INVOLVEMENT OF CELLULAR TRANSCRIPTION FACTORS YY1 AND SPX IN REGULATING TRANSACTIVATION OF HERPES SIMPLEX VIRUS TYPE 1 γ_1 GENE PROMOTERS

by

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ABSTRACT

The goal of this research was to identify and characterize cellular factors involved in activating herpes simplex virus type 1 (HSV-1) γ_1 genes. Studies of two model γ_1 gene promoters, from the major capsid gene VP5 and glycoprotein D (gD), enabled us to identify two cellular factors which effected the activation of these genes. Cellular transcription factor YY1 was identified as the protein which bound to a DNA element which was needed for activating VP5 following HSV-1 activation or cotransfection with HSV-1 transactivating proteins ICP0, ICP4, and ICP27. YY1 also bound to the regulatory regions of several other HSV-1 genes including the gD promoter. Therefore the role of YY1 binding to gD was evaluated, following specific mutational inactivation of the YY1 site, in transient expression assays in which the viral promoter construct was induced by superinfection with HSV-1 or cotransfection with ICP0, ICP4, or ICP27. During the course of these experiments, we found that a second cellular protein, a member of the Sp1 family ("SpX"), bound the gD promoter at a site overlapping the YY1 site. YY1 and SpX bound independently to this cis-acting element which is located at -75 to -53 in the gD promoter. The function of SpX binding site also was examined in transient expression assays. The results of these assays demonstrated that both YY1 and SpX activate the gD promoter with SpX playing the major role. Cotransfection experiments established that a functional SpX site was sufficient to permit gD promoter activation by ICP0 and ICP4.

CHAPTER 1: INTRODUCTION

INTRODUCTION TO HERPES SIMPLEX VIRUS TYPE-1

I. Historical Perspective

The ancient Roman scholar Herodotus provided the earliest specific description of the symptoms of herpes simplex virus infection. He described "the herpetic eruptions which appeared about the mouth at the crisis of simple fever and the weals of febrile urticaria" at approximately 100 A.D. (129). Even earlier, Greek physicians applied the term "herpes"--meaning "to creep"-- to any disease producing spreading cutaneous lesions (226). Hutfield and other historians attribute the classical description of genital herpes to the French physician John Astruc, who published "De Morbis Venereis" in 1736 (129, 226). According to Hutfield's history, Diday and Doyon, the authors of the first published book on the genital herpes (published in 1886), believed that either a nerve-associated or blood-borne "trigger mechanism" reactivated the herpetic lesions. Previously, physicians had considered herpes outbreaks as the consequences of nerve disorders, "humors," sexually transmitted diseases, or overindulgence in sex (129).

The study of herpes infections by Grüter, Löwenstein, Luger, and Lauda at the turn of the 18th century shifted from patient observation to experimental studies to indentify the agent responsible for infectious transmission (reviewed in 226). By the late 1940's and early 1950's herpes simplex viruses were established as the

causative agents of numerous diseases affecting the skin, mucous tissues, eyes, and occasionally, the brain or internal organs of the host (e.g., 227, 336).

As early as 1921 Lipschutz suggested that two "etiologically different," although biologically related, herpes viruses existed: herpes febrillis and venereal herpes. However, this hypothesis was controversial (226). The definitive work of Nahmias and Dowdle, which clearly identified two antigenically and biologically related, but distinct, types of "herpes hominis" viruses, vindicated Lipschutz in 1968 (226). Herpes hominis type 1 was responsible for non-genital infections and now is called herpes simplex virus type 1 (HSV-1). Conversely, the viral agent of genital herpes was classified as herpes hominis type 2, now known as HSV-2 (Table 1-1). Clarifying the similarities and differences between HSV-1 and HSV-2 continues to be a focus of research (e.g., (154, 195, 305)).

The phenomenal amount of HSV research accomplished since the 1960's renders nearly impossible writing a historical summary that is both brief and comprehensive (for recent reviews see 262 and 266). The explosion of research during the last 35 years resulted from newly developed techniques in molecular biology that have expanded the horizons of what is possible to learn about the virus. For example, the spread of individual strains of HSV-1 and HSV-2 now can be carefully tracked, thus explaining more of the epidemiology of herpes simplex virus infections (e.g., 280, 341). Increased knowledge about the macromolecular synthesis by the viruses triggered the development of antiviral therapies which are

effective in many, though not all, HSV infections (337). New techniques enabled researchers to probe the mechanisms of viral replication and pathology (e.g., 4, 51, 54, 119, 320). Some of the information gathered in the last few years is presented in the following sections of this introduction.

II. Classification

Herpes simplex virus type 1 (HSV-1) is a member of the family herpesviridae. All herpesviridae share common structural features including of a central core of double-stranded DNA surrounded by a protein capsid composed of 162 capsomeres which has a diameter of approximately 100 nm and is called a nucleocapsid (Fig. 1-1). A lipoprotein envelope coats the nucleocapsid, and an amorphous tegument is between the nucleocapsid and envelope. The nucleocapsid is assembled in the cellular nucleus. By electron microscopy the viron envelope appears trilaminar and is composed of host cell membranes which contain viral glycoproteins (reviewed in 225, 263).

In addition to structural features, two other characteristics distinguish the herpesviridae from other DNA viruses. A hallmark of herpesviridae is their ability to produce life-long latent infections after primary infection of the host. Latent herpesviruses can be reactivated through various physical and chemical stimuli resulting in the production of infectious viruses throughout the life of the host (224, 311). A second distinguishing feature of the herpesviridae is the ability of the

de-proteinated viral genome to produce infectious virus when introduced into the host cell (288).

The family herpesviridae are grouped by biological traits into three subfamilies: alphaherpesviruses, betaherpesviruses, and gammaherpesviruses. HSV-1 is one of the eight human herpesviruses and is a member of the alphaherpesviruses (65). The other alphaherpesviruses which infect humans are HSV-2, which shares 50% homology with HSV-1 at the nucleic acid level (154), and varicella zoster virus (334). The alphaherpesviruses are characterized by their ability to grow rapidly in tissue culture in a wide variety of cell types. Alternative animal models have been established because virus replication is not restricted to the host species. Arrest of host cell macromolecular synthesis follows productive infection, which ultimatily results in cell destruction. Characteristically, alphaherpesviruses productively infect epithelial cells while latency is established in neuronal cells primarily, but not exclusively, in the sensory ganglia.

The two herpes simplex viruses, HSV-1 and HSV-2, are closely related. However, the two viruses can be distinguished from one another, and from the other herpesviruses, by specific biochemical and biological properties. These characteristics include differences in the genome and different antigenic proteins (154, 226, 305). Slight differences in tissue tropism produce a closely related but distinct spectrum of disease (Table 1-2, reviewed in 182).

In comparison with the alphaherpesviruses, the betaherpesviruses and the

gammaherpesviruses propagate slowly and have a limited host range, infecting only animals from the same order or family to which the natural host belongs. The human betaherpesviruses include human cytomegalovirus, human herpesvirus (HHV)-6, and HHV-7 (15, 86, 189, 269, 300). The betaherpesviruses productively infect several cell types within the host, and the site of viral latency is controversial. Cells infected with betaherpesviruses frequently form cytomegalia or syncytia of cells through fusion.

The prototypic human gammaherpesvirus is Epstein-Barr virus (EBV, (73)). However, the recently discovered HHV8, or human kaposi-associated herpes virus, tentatively has been assigned to the gammaherpesvirus group based on genomic similarity to EBV and another member of the group, herpes saimiri (38). Gammaherpesviruses productively and latently infect B or T lymphocytes. However, *in vitro* some gammaherpesviruses productively infect epithelioid and fibroblastic cells (263). Both human and nonhuman gammaherpesviruses are associated with oncogenesis in the host (e.g., 153, 303).

HSV-1 BIOLOGY

I. Diseases

Spread of HSV-1 occurs through direct contact with infected tissue. The usual site for primary infection is the oropharyngeal mucosal epithelium. The majority of primary infections by HSV-1 in immunocompetent individuals are asymptomatic.

More than 80% of a given population is infected with HSV-1 by adulthood. The clinical symptoms which do occur in the immunocompetent host are rarely life-threatening and include sore throat, fever, mucosal ulcers or lesions, and malaise (reviewed in 182, 338). During the normal course of disease following the primary infection the HSV-1 virus becomes latent in the trigeminal ganglia and periodically reactivates resulting in recurrent vesicular stomatitis. However, primary HSV-1 infections of neonates and other immunocompromised individuals can lead to fatal disseminated multi-organ infection or encephalitis (e.g., 195). Although infrequent, primary HSV-1 infection may also result in fatal meningoencephalitis (e.g., 227).

A defining characteristic of the non-fatal HSV-1-associated diseases is the recurrent nature of the symptoms. Classically, HSV-1 results in recurrent vesicular stomatitis of the facial mucosa and epithelium. The vesicles are less prominent in the mucosa and instead appear as shallow ulcers. In addition, recurrent HSV-1 infections can spread to the optic neurons and result in eye infections. Herpes simplex keratoconjunctivitis is second only to trauma as a cause of corneal blindness in the United States (183).

In addition to oropharyngeal mucosal tissues, primary infection sites for HSV-1 also include abraded epithelium and genital mucosa. Examples of skin infections by HSV-1 are recurrent eczema herpeticum or herpes gladitorium (336). Genital infections resulting from HSV-1 are similar to those caused by HSV-2.

HSV-2 is the major cause of virally-associated genital lesions which produces recurrent infections of the genitalia (129, 338). HSV-2, like HSV-1, also infects any mucosal tissue or abraded epithelium and is associated with a spectrum of diseases similar to HSV-1. The infant mortality rate following neonatal infection is higher with HSV-2 than with HSV-1 (195). Interestingly, the incidence of genital infections in women which are attributable to HSV-1 rather than to the more common cause, HSV-2, has increased in recent years (280, 341).

II. Primary Infection, Latency, and Reactivation

An HSV-1 primary infection initiates with the virion binding to the extracellular matrix of epithelial of cells. The initial binding is followed by a stable receptor-mediated binding to the cell and fusion of the viral envelope with the cellular plasma membrane (reviewed in 182, 263). Once the de-enveloped viral particle transports to the nuclear envelope, the viral DNA is imported into the nucleus and the viral genes are activated. Following viral protein expression, the viral nucleocapsids assemble in the nucleus within large inclusion bodies (217). The nucleocapsid associates with the tegument and leaves the nucleus and obtains an envelope made of cellular phospholipids and viral glycoproteins. How this occurs is currently a center of controversy (35, 39, reviewed in 263). Once the nucleocapsid is enveloped, the viral glycoproteins contained within the viral envelope undergo further processing which results in a fully infectious mature virion (39, 162). *In*

vitro, infectious virions are present within 12 hours of the initial infection (119). Virions leave the infected cell either by exocytosis to the extra-cellular environment or by a yet to be determined method of direct cell-to-cell infection. Ultimately, the production and exiting of the virions destroys the infected cell.

HSV-1 enters the nervous system by direct cell-to-cell infection from productively infected epithelial cells to the sensory neurons. The virus particles travel retrograde up the neuronal cells until the particles reach the ganglia and the virus establishes latency in the form of an episome (210, 294, reviewed in 182, 263, 338). In a facial infection, HSV-1 latency is established in the trigeminal ganglia, while in a genital infection, latency is established in the sacral root ganglia. In addition to the ganglia, HSV-1 genomes have been detected in vascular tissues (13, 346) and in the brain (139).

Cytopathology is not apparent during latency. The viral DNA is uncoated within the nucleus, but is maintained as an essentially silent circular episome (210). While specific viral RNAs are synthesized during latency, viral protein synthesis has not been irrefutably documented for HSV-1 (96, 165, 306, 312, reviewed in 234).

Reactivation of the latent HSV-1 genome leads to expression of the necessary genes to produce infectious virions. Physical or emotional stress reactivates HSV-1, as do UV light, illness, tissue damage, and immune system suppression (224). After reactivation, the infectious virions move down the sensory neurons and infect epithelial cells at the periphery. Generally, viral eruption and productive infection

cases are near the original site of the primary infection. However, during reactivation the virus can spread to other locations such as down the ophthalmic branch of the trigeminal ganglion to the eye or up from the trigeminal ganglion to the brain (reviewed in 182, 338).

Animal models which are used to determine the *in vivo* consequences of HSV-1 include mice, rats, and rabbits. For example, nasal inoculation of mice established that HSV-1 infections of the oronasal mucosa could ultimately infect the eye via the trigeminal ganglia (294) The effectiveness of protective vaccines is evaluated using animal models (e.g., 91, 229). In addition, unlike some other viruses, the HSV-1 virus readily infects many of the mammalian cell lines used in tissue culture (reviewed in 263). Thus, virion components which are essential for infectivity were indentified by *in vitro* infection of cultured cells by HSV-1 mutant viruses (e.g., 31, 125). Other techniques used in studying HSV-1 include explantation of neurons to study reactivation of the virus and cell-free systems to examine the molecular biology of HSV-1 (e.g., 271, 311).

HSV-1 MOLECULAR BIOLOGY

HSV-1 STRUCTURE

I. Genome

The HSV-1 genome is 152.26 kilobases long (202, 204, 243) and contains at least 72 open reading frames (ORF) depending on the strain (45, 202). The genomic structure consists of a 107.9 kb unique region (UL) flanked by 9.2 kb inverted repeats and a 13.0 kb unique region (US) flanked by its own 6.6 kb repeat sequences (204). The long repeat (R_L) regions contain the *ab* and *b'a'* sequences. The short repeat (R_S) regions contain the sequences *a'c'* and *ac* (203, 243).

Two other structural features are of interest in the HSV-1 genome and may be seen in the map of the functional organization of the HSV-1 genome provided in Fig. 1-2 (263). First, the HSV-1 genome contains three origins of replication initiation (oris). Two oris are present in the R_S regions, while the third is located in the UL region (263). Mutational analysis indicates that any single ori is sufficient for DNA synthesis. In a second feature, the genes encoded within the R_L and R_S are diploid and include genes for IE proteins ICP0 and ICP4 (203, 243). Also present in the repeat regions of some, but not all, strains of HSV-1 is the ICP34.5 gene (45).

The genome of HSV-1 is present in the nucleocapsid as one of four possible

stereoisomers (Fig. 1-3). The prototypic stereoisomer is defined as R_L-UL-R_L-R_S-US-R_S (reviewed in 263). The inverted stereoisomers are produced in equimolar amounts during a lytic infection (53, 263). One mechanism proposed for the inverting of the original genomic stereoisomer present within the infected cell is recombination between the *pac* homology sequences which are generated during the synthesis of HSV-1 DNA concatamers (52). The new viral DNA concatamers are synthesized from the original genomic template via a rolling circle process (52, 66) once the viral DNA has entered the cell nucleus and the genome assumes a circular form (87). The viral concatamers are cleaved into monomeric genomes following recombination and packaged into preformed capsids (e.g., 6, 304). Recently, Zhang, et al., (356) demonstrated that inverted copies of the original genomic stereoisomer are present early during concatamer synthesis.

II. Virion structure

The HSV-1 virion consists of an enveloped nucleocapsid with a tegument between the capsid and envelope. The selective extraction of capsid components with 2.0 M guanidine hydrochloride demonstrated that both the hexons and pentons of the capsid are composed of the major capsid protein VP5 (UL48, 231). Between the hexons and pentons are triplexes, also called intercapsomeric fibrils, which are composed from one molecule of VP19C (UL38) and two molecules of VP23 (UL18, 231). VP19C also binds HSV-1 DNA according to data from

electrophoretic gel mobility shift assays (23). The proteins VP24 (UL26) and VP26 (UL35) also are components of the completed nucleocapsid (230, 263, 279, 322, 359).

Two additional proteins are necessary for capsid assembly although they are absent from the DNA-containing nucleocapsid. VP21 (UL26) and VP22a (UL26.5) serve as the scaffolding or support within the capsid during assembly (260, 291). Without VP22a and VP21, the other capsid proteins assemble into sheets or other aberrant structures (57, 320).

The tegument is an amorphic mass of proteins between the envelope and capsid. One major component of the tegument is VP16 (αTIF, Vmw65, ICP25, (11, 37, 263)). Other proteins in the tegument are the viral-host shut-off (*VHS*) protein (253), VP13/14 and VP1/2 (72, 225). Depending on which cell lines are used for viral infection and virion assembly, other proteins also can be present in the tegument (208, 318, 349, 350). The regulatory proteins ICP0 and ICP4 are found in the tegument of complete virions from primate cell lines HEp2 and Vero although not in virions assembling in baby hamster kidney cells, BHK-21 (347). Chemical cross-linking studies demonstrated that four tegument proteins, including VP16, associate with the intravirion domains of the envelope viral proteins gB, gD, and gH, but not with gC (360).

