for the repressor to be available in the RV*lac*-infected cell for the regulation of expression of operator-containing HCMV promoters of all kinetic classes in the context of the viral genome.

The next question addressed in this study was to determine if the *lac* repressor produced in RV*lac*-infected cells would recognize its cognate operator sequence, and regulate expression from operator-containing promoters. Initially, this question was approached by analyzing the *lac* repressor-operator interaction using operator-containing heterologous promoters and RV*lac* in transient assays. To this end, CAT reporter constructs driven by *lac* operator-containing RSV-LTR promoters (pOp13CAT and pOpRSVICAT) were used (see Figure 8A). The number and relative position of the operator sequences were different for these two constructs. pOp13CAT contains three tandem operator sequences immediately downstream of the RSV promoter, while pOpRSVICAT contains two operator sequences, one of which is located adjacent to the TATA box, and the other one is present downstream of the RSV promoter. Approximately 80-100% reduction in CAT activity was observed in cells transfected with these constructs and infected with RV*lac* (Figure 8B), suggesting that 1) the repressor polypeptide was expressed at significant levels from the MIEP-*lac i* gene cassette in the viral genome, 2) presumably formed homotetramers, prior to and/or after migrating into the nucleus, and 3) the functional repressor was present in sufficient amounts for efficient repression of the operator-containing promoters. From Figure 8B, it is evident that a more effective repression of promoter activity occurred when the *lac* operator sequence is positioned closer to the TATA box. In RV*lac*-infected cells, CAT activity 80 to 100 times less than that obtained in the presence of wild type virus AD169 infection represented a highly significant level of repression. This may, at least in part, be explained by the presence of multiple copies of the operator sequence. Sadler et al. (1980) have reported that the half-lives of operator-repressor complexes increase with increasing numbers of

operators in tandem, thereby enhancing the repressor capabilities to sterically block the binding of the RNA polymerase to the transcription start site. However, the ability of IPTG to derepress such operator-containing promoters may be reduced.

When cells transfected with pOpRSVICAT and infected with RV*lac* were exposed to IPTG, at concentrations ranging from 10 to 100 uM for 7 hs, CAT activity rose to levels 4 times of those of cells left untreated (Figure 9). This represents a modest level of derepression (about 40% of the level of promoter activity in AD169-infected cells), suggesting that the repression of this operator-containing promoter was not fully reversible. Several factors can contribute to this phenomenon. First, reversion with IPTG may be less easily achieved due to the presence of two operator sequences. Secondly, because of the limited permeability of the eukaryotic plasma membrane to IPTG [in *E.coli*, IPTG enters the cell using the inducible *lac* permease system (Kennedy, 1970)], significant amounts of the inducer may have been difficult to obtain inside the cell at the concentrations tested. Furthermore, the amounts of IPTG transferred from the cytoplasm to the nucleus may have been insufficient to act upon the repressor-operator complexes on the promoter in pOpRSVICAT. Lastly, the time of exposure to IPTG was probably insufficient, and was reflected in the magnitude of the response. From these experiments, it was concluded that higher concentrations of IPTG and longer times of exposure to the inducer needed to be tested in subsequent experiments in order to obtain a more effective derepression.

The next step represented a central goal of this project. This was to construct a *lac* operator-containing HCMV promoter (Specific objective 2) in order to insert it into the endogenous locus in the HCMV genome (Specific objectives 3, 4, and 5) for future

analysis of conditional expression in the context of a natural infection, and to test its susceptibility to RV*lac*-mediated repression and derepression by IPTG in CAT reporter constructs in transient assays (Specific objective 6). The *lac* operator chosen was the 24-base pair sequence 5'GAATTGTGAGCGGATAACAATTTC3'. In terms of its structure, this operator sequence is an imperfect palindrome. Simons et al. (1984) showed that operators containing imperfect palindromes bind the *lac* repressor with an 88 % shorter half-life than perfect palindromes. This may result in a more effective derepression mediated by IPTG. Thus, an imperfect palindromic operator sequence was chosen for this study in order to be able to dissociate the repressor-operator complexes more easily in the presence of IPTG. This is part of concerted efforts that needed to be made to achieve significant levels of *lac* repressor-mediated repression that can be efficiently reversed by IPTG. The 24-base pair operator sequence was inserted at different positions within the HCMV US9 promoter (see below). The US9 gene was targeted for conditional expression because this gene is known to be dispensable for growth of the virus in primary human fibroblasts (HFF) in culture (Jones and Muzithras, 1992). Thus, the lack of expression of the US9 gene in the repressed state would not affect virus replication in HFF cells. This characteristic allows for the analysis of parameters of repression and derepression without having virus growth dependent on IPTG. Moreover, the US9 gene is located adjacent to the site of insertion of the MIEP-*lac i* gene cassette in RV*lac*. Therefore, recombinant viruses with the double insertion of the *lac i* gene and the *lac* operator sequence can be generated after a single recombination event. By utilizing a virus expressing the *lac* repressor and containing an operator-modified US9 promoter, the