The envelope of the virion is derived from cellular phospholipids and numerous viral glycoproteins are located in the virion envelope. Glycoproteins gD, gB, gH, gK, and gL are defined as essential to virus replication by virtue of being required

for replication *in vitro* (31, 51, 85, 126, 184, 265). Important auxiliary roles are played by several of the so-called dispensable glycoproteins such as gC, gM, and gE/gI (e.g., 8, 31, 51, 62, 63). In addition, other glycoproteins of unknown function are present in the envelope, for example gG and gJ (8). The following glycoproteins are present as complexes within the envelope which may important to their function: gE/gI, gH/gL, and multimers of gB (62, 125, 360).

In addition to the infectious virions, there are other viral particles produced during an infection which lack one or more of the virion components. For example, L particles contain only tegument enclosed in an envelope (208, 318). On the other hand, type A capsids contain capsid shell proteins but lack scaffolding proteins, tegument, envelope, and viral DNA (e.g., 35, 291, reviewed in 225, 263). While neither particle is infectious alone, L particles enhance the infectivity of virions when included in the viral inoculum (206). Up to 10^4 to 10^5 particles may be made within an infected cell, but only 10^2 to 10^3 particles become assembled into infectious virions (65).

HSV-1 REPLICATION

I. Virion Entry

At the molecular level, productive HSV-1 infection initiates with the transitory binding of the virion to cellular heparin sulfate and chondroitin sulfate glycosaminoglycans ((Fig. 1-4) 31, 344). However, the ability to infect of heparin

sulfate deficient mouse cells or heparin and chondroitin sulfate deficient mouse cells by wild-type (wt) HSV-1 demonstrates that alternate means exist for the virion to bind the cell (9, 98). Binding to the proteoglycans is mediated by the glycoprotein gC (31, 344, 354). Yet, viruses deficient in gC enter non-polarized cells efficiently and spread from cell to cell *in vitro*. Another glycoprotein, gB, plays a undetermined role in the initial transitory binding by the gC deletion viruses (305). The binding of the virion activates cell surface components. These transitory associations position the virion close to the cellular plasma membrane permitting the occurrence of the essential next step which is stable receptor-mediated binding between specific viral glycoproteins and cell surface molecules.

Entry of the virion into the cell is inhibited by soluble gD but not by soluble gB, indicating that virion attachment and entry requires gD specific interactions with receptors which are present on the cell surface in limited quantities (143, 184). Brunetti et al. (27) demonstrated that gD interacts with either the 275-kDa mannose-6-phosphate/insulin-like growth factor receptor (M6P/IGFIIR) or the 46-kDa cation-dependent mannose-6-phosphate receptors which are present in the plasma membranes of many mammalian cell lines. Mannose-6-phosphate receptor specific antibodies and soluble M6P/IGFIIR inhibit up to 75% of the infectivity of wt HSV-1 on Vero cells (26, 27). In contrast, mouse cells are fully infective for wt HSV-1 when both of the mannose-6-phosphate receptors are absent (26). Thus, while the mannose-6-phosphate receptors are involved in gD-mediated virion

attachment, other routes exist for receptor-mediated entry into the cell.

The virion can enter the cell by endocytosis or by fusion. If the virion enters the cell by endocytosis the viral particle is degraded within the lysosomes without causing infection (34). However, infection takes place if the HSV-1 virion envelope fuses with the plasma membrane of permissive cells. Although the process is not well understood, fusion requires at least three glycoproteins, gB, gH/gL, and gK (31, 85, 126, 128). Interestingly, gK is required not only for virion envelope fusion early during infection, but at the end of infection to facilitate virion export out of the cell (127). In the case of HSV-1, fusion is pH independent (305).

Depending on the HSV-1 viral strain or host cell type involved, additional glycoproteins are also important for initiating virion entry into the cell. For example, although the gE/gI complex is dispensable *in vitro* for the initial fusion of a virion from the supernate, the gE/gI complex is required for direct transfer of the virus from an infected cell to an uninfected cell (62, 63). Additionally, infection of mice with viruses containing disruption of either the gI or the gE gene show that both gE and gI are required for full infectivity and viral spread within ear epithelial tissue (8). Another glycoprotein, gM, also is required by some viral strains for cell-to-cell infection (7, 51). The difference in glycoprotein requirements for initial *in vitro* virion entry as compared with direct cell-to-cell spread implies that the two mechanisms are different.

The viral particle is released directly into the cytoplasm following fusion with

the plasma membrane. An intact cytoskeleton is required for the translocation of the viral particle through the cytoplasm to the pore of the cellular nucleus since translocation is inhibited by drugs such as nocodazale which depolarize the cellular microtubular structure (167). The viral DNA and the tegument proteins are imported into the cellular nucleus, while the capsid proteins remain in the cytoplasm and are degraded into amino acids.

II. Transcriptional Control of the HSV-1 Genes

A. Introduction to the HSV-1 gene classes.

During the productive infection cycle, the HSV-1 genes are expressed in three definable classes: immediate-early, early, and late (Fig. 1-5, (119, 120, 317)). The classes are defined by the requirements for, and the order of, their expression. The optimal expression of the immediate-early (α or IE) class of HSV-1 genes does not require *de novo* viral protein production; however, cellular factors and the presence of virion components are necessary (11, 37, 90, 205). The full expression of both the early (β or E) genes and the late (γ or L) genes requires the presence of viral α proteins, in particular ICP0, ICP4, and ICP27 (18, 50, 54, 64, 315). As a result, both β and γ gene expression are prevented in the presence of cycloheximide and other protein synthesis inhibitors (119, 317, 357). The γ genes are differentiated from the β genes by requiring viral DNA replication in addition to the presence of α proteins for normal expression (117, 119, 317). Active viral DNA replication appears to be required for late gene expression because the addition of increasing

numbers of viral genomic templates does not trigger γ_2 gene expression (93, 145).

Examination of *in vitro* HSV-1 infections utilizing metabolic inhibitors has provided a general time-line for viral gene expression (119, 120, 331). The accumulation of specific proteins may differ from the rate of mRNA transcription initiation depending on the posttranscriptional regulation (e.g. 144). The production of α gene peptides begins at 2 hours post-infection (hpi) and is repressed by 6 hpi. However, the alpha gene mRNAs are present by 1 hpi, remain high through 6 hpi, and are undetectable by 12 hpi. The beta gene protein products peak at 5-7 hpi, while the β gene mRNAs are present by 3 hpi, peak at 6-8 hpi, and slowly taper off by 12 hpi. Viral DNA synthesis follows β gene expression, initiating at 3 to 4 hpi and peaking at 12 hpi. While the height of gamma gene protein production is from 12 to 17 hpi, the γ_1 gene mRNAs are present by 3 hpi. Transcription of γ_1 genes increases, and γ_2 gene transcription, initiates following the start of viral DNA replication.

B. The α Genes

There are five known α genes, four which participate in regulating the transcription of the other viral genes. Two of these proteins, ICP4 (infected cell protein 4) and ICP27 are essential (54, 77, 267). ICP4 (α4, Vmw175, IE175) is required for transcription of the genes in the β and γ classes, and represses the transcription of the α genes, including its own gene (54, 236). ICP27 (α27, Vmw63, IE63) plays several roles, including regulating the processing of viral and cellular mRNAs (107, 109, 274) and enhancing viral DNA replication (254). Two

α products, ICP0 and ICP22, enhance the transcription of the β and γ class genes in a cell-specific manner. ICP0 (α0, Vmw110, IE110) synergistically enhances the activation associated with ICP4 (77, 281, 315) and independently activates many cellular and viral genes (56, reviewed in 263). ICP22 (α22) is associated with incomplete phosphorylation of the C-terminal repeat domain of the RNA polymerase II enzyme (RNA Pol II) which in some cell types correlates with enhanced γ gene transcription (256).

The fifth α gene product, ICP47 (α47), however, does not regulate viral gene expression, but rather assists the virus in evading the host's immune surveillance system (158, 353). ICP47 inhibits the presentation of HSV-1 antigens by the major histocompatibility complex I (MHC-I) by preventing the transportation of viral peptides into the endoplasmic reticulum and thus preventing the peptide from binding to the MHC-I complex (86a, 114a).

Because of the importance of ICP0, ICP4, and ICP27 in regulating mRNA transcription and processing from all three classes of viral genes, these proteins will be described in greater detail before discussing HSV-1 gene transcription. ICP0, ICP4, and ICP27 are nuclear phosphoproteins (339) and are both guanylated and adenylated (16, 17, 248). These viral proteins do not appear to be kinases. However, changes in the phosphorylation state of these proteins could result in altering their function in a manner similar to the changes demonstrated for many other proteins (e.g., 124, 137). All three proteins also contain a zinc-finger domain (79, 108, 220), a feature which is important in protein-protein or protein-DNA

interactions (111, 329).

Many protein-protein interactions are mediated by ICP0, ICP4, and ICP27. For example, both ICP4 (213) and ICP0 (40, 46) form homodimers. ICP4 also heterodimerizes with ICP0 (351) and likely heterodimerizes with ICP27 (270). ICP0, ICP4, and ICP27 also associate with specific cellular proteins. As a example, early during the infection ICP0 colocalizes with the nuclear protein structure ND10 (161, 199, 313). ICP0 also immunoprecipitates with another unidentified 130 kDa nuclear protein (211). The presence of ICP27 in the cell nucleus triggers a redistribution of the small nuclear ribonucleoproteins (snNRPs), subunits of the cellular mRNA complexes, into fewer and larger granules as opposed to the speckled distribution of snNRPs appearing in uninfected cells (245, 273).

Examples of the complex interplay between ICP0, ICP4, and ICP27 appear in the nuclear importation process. *In situ* immunofluorescence studies of cells infected with wt or mutated HSV-1, or cotransfected with plasmids expressing wt or mutated ICP0, ICP4, or ICP27 revealed that these proteins regulate the subcellular localization of each other (221, 351, 361, 362). ICP27 inhibits the transportation of ICP0 and ICP4 to the nucleus during early infection. The level of inhibition is greater with increasing concentration of ICP27 or multiplicity of infection (moi). In contrast, ICP4 enhances ICP0 translocation to the nucleus (361, 362]). However, in a wild-type HSV-1 (KOS) infection, or when expressed individually from plasmids, all three proteins reach in the nucleus by 5 hpi (220,

361, 362). The physiological significance of delaying the subcellular transport of ICP0 and ICP4 by ICP27 remains unclear. However, the effects of ICP27 on the importation of ICP0 and ICP4 must be considered when evaluating cotransfection expression assays which compare the results of combining these proteins.

ICP4, the major transcriptional regulatory protein of HSV-1, is essential for activation of the β and the γ genes (e.g., 54, 76, 77, 169). In addition, ICP4 ultimately represses the transcription of the α genes (54, 236). However, low concentrations of ICP4, comparable to those occurring early during infection, actually activate transcription of the α genes (54, 281, 351).

In contrast with either ICP0 or ICP27, ICP4 binds to specific sequences of DNA. Binding of ICP4 to the consensus site (ATCGTCNNNNYCGRC, (81, 214)) bends the DNA helix (80). In addition to binding to the consensus site, ICP4 also binds at low affinity to numerous DNA nonconsensus sequences (99, 214). The ability of ICP4 to downregulate α gene transcription requires the presence of an ICP4 consensus site in the correct orientation and spanning the transcription initiation point (59, 160, 259). However ICP4 binding at other locations enhances gene activation (47, 99, 100, 298).

The activation of β and γ genes by ICP4 does not require ICP4 consensus binding sites, in spite of the existence of ICP4 consensus sites in the regulatory regions of several β and γ genes (133, 297). For example, a mutated virus producing an ICP4 peptide incapable of binding DNA still expresses wild-type levels of β and γ gene products in a normal kinetic pattern (289). However, by

binding to DNA, ICP4 enhances the binding of the TATA-binding protein (TBP) to the TATA box DNA sequence and enhances the stability of the TFIIB interaction with TBP, thus preferentially increasing viral gene transcription (47, 298). In addition, ICP4 contains a serine-rich protein domain which is homologous to the N-terminal domain of a newly identified cellular coactivator, PC4. The N-terminal domain is important to the interaction between PC4 and various cellular general, or basal, transcription factors (GTFs) (88). Intriguingly, the PC4-like domain of ICP4 is the most critical region for enhancing the binding of TBP and TFIIB (47, 298) suggesting the ICP4 and PC4 share a common mechanism.

Without ICP4, ICP27 is not sufficient to activate γ gene production, as demonstrated by the complementation with ICP27 of viral mutants containing both an ICP27 gene deletion and mutated or nonfunctional ICP4 gene (270). Nevertheless, ICP27 is essential to the normal replication of the HSV-1 virus and is associated with many regulatory activities (e.g., 281, 315). Among the functions attributed to ICP27 are (i) activating at least two β genes without ICP4 (270), (ii) increasing the efficiency of viral DNA synthesis, independent of its role as a gene activator (255), (iii) posttranslational modification of ICP4 (315), (iv) the possible modification of ICP0 (270), and, (v) most importantly, altering post transcriptional processing (207, 274). ICP27 binds to RNA (25) and enhances the processing of the 3' polyadenylation site (poly(A) site) in a sequence specific fashion at γ gene poly(A) sites, even though γ gene poly(A) sites are poorly adenylated in the uninfected cell (207). ICP27 increases the stability of rapidly degraded mRNA

transcripts such as the β interferon transcript when the poly(A) site from a HSV-1 γ gene is present (25). However, ICP27 also represses the processing of specific mRNAs by inhibiting the splicing of introns in a gene-specific manner (25, 107, 109, 274). By downregulating splicing efficiency of mRNA transcripts, ICP27 contributes to the downregulation of HSV-1 α genes and cellular genes which contain introns.

Although ICP0 is not essential for HSV-1 replication, the loss of ICP0 inhibits replication of the mutated viruses at low moi in most cell lines (33, 41) and reduces the ability of the virus to reactivate from latency (30, 32). In some cell lines, a cellular function substitutes for ICP0 in virus replication (33). The presence of ICP0 synergistically enhances ICP4-mediated transactivation of many HSV-1 β and γ genes, but ICP0 is not always required for wild-type transactivation by ICP4 (e.g., 78, 236). ICP0 appears to function through direct or indirect protein-protein interactions. For example, point mutations in the zinc finger domain of ICP0 demonstrated that this domain is required for interactions with ICP4 (79), and with cellular proteins located within the ND10 structure of the cell nucleus (74, 211). Interestingly, ICP0 acts as a promiscuous transactivator for many viral and cellular genes in transient assays (56, reviewed in 263).

C. Regulation of α Gene Transcription

The transcription of the HSV-1 α genes begins once the viral DNA has entered the cell nucleus. Although α gene expression can occur in the absence of viral

proteins (288), during an HSV-1 productive infection the transcription of the α genes is initiated by interactions between cellular proteins and the viral tegument protein, VP16 (αTIF, ICP25, Vmw65, (e.g., 11, 37, 197)). VP16 directly interacts with a cellular protein called HCF (C1, CCF, VCAF-1, (90, 150, 170, 335, 340, 345)). At the same time, another cellular protein, Oct-1 (αH1, OTF-1 (205, 235, 246)), binds to a DNA sequence characteristic of the α gene promoter, the TAATGARAT motif (191, 235). The VP16-HCF complex then forms a complex with the Oct-1 protein to form an transcription activation complex (150, 168, 310). Recent data shows that at least one other cellular component is present in the activation complex (5, 170). The activation complex stabilizes the cellular RNA polymerase II (RNA Pol II) initiation complex by interacting with the TFIID and TFIIB components and thus increases the transcript initiation frequency for the α genes (187, 314). The RNA polymerase II for transcribes the α genes without further viral modification.

The gene activation promoted by the VP16 complex requires a specific DNA binding factor such as Oct-1. Replacing Oct-1 with Oct-2.4 or Oct 2.5, closely related members of the octamer binding family which are expressed in neurons, inhibits transactivation by VP16 (172, 185, 186). This requirement for a specific DNA binding protein may operate in the establishment of HSV-1 latency in neuronal cells.

Deciphering the molecular interactions between the VP16 protein and the various general transcription factors has depended on extensive use of a fusion

protein system in which the activation domain of the VP16 peptide (a highly acidic region in the C-terminus) is fused to the DNA binding domain of the bacterial activator GAL4 (268). The resulting fusion protein is called GAL4-VP16. The targets of the VP16 activation domain defined thus far by the GAL4-VP16 system include TFIID (156, 314), TFIIB (187), and TFIIA (327). The overall effect of VP16 interacting with these different cellular proteins is to increase the numbers of effective RNA Pol II preinitiation complexes.

The production of α gene transcription is tightly regulated with α gene mRNA being nearly undetectable by 12 hpi (331). ICP4 is required for α gene transcription downregulation (e.g., 54, 236). The initial model for repression suggested that the binding of ICP4 to an ICP4 consensus site overlapping the transcription initiation site inhibited the formation of the RNA Pol II transcription complex (60, 160). However, Everett and Orr (75) have engineered a mutant virus which lacked the ICP4 site spanning the ICP0 initiation site. This mutated virus downregulated ICP0 with wild-type kinetics. Clearly, the overlapping ICP4 binding site was not the entire mechanism by which ICP4 regulated gene repression. Elaboration of this model comes from recent reseach which demonstrated that the ICP4 binding site also must be in the correct orientation for ICP4-associated repression (100). This result hints that protein-protein interactions are important in α gene repression as well. In addition, ICP27 contributes to α gene downregulation by repressing the splicing of the α gene transcripts (107, 109, 274).

D. The β Genes

HSV-1 β gene products are involved in three main functions: inhibiting host cell macromolecule synthesis, processing nucleotides for viral replication, and synthesizing viral DNA. The major host cell shut down gene is UL41 (*vhs*) (253). The *vhs* product enhances the degradation of all classes of mRNA including viral mRNA and thus also contributes to the regulation of viral gene expression (146, 171). Examples of viral enzymes in this class are thymidine kinase (TK, UL23) and ribonucleotide reductase (RR, UL39, and UL40). HSV-1 TK phosphorylates not only deoxypyrimidines as does the host thymidine kinase, but also purine pentosides, an ability which was exploited in creating several antiviral drugs such as acyclovir (337). The HSV-1 RR enzyme reduces ribonucleotides to deoxyribonucleotides, salvaging them for viral DNA synthesis (134).

Many of the functions needed to synthesize viral DNA are also encoded as β genes. The subunits of viral DNA polymerase is composed of two β gene products, the catalytic polymerase subunit (UL30) and the processivity subunit (UL42), both essential to the function of the enzyme (61). The HSV-1 helicase-primase is formed by three β polypeptides (UL5, UL8, and UL52) which associate as a complex (292). Additionally, two DNA binding proteins are also produced by HSV-1 β genes and are required for DNA synthesis, the origin binding protein (UL9), and the major single-stranded DNA binding protein, ICP8 (UL29) (264, 343). While HSV-1 is not known to encode a topoisomerase, a cellular topoisomerase, p107, is preferentially associated with viral DNA by 6 hpi (70).

E. Transcriptional Regulation of the β Genes

The transcription of the β class genes requires one or more of the α regulatory proteins, ICP0, ICP4 or ICP27. Most frequently, ICP4 is needed for activation (54, 56, 133, 270). Beyond this requirement, little else is agreed upon as required for β gene regulation. The many contradictory results reflect the presence of redundant mechanisms for regulating HSV-1 gene expression.

Historically, the thymidine kinase or tk gene has been considered the prototypical β gene (263). The principles accumulated from numerous studies apply to the structure of some, but not all, of the β genes. The tk promoter DNA sequences which are upstream from the TATA box are necessary for maximum gene expression levels, but are not required to regulate the β gene expression pattern (131, 132, 133, 200). In common with several other β genes, the upstream region of the tk promoters includes binding sites for the cellular transcription regulatory factors Sp1 and the CCAAT box factor. Mutating the Sp1 and CCAAT sites reduces the levels of gene expression, while not affecting the expression pattern (21, 132). Although ICP4 consensus binding sites are not needed, the ICP4 protein is required for the classic β gene expression pattern (133, 237). The mechanism by which ICP4 induces tk expression does not require the tk transcription initiation site (48). Although a TATA box DNA sequence is required for wild type tk expression, the TATA box sequence from the gC gene, a γ gene, can be substituted into the tk promoter with little change in either the level or timing of expression (309). In short, specific *cis*-acting DNA sequences have not

yet been definitively identified as required for, or characteristic of, the β gene expression.

Nevertheless, the need for the α proteins, especially ICP4, in activating and regulating the β genes is clear (e.g., 133). ICP4 enhances the stability of the TFIID and TFIIB complex (298) in the presence of the ICP4 binding site, and perhaps it retains this ability in the absence of known ICP4 binding sites. Enhancing the stability of the TFIID complex increases the frequency at which the RNA Pol II complex initiates transcription of a gene. Cook, et al., show that ICP4 increases the stabilization of the TFIID and TFIIB complex inversely to the ability of the TFIID complex to form in the absence of ICP4 (47).

In the absence of the ICP4 protein, ICP27 is capable of activating transcription from two β genes, tk and ICP6, which is likely another example of redundancy within the regulation of the HSV-1 genes (270). This work does illustrate that ICP27 functions as more than a post-transcriptional regulator.

The downregulation of the β proteins is associated with a decrease of β gene transcripts. Two mechanism account for this decline. First, the overall levels of the α gene products are decreasing in response to auto-downregulation by ICP4 and ICP27. Thus, the rate of transcription initiation from the β genes is reduced. Secondly, increasing amounts of the *vhs* protein, a β gene product, are produced which leads to a general degradation of all classes of mRNA, both viral and cellular (146). The increased breakdown of the β transcriptions combined with lower transcription initiation rate leads to the decrease in β gene production.

F. The γ Genes

The γ genes predominantly produce structural proteins which are found in the infectious virion such as the major capsid protein VP5, the envelope glycoprotein gD, and the tegument protein VP16. As discussed in detail in the section describing viral entry, the viral envelope glycoproteins function during attachment and fusion of the virion. The roles played by the viral capsid and tegument proteins were described in the section concerning viral infection and virion assembly. Some of the γ proteins play multiple roles during infection. For example, VP16 is required both for tegument structural integrity and for efficient transcriptional initiation of the α genes.

Several of the γ gene products elicit host immune antibodies, for example, gD and gB. Chief among these is glycoprotein D (gD), which triggers the production of neutralizing antibodies in humans and in mice (49, 301, 307). The immunogenicity of gD makes it the focus of attempts to develop vaccines (91, 229). In addition, the host response to gD is implicated as a contributing immunopathological factor in the development of blindness resulting from herpes stromal keratitis (e.g., 4, 182).

Many of the γ gene products are extensively modified. Some require association with other specific viral products for the correct posttranslational processing and transportation. For example, importing the VP5 peptide into the cellular nucleus requires VP22a (121, 232). The viral glycoproteins are both O-linked and N-linked glycoslated at different stages of virion maturation and at least one viral protein is

myristilated by cellular factors (e.g., 7, 36, 82, 192, 301, 305). The glycoproteins gH and gL exist as a heterodimer and both peptides must interact for proper folding of each other (125, 265). Several of the viral proteins must undergo proteolytic cleaveage such as VP21, VP22a, and VP24 (247, 248, 319).

G. The regulation of γ gene transcription

The levels of γ gene products such as gC, gD, and VP5, depend predominantly, although not entirely, on transcriptional regulation (94, 144, 299). In addition to the presence of viral α proteins, in particular ICP0, ICP4 and ICP27, the concurrent synthesis of viral DNA is required for the optimal expression of the γ genes (50, 94, 117). The γ genes are further divided into two sub-classes -- γ_1 (leaky-late or βγ and γ_2 (true late) -- based on dependence for viral DNA replication prior to the initiation of gene transcription. The transcription of the γ_2 genes stringently requires viral DNA replication. Thus, γ_2 genes are essentially silenced by the presence of DNA synthesis inhibitors such as phosphonoacetic acid (PAA). On the other hand, γ_1 genes are expressed at low levels prior to viral DNA replication or in the presence of PAA. The production of γ_1 genes is upregulated to higher levels following the start of viral DNA synthesis (e.g., 50, 94, 117, 241, 331).

Viral alpha proteins are requisite for the effective activation of the γ promoters as demonstrated by studies using cycloheximide reversal (119, 357), amino acid analogs (120, 241), transient expression assays (18, 50, 55, 78, 281, 315), and

HSV-1 viruses containing mutations in the alpha genes (e.g., 32, 54, 64, 77, 267, 328). Transient expression assays in which expression plasmids for ICP0, ICP4 and ICP27 are used to induce the tested gamma gene promoter illustrate these proteins are sufficient to initiate transcription from γ gene promoters (e.g., 18, 50, 55, 78).

The role of DNA binding by ICP4, the major viral transactivator, in gamma gene activation is complex. While ICP4 binding sites exist in many of the gamma gene regulatory regions, recognizable ICP4 Sites are not present in others. Even when present, the ICP4 sites are not a requirement. For example, the gD regulatory region contains three ICP4 consensus binding sites, but any or all of the ICP4 sites can be mutationally inactivated, and the kinetics and production of gD mRNA by the mutated viruses remain indistinguishable from that by the wild-type virus (297). Mutations in the ICP4 protein inactivating its DNA binding ability frequently reduce the γ gene activating function of the protein (e.g., 99, 290). However, Shepard, et al., described a mutated virus which produces an ICP4 peptide incapable of binding DNA. HOwever, the virus could still express several γ proteins with a wild type pattern (289).

Recent results from Gu and DeLuca (99) offer an explanation for some of the contradictory results described above. While examining the gC (a γ_2 gene) promoter and 5' leader sequence in a reconstitution study, they determined that ICP4 binding to DNA sequences 3' of the transcription start site (*inr* region) was necessary for gC promoter activation. ICP4 bound to very degenerate consensus sites and its binding elsewhere readily compensated for the loss of an individual

site. The binding of ICP4 in the presence of either the gC TATA box or the gC *inr* was sufficient for activation of the gC promoter in the reconstitution system. Both the TATA box and *inr* region were required for optimal activation. The TATA box binding protein TBP could not substitute for the TFIID complex, showing that TAFs are required for ICP4 activation of the gC promoter. In addition, mutation of the *inr* decreased promoter activation by ICP4 which suggests an interaction between the *inr* region and the ICP4 protein.

Studies using chimeric constructs in which different DNA sequences from both β gene and γ_2 gene regulatory regions were fused together have delineated elements important to the activation of several γ_2 genes, the TATA box and sequences downstream from the TATA box. Within the downstream region, the 5' transcribed noncoding region (5' leader sequence), there are two elements influencing γ_2 activation. The first is the transcription start site, the *inr* region, which spans approximately from -10 to +10 bp. The second region is located at approximately +30 to +40 bp in the 5' leader sequence and is known as the downstream activating sequence (DAS). In spite of the similarity in location between several of the γ_2 genes in the *inr* region and the DAS, there are no obvious sequence homologies in these elements from gene to gene.

Furthermore, even though γ_2 genes have a TATA motif, the TATA boxes are not universally interchangeable between the γ_2 genes. For example, while the TATA box from gC gene can effectively substitute for the UL24 TATA box, the

US11 TATA box did not function in place of the UL24 TATA box (152).

The DNA sequences which regulate the activation of the γ_1 transcription are a current focus of research. Two models have been proposed which would explain the initiation of γ_1 transcription prior to viral DNA synthesis. The first model suggests that the γ_1 genes are chimeras which contain both β and γ_2 gene regulatory elements. The second model implies that the γ_1 genes are not merely chimeras, but contain unique elements which trigger the γ_1 pattern of expression. The two proposals are not mutually exclusive.

Several studies provide evidence that γ_1 regulatory regions contain both β and γ_2 elements (e.g., 83, 118, 132, 200, 309, 333). In studies using mutated HSV-1, sequences from either a β gene, *tk*, or a γ_2 gene, $\gamma_2$42, were exchanged. When the sequence upstream from and including the TATA box of the gene was fused to the $\gamma_2$42 sequence which was downstream from the $\gamma_2$42 TATA box, the hybrid regulatory region demonstrated γ_1 expression characteristics (200). A further example is the examination of the hybrid gE/gC promoters using recombinant virus (283). In this case, γ_1-type gene expression was produced when gE (a γ_1 gene) sequences upstream from the TATA box were fused to the gC (a γ_2 gene) TATA and downstream region. Conversely, when the gC sequences upstream from the TATA box were fused to the γ_1 gE TATA and downstream sequences this promoter was not activated in a γ_1 pattern, but rather in a γ_2 pattern like gC, in spite of the presence of a γ_1 TATA and downstream region. Thus the data support

the prediction that the γ_1 or β-like sequences upstream from the TATA box are responsible for the early production of the γ_1 genes.

However, evidence exists to support a second model where the γ_1 promoter is not merely a chimera of β and γ elements, but contains structures unique to itself (242). Recent work with U_L38 (a γ_2 gene) supports this model. When viral DNA replication is inhibited, transcription of a γ_2 gene, UL38, still occurs if sequences from a γ_1 gene, VP16, which are downstream from the VP16 TATA box, replace the γ_2 downstream region (104, 105). In this work, the point of interest is that γ_1 gene sequences downstream from the TATA box, rather than upstream, resulted in the change to β-like or γ_1-like transcriptional activation. Studies of the γ_1 VP5 gene regulator region using recombinant HSV-1 viruses showed that deleting an upstream Sp1 binding site resulted in the loss of the characteristic γ sensitivity to viral DNA replication (122). In this research, an upstream sequence affected the gene response to viral DNA replication, rather than the downstream sequences predicted by the chimeric model.

Evidence, therefore, exists for either the model of the γ_1 promoter as a chimera of β upstream and γ_2 downstream elements, or the model of the γ_1 promoter as containing elements unique to itself. While these differing results simply may reflect differences in experimental systems, they may also reflect the presence of more than one regulatory mechanism among the genes grouped together as γ_1 genes. There remain many questions about γ_1 gene regulation.

CELLULAR FACTORS AFFECTING HSV-1 γ GENE TRANSCRIPTION

I. Cellular Basal Transcription Factors

The formation of eukaryotic transcription complex begins with the binding to the TATA box by the particular TFIID complex which is specific to the promoter. In the classic model of transcription, the assembly of the transcription complex proceeds step-by-step to completion (reviewed in 28, but see 159 for another model). The TATA box is bound by the TATA box binding protein (TBP, (233)) and specific TBP-associated factors (TAFs) which comprise the TFIID complex (see Fig. 1-6, (69)). The presence of various TAFs are necessary for TBP to respond to specific transcriptional activators (e.g., 20, 44, 69, 88, 166, 251). TFIID enhances the binding of TFIIB (e.g., 112, 175) which in turn recruits TFIIE or TFIIF simultaneously with RNA Pol II (28, 261). These proteins, TFIID, TFIIB, TFIIE/F, and RNA Pol II, comprise a "minimal initiation complex" which is capable of transcribing some promoters (239, 275). TFIIE and TFIIH are required more effective transcription initiation, possibly affecting a conversion of the preinitiation complex into an active elongation complex (28, 114).The presence of TFIIA and TFIIJ are required for the normal activated transcription of certain eukaryotic genes (e.g., 308).

The initiation of transcription is an ATP-dependent step which allows RNA Pol II to move forward, or downstream, on the DNA template. (reviewed in 276). Bending and other local distortions of the DNA enhance transcription by reducing

the energy required to melt the helix into single-stranded templates (321). The RNA Pol II enzyme, a complex of at least 10 subunits (reviewed in 342), requires TFIIF for efficient mRNA elongation, but separates from the rest of the general, or basal, transcription factors following the initiation of transcription (GTFs, reviewed in 276).

Transcription is characterized as either basal or induced. Basal transcription requires only the general transcription factors necessary to position RNA Pol II on the DNA template (198). In activated transcription, additional factors, called activators, increase the productivity of the RNA Pol II complex by increasing the rate of complex formation, enhancing complex stability, or aiding in elongation (e.g., 216, 249).

The largest subunit of the RNA Pol II complex possesses the unusual feature of a repetitive C-terminal domain (CTD) consisting of 26 to 52 copies of a seven amino acid sequence. While RNA Pol II complexes lacking the CTD can initiate accurate basal transcription (e.g., 29), deletions in the CTD reduce the response of RNA Pol II to signals from activators (277). The CTD is phosphorylated following the binding of the RNA Pol II complex to the DNA template (2, 244), suggesting that phosphorylation is important to the start of activated transcription (89, 282).

Parvin et al. (240) demonstrated that the GTFs which are required for basal transcription vary for different promoters. The required GTFs for the γ_1 promoters have not been specifically determined. However, TFIID, TFIIB, and TFIIE/F were

required for full ICP4-associated transactivation of gC, which is a γ_2 gene, as shown by reconstitution studies using purified factors. ICP4 will not substitute for any of the GTFs (99). Since ICP4 is required for transcription of gD, and other γ_1 genes, the same GTF requirements may apply. Cook et al., suggests that ICP4 may interact with TFIID via a TAF or coactivator protein (47).

Presumably, a typical eukaryotic preinitiation complex forms on the gD and VP5 viral DNA promoters, since HSV-1 uses the eukaryotic RNA Pol II complex to transcribe mRNAs from these γ_1 genes. In common with many eukaryotic promoters transcribed by RNA Pol II, the gD and VP5 genes both contain TATA boxes at approximately -25 to -30 bp. TFIID binding on the γ_1 DNA is stabilized by TFIIB and ICP4, assuming that the preinitiation complex formation on gD and VP5 promoters is similar to that on the gC promoter (99).