feasibility of the *lac* repressor-based system to study HCMV gene expression in the context of the viral genome can be assessed.

Initially, a single *lac* operator sequence was inserted at a unique *Sst*II site in the leader sequence of the US9 gene, forty nucleotides downstream of the transcription start site (position +40). This approach was undertaken because it represented a quick and convenient insertion of the *lac* operator sequence into the US9 promoter-leader region. Although the majority of the reports to date have favored the insertion of operators within promoter sequences, *lac* operators have also been found to be biologically functional when positioned downstream of the promoter. Deuschle et al. (1986) observed that a *lac* repressor-operator complex can be formed and block transcriptional elongation when the operator was inserted downstream of the coliphage T5 promoter. A 90 % reduction of promoter expression was observed.

Alternatively, a single *lac* operator sequence was positioned 16 or 6 nucleotides upstream of the transcription start site in the US9 gene. The criterion was to place operator sequences at different positions relative to the TATA box. In the US9 promoter, the operator at -16 is 6 nucleotides downstream of the TATA box, while the one positioned at -6 is 16 nucleotides downstream of the TATA box. It has been suggested that the *lac* repressor bound to an operator immediately downstream of the TATA element sterically impairs the binding of the RNA polymerase to the transcription start site (Brown et al., 1987). The position of the operator has been demonstrated to be critical for effective repression mediated by the *lac* repressor (Rodriguez and Smith, 1990), (Hu and Davidson, 1987).

Another aspect to be considered for the construction of the operator-containing US9 promoters was the number of operator sequences that needed to be inserted into the promoter. Reports on the use of the *lac* repressor-operator system in mammalian cells consistently show that tighter repression can be obtained with numerous operators in tandem array. This could be due to an enhanced stability of the repressor-operator complexes as a result of increased protein-protein interactions. Operators in tandem in specific spatial arrangements are thought to lead to the formation of intervening DNA loops upon binding of the repressor tetramers, which increases the stability of the interactions, leading to higher levels of repression (Besse et al., 1986), (Mossing et al., 1986), (Kramer et al., 1987). However, these tandem operator insertions can have an intrinsic impact on promoter expression, leading to reduced promoter activity even in the absence of the repressor. For example, when analyzing the effect of several operator insertions on SV40 early promoter activation relative to the parental promoter upstream of the CAT gene, Hu and Davidson (1987) showed that 1) a single operator positioned between the transcription start site and the TATA box decreased CAT activity by about 25%, 2) two tandem operator sequences resulted in 70% reduction in CAT activity, and 3) three operators decreased CAT activity by approximately 85%. This indicates that the operator insertions *per se* resulted in decreased promoter activity. For the system to operate efficiently, it is desired that the operator have a minimal impact on promoter expression. This seems to be more easily achieved with single operator insertions.

In addition, the presence of several operators may result in lower levels of reversion of repression mediated by IPTG. This was observed by Rodriguez and Smith (1990), who studied an operator-containing late vaccinia virus promoter using the

luciferase reporter system. When a single operator was inserted, IPTG induced luciferase activity to approximately 90% of the control levels. In contrast, when two operators were present, reversion was only 50%.