The TFIID complex contains various TAFs which interact with specific proteins. A key molecule is $TAF_{II}250$ which links many TAFs with TBP, and interacts with other regulatory proteins (44, 88, 92, 116, 250, 287, 332). For example, Drosophila $TAF_{II}110$ ($dTAF_{II}110$), and the human homolog $hTAF_{II}110$, interact with $dTAF_{II}250$ and bind directly to the glutamine-rich activation region of Sp1 (92, 116, 250, 332). In contrast, the acid-rich activation domain of HSV-1 VP16 binds to $dTAF_{II}40$ which interacts with $dTAF_{II}60$ and $dTAF_{II}250$ (95). Thus the ability of the TFIID complex to respond to different upstream activator proteins depends on the specific TAFs within the TFIID complex (24).

Factors also have been isolated that inhibit the formation of the preinitiation complex (3). The Dr1 protein represses both basal and activated transcription by binding to TBP and preventing the association of the other GTFs, while allowing TBP to bind to the TATA box (135). In contrast, the DNA topoisomerase I represses basal transcription, but stimulates transcription in the presence of an activator (212).

II. Cellular Activators

Activators bind to DNA enhancer sequences which are defined as "a *cis*-acting sequence that increases the utilization of (some) eukaryotic promoters, and can function in either orientation and in any location (upstream or downstream) relative to the promoter" (179, 216, 223). Activators can be present in nearly all cell types, or specific to certain cell types. Examples of general activators are Sp1, YY1, and Oct-1, present in numerous cell types including epithelial and non-differentiated cells. In contrast, Oct-2 although closely related to Oct-1 is cell type specific, present in neurons and lymphoid cells (151). YY1 and Sp1 will be described in greater detail because they are pertinent to the work presented here.

One of the most intriguing points about YY1 is the multitude of roles it plays and the many mechanisms by which it accomplishes its regulatory tasks. YY1 is nearly ubiquitous in the cells of higher mammals. Genes regulated by YY1 are as

different as granulocyte macrophage-colony stimulating factor (GM-CSF, (352)), fetal globin proteins (103), immunoglobin heavy chain (238, 257), and β-casein (97, 209, 252). A recent review of the numerous genes influenced by YY1 is found in Shrivastava and Calame, 1994 (295). YY1 functions both as a repressor and as an activator depending on the context. Repression and activation can occur at the same gene (293). Further, YY1 can act as an initiator protein for some, but not all, TATA-less genes (140, 285). As an example, accurate initiation of basal transcription from the adeno-associated virus P5 promoter proceeds in the presence of only three factors, YY1, TFIIB, and RNA Pol II, suggesting that YY1 is capable of recruiting TFIIB and RNA Pol II to some templates (324).

Near the end of 1991 several groups independently cloned homologs of YY1: the human homologs were named YY1 (293) and NF-E1 (238), the mouse homologs were δ (110), and UCRBP (84) and the chicken homolog was F-ACT1 (176). To add confusion to the literature, prior to the cloning of YY1 various regulatory activities had already been assigned to proteins such as common factor 1 (CF1, (257, 258)), MAPF1 (326), μE1 (330), and LBF (Chapter 2 and 3, (43, 215)). These proteins were later determined to be identical to, or to be homologs of, of YY1.

YY1 is a 414 amino acid protein containing four zinc fingers of the Cys_2His_2 type, an acid-rich domain, and 11 consecutive histidines (111, 293). The binding of YY1 to its consensus binding site (G/C)NCCATNTT, (295)) binds the DNA helix (228). There are eight possible phosphorylation sites in YY1 and the DNA binding

ability of YY1 is abolished by phosphatase treatment (12). Modeling of the protein suggests that YY1 is highly flexible which probably assists in its multiple functions (110).

One common mode of regulation by YY1 is illustrated in the regulation of the c-*fos* gene (102). YY1 and a second protein, the serum response factor, compete for binding to the same DNA element, the serum response element or SRE. When YY1 is bound to the c-fos promoter SRE, gene expression is repressed. Competitive repression of gene activation by YY1 through the inhibition of the binding of a second protein regulates other genes such as the α-actin gene (176, 177, 193) and the rat serum amyloid A1 gene (190). In another kind of YY1 regulation, Sp1 and YY1 compete for binding to overlapping sites at the *inr* site of the murine *COXVb* promoter which is a TATA-less promoter. However, instead of repressing gene activation, as in the previous examples, YY1 plays a critical role in initiating *COXVb* expression, while Sp1 was unable to transactivate the *COXVb* gene (10).

A feedback regulation cycle appears to exist between YY1 and the c-*myc* gene. YY1 activates the c-*myc* promoter, producing higher levels of c-Myc protein production (258). However, c-Myc binds to YY1 and inhibits both the repressor and activator functions of YY1 (296). For instance, during myogenesis proliferating myoblasts contain high levels of YY1. YY1 both represses the production of α actin and enhances the production of c-Myc. As the levels of c-Myc increase, the levels of functional YY1 decrease, relieving the repression of the α-actin gene and leading to the final differentiation of the myoblasts (178).

YY1 represses the P5 promoter of the adeno-associated virus when binding to the promoter alone (293). However, the presence of the adenovirus E1A protein relieves the YY1 repression (293), either by direct binding between E1A and YY1 (180) or by the binding of YY1 to a cellular protein associated with E1A (173). Furthermore, when YY1 is present the activation of the P5 promoter associated with E1A is enhanced as compared to the activation associated with E1A alone (285, 293). Other viral genes are regulated by YY1, including ones in Moloney murine leukemia virus (84), HSV-1 virus (Chapter 2, (43)), Epstein-Barr virus (218, 219), pseudorabies virus (163), cytomegalovirus (188), human papilloma virus (19, 201) and human immunodeficency virus (HIV) (196).

In addition to binding DNA, YY1 binds directly to a number of proteins (e.g., 136, 174). With the c-*fos* gene YY1 can bind not only to its own DNA binding site but to the ATF/CREB protein which further contributes to the repression of the c-*fos* promoter (358). Sp1 and YY1 also physically interact, suggesting an explanation for why the ability of YY1 to activate transcription from TATA-less promoters is increased by the presence of nearby Sp1 sites (174, 218, 284).

Sp1 is the prototype protein of a family of related transcription activators which include at least 4 members (106, 157). Sp1 is a single polypeptide having two posttranslational forms with apparent molecular masses of 95 kDa and 105 kDa (137, 138, 148). Sp1 was identified as a transcription factor which altered the activity of RNA Pol II such that the enzyme preferentially recognized only

promoters containing SV-40-like sequences (67, 68). The dependence of Sp1-mediated activation on promoter structure suggested that a specific DNA sequence, initially determined to be CCGCCC, served as a target for Sp1 (e.g., 68, 149). Nonconsensus binding sites for Sp1 include GGAGGG (58, 147, 325, 348) or CACCC (22, 106). Examples of cellular genes activated by Sp1 include the c-myc gene (58, 113) and the rat growth hormone gene promoter (278). Sp1 also is required by numerous TATA-less genes (e.g., 323). While the mechanism of Sp1 activation is still unknown, Sp1 interfaces with TBP through a bridge of two TAFs: $TAF_{II}110$ which in turn binds $TAF_{II}250$ (92, 332).

The Sp1 family members possess three zinc finger domains of the Cys_2His_2 type and have at least 72% amino acid identity. These polypeptides contain conserved glutamine-rich activation domains, are posttranslationally glycosylated, and are phosphorylated at serine/threonine motifs (106, 138, 157). Another closely-related protein, BTEB, contains highly homologous zinc fingers (72% amino acid identity) but lacks the glutamine-rich region, having instead an acidic domain. BTEB recognizes the Sp1 consensus sequence with an affinity similar to Sp1 (130, 302). Numerous other proteins also contain Cys_2His_2 type zinc fingers and bind to guanine-rich DNA sequences (14), thus complicating the assignment of a specific protein of the responsibility for an activity associated with a GC-rich binding site.

At least two proteins associate with Sp1 and inhibit its ability to activate promoters, p74 (222) and Sp1-I (42). The retinoblastoma-susceptibility gene

product (pRB) stimulates Sp1-mediated transactivation by binding to Sp1-I and releasing Sp1 (42, 155). Further regulation of Sp1 is provided by the cyclins. Cyclin D1 specifically represses Sp1 activation in an pRB-independent manner, while Cyclin C increases transcription mediated by Sp1 and other factors in a pRB-dependent fashion (286).

Other factors influence the activation of specific genes by Sp1. For example, the binding of Sp1 to the low density lipoprotein receptor gene promoter is increased by the presence of the sterol regulatory element-binding protein thus enhancing the expression of the gene (272). In other cases, Sp1 can be targeted to DNA by associating with other DNA binding proteins, such as the human neurofilament (H) protein (71). Sp1 also self-associates in a fashion that permits the looping of the DNA between widely spaced Sp1 sites, enhancing gene expression with an increasing numbers of sites (316). Alternatively, in the rat growth hormone gene promoter the binding sites for the Pit-1 protein and Sp1 overlap and are mutually exclusive, yet both proteins are required for the activation of this gene (278). The authors speculate that each protein plays a unique role in regulating the sequential assembly of the RNA Pol II complex. As a final example of factors affecting Sp1-mediated activation, both the production and phosphorylation of Sp1 is increased during SV40 infection, thus enhancing viral gene activation (137).

Sp1 targets a viral protein activator, E2, to a bovine papillomavirus type 1 promoter, thus increasing E2 function even when the Sp1 and E2 sites are widely

separated or in the absence of E2 binding sites (181). Interactions between viral proteins and Sp1 also activates the expression of certain genes of the parvovirus minute virus of mice (164) and HIV (123, 141, 194). Additional viral genes requiring Sp1 for maximum expression include the HSV-1 gene VP5 (122) and the Epstein-Barr virus immediate-early gene BRLF1 (355). However, HSV-1 ICP4 represses Sp1-activated transcription from its own promoter, where a strong ICP4 binding site overlaps the transcription initiation site (100, 101). Thus, context affects the outcome of the interactions beween Sp1 and viral proteins just as it does for YY1.

SCOPE OF THE STUDY

I. Research Aims

The long term goal of our laboratory group is identifying the molecular mechanisms which activate and regulate the transcription of the HSV γ_1 genes. When this research was undertaken in 1990, little was known concerning the cellular factors required for transactivation of the γ_1 genes. The goal of the research presented in this thesis was to identify and define the functions of specific cellular factors which bind the promoters of two HSV-1 γ_1 class genes, VP5 and gD.

II. Results of this Study

Specifically, this study:

(1) determined that the cellular factor which bound the VP5 promoter at -60 bp also bound the regulatory regions of several other HSV-1 genes, including the gD promoter.

(2) identified YY1 as the protein binding the VP5 and gD promoters.

(3) discovered that a second cellular protein bound to the gD promoter at a site overlapping the YY1 site.

(4) identified that this second protein was a member of the Sp1 protein family ("SpX").

(5) established that both YY1 and SpX contribute to the transactivation of the gD promoter in the presence of ICP0, ICP4, and ICP27, with SpX playing the major role.

III. Importance of the study

This research identified cellular factors whose association with specific HSV-1 γ_1 genes was previously unknown. The influences that cellular YY1 and SpX have

on gD gene transactivation were established, increasing the understanding of the interactions between HSV-1 and the host cell. The data presented here thus becomes a basis for future research.

CHAPTER 2: Article #1

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Transactivation of the Major Capsid Protein Gene of Herpes Simplex Virus Type 1 Requires a Cellular Transcription Factor

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ABSTRACT

The purpose of this investigation was to identify and characterize the regulatory elements involved in the transcriptional activation of the $\beta\gamma$ (leaky-late or γ_1) genes of herpes simplex virus type 1 (HSV-1) by using the major capsid protein (VP5 or ICP5) gene as model. Gel mobility shift assays with nuclear extracts from uninfected and infected HeLa cells enabled us to identify two major protein-DNA complexes involving the VP5 promoter. The mobilities of these two complexes remained unaltered, and no unique complexes were observed when infected nuclear extracts were used DNase I and orthophenanthroline-Cu^+ footprint analyses revealed that the two complexes involve a single binding site, GGCCATCTTGAA, located between -64 and -75 bp relative to the VP5 cap site. To determine the function of this leaky-late binding site (LBS) in VP5 gene activation, we tested the effect of mutations in this region by using transient expression

of a *cis*-linked chloramphenicol acetyltransferase gene. Deletion of the above sequence resulted in a seven-to eightfold reduction in the level of transactivation of the chloramphenicol acetyltransferase gene by superinfection with HSV-1 or by cotransfection of HSV-1 immediate-early genes. From these results, we conclude that the LBS sequence and a cellular factor(s) are involved in the transactivation of the VP5 gene. A search of published gene sequences revealed that sequences related to the LBS exist in a number of other HSV-1, cytomegalovirus, retrovirus, and cellular promoters. Sequence homologies of binding sites and results of unpublished competition binding studies suggest that this leaky-late binding factor may be related to, or the same as, a ubiquitous cellular transcriptional factor called YY1 or common factor-1 (also known as NF-31, δ, and UCRBP).

During productive infection of cells in culture, the genes of herpes simplex virus type 1 (HSV-1) are expressed as three major classes that are coordinately and sequentially regulated. On the basis of the kinetics of their expression and sensitivity to metabolic inhibitors, these classes have been referred to as α or immediate-early (IE), β or delayed-early, and γ or late (26; for a review, see references 53 and 62). This differential regulation of gene expression is mediated largely at the transcriptional level (20, 24, 30, 62, 65), although posttranscriptional processes apparently also play a role (24, 30, 58, 65). The late genes have been further subdivided into two classes on the basis of the stringency of their requirement for viral DNA replication (53, 62). Thus, the $\beta\gamma$ genes (also called γ_1 or leaky-late) are first transcribed and expressed at relatively low levels prior to viral DNA replication; following replication they are expressed at more abundant levels. This contrasts with the true late, or γ_2, genes whose expression exhibits a more stringent requirement for viral DNA replication (25, 53, 62). The studies described in this paper focus on the regulation of the $\beta\gamma$ class by using primarily the VP5, or ICP5, gene as a model. This gene codes for a 155-kDa polypeptide which serves as the major capsid protein of the virus.

The molecular mechanisms involved in both the initial activation of expression by the $\beta\gamma$ genes and the subsequent acceleration of their expression after DNA replication are not well understood. For the initial activation it is clear, however, that viral IE proteins are required. This was first suggested in studies using cycloheximide reversal (26, 66) and amino acid analogs (27) and subsequently by investigations with IE gene

mutants of HSV-1 (4, 8, 11,15, 20, 36, 46, 48, 50 51, 54, 63, 66). The latter studies indicated that at least three of the five IE proteins, ICP0, ICP4, and ICP27, are involved.

More specific information on the requirements for viral IE proteins has come from various transient expression experiments. The initial findings from these were that promoters of genes frequently assigned to the $\beta\gamma$ or leaky-late class (e.g., VP5, gD, and gB) exhibit low activity when transfected into uninfected cells but that they are strongly activated by HSV-1 superinfection or by cotransfection of HSV-1, IE genes (3, 6, 8, 9, 55, 59). The latter experiments confirmed that three IE proteins, ICP0, -4, and -27, are required for full transactivation of $\beta\gamma$ genes such as VP5 (6, 16, 55, 59). Exactly how the three IE proteins function in this context is not clear, although at least part of the activity of ICP27 appears to be at the posttranscriptional level (58). moreover, with the exception of ICP4 binding sites in the gD promoter (60), direct binding of IE proteins to regulatory regions of the $\beta\gamma$ genes has not been demonstrated.

Studies using inhibitors of viral DNA synthesis have clearly demonstrated that the $\beta\gamma$ genes require, in addition to IE gene function, viral DNA replication for their full expression (6, 20, 25, 45). Transcription from $\beta\gamma$ promoters, such as those of VP5, gD, and gB, is initiated early during infection along with those of typical early, or β, genes such as the thymidine kinase (TK) gene, but in the absence of viral DNA synthesis, the usual postreplicative increase in their expression does not occur (20, 65, 66). Exactly how expression of both the $\beta\gamma$ and γ genes is coupled to DNA replication is not clear. However, the structural requirements for this coupling appear to be a minimal TATA box promoter, some downstream sequences, and a replication function provided in *cis* (29).

In addition, it seems that ongoing replication and not simply amplification of the template is the key ingredient provided by viral DNA synthesis (29).

Transfection studies have also provided insight concerning the *cis*-acting DNA sequences required for transactivation of $\beta\gamma$ promoters. Transient expression assays with the VP5 promoter coupled to the bacterial chloramphenicol acetyltransferase (CAT) gene demonstrated that sequences located no more than -125 bp from the cap site are required for maximum transactivation either by superinfection or by cotransfection of IE genes. sequences located between -4 and -75 were found to suffice for transactivation, but at a 2.5 to 5-fold-lower level of that obtained with the complete -125 sequence. In addition, these investigations identified a region located between -75 and -168 that exerted a silencer effect on transcription from a VP5 promoter placed downstream from a simian virus 50 enhancer (2, 3, 6). These studies, however, did not disclose the precise regulatory sequences or the proteins that interact with them.

A detailed analysis of the gD promoter revealed that sequences upstream to -33 (essentially only a TATA box and downstream regions) sufficed for true late gene type of activation by a *cis*-linked origin of replication, whereas DNA sequences located between -33 and -83 were essential for $\beta\gamma$ type of transactivation by virus superinfection (13, 14, 29). Deletion analysis further localized the region required for transactivation to two G-rich sequences, one located between -63 and -73 and one of lesser importance located between -43 and -55 (14). However, in this work the proteins that interact with these sequences were not identified.

From the foregoing discussion, it is apparent that the general requirements for

transactivation of certain $\beta\gamma$ promoters have been established. However, we still do not know what cellular factors are involved, what specific promoter sites are required, how individual IE proteins function in the activation process, and why DNA replication is required for maximum $\beta\gamma$ gene expression. The investigations we report here provide new insight into the first two questions. By using gel mobility shift and DNA footprinting analysis we have identified a regulatory sequence, present in the VP5 promoter and in several other HSV-1 genes, that binds one or more cellular factors. We further show, by deletion analysis and transient expression assays, that this binding site is essential for fully activated expression of the VP5 gene. From these studies we conclude that transcriptional activation of the VP5 gene, and probably several other $\beta\gamma$ genes, requires not only the action of viral IE proteins, but also the binding of a cellular factor(s) to a specific promoter regulatory site.

MATERIALS AND METHODS

Cells and virus. HeLa cells (ATCC CCL2) and HEp-2 cells (ATCC CCL23) were propagated at 37°C under 5% CO_2 atmosphere in Dulbecco's modified Eagle medium (DEM; GIBCO Laboratories, Inc) containing 10% heat-inactivated calf serum (Hyclone Labs, Inc.), penicillin (100 U/ml), and streptomycin (100 μg/ml). The *vhs*-1 mutant of HSV-1 (KOS), defective in a virion-associated host shutoff function which maps in the UL41 gene (33, 49), was kindly provided by G. S. Read, Loyola University of Chicago. It was propagated in HEp-2 cell monolayers as previously described (40).

Plasmids and DNA fragments. Plasmid VP5(-168)CAT, containing the HSV-1 VP5 promoter fragment -4 to -168, relative to the cap site, linked to the reporter gene for bacterial chloramphenicol acetyltransferase (CAT), was kindly provided by E. Blair and E. Wagner, University of California, Irvine (3, 6). Plasmid pGTSa2, prepared in this laboratory and containing the 4.0-kbp *Sal*I D fragment of HSV-1 (KOS) cloned in pBR322, was used to prepare gD promoter fragments. The latter contains the 23.7-kbp *Bgl*II HM fragment of HSV-2 cloned into pBR322 and encodes three IE proteins, ICP0, -4, and -27. Plasmid DNA was prepared by the alkaline lysis procedure (1) with modifications (34). Plasmids used for transfection were further purified by CsCl buoyant density centrifugation. DNA fragments from restricted plasmids were isolated by the DEAE paper electroelution method (12). Fragments were labeled at their 3' ends by Klenow fill-in (34) using α-^{P}-labeled deoxyribonucleoside triphosphates (NEN Research Products).

Oligonucleotides. A double-stranded oligonucleotide containing the VP5 leaky-late binding site (LBS) and having the sequence CCAGGATCCAGGGCCATCTTGAATGGATCCTGG was synthesized at the oligonucleotide facility of the Vollum Institute, Oregon Health Sciences University. A control double-stranded oligonucleotide (C2) having the sequence CCTTGCCACATGACCTGTTCCT that lacks an LBS was kindly provided by K. Riggs, Columbia University.

Enzymes and coenzymes. Enzymes, coenzymes, and their sources are as follows: restriction endonucleases (Bethesda Research Laboratories,l Inc ([BRL]; New England

Biolabs, Inc; and Boehringer Mannheim GmbH), used with either the 10X reaction mixes provided by BRL or the appropriate amounts of KGB buffer (34); exonuclease BAL 31 (U.S. Biochemical Corp.); Klenow fragment of DNA polymerase, T4 DNA ligase, and RNase A (BRL); and bacterial CAT, S-acetyl coenzyme A synthetase, and coenzyme A (Sigma Chemical Co.).

Nuclear extracts and mobility shift assays. Nuclear extracts were prepared from HeLa S monolayers that were either uninfected (mock infected) or infected with HSV-1, multiplicity of infection of 10 to 15, for 8 h (10). For mobility shift assays (18, 19), 0.1 to 1 ng of DNA probe, 0.7 to 3.5 $\lambda\mu$g of poly(dI-dC) · poly(dI-dC) (Pharmacia), and nuclear extract (1 to 5 ηg of protein) were mixed in a reaction buffer providing final concentrations of 6mM tris-HCl (pH 7.9), 40 mM KCl, 2mM EDTA, 0.2 mM dithiothreitol, and 8% (vol/vol) glycerol in a total volume of 20 or 25 μl. Following a 30-min incubation at room temperature, 2 μl of 0.25% bromphenol blue was added and the samples were electrophoresed at 4°C through polyacrylamide gels made with 4% acrylamide-0.13% bisacrylamide in a Tris-borate buffer (25 mM Tris base, 25 mM boric acid, 1 mM EDTA). Gels were dried under vacuum and exposed to Fugi RX film with intensifying screens. For competition binding assays, the above conditions were used but with 100- to 300-fold-excess unlabeled competitor DNA added 10 min before the radiolabeled probe. The probe was then added, and the incubation was continued for an additional 20 min at room temperature. A high level of competitor was needed for effective competition because of the relatively high concentration of the leaky-late binding factor (LBF) we and others (52) have found to be present in nuclear extracts.

DNA footprinting. Mobility shift assays were carried out as described above but scaled up 10- to 30-fold. After the protein binding reaction the samples were treated for 30 to 20 s with DNase I at 3.5 ng/μl in the presence of 3.5 mM $MgCl_2$. The reactions were stopped by addition of EDTA to 2.5 mM, and the samples were loaded onto a polyacrylamide gel and electrophoresed as described above for the mobility shift assay. Alternatively, following the mobility shift procedure, the gels were subjected to the orthophenanthroline-Cu^+ footprinting procedure (32). Protein-DNA complexes and free DNA probe were excised as gel strips and inserted into a 2% agarose slab gel. Following the addition of 5 μg of *Escherichia coli* tRNA per strip, the DNA fragments were electrophoresed onto DEAE paper strips and isolated as described above (12). Dried DNA samples, containing 5 x 10^3 to 10 x 10^3 cpm, were run on DNA sequencing gels (made with 8% acrylamide) along with the probe that had been subjected to Maxam and Gilbert cleavage reactions (35).

Construction of VP5 promoter mutations. Plasmid VP5(-168)CAT (90 μg) was linearized at the *Sal*I site at the 5' end of the VP5 promoter and then digested with 2.25 U of BAL 31 exonuclease for 23 5o 38 s (34). This produced deletions of various sizes that extended through the VP5 binding site region. The deleted ends were polished with a Klenow fill-in reaction, and *Bgl*II linkers (New England Biolabs, Inc.) were added by blunt-end ligation (34). Following cleavage with *Nco*I in the CAT gene, fragments of 617 to 642 bp containing the deletions (fragment 1) were isolated by agarose gel electrophoresis. Fragment 2 was prepared by a partial digestion of VP5(-168)CAT with *Bst*NI to cleave at one of two sites located 5' to the VP5 binding site. Following Klenow

polishing, *Bgl*II linker attachment (34), and *sal*I digestion, fragments of 78 and 94 bp were isolated by agarose gel electrophesis (fragment 2). Fragment 3 was prepared by restriction of VP5(-168)CAT with *Nco*I and *Sal*I and isolating the 3.04-kbp *Nco*I-*Sal*I vector fragment. Finally, fragments 1, 2, and 3 were ligated together to produce VP5CAT plasmids with deletions in the VP5 promoter (see Fig. 2-4A). Competent *E. coli* HB101 cells (BRL) were transformed with the resulting mutated plasmids, and 40 ampicillin-resistant clones were picked. From restriction and Maxam and Gilbert (35) sequencing analyses, eight mutant clones that contained the desired range of deletions in the VP5 promoter were selected for further study.

Transfections and CAT assays. HeLa cells were seeded in 35-mm well cluster dishes 20 h prior to transfection at 3 X 10^5 cells per well in DMEM containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (DMEM-10% IFBS-P/S). The medium was changed after 17 h, and 0.5 ml of calcium phosphate-precipitated DNA was added 20 h after seeding (22). Each 0.5-ml sample contained either 5 μg of target plasmid and 11 μg of cotransfected effector plasmid (pGR150B). Four hours after adding the DNA, the cells were shocked by adding to their medium 1 ml of 15% (vol/vol) glycerol in DMEM-10% ICFS-P/S (21). After 1 min, the cells were washed with PBS-1 (0.14 M NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4), overlaid with DMEM-10% ICFS-P/S, and incubated further at 37°C. The transfected cells were either superinfected 20 h after transfection with the *vhs*-1 mutant of HSV-1 (KOS) at a multiplicity of infection of 3 and harvested 26 h later for CAT assay or transfected and harvested for CAT assay 44 h after transfection. Cell washing, lysis, and CAT assay

were performed by the method of Nordeen et al. (43). The CAT assay consisted of measuring incorporation of ^{3}H-Na-acetate (NEN research Products, Inc.; 3.3 Ci/mmol, 10 mCi/ml) into acetylchloramphenicol by using a coupled reaction with acetyl coenzyme A synthetase. The assay was verified by thin-layer chromatography and autoradiography.

RESULTS

Two major protein-DNA complexes are formed with the VP5 promoter. To identify and characterize potential regulatory complexes formed with VP5 promoter, we carried out a series of mobility sift analyses by using a ^{32}P-labeled VP5 promoter fragment (-4 to -168 bp relative to the cap site) and various amounts of nuclear extracts from uninfected and HSV-1-infected HeLa cells. Complexes formed were analyzed by electrophoresis through polyacrylamide gels (Fig. 2-1). Two major complexes were observed, one of lower mobility (complex A) and one of higher mobility (complex B), when nuclear extracts from either uninfected or infected cells were used. Although viral proteins are known to transactivate the VP5 promoter, no complexes unique to the infected-cell extracts were observed, even at high concentrations of nuclear extract. In addition, the relative mobilities of the two complexes remained unchanged when the nuclear extract from infected cells was used. The only difference we have observed between uninfected and infected cell complexes is a six- to ninefold increase in the ratio of complex B to A with some nuclear extract preparations. As discussed below, this may simply reflect differing amounts of proteolytic activity in the extracts.

DNA footprinting analysis reveal that a common core sequence is involved in both VP5 promoter complexes. To define the DNA sequences involved in the formation of the two VP5 promoter complexes, we first carried out DNase I footprinting analyses on complexes A and B with nuclear extracts from uninfected and infected HeLa cells. The DNase-treated complexes were resolved on mobility shift gels and excised, and the DNA was extracted and analyzed on DNA-sequencing gels. With infected-cell nuclear extracts, both complexes were found to protect a similar region, nucleotides -66 to -74. With uninfected nuclear extracts, a similar footprint was obtained (data not shown). Since the footprints obtained by the DNase method were somewhat weak, probably because of the short half-life of the complexes (see Discussion and reference 64), we subsequently employed the orthophenanthroline-Cu^+ footprinting method (32). This more clearly showed, with either uninfected or infected nuclear extracts, that essentially the same promoter sequence was protected in both complexes (Fig. 2-2A and B). A summary of the footprint mapping is presented in Fig. 2-2C. These results indicate that a cellular protein(s) forms both complexes A and B and that these two complexes encompass a common sequence, GGCCATCTTGAA, which we call the LBS (the leaky-late, or $\beta\gamma$,binding site), located between -64 and -75, in the VP5 promoter.

The observed promoter complexes are unique t the VP5 gene and several other genes of the $\beta\gamma$ class. To obtain information on the binding site specificity of the protein(s) involved in the VP5 complexes, we carried out a series of binding assays using as competitors unlabeled DNA fragments from a number of different HSV-1 genes as well as from several other viral and cellular genes. Some of the more significant

competition binding results with the labeled 164-bp VP5 promoter fragment as probe are depicted in Fig. 2-3A, and all competition results are summarized in Table 2-1. Of the HSV-1 gene sequences that contained potential homologs to the VP5 LBS (Table 2-2), good competition was observed by using excess unlabeled promoter fragments from the VP5 and glycoprotein D (gD) genes and with a fragment from the nontranslated leader region of the glycoprotein B gene that contains an LBS homolog at +137 (gB_a) (Fig. 2-3A, lanes 3 to 7). A gB promoter fragment containing a partial match to the VP5 LBS at -249 (gB_b) showed little competition (lanes 8 and 9). Likewise, a fragment from the UL37 promoter containing a 9 of 11 bp match to the VP5 LBS at -137 showed negligible competition, whereas a sequence from an internal position (+750) in the UL37 gene that has a 10-bp perfect match to the VP5 site was found to compete well (data not shown; Tables 2-1 and 2-2). Promoter fragments from various viral and cellular genes that lacked substantial homology to the VP5 LBS sequence showed little or no competition. Among these are the promoter regions from HSV-1 genes belonging to various kinetic classes: TK (DE or β) and VP16 ($\beta\gamma$), shown in Fig. 2-3B and ICP47 (α or IE) and gC (γ_2), presented in Table 2-1. In other experiments now shown, promoter fragments from adenovirus type 2 major late and cellular epsilon globin promoters also failed to compete (Table 2-1). Since the VP5 LBS overlapped a potential NF-1 site (Fig. 2-2C), an adenovirus type 2 fragment containing an NF-1 site was used as a competitor. Since this DNA failed to compete significantly (Fig. 2-3B, lanes 9 and 10), it is unlikely that the observed complexes involve NF-1. These results suggest that the DNA sequences involved in the formation of VP5 promoter complexes A and B may be unique to a

subset of HSV-1 genes that includes several $\beta\gamma$ genes and that transcription factors common to other viral and cellular promoters, such as Sp1, CBF, and NF-1/CTF, are not likely to be involved in the formation of these complexes.

To demonstrate that the gD and gB fragments that competed for complex formation by the VP5 promoter (Fig. 2-3) bind the same (or very similar) protein(s), we ran ^{32}P-labeled VP5, gD, and gB DNA fragments in a gel shift assay with nuclear extracts from uninfected HeLa cells and a synthetic oligonucleotide containing an 11-bp match to the VP5 LBS as a competitor (Fig. 2-4). An oligonucleotide lacking the LBS sequence (C2) was used as a negative control. First, it can be seen that the gD and gB fragments form two major complexes of relative mobilities similar to those formed by the VP5 probe (lanes 2, 7, and 12). Second, it is apparent that the LBS sequence (lanes 3, 4, 8, 9, 13, and 14), but not the control oligonucleotide, C2 (lanes 5, 10, and 15), competes for the complexes formed by the VP5, gD, and gB promoter/leader DNA fragments. These results strongly suggest that the same or very similar protein(s) forms the A and B complexes with these three DNA probes.

When a nuclear extract from infected cells was used with the labeled gD promoter, the mobilities of complexes A and B remained unchanged. However, a new complex of higher molecular weight was observed (data not shown). Since this new complex had approximately the same mobility as a gD complex formed with a cloned ICP4 protein (a generous gift from K. Wilcox) and since the gD fragment used contains a known ICP4 binding site (60), it is likely that the new complex represents an ICP4 complex.

Mutational analysis of the VP5 binding site. To assess the role of the VP5 binding site, or LBS, in transcriptional regulation, a series of mutated VP5 promoter constructs were generated from plasmid VP5-CAT by progressive BAL 31 nuclease deletion from *Bst*NI sites located at approximately-76 and -93 and ligation of the resulting fragments with *Bfl*II linkers. The deletion strategy is described in Materials and Methods, and a summary of the deletion and insertion mutations used is presented in Fig. 2-5A.

To correlate complex A and B formation with promoter activity, we tested the mutated VP5 promoter constructs in a competition gel shift assay (Fig. 2-5B). The results showed that constructs 6 (12-bp insertion between -76 and -77) and 2 (deletion -83 to -93 [data not presented]) competed as well as the wild-type VP5 promoter, whereas deletion 10 (-70 to -93) showed weak competition and deletion 13 (-50 to -93) failed to compete significantly for protein binding (Fig. 2-5B). These results are compatible with the mapping of the LBS as determined by footprint analyses (Fig. 2-2).