Taking into consideration all of the evidence presented above, and because it was hypothesized that the operator sequence at +40 may interfere with the translation of the US9 mRNA (see below), it was therefore concluded that a single *lac* operator sequence at either -16 or -6 should be inserted into the endogenous US9 promoter for future analysis of conditional promoter expression. However, the US9 promoter-leader containing the operator sequence at +40 was included in the experiments on transient expression of operator-containing promoters in CAT reporter constructs (see below), to compare its expression with that of the wild type US9 promoter, as well as with that of the US9 promoters with operator sequences at –16 and –6. If the operator insertion at +40 proved to have no intrinsic effect on expression, and if it was efficiently acted upon by the *lac* repressor and IPTG, it would then be considered for insertion into the viral genome for future analysis.

By inserting the operator-containing US9 promoters into the viral genome, and assessing repression by the *lac* repressor and derepression by IPTG, the feasibility of this conditional expression system for the study of HCMV gene function during a natural infection of cells in culture can be evaluated. As explained at the beginning of this chapter, a HCMV gene can be targeted for conditional expression by inserting its promoter containing an operator into the endogenous locus by homologous recombination, provided a screening method is available to isolate the resulting recombinant virus. The operator-containing promoter in the endogenous locus can be

acted upon by the *lac* repressor produced from the same recombinant virus, or a coinfecting recombinant HCMV. Thus, targeted gene expression and virus replication can be assessed in the repressed state. Addition of IPTG reverses the repression, and allows for expression of the target gene. In this study, the operator-containing US9 promoter and the *lac i* gene were constructed into a recombination vector (pRV*lac*US9op) for insertion into the US9-US10 intergenic region in the HCMV genome by a one step homologous recombination event leading to the generation of a single recombinant HCMV. Although one pool of virus was identified to contain recombinant virus, the isolation of the virus with the double insertion was unsuccessful. The reason(s) for failing to isolate this recombinant virus is unknown. Possibly, the *lac* repressor produced from the recombination vector pRV*lac*US9op in cells transfected with parental virus RV134 and pRV*lac*US9op would bind to the operator sequence in the recombination vector. The repressor-operator interaction could therefore interfere with homologous recombination into the RV134 DNA, leading to aberrant or lack of recombination. Hence, appropriate recombination becomes a very rare event, which would be reflected in an extremely small number of the double insertion recombinant virus resulting from the transfection. Consequently, isolation of such a recombinant virus would be very difficult to achieve. These results suggested that future studies should address conditional expression of the operator-containing US9 promoters in the context of the viral genome by generating one recombinant virus containing the operator-containing US9 promoter and another recombinant virus that expresses the *lac* repressor, to be used in coinfection experiments. This approach has been successfully applied to vaccinia virus (Rodriguez and Smith, 1990). Their report included the generation of a recombinant virus expressing the

repressor protein and a series of viruses containing operator sequence(s) at different positions between the vaccinia virus late 4b promoter and the luciferase reporter gene. The data from coinfection experiments demonstrated optimal inhibition of luciferase activity which could be restored after treatment with IPTG.

Before new approaches are applied to address conditional expression of the operator-containing US9 promoters in the viral genome, it was necessary to determine if the operator insertions at –16 and –6 in the US9 promoter in the endogenous locus had an impact on US9 gene expression in the absence of the repressor. In order to answer this question (see below), recombinant viruses RVUS9op-16 and RVUS9op-6 were generated. These viruses contain a single operator sequence located at –16 or –6, respectively, in the US9 promoter in the viral genome. In addition, a recombinant virus (RV0) containing the linker sequence used to perform the insertions between US9 and US10 in the viral genome was generated, to control for the impact of such a sequence on endogenous US9 promoter expression.

The analysis of the growth properties of the recombinant viruses generated (RV*lac*, RVUS9op-16, RVUS9op-6, and RV0) by one step growth curves showed that there were no significant differences in the rate of virus replication of the recombinant viruses relative to the parental virus RV134. This implies that the *lac* repressor based system can be integrated into the HCMV genome without impacting on the production of virus progeny.