The VP5 promoter binding site (LBS) is required for optimal transactivation by superinfecting HSV-1 or by cotransfected IE genes. To analyze the role of the A and B complexes in the regulation of VP5 gene expression, we tested the wild-type and mutated VP5 promoter constructs in transient expression assays for bacterial CAT expression. HeLa cells were transfected with the VP5-CAT plasmids; 20 h later they were superinfected with HSV-1 KOS (*vhs*-1 mutant) and harvested 26 h after that for CAT enzyme assay (Fig. 2-6A). The host shutoff mutant, *vhs*-1 (33, 49), was used to achieve greater CAT mRNA stability. In agreement with earlier findings from several laboratories (6, 8, 9, 16), the wild-type as well as mutated VP5 promoters were

essentially inactive without viral superinfection (Fig. 2-6A, bars labeled U); CAT activities were approximately the same as those of the controls without transfected CAT plasmids. Virus superinfection, however, stimulated activity of the wild-type promoter 425-fold over control levels. A small deletion to the left of the NF-1 homology (construct 2, -83 to -93 [data not shown]) as well as disruption of the NF-1 sequence with a 12-bp linker insertion (construct 6) had only a small effect on CAT activity. However, a deletion that extended through the potential NF-1 site and half of the VP5 binding site (construct 10) reduced virus transactivation 4.6-fold. A more extensive deletion that removed all of the VP5 binding site (deletion 13) produced an 8.3-fold reduction in the level of virus-transactivated CAT gene expression.

Cotransfection experiments have very clearly demonstrated that at least three HSV-1 IE (α) genes are required to transactivate the VP5 promoter (6, 16, 55, 59). To determine the role of the LBS sequence in this process, cells were cotransfected with the VP5-CAT constructs and plasmid pGR150B that contain IE genes for ICP0, ICP45, and ICP27 (kindly provided by G. Hayward, Johns Hopkins University). CAT activity was determined 44 h later (Fig. 2-6B). Although the level of CAT expression in this case was not as great as that observed with virus superinfection, the results were qualitatively similar: (i) a 12-bp *Bgl*II linker insertion at -80 (construct 6) reduced CAT expression approximately twofold; (ii) deletion 10, which removed one-half of LBS, reduced CAT expression about fivefold; and (iii) deletion of all of the VP5 binding site (deletion 13) reduced CAT gene expression sevenfold relative to that of the wild-type promoter.

The results of the foregoing experiments using VP5 promoter mutations indicate

that the VP5 LBS sequence is required for maximum levels of virus transactivation. Since disruption of the potential NF-1 sequence with a 12-bp insertion had only a small effect on transactivated VP5 promoter activity, it appears that this site plays a minor role, if any, in VP5 transactivation under the conditions used.

DISCUSSION

Two major complexes are formed on the VP5 promoter with cellular proteins. In this investigation we have examined DNA-protein interactions that occur on promoter/regulatory sequences of the major capsid protein (VP5) gene and several other genes of the $\beta\gamma$ (leaky-late) class of HSV-1. Gel mobility shift assays revealed that two major complexes were formed on the VP5 promoter when either uninfected or infected-cell extracts were used. Similar complexes were also observed with gD and gB gene fragments. Since no significant changes in the mobilities of the complexes were observed when infected-cell extracts were used, we conclude that a cellular factor(s) is involved in both cases. We were surprised to find no evidence for virus-specific promoter complexes, since the region of the VP5 promoter used in this study contains the elements necessary for transactivation by viral proteins (Fig. 2-6) (3, 6). These observations suggest, therefore, that any viral regulatory proteins that interact with this region of the VP5 promoter do so either directly by binding weakly to the DNA or indirectly by binding weakly to cellular factors, by binding to cellular factors that weakly bind to the promoter, or by altering the activity of one or more cellular transcription factors.

From both our footprinting results and our oligonucleotide competitions it appears that VP5 complexes A and B involve essentially the same base sequence. However, the reason for the different mobilities of the complexes is not known. It could be that two different cellular proteins bind the same sequence, the complex of lower mobility involves an additional protein, or a breakdown product of the protein forming complex A forms complex B. Evidence supporting the latter possibility has been presented to explain the formation of two complexes with the μE1 site of the immunoglobulin heavy chain enhancer (64). (As discussed below, the cellular factor that binds to the μE1 site, NF-μE1 or simply NF-E1, may be related to, or the same as, the VP5 binding factor, LBF). The formation of two complexes involving one binding site appears not to be restricted to the VP5 promoter, since DNA fragments that contain only one LBS from other $\beta\gamma$ genes (gD and gB) also produce two complexes (Fig. 2-4; Table 2-2).

The VP5 promoter binding site, LBS, is required for transactivation by viral factors. We have shown by both chemical and enzyme footprint analyses that the two VP5 promoter complexes involve a unique sequence, GGCCATCTTGAA, located between -64 and -75 bp relative to the mRNA start site. By using transient expression assays with plasmids containing mutations in the VP5 promoter, we further showed that this sequence, the LBS, is required for maximum levels of transactivation of the VP5 promoter by either superinfecting HSV-1 or cotransfected IE genes for ICP0, -4, and -27. When the LBS was completely deleted from the promoter, transactivation levels were reduced seven- to eightfold. But, since a promoter deleted in this sequence (deletion 13) could still be transactivated, albeit at levels considerably lower than those of the wild-

type promoter (Fig. 2-6), it appears that other regions of the VP5 promoter may also be involved in the transactivation process. This observation suggests that a multiplicity of factor interactions may be required for maximum transactivation of the VP5 promoter.

Homologs of the LBS may be involved in the regulation of other viral genes. A search of published herpesvirus DNA sequences (7, 37, 38) revealed that sequences bearing close homologies to the VP5 LBS occur in promoters as well as in internal sites of a number of genes. Some of these are listed in Table 2-2. So far, we have identified sequences in three HSV-1 genes (gD, gB, and UL37), in addition to the homologous VP5 sequence, that compete for LBF binding, and we have shown that an oligonucleotide containing the VP5 LBS effectively competed with two of these sequences (gD and gB) for complex formation (Fig. 2-4). Of these three genes, gD and gB are frequently assigned to the $\beta\gamma$ class, and UL37 genes remains to be firmly established. In the case of gD, however, deletion analyses by Everett (14) indicated that the promoter region containing the LBF binding site is very critical for viral transactivation of the gene. Potential $\beta\gamma$ promoters that failed to compete against the VP5 complexes, UL14/15, UL24, and VP16 (Table 2-1), lack a close match to the LBS sequence. Of further interest is the occurrence of 11 copies of an LBS homolog, GCCATRT, within the first intron of the ICP0 gene. The role of these sequences in ICP0 regulation is currently under investigation.

Of related interest is the existence of multiple copies of LBS homologs in the IE genes of the cytomegalovirus (CMV) genomes. For example, in the IE gene I of MCMV, one copy of the sequence GCCATGT is found at -247 bp, and three copies of the

sequence GCCAT$^{C}_{A}$TTG are found between +133 and +180 in the first exon (Table 2-2). Even more bizarre is the occurrence of 9 copies of an LBS homolog, GCCATNT, in the enhancer/promoter region and the first exon of human CMV (HCMV) genome, and 24 copies of this same sequence in the simian CMV (SCMV) enhancer/promoter and first intron (28). Although we don't yet know what role these sites may play in CMV gene regulation, we have found that three LBSs found in the murine CMV (MCMV) IEI gene first exon do indeed bind the LBF, and the complexes formed are fully competed by the VP5 LBS oligonucleotide u(39). Also in the Epstein-Barr virus BZLF promoter, two inverted copies of an LBS homolog exist and these have been shown to confer negative regulation on BZLF expression (42).

Computer search also showed that homologies to the VP5 LBF exist in promoters (LTRs) of a number of retroviruses including simian immunodeficiency virus type 1 (SIV-1) and simian retrovirus type 1 (SRV-1) (Table 2-2). All of the LTRs containing homologies have a 7- to 14-bp match to the VP5 LBS located approximately-130 to -180 bp upstream from the mRNA start sites. Although we do not know as yet if these all bind the cellular factor, LBF, we found that a fragment of the SRV-1 LTR that contains a 14-bp perfect match to the VP5 LBS formed two complexes of similar mobility to the VP5 complexes, and an oligonucleotide containing the VP5 LBS effectively competed with the SRV-1 fragment for LBF binding (39). It will be of interest to learn what extent the LBS homologs in these viruses play a role in regulation and if the LBS confers the ability to be transactivated by HSV-1.

The cellular factor, LTF, may be identical or closely related to a ubiquitous

transcription factor. We noted that the VP5 promoter binding site, LBS, bears a close sequence homology to the binding sites reported for a cellular factor variously called YY1 (57), CF1 (52), NF-31 (44), δ (23), or UCSRBP (17). This factor appears to be somewhat unique in that it can exert either a positive or a negative control over transcription, depending on the particular gene and binding site involved. Calame's laboratory has presented evidence that common factor 1 (CF1), which binds to homologous sequences in the murine *c-myc* and skeletal α-actin promoters and acts as a transcriptional activator (Table 2-2), is the same factor NF-E1, or simply NF-E1 (52 and references therein).

Four groups have independently cloned a cDNA encoding this factor and characterized the protein. Shi et al. have termed this factor YY1 and have shown that it binds to two sites in the adeno-associated virus P5 promoter and cap site regions here it acts as a transcriptional repressor; in the presence of adenovirus E1A protein, however, it acts as a transcriptional activator (57). Hariharan et al. called the factor δ and demonstrated that it positively regulates certain ribosomal protein genes (23). Park and Atchison termed it IF-E1, a factor that apparently both positively and negatively regulates immunoglobulin in gene transcription by binding to sites in the kappa and heavy chain enhancers (44 and references therein). Flanagan et al. called the factor UCRBP and demonstrated its role in repressing transcription from a site in the Moloney murine leukemia virus LTR (17).

As shown in Table 2-2, the VP5 LBS sequence matches the known binding sites for this factor (i.e., CF1, YY1, etc.) from 6 of 7 to 11 of 11 positions. This similarity

of LBF to CF1 extends beyond its binding site homology, for we have found that oligonucleotides containing CF1, NF-μE1, and YY1 binding sites fully compete for complex formation by the VP5 LBS (41). In the case of VP5 (Fig. 2-6) and possibly also gD (14), it seems that the cellular factor by itself does not cause repression, since deletion of the LBS site did not activate gene expression from these promoters. However, the LBS sequence in the context of these two genes appears to function as a positive control element in the presence of HSV-1 IE proteins (Fig. 2-6B) (14). This may indeed be analogous to the activity of YY1 in the presence of adenovirus E1A on the adeno-associated virus P5 promoter (57).

In summary, the key findings presented in this paper are the following. (i) We have identified a regulatory sequence, GGCCATCTTGAA, which we call the LBS, in the promoter of the major capsid protein gene, VP5. This site binds a cellular factor (or factors), the leaky-late binding factor, LBF. (ii) By mutational analysis of the LBS we have demonstrated that it is required for maximum levels of VP5 transactivation either by superinfecting HSV-1 or by cotransfected IE HSV-1 genes, ICP0, -4, and -27. (iii) Homologs of the LBS consensus sequence, GCCATNT, occur throughout the HSV-1 genome, and we have shown that the LBS sequences found in the gD, gB, and UL37 genes also form complexes with the LBF. (iv) Finally, we have identified homologs of the VP5 LBS in regulatory regions of MCMV, SCMV, and HCMV genomes and in a number of retroviruses LTRs. From results to be presented elsewhere, we have evidence that the cellular factor, LBF, described in this paper is the same, or very similar to, a previously described cellular factor variously referred to as YY1 (57), CF1 (52), NF-31

(44), δ (23), or UCRBP (17).

The results presented in this paper also provide new insight into the regulation of the HSV-1 major capsid protein, or VP5, gene and our data suggest that this regulation is very likely relevant to several other HSV -1 genes of the $\beta\gamma$ (leaky-late or γ_1) kinetic class. Finally, we have provided the first evidence that a cellular transcription factor is required for full activation of VP5 gene expression by either a superinfecting virus or by HSV-1 IE genes ICP0, -4, and -27. Exactly how the promoter complexes formed by the LBF interact, either directly or indirectly, with the viral IE proteins is being investigated in this laboratory.

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CHAPTER 3: Article #2

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YY1 is the Cellular Factor Shown Previously to Bind to Regulatory Regions of Several Leaky-Late (ßγ, γ_1) Genes of Herpes Simplex Virus Type 1

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Running Title: YY1 binds to sites in certain HSV-1 βγ Genes

Abstract

We have recently reported that a cellular factor, the leaky-late binding factor (LBF), binds to sites in a number of leaky-late (βγ or γ_1) genes of the herpes simplex virus type 1, and that an LBF site is essential for maximum viral transactivation of the major capsid protein (VP5) gene. Results of binding competition, partial proteolysis, and monoclonal antibody inhibition assays presented here establish that LBF is identical to the transcription factor YY1. This, along with our previous observations, suggests that YY1 plays a role in HSV-1 gene regulation.

We recently reported that a nuclear factor from HeLa cells, which we call the leaky-late binding factor (LBF), binds to promoter and internal sites in a number of herpes simplex type 1 (HSV-1) genes of the leaky-late ($\beta\gamma$ or γ_1) kinetic class. These include the major capsid protein (VP5), glycoproteins D (gD) and B (gB), and UL37 genes (4). We further demonstrated that the LBF binding site (LBS), having the consensus sequence GGCCATNTT, is required for maximum transactivation of the VP5 promoter by superinfected HSV-1 or cotransfected HSV-1 immediate early genes ICP0, ICP4, and ICP27 (4). Analyses of binding site sequences suggested that the LBF might be related to the murine transcription factor CF1 (14), also known as URCBP (6), δ (7) and its human counterpart YY1 (16, 17), also known as (9,11), NF-E1 (13), MAPF-1 (18).

Competitions by CF1 and LBF binding site containing oligonucleotides. To determine if LBF is related to common factor 1 (CF1), a factor shown previously to bind to several cellular promoters including that of c-*myc* (14), we performed gel shift competition assays as previously described (4) using ^{32}P-labeled promoter fragments from the VP5, gD, and human c-*myc* genes (Table 3-1). The human c-*myc* promoter, like that of its murine counterpart, contains a CF1 site (at -236 bp relative to its P_1 cap site (8)). Double-stranded oligonucleotides containing or lacking LBF or CF1 sites (Table 3-2) were used as competitors. As we have shown previously, the VP5 and gD promoter fragments yielded two major complexes, A and B, (Fig. 3-1). These complexes footprint over the same site in the VP5 promoter (4). Evidence discussed later suggests that complex B is formed by a proteolytic cleavage product of

the protein that forms complex A. The c-*myc*-353 fragment likewise produced two complexes having relative mobilities similar to those observed with the two HSV-1 promoter DNAs (Fig. 3-1, lane 13). The pattern of complexes formed by the three DNA probes was the same whether uninfected or 8 hour HSV-1 infected nuclear extracts were used. (Some assays, especially those using the gD promoter, showed the presence of additional minor complexes. Except for some complexes characteristic of the gD probe, these were not consistently reproducible.) Most importantly, excess amounts of unlabeled oligonucleotides containing either the VP5 LBF or the murine CF1 site effectively inhibited formation of complexes A and B by each of the three labeled promoters (Fig. 3-1). A control oligonucleotide (C2), lacking an LBS or CF1 site, failed to compete significantly (Fig. 3-1). These results suggest that the two major DNA-protein complexes observed with the VP5, gD, or c-*myc* promoter fragments are formed by the same or similar cellular proteins, and this protein(s) has a binding specificity similar to murine CF1.

LBF may be related to transcription factor YY1. Because of binding site similarities and recent reports indicating that CF1 is the murine counterpart of YY1 (13), we tested two oligonucleotides containing known YY1 binding sites from adeno-associated virus (YY1a from the P5-60 site and YY1b from the P5+1 site, Table 3-2) as competitors of LBF complex formation (Fig. 3-2). Excess amounts of either the P5-60 or P5+1 oligonucleotide inhibited formation of the VP5 promoter complex to a similar extent as that observed using the LBS oligonucleotide (Fig. 3-2, lanes 3, 4, and 5). Oligonucleotide C2, lacking a YY1 binding site, showed no significant

competition. Similar results were obtained when the two oligonucleotides containing YY1 binding sites were used as competitors with labeled gD or c-*myc*-353 promoter DNAs (data not shown). In addition, when HIS-YY1, a bacterially-expressed recombinant YY1 protein having the same binding affinity as purified YY1 (17), was incubated with labeled VP5 promoter DNA, the complex formed had the same mobility as complex A formed by the nuclear extract (see Figs. 3-3 and 3-4). Formation of this complex was inhibited by the LBS oligonucleotide to a similar extent as that observed with the YY1 oligonucleotides (P5-60, P5+1) (data not shown). These results strongly suggest that LBF is the same as, or is closely related to, YY1.

Partial proteolysis with Staphylococcus aureus V8 endoprotease. To further establish if the protein(s) forming complexes A and B with the VP5, gD, and c-*myc* promoters were related to YY1, we carried out a partial proteolytic digestion of the complexes (10, 14, 15) using V8 protease. The resulting subcomplexes were separated and identified by gel shift analysis (Figs. 3-3A and B). Each of the three probes, when incubated with uninfected nuclear extract and increasing amounts of V8 endoprotease, produced a similar and reproducible pattern of proteolytic products that retained DNA-binding ability. These subcomplexes had similar relative mobilities for all three DNA probes (Fig. 3-3A). A nuclear extract from HSV-1 infected cells gave similar results (data not shown). When recombinant YY1, HIS-YY1, was incubated with labeled VP5 promoter DNA and subjected to partial proteolysis, the pattern of subcomplexes was strikingly similar to that produced by the nuclear extract (Fig. 3-3B). These results provide further evidence that the nuclear protein(s) forming

complexes A and B is(are) similar for all three promoters and that this protein is structurally similar to YY1.