In order to test the system in transient assays, expression of the operator-containing US9 promoters was assessed in CAT reporter constructs (Specific objective 6). This included the analysis of promoter activity in the presence of HCMV immediate-

early proteins IE1 and IE2, or wild type virus AD169, to investigate the impact of the operator insertion on basal promoter expression. To determine the viability of this system to conditionally express an HCMV promoter, the analysis on basal operator-containing US9 promoter activity was followed by experiments addressing RV*lac*-mediated repression and restoration of activity of the operator-containing promoters by IPTG. The operator-containing promoter-leader sequence was tested in a CAT reporter construct (pUS9opCAT), in the presence of HCMV immediate-early proteins IE1 and IE2. From this experiment it was concluded that this operator location was not appropriate for the analysis of conditional expression of this promoter, since the operator insertion *per se* completely abrogated CAT activity (Figure 20). Very low levels of CAT activity were also obtained when cells transfected with pUS9opCAT were infected with AD169 (Figure 21). A possible explanation for these results is that, given the palindromic structure of the operator present in the leader sequence, the formation of a hairpin structure may have reduced the translatability of the CAT mRNA. Consequently, additional sites of insertion for the operator needed to be tested. Thus, the operator insertions at –16 and –6 in the US9 promoter were analyzed.

To resolve the question of whether the operator insertions at –16 and –6 were adequate for the objectives of this project, it was critical to determine the extent to which the operators affected promoter activation in the absence of the repressor. To answer one aspect of this question, the operator-containing US9 promoters were cloned into a CAT reporter plasmid and tested for activation by IE1 and IE2. Figure 22 shows that the operator positioned at -16 (in pUS9op(-16)CAT) had the least impact on promoter expression. Activation of this promoter was 70% that of the wild-type promoter. In

addition, promoter expression in pUS9op(-16)CAT in the presence of all viral proteins (Figure 23) showed a lesser impact of the operator insertion, when compared to insertion at –6, relative to the wild type promoter in pUS9CAT. Clearly, these results suggested that the US9 promoter with the operator adjacent to the TATA box (at position -16) was the most appropriate promoter for an accurate analysis of repression by the *lac* repressor, due to a lesser effect on basal expression of the promoter by the operator insertion. However, because different outcomes can be expected in terms of the intrinsic influence of the operator at different positions relative to the TATA box when the promoter is analyzed in the context of the HCMV genome, both operator-containing US9 promoters were inserted into the endogenous locus to generate recombinant viruses RVUS9op-16 and RVUS9op-6, as described above.

In order to determine the impact of the operator insertion on endogenous US9 promoter expression, US9 mRNA was measured in cells infected with RVUS9op-16 and RVUS9op-6. It was desired that basal promoter activity would not be affected significantly by the operator insertion, in order to use these variant promoters to conditionally express the US9 gene using the *lac* repressor and IPTG. The results presented in Figure 24 demonstrate that the operator insertions at –16 and –6 in the US9 promoter do not have a major impact on endogenous promoter expression. Although a 2-fold increase in US9 mRNA levels was observed in cells infected with RVUS9op-16 or RVUS9op-6, relative to the highest levels of US9 mRNA achieved in cells infected with AD169 or RV134, these changes do not reflect a significant influence of the operator insertion on basal promoter expression. It is worth noting that the operator insertion at –6 may be more suitable to assess *lac* repressor-mediated repression, since US9 mRNA

levels in cells infected with RVUS9op-6 were equivalent to wild type levels at 24 hs post infection, and increased approximately 2-fold at later times. Thus, basal levels of promoter expression were not decreased as a result of the operator insertion. On the other hand, US9 mRNA levels in cells infected with RVUS9op-16 were lower than wild type levels at 24 hs, so this operator insertion resulted in a reduction of basal promoter activity at early times. These data suggest that the operator insertions in the endogenous US9 promoter, in particular, when the operator is positioned at –6 relative to the transcription start site, may be appropriate for the application of the *lac* repressor-based system to study US9 gene expression in the context of the viral genome.

Furthermore, it was interesting to observe that US9 mRNA at late times was higher than that at early times in cells infected with any of the recombinant viruses. This suggests that insertions within and upstream of the endogenous US9 promoter have an impact on the kinetics of US9 gene expression. This early-late pattern of gene expression may be of no relevance for the use of the *lac* repressor-based system to conditionally express the US9 gene, but it may be the subject of future studies on the regulation of endogenous US9 promoter expression. The decrease in US9 gene expression from RV134 at early times was also observed by Jones et al. (1991), although they reported that this was a slight reduction.