A monoclonal antibody to YY1 inhibited formation of complexes A and B. To further establish if LBF was the same as YY1, aliquots of HeLa nuclear extract were incubated with increasing amounts of a monoclonal antibody to YY1 (anti-YY1) for 5 to 10 minutes at room temperature prior to complex formation with labeled VP5 promoter DNA (16). The resulting complexes were analyzed by gel mobility shift assay (Fig. 3-4). Increasing amounts of anti-YY1 inhibited the formation of complex A and, to a lesser but significant extant, complex B (Fig. 3-4, lanes 3 and 4). The same amount of a control monoclonal antibody, anti-HSV ICP4 (lanes 5 and 6), or two other non-related antibodies, anti-gB or anti-HSV ICP0 (data not shown), had little effect. Complex formation by the recombinant YY1 protein, HIS-YY1, was inhibited to a similar extent by anti-YY1 but not by the control antibodies (Fig. 3-4, lanes 7-11, data not shown). These results provide further evidence that the protein forming complex A of the LBF is the same as transcription factor YY1. Additionally, these results are consistent with the hypothesis that complex B is formed by a proteolytic product of the protein that generates complex A, factor YY1.

Relation of Complexes A and B to Factor YY1. We have consistently observed two major complexes, A and B, in gel shifts with HeLa nuclear extracts and DNA probes containing LBF or YY1 sites. Our previous DNA footprint analyses (4), and the observation that a 27 bp oligonucleotide containing a single VP5 LBF site formed the two complexes (L. Mills, unpublished results), indicate that these two

complexes involve the same binding site. Furthermore, observations strongly indicate that the protein forming the smaller complex (B) is structurally related to the one forming the larger complex (A), and that the protein forming complex A is YY1: (i) formation of the two LBF complexes on VP5, gD, and c-*myc* promoter fragments was inhibited by oligonucleotides containing binding sites for LBF, CF1, or YY1 (Figs. 3-1 and 3-2); (ii) formation of an HIS-YY1 complex with the VP5 promoter fragment was inhibited by an oligonucleotide containing YY1 binding sites (data not shown); (iii) partial proteolysis with V8 endoprotease yielded the same pattern of protein-DNA subcomplexes with either HeLa nuclear extracts or HIS-YY1 (Fig. 3-3); (iv) a monoclonal antibody specific for human YY1 inhibited LBF complex formation by a HeLa nuclear extract (Fig. 3-4).

These results further suggest that either two structurally related forms of YY1 exist in the cell, or that the smaller complex is formed by proteolysis of YY1 before and/or during nuclear extract preparation. The latter possibility appears to be the most likely since: (i) only a single YY1 mRNA has been detected in HeLa cells by norther blot hybridization (17); (ii) only a single YY1 polypeptide has been detected in western blots (16); (iii) a complex having mobility similar to complex B was shown to be formed by proteolysis of F-ACT1 (a chicken homolog of YY1) (9, 10); and (iv) incubation of a HeLa nuclear extract with a crude, control antibody preparation containing endogenous protease activity resulted in an increase in complex B and decrease in complex A with the HeLa nuclear extract and the conversion of complex A to complex B when HIS-YY1 was used (L. Mills, unpublished observation).

In summary, we have presented several lines of evidence supporting the conclusion that the cellular protein, previously referred to as the leaky-late binding factor, or LBF (4), is the same as transcription factor YY1 (17), also known as CF1 (14), UCRBP (6), δ (7), NF-E1 (13), F-ACT1 (9, 11), and MAPF-1 (18). This factor has been shown to act as either a repressor (2, 6, 9, 11, 12, 13, 17) or activator (7, 14, 16, 17) of viral and cellular gene transcription. The results of studies presented here, taken together with our previous analyses of the VP5 promoter in transient assays (4), and preliminary results from analyses of point mutations in the YY1 binding site of the HSV-1 gD promoter (L. Mills, unpublished results), indicate that factor YY1 also plays a role in the transactivation of certain HSV-1 βγ genes by viral factors.

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CHAPTER 4: Article #3

An Sp1-related Protein and YY1 Are Involved in Transactivation of the Herpes Simplex Virus Type-1 Glycoprotein D Gene

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Abstract

The herpes simplex virus type 1 (HSV-1) glycoprotein D (gD) promoter contains a binding site for the cellular transcription factor YY1. We have identified an additional cis-acting site upstream from and overlapping the YY1 site. Electrophoretic gel mobility shift analyses using Sp1 specific oligonucleotides and antibodies established that Sp1 and Sp1-related proteins (collectively called "SpX") bound to this site. We found that SpX and YY1 bind to these sites, located between -75 bp to -53 bp in the gD promoter, in an independent and possibly mutually exclusive fashion. To examine the function of the SpX and YY1 sites in gD transactivation site-directed mutations were created in the SpX and YY1 sites and the resulting promoters examined in transient expression assays. The results established that both the SpX and YY1 sites were involved in gD gene transactivation following viral induction, with the SpX site playing the major role. Studies of the transactivation levels following cotransfection with HSV-1 immediate-early genes suggest that wild-type transactivation involves the proteins ICP0 and ICP4 interacting directly or indirectly with SpX.

Introduction

During the productive infection cycle, the genes of herpes simplex virus type 1 (HSV-1) are expressed as three sequential interdependently regulated classes: immediate-early, early, and late (23, 49, 58). The expression of the immediate-early class (IE or α) genes does not require *de novo* viral protein synthesis, but does requires cellular and virion components (for references see review 49). The expression of the early (E or β) genes and late (L or γ) genes requires the presence of specific IE class HSV-1 gene products (1, 49, 58). The late genes require viral DNA synthesis for normal levels of expression, and are subdivided into two sub-classes, true late and leaky-late, based on the stringency of their requirement for viral DNA replication. Expression of the true late (γ_2) genes requires ongoing viral DNA synthesis, while leaky-late (γ_1 or $\beta\gamma$) gene expression occurs at low levels prior to viral DNA replication, and is strongly upregulated once replication occurs (6, 14, 21, 59).

The expression of γ_1 genes is the focus of the work presented here. Previous experiments using IE gene mutants, metabolic inhibitors, and transient expression assays have demonstrated that γ_1 promoters are essentially silent in the absence of viral IE proteins (2, 6, 7, 12, 24, 50). Three of the five major IE proteins, ICP0, ICP4, and ICP27, are required for maximum activation of γ_1 promoters (for a review see 49, 58). The molecular interactions by which ICP0,

ICP4 and ICP27 regulate γ_1 gene expression are becoming understood in greater detail (for examples see 12, 19, 60, 61). However, the roles played by various cellular factors in γ_1 gene regulation still need clarification (37, 43, 49, 56). Furthermore, the function and identity of the DNA regulatory elements affecting γ_1 class expression are understood in only a limited manner (17, 22, 25, 38).

In previous studies, we found that the cellular factor YY1 binds to several γ_1 promoters including those of the genes for the major capsid protein (VP5) and glycoprotein D (gD). Using deletion analysis we demonstrated that the YY1 binding site in the VP5 promoter was required for maximum gene expression in transfected HeLa cells following HSV-1 superinfection or IE gene cotransfection (5, 41). In contrast, studies by Huang and Wagner (25) using rabbit skin cells infected with recombinant viruses failed to demonstrate a significant role for YY1 in the regulation of VP5 promoter activity. Instead, their results indicated that an Sp1 binding site located downstream from the YY1 site was more important than the YY1 site for the expression of VP5.

The role played by cellular factors in the regulation of another γ_1 gene, gD, is also unclear. Previous studies by Everett, using deletion analyses, revealed several *cis*-acting regions in the gD promoter required for transactivation by HSV-1 regulatory proteins in transient expression assays (10, 11). Of particular importance was a region between -42 and -73 base pairs from the cap site (bp) of the gD promoter. We found that YY1 binds within this region at -76 to -55 bp (41). This region contains two G-rich elements (called the G1 and G2 regions).

The G1 region contains a potential Sp1 binding site at -72 to -64 bp which matches the Sp1 binding site found in the c-*myc* promoter (8). To determine if Sp1 or Sp1 related proteins bound to the G1 region, we conducted electrophoretic gel mobility shift assays using Sp1-specific oligonucleotides and antibodies. In addition, we carried out transient expression assays using reporter plasmids which contained site directed mutations to examine the role of the YY1 binding site and the G1 region in gD gene expression. The results presented here demonstrate that (a) YY1 and Sp1 and/or Sp1-related proteins (collectively called "SpX") bind to adjacent and overlapping sites located at -73 to -53 bp in the gD promoter; (b) SpX and YY1 bind independently and mutually exclusively to their respective sites; and (c) Both SpX and YY1 contribute in a non-synergistic fashion to the activity of the gD promoter, with SpX playing the major role.

MATERIALS AND METHODS

Cells and Virus. HeLa cells (ATCC CCL2) were maintained at 37°C under 5% CO_2 in Dulbecco's modified Eagle medium (DMEM, GIBCO Laboratories, Inc.) containing 10% heat inactivated bovine calf serum (IBS, Hyclone Labs, Inc.). DMEM containing penicillin and streptomycin (100 units/ml and 100 μg/ml respectively, GIBCO BRL) and 10% (v/v) inactivated fetal bovine serum (DMEM-10% IFBS-P/S) was used during propagation for transient assays.

Wild type HSV-1(KOS) and HSV-1 (*vhs*-1), an HSV-1(KOS) mutant defective in a virion-associated host shut off function (32, 47) gene, were grown in HEp2 cells and titered on Vero cells as described previously (5). HSV-1(*vhs*) was kindly provided by G. S. Read, University of Missouri at Kansas City.

Oligonucleotides. Double stranded oligonucleotides and their sources are indicated in Table 4-1.

Construction of Plasmids. The plasmid pgDCAT contains a 403 bp fragment of the wild-type (wt) glycoprotein D (gD) promoter (-392 bp to +11 bp relative to the cap site) of HSV-1 (Glasgow strain 17+), fused to the bacterial chloramphenicol acetyltransferase (CAT) gene (Fig. 4-1, (10, 13, 39). Plasmid pgD-67/-56CAT is similar to pgDCAT, but contains a 12 bp deletion (-67 bp to -56 bp inclusive) with an *Xho*I linker insertion (13). The CAT gene cassette is followed by 100 bp of the termination sequence from the HSV-1 ICP4 gene. The basic vector is pUC9. Both plasmids were gifts from R. Everett, Glasgow. For clarity in this research, pgDCAT will be referred to as wt-gD-392CAT.

The wt-gD-392CAT plasmid served as the prototype wt gD promoter plasmid. Both the wt-gD-392CAT and the pgD-67/-56CAT plasmids were used for constructing the following site directed mutations (Fig. 4-1): (a) gDY-392CAT which contained a 5 bp mutation centered at -60 bp in the YY1 binding site; (b) gDS-392CAT which contained a 4 bp mutation centered at -70 bp in the Sp1 binding site, and (c) gD2m-392CAT which contained a 5 bp mutation centered at

-68 bp in the Sp1 site and a 5 bp mutation centered at -60 bp in the YY1 binding site. In addition, a gD-75 series was constructed in which the mutations corresponded to those in the above gD-392 plasmids but with the gD promoter sequence truncated at -75 bp just upstream of the SpX site (gDwt-75CAT, gDY-75CAT, gDS-75CAT, and gD2m-75CAT, Fig. 4-1). The sequences of the gD promoter mutations were verified by dideoxyribonucleotide sequencing (52) using the following primers based on the wild-type gD sequence: (a) EgI, centered at -112 bp (CCCGGCCGTGTGACACTATCGTCCATA), and (b) HIII, centered at +3 bp (CGCTGAAGCTTATGACCGAACAATCC, MMI Core Facility, Oregon Health Sciences University).

Enzymes. Enzymes and their sources are as follows: Restriction endonucleases (Bethesda Research Laboratories [BRL] and New England Biolabs, Inc.[NEB]) were used with either the 10x reaction buffers provided by the manufacturers, or the appropriate amounts of KGB buffer (51); Klenow fragment of DNA polymerase, T4 ligase, and RNase A (NEB or BRL); Sequenase Version 2.0, buffers, and nucleotides (U.S. Biochemicals); and bacterial chloramphenicol acetyl transferase (CAT) *S*-acetyl coenzyme A synthetase, and coenzyme A, (Sigma Chemical Co.).

Nuclear Extracts. Nuclear extracts were prepared from uninfected, mock-infected, or HSV-1 (KOS) infected HeLa cells (moi = 10, harvested at 8 h post infection) by one of the following techniques: (a) the method of Dignam, et

al. (9) as previously described (5), or (b) the Nonidet-P40 (NP40) lysis method (40). With both methods nuclear proteins were eluted at 420 mM NaCl. The protein concentration of the nuclear extracts was determined by Bradford protein assay (Bio Rad Laboratories).

Electrophoretic Mobility Shift Assays. Electrophoretic mobility shift assays (EMSAs) were performed essentially as described (41) with the following modifications: The reaction mixtures contained 8 mM n-(2-hydroxyethyl)piperazine-N'-(2-ethane sulfonic acid) HEPES, pH 7.6, 0.8 mM EDTA, 8% (v/v) glycerol, 0.75 mM $MgCl_2$, 0.1 mM $ZnSO_4$, 0.2 mM 1,4-dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Company) or 0.2 mM 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF, CALBIOCHEM), 40mM KCl, 1 to 3 μg of poly-(dI-dC)•poly-(dI-dC) (Pharmacia Biotech) per 16 μl reaction volume. The running buffer for the 4% acrylamide-0.13% bisacrylamide gels was non-recirculating buffer, DBB-1 (3) with a final concentration of 25 mM Tris, 0.10 M glycine, and 1 mM EDTA, pH 8.5 to 8.6. For competition binding assays, 25 to 500-fold molar excess of the unlabeled oligonucleotide competitor DNA was added to the radiolabeled DNA in the reaction buffer prior to the addition of nuclear extract or purified protein. The gels were run at 4°C and at 250V for 2 to 2.5 hours, depending on the size of the labeled DNA fragment. The gels were dried under vacuum and exposed to Kodak XAR or Fuji RX film with Dupont intensifying screens.

Antibodies and Peptide. Polyclonal anti-Sp1 antibody and Sp1 control peptide were purchased from Santa Cruz Biotechnologies. The following antibodies were gifts that are deeply appreciated: Monoclonal anti-YY1 antibody from Yang Shi (Department of Pathology, School of Medicine, Harvard University), and polyclonal anti-HSV-1 ICP4 from W. McClements (Merck, Sharp, and Dohme Laboratories).

Transfections and CAT Assays. Transfections and CAT assays were carried out essentially as previously described (5). Briefly, HeLa cells were seeded at 3 x 10^5 cells per 35 mm^2 well approximately 20 hours prior to transfection. DNA was transfected using the calcium phosphate method (15). All samples were done in duplicate for each transfection. For HSV-1 superinfection studies, each sample contained 8 μg of target plasmid and 8 μg of carrier plasmid (either pUC18 or pUC19). For cotransfection studies, each sample contained 5 μg of target plasmid and 1 μg each of the tested effector plasmid containing an HSV-1 immediate-early gene. Carrier plasmid DNA was added as needed to bring the total volume of transfected DNA to 10 μg for each sample. The effector plasmids containing genes for HSV-1 immediate-early proteins were the following: pGH94 (ICP0), pGH78 (ICP4), and pGR215 (ICP27). The cells were shocked at four hours post-transfection with 1 ml 15% (v/v) glycerol in DMEM, rinsed with PBS-A and then overlaid with 3 ml DMEM -10% IFBS-P/S.

For superinfection assays, the cells were superinfected at 20 h post-transfection with the *vhs*-1 mutant of HSV-1(KOS) at an m.o.i. of 3 and harvested 22 to 24 h post-infection for CAT assay. For cotransfection assays, the media was changed at 24 h post-transfection and the cells were harvested 44 h post-transfection. To analyze CAT activity, we used the method of Nordeen, et al. (45) which measured the incorporation of [^{3}H]-Na-acetate (NEN Dupont, 3.3 μCi/mmol, 10 mCi/ml) into acetyl-chloramphenicol via a coupled reaction with acetyl coenzyme A synthetase. Incorporation was measured in a Beckman LS 6500 scintillation counter.

RESULTS

Members of the Sp1 family bind to a site that overlaps the YY1 binding site in the gD promoter. During our investigation of the role of YY1 in the regulation of the gD gene expression, it became apparent that several other proteins bound to the gD promoter sequences located between -121 to +11 bp. Within the previously identified G1 region (11) we noted the presence of a potential Sp1 binding site (GGGAGG at -70 bp) which is adjacent to and overlapping the YY1 site (41) and matches Sp1 binding sites previously identified in the human immunodeficiency virus (HIV) long terminal repeat (29), the pseudorabies virus immediate early gene promoter (57), and the human *c-myc*

promoter (8). To facilitate the formation of Sp1 or Sp1-related complexes on the gD promoter (see 18, 27, 30), we carried out gel mobility shift assays using the labeled gD promoter fragment (from -121 to +11 bp) in the presence of 0.75 or 1.25 mM Mg^{2+} with either uninfected or HSV-1 infected nuclear extracts (Fig. 4-2, data not shown). Four cellular complexes, A, B, C and D, were revealed in addition to the previously identified YY1 and YY1 breakdown product (Fig. 4-2, lane 1, (33, 34, 41)). The breakdown products from the nuclear extracts (marked bd in figures) which were observed in the assays varied in quantity and identity depending on the method of preparation used to generate nuclear extracts. (See **Materials and Methods**.)

To establish if any of the complexes A, B, C, or D were related to Sp1, we carried out gel mobility shift assays in the presence or absence of Sp1-specific oligonucleotides or antibodies (Fig. 4-2). Addition of an oligonucleotide containing the Sp1 consensus sequence, GGGCGG, (Sp1 oligonucleotide, Table 4-1) inhibited the formation of the complexes A, B, C, and D (lane 2 and 3). However, the Sp1 oligonucleotide had little effect on the YY1 complex (lane 2 and 3). Addition of the polyclonal Sp1-specific antibody (anti-Sp1) resulted in supershifting and inhibiting complexes A and C (lane 4, supershifted complex marked with a dot). Increasing amounts of anti-Sp1 resulted in the inhibition of complexes B and D (lanes 5 and 6). The addition of an Sp1 peptide which lacked the DNA binding domain obliterated the effects of the anti-Sp1 (lanes 7 to 9). This result confirmed that the effects of anti-Sp1 were due to the presence of Sp1 or Sp1-related proteins

in complexes A to D. However, the presence of anti-Sp1 did not affect the YY1 complexes (lanes 4 to 9).

The persistence of the complex marked with an arrow in Figure 4-2 lanes 2 to 3) is due in part to the presence of the HSV-1 ICP4 complex at the same location as an SpX complex, which was established by comparing HSV infected nuclear extract with mock infected nuclear extract in the presence of anti-ICP4 specific polyclonal antibody (data not shown). The reason for the apparent inhibition of ICP4 binding by anti-Sp1 is not clear.

The data from the Sp1 oligonucleotide and anti-Sp1 assays indicated that the complexes A through D which bound to the gD promoter were Sp1 or Sp1-related proteins. These different protein complexes collectively will be called "SpX" for convenience.

YY1 and SpX bind independently and mutually exclusively to the gD promoter. We wanted to determine whether YY1 and SpX binding to the gD promoter was cooperative or independent, thus we added increasing amounts of site specific oligonucleotides to gel shift assay reactions containing a limiting amount of radiolabeled gD probe (Fig. 4-3A). Note that in figure 4-3A, the control lanes (lanes 2, 6, and 10) each contained only HSV-1 infected nuclear extract with no additional oligonucleotides. The YY1 site oligonucleotide (Table 4-1) inhibited the formation of the YY1 complex at 25-fold molar excess (lane 3) but did not inhibit formation of any of the SpX complexes at 500-fold (lane 9). The presence of the Sp1 oligonucleotide inhibited the formation of SpX complexes A to D as

was expected (lanes 6 to 9). As SpX complex formation was inhibited, the intensity of the YY1 complex increased (lanes 6 to 9). A control oligonucleotide, C2 (Table 4-1) did not affect either the YY1 or the SpX complexes (lanes 10 to 13). Complexes were not seen which could be inhibited by both the YY1 and the Sp1 oligonucleotides. On the other hand, inhibiting SpX complex formation by the Sp1 oligonucleotide resulted in enhanced formation of the YY1 complex by increasing the amount of DNA probe available for YY1 complex formation. The data imply that SpX and YY1 form independent and possibly mutually exclusive complexes on the gD promoter fragment.

The following experiment was performed to determine how the presence of purified Sp1 affected the formation of the SpX or the YY1 complexes in nuclear extracts. Nuclear extract from uninfected HeLa cells was allowed to react with radiolabeled DNA for the usual 15 minutes to allow the YY1 and SpX complexes to form (Fig. 4-3B). Then increasing amounts of the following proteins were added to the DNA binding mixture: (a) purified recombinant human Sp1 (rhSp1, lanes 2 to 5), (b) HSV-1 infected HeLa nuclear extract (lanes 7 to 9), or (c) BSA (lanes 11 to 14). Infected nuclear extract was used because the slight difference in complex formation between the infected and uninfected extracts helped confirm the addition of two distinct aliquots of extract. Addition of increasing amounts of rhSp1 caused an increase of the SpX complexes (especially SpX-A and SpX-C) and a concomitant decrease in the YY1 and SpX-D complexes (lanes 1 to 5). Increasing amounts of nuclear extract resulted in increasing levels of SpX

complexes A, B, and C (lanes 7 to 10) but did not significantly increase YY1 or SpX-D. BSA had little effect on complex formation (lanes 11 to 15). These results indicate that rhSp1 can compete with YY1 for binding to the gD promoter, demostrating the SpX blocks YY1 binding and suggesting that SpX and YY1 binding might be mutually exclusive.

The sequences located from -75 bp to -53 bp within the gD promoter are sufficient for the formation of both the YY1 and the SpX complexes. We examined the binding of YY1 and SpX to their respective sites in isolation from nearby gD promoter sequences by using a radiolabeled oligonucleotide, gDSY, containing the sequences located from -75 bp to -53 bp of the gD promoter (Table 4-1) as a probe in gel mobility shift assays (Fig. 4-4). Again, the addition of the YY1 oligonucleotide inhibited only the YY1 complex, whereas the SpX complexes were unaffected (lane 2 to 4). Low amounts of YY1 and SpX breakdown products were present in this particular nuclear extract and are not visible in the exposure shown although visible in other gels. The addition of the Sp1 oligonucleotide inhibited only the SpX complexes (lane 5 and 6). Similar results were obtained when specific antibodies were used. Anti-YY1 inhibited only the YY1 complex while anti-Sp1 supershifted and inhibited only the SpX complexes (lanes 7 to 10). The increased intensity of the supershifted complex in this figure as compared with figure 4-2 reflects the increased amount of nuclear extract present in this particular reaction. These results confirmed that the formation of either the YY1 complex or the SpX complexes did not require the simultaneous binding of both proteins to the

DNA, but instead YY1 or SpX complex formation occurred independently. In addition, the results also established that the sequences located within -75 bp to -53 bp of the gD promoter was sufficient for the binding of YY1 or SpX.

Mutation of the SpX or YY1 sites greatly reduces binding by these proteins. We wanted to determine what influence SpX or YY1 binding had on gD transactivation so reporter plasmids were constructed which contained site-directed mutations in the YY1 site, SpX site, or in both the YY1 and SpX sites (See **Materials and Methods**). The first series of plasmids, the gD-392 series, contained the gD promoter sequence from -372 to +11 bp fused to the CAT gene and were derived from the plasmid wt-gD-392CAT (Fig. 4-1). A second series of plasmids, the gD-75 series, also was constructed and contained site mutations corresponding to the mutations in the gD-392 series. However, for the gD-75 series, the gD promoter sequence was truncated at -75 bp which is just upstream from the SpX binding site (Fig. 4-1). The effects of the mutations on protein binding were determined by gel shift analysis with and without oligonucleotide inhibitors (Figs. 4-5A and B).

Figures 4-5A and 4-5B illustrate the limited protein binding which occurred on the mutated gD-392 promoter fragments as compared with the wild-type gD-392 promoter fragment. Mutation of the YY1 site at -60 bp (gDY-392CAT) essentially eliminated YY1 binding but retained SpX binding (Fig. 4-5A, lanes 2 to 6 and 8 to 12). In contrast, mutation of the SpX site at -70 bp (gDS-392CAT) severely reduced SpX binding but retained YY1 binding (Fig. 4-5B, lanes 2 to 6

and 8 to 12). Mutation of both the YY1 and SpX sites (gD2m-392CAT) severely reduced SpX binding and essentially eliminated YY1 binding (Fig. 4-5B, lanes 2 to 6 and 14 to 18). With both the gDS-392CAT and the gD2m-392CAT mutations a small amount of residual SpX binding persisted (lanes 8 to 18). Competition binding using a gD promoter fragment spanning from -60 bp to +11 bp showed this residual complex to be the result of weak SpX binding to sequences downstream of -60 bp (data not shown).

The wild-type and mutated promoter fragments from the gD-75 series of plasmids also were analyzed by gel mobility shift assays. The assays demonstrated that the protein binding characteristics of the gD-75 series promoters corresponded to those observed for the equivalent promoter in the gD-392 series (data not shown). The results of these DNA binding studies further support the previous finding that SpX and YY1 bind the gD promoter independently.

SpX binding is sufficient for gD transactivation. The effects of mutating the SpX or YY1 sites on gD transactivation were analyzed by transient CAT expression assays (**Materials and Methods**). To induce CAT expression from the wild-type and mutated promoters it was necessary either to superinfect with HSV-1 or to cotransfect with HSV-1 immediate early gene expressing plasmids. To perform the superinfection assays the wild-type or mutated reporter plasmids were transfected via the calcium phosphate method and the transfected cells were superinfected twenty hours later with HSV-1 *vhs*. This virus strain, a host shut-off

mutant, was used to achieve greater stability of the CAT mRNA (47). Cells were harvested at 20 to 22 h following superinfection. Cell lysates were analyzed for CAT activity by the method of Nordeen, et al. (45).

To serve as a control, the basal activity of the wild-type and mutated promoters in the gD-392 and the gD-75 series was established by mock-infecting the transfected cells without virus present. Without viral induction the wild-type and mutated gD promoters in both the gD-392 and the gD-75 series were essentially inactive (Fig. 4-6, Table 4-2) as predicted from previous work (10, 11).

Following HSV-1 superinfection, CAT activity levels from wt-gD-392CAT were stimulated 91-fold over the activity from the uninfected wt-gD-392CAT (Fig. 4-6, Table 4-2). When compared with the virus-stimulated wt-gD-392CAT, induced CAT activity from gDY-392CAT was increased 1.8-fold over the wt levels. However, mutating the SpX site reduced the CAT activity from the superinfected gDS-392CAT. When both the YY1 and SpX sites were mutated, the CAT activity was transactivated to only 2% of the superinfected wt-gD-392CAT level.

Although the removal of the upstream elements from -392 bp to -75 bp resulted in an increase in CAT activity expressed from the wild-type promoter (gDwt-75CAT) as compared with the prototype wild-type promoter in wt-gD-392CAT, the truncation of the mutated promoters did not necessarily result in an increased activity following superinfection. For example, the CAT activity

transactivated from the gDY-75CAT was reduced relative to the wt-gD-392CAT, in contrast to the increase observed when the YY1 mutation was in the context of the longer promoter. Likewise, the CAT activity expressed from gDS-75CAT was approximately half of the CAT activity observed from the longer SpX mutation gDS-392CAT. The activity from gD2m-75 CAT remained low, less than 6% of the wt-gD-392CAT activity.

These results indicated that both SpX and YY1 played a positive role in virus transactivation of gD with SpX providing the major portion of the transactivation. The role of the upstream elements in gD transactivation remains unclear.

Mutation of the SpX site results in reduction in gD promoter induction by ICP0 and ICP4. The mechanism by which the SpX and YY1 binding sites function in transactivation was investigated further by cotransfecting the wild-type and mutated gD-392 reporter plasmids with one or more effector plasmids which expressed the HSV-1 IE genes ICP0, ICP4 or ICP27. These IE proteins have been found to be the major transactivators of γ_1 promoters, including gD (13). We specifically addressed the following questions: (a) What IE protein, or combination of IE proteins, is involved in activation of the gD promoter through the SpX and YY1 sites? (b) To what extent do the SpX and YY1 sites, either singly or in combination, contribute to this activity? For these experiments we used the gD-392

promoter series of plasmids since the longer promoter should more closely approximate environment of the viral genome.

The maximum effect activation of the gD-392 series plasmids which was induced by any single cotransfected IE gene was only twice the CAT levels observed without a cotransfected effector plasmid (Table 4-3). Cotransfection with the combinations of ICP0 plus ICP27 (0+27) or ICP4 plus ICP27 (4+27) also induced relatively low levels of activation from any of the gD-392 plasmids.

As previously observed, ICP0+4+27 provided the maximum stimulation for the wild-type gD promoter, wt-gD-392CAT (Fig. 4-7, (13)). The combination of 0+4+27 also provided maximum induction for the mutated gD-392 promoters, producing a pattern similar to that seen following virus superinfection. Thus, the YY1 mutation resulted in a level of CAT expression that was increased 2-fold relative to that from wt-gD-392CAT, while the mutation of the SpX site and the mutation of both sites impaired CAT expression.

Both 0+4+27 and 0+4 activated gDY-392CAT above the optimal induction of wt-gD-392CAT (Fig. 4-7). In contrast, neither gDS-392CAT and gD2m-392CAT were efficiently transactivated by 0+4+27 or by 0+4. Interestingly, the presence of an intact YY1 site in the wt gD promoter actually reduced the response to 0+4 when compared with the promoter with an nonfunctional YY1 site.

These data show that the ability of ICP4 plus ICP0 to induce transactivation of the gD promoter is inhibited by mutations in the SpX or YY1 sites. Here we

have shown that a functional SpX site at -70 bp is required for effective activation of the gD promoter, while inactivating both the Sp1 and YY1 sites almost obliterates transactivation ICP0, 4, and 27.

DISCUSSION

One or more members of the Sp1 protein family ("SpX") (18, 27, 30), bind to the sequences located at -75 bp to -63 bp in the gD promoter, within the region previously called the G1 region by Everett (11), as demonstrated by gel mobility shift assays using Sp1-specific antibodies and oligonucleotides (Fig. 4-2, 4-3, and 4-4). The SpX binding site (-75 bp to -63 bp) overlaps the YY1 site which is located at -66 bp to -55 bp (41). YY1 and SpX complexes form independently and may compete for binding on the gD promoter as was shown by oligonucleotide inhibitions assays (Fig. 4-3A), rhSp1 competition (Fig. 4-3B), and analysis of mutated gD promoter DNA fragments (Fig. 4-5). Both the SpX and YY1 binding sites play a positive role in transactivating the gD promoter, with the SpX site playing the major role in expression (Table 4-2 and 4-3, Fig. 4-6 and 4-7). When both the SpX and YY1 sites were mutationally inactivated, neither superinfection by HSV-1 nor cotransfection with HSV-1 IE proteins ICP0, ICP4 or ICP27 could effectively transactivate the gD promoter. Cotransfection with IE expression plasmids established that mutation of the SpX site resulted in a reduction of gD promoter transactivation by ICP0 and ICP4. These results showed that while YY1

and SpX binding played a positive role in gD transactivation, SpX binding was a major factor in gD promoter transactivation by the HSV-1 proteins ICP0 and ICP4.

Although the identity of the individual SpX complexes could not be established with certainty, the results presented here offered some clues. Complexes SpX-C and SpX-A specifically increased in the presence of purified recombinant human Sp1, which suggested that these two complexes are composed of proteins of the same molecular weight as Sp1 (Fig. 4-3B). In addition, both SpX-A and SpX-C reacted with lower titers of the anti-Sp1, again suggesting that these complexes contain Sp1. Since SpX-A increases with increasing concentration of rhSp1 or nuclear extract, SpX-A may be a multimeric complex of Sp1.

Both Sp1 and YY1 are cellular transcription regulators influencing both cellular and viral gene expression. Sp1 is an activator, as are most of the currently identified members of the Sp1 protein family (18, 27, 30). YY1 can be both an activator or a repressor of gene expression, depending on context (40, 46, 53, 54).

In theory, the presence of YY1 or Sp1 binding sites could have provided activation of the gD promoter in uninfected cells. Instead, basal activation of the gD promoter in the absence of viral proteins is very low, only 3 to 4 times the value of the CAT assay background control. However, mutations in the SpX and YY1 sites reduced the basal expression levels indicating that these cellular proteins did provide a low level of constitutive activation in uninfected cells.

In spite of the presence of binding sites for the potent cellular transcription factors Sp1 and YY1, the activation of the gD promoter requires the presence of HSV-1 viral proteins ICP0, ICP4, and ICP27 (for examples see 12, 13, 55). Both Sp1 and YY1 can regulate gene expression of viruses other than HSV-1 through interactions with viral proteins. For example, in the presence of viral proteins, Sp1 activates genes in the human immunodeficiency virus (HIV, 26, 