After analyzing the intrinsic impact of the operator insertions on basal US9 promoter expression, both in transient assays and in the context of the viral genome, it was of critical importance to examine the ability of the *lac* repressor produced in RV*lac*-infected cells to repress the expression of the operator-containing US9 promoters, in order to demonstrate the susceptibility of an HCMV-specific promoter to regulation by

the *lac* repressor. It was reasoned that because the operator insertion in pUS9op(-16)CAT had a lesser impact on basal promoter expression in transient assays, this reporter construct would be more suitable for the analysis of *lac*-mediated repression in transient assays. The procedure involved transfecting cells with the CAT reporter construct and subsequently infecting with wild type virus AD169 or RV*lac*. A highly significant reduction in CAT activity was observed in the presence of the *lac* repressor, at 24, 48, and 72 hs post infection (Figure 25). The repression observed was 10-fold, which is in agreement with reports by others (Hu and Davidson, 1987), (Deuschle et al., 1990), when using single operator insertions. Levels of repression of 30- to 50-fold have been reported when two operators in tandem were used (Hu and Davidson, 1987). The level of regulation by the *lac* repressor obtained in mammalian cells so far is substantially lower than that within the *lac* operon in *E.coli*. In bacteria, repression of expression can be 1000-fold (Gilbert and Muller-Hill, 1970). One possibility for this significant difference is the DNA context in which the operator is located. The different molecular configurations of bacterial versus eukaryotic DNA (in this particular case, the viral US9 promoter) may influence the formation and/or the stability of the operator-repressor complex, thus affecting the magnitude of the repression achieved. Another explanation relates to the transport of the *lac* repressor to the nucleus of the eukaryotic cell. It is possible that higher levels of repression were not obtained because of insufficient amounts of functional tetrameric repressor present in the nucleus of the RV*lac*-infected cells. Although significant levels of the 37 kD polypeptide were detected at 24, 48, and 72 hs in RV*lac*-infected cells (see Figure 7), the efficiency of transport of the repressor

from the cytoplasm to the nucleus, as well as the sufficiency of functional tetramers to act upon the operator sequence are unknown.

In contrast with these observations are the calculations by Figge et al. (1988), who stated that "....the concentration of repressor molecules required to achieve 99.9% operator saturation with repressor would be $5x10^{-8}$ M, which corresponds to 2000 repressor tetramers per nucleus. We previously calculated that our cells (CV-1P cells) each contain approximately $4x10^{4}$ repressor tetramers. If 10% of these are contained within the nucleus as shown by Hu and Davidson (1987), then there is sufficient repressor present to achieve >99.9% saturation of a single chromosomal operator site".

However, in the paper by Figge et al., a single operator-containing SV40 hybrid early promoter-CAT gene cassette had been stably integrated into high molecular weight DNA. In this study, as well as in others using plasmids bearing operator-modified promoters, the number of operator sequences within the nucleus is likely to be much higher. Therefore, the amount of repressor required for efficient saturation of all the operator sites within the nucleus may be substantially higher as well. Finally, although the magnitude of the response is far lower than in bacteria, the repression obtained is probably stringent enough to assess regulation of expression of the operator-containing US9 promoter accurately.

To assess IPTG-mediated derepression of pUS9op(-16)CAT in RV*lac*-infected cells, concentrations of IPTG ranging from 0 to 2 mM were used, and cells were exposed to IPTG for 70 hs following infection with RV*lac*. According to the reports on IPTG induction in mammalian cells, the conditions chosen in this study represent a medium dose of IPTG (high doses range from 5 to 50 mM, and toxicity has been reported at

concentrations higher than 50 mM), and a maximal time of exposure (in other studies, exposure to the inducer ranged from 7-12 hs to up to 3 days). A significant level of derepression (about 80%) of pUS9op(-16)CAT was achieved with 0.8 and 2 mM IPTG (Figure 27). This degree of derepression is concordant with those obtained by others (60-80%) (Hu and Davidson, 1987). Although the repressed phenotype was not fully reversed, the results obtained may still be significant. A plateau seems to have been reached at 0.8 mM IPTG, but testing higher concentrations of IPTG may result in 100% reversion.

The amounts of IPTG required to obtain 80% derepression in these experiments are about 10-20 times higher than those that result in maximum induction in *E.coli* (0.1 mM) (Cho et al., 1985). A possible explanation is that the amount of IPTG that actually enters into the cytoplasm, and is subsequently transferred to the nucleus, may be significantly less than the concentrations present in the culture media. In addition, the rate at which IPTG binds the operator-bound repressor in the nucleus may be slower than in *E.coli*, resulting in derepression with slow kinetics compared to the induction process in bacteria.

Overall, the IPTG derepression experiments suggest that IPTG can enter the RV*lac*-infected cells, and act upon the repressed operator-containing US9 promoter to significantly relieve the block on promoter expression, leading to activation to 80% of the parental promoter expression levels. These conclusions strictly apply to the transient expression system. In other words, for repressed operator-containing US9 promoters in the viral genome, the conditions for IPTG derepression would have to be optimized.

All the observations discussed above relate to the central objective of this project, i.e., the analysis of *lac*-mediated repression of operator-containing US9 promoters in transient assays using CAT reporter constructs, and the ability of IPTG to reverse the repressed phenotype. Future studies, however, will analyze the *lac* repressor-based system in the context of the HCMV genome.

CHAPTER VI

CONCLUSIONS

An *Escherichia coli lac* repressor-based system was developed to study HCMV gene expression in the context of the viral genome. Two major aspects were investigated: a *lac* repressor expressed from recombinant HCMV, and an operator-containing HCMV specific promoter for which *lac* repressor-mediated repression and restoration of promoter activity by IPTG can be efficiently achieved. The system was first tested in transient expression assays, to assess the feasibility of this approach to conditionally express a HCMV specific promoter. The repressor produced from recombinant HCMV (RV*lac*) efficiently repressed operator-containing heterologous promoters in CAT reporter constructs in transient assays. The repression observed was reversed by IPTG. Different operator insertion sites within the HCMV US9 promoter were tested to assess the impact of the operator sequence on basal promoter expression, both in transient assays (using CAT reporter constructs), and when these promoters were inserted into the viral genome. Basal promoter expression was affected to a lesser extent in transient assays when the operator sequence was inserted at –16. Thus, this promoter was chosen for the analysis of *lac* repressor-mediated repression in transient assays. The viability of this system to control an HCMV promoter was demonstrated when efficient repression of the operator-containing US9 promoter was observed in the presence of RV*lac*, and repression was reversed after the addition of IPTG. Moreover, the operator insertions in the endogenous US9 locus did not affect US9 basal promoter expression significantly. Interestingly, the operator insertions affected the kinetics of US9 gene expression in the context of the virus: an increase in the levels of US9 mRNA at late times post infection

was observed. These observations provide the basis for future investigation of the US9 promoter sequences affected by the operator insertions that resulted in changes in US9 promoter expression. Overall it was concluded that the *lac* repressor-based system can be applied to study HCMV gene expression. Future studies may address the conditional expression of other HCMV promoters, adapting this system so that putative essential HCMV genes can be targeted.

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VITA

I was born in Buenos Aires, Argentina. I obtained my Bachelor's (Biology major) and Master's (in Biotechnology) degrees from the University of Buenos Aires in 1985 and 1988, respectively. After my graduation, I was awarded a four-year graduate research fellowship by the National Council of Scientific Research of Argentina (CONICET). I did basic research on the role of thyroid hormones in thermogenesis at the University of Buenos Aires School of Medicine, where I also served as a teaching assistant for medical students. I presented my work in numerous national and international meetings, and I published seven papers as a co-author (four of which as first author) in major journals, such as Neuroendocrinology and Acta Endocrinologica.

I came to the United States in 1992, in order to pursue a Doctoral degree in Biomedical Sciences. My interests were to do basic research concentrating on regulation of gene expression. The research I performed in Dr. Stenberg's laboratory (Eastern Virginia Medical School, Department of Microbiology and Molecular Cell Biology, 700 West Olney Road, Norfolk, Virginia 23507) allowed me to fulfill my expectations. The studies presented in this dissertation show that I was able to gain ample experience in the area of my interest. I presented my work in several local and international meetings. As a result, I obtained several awards, from Eastern Virginia Medical School, the American Society for Microbiology, and at the 22nd International Herpesvirus Workshop. I have been a scholar member of the Honor Society of PHI KAPPA PHI since 1994. At present, a manuscript including these studies is in preparation for submission for publication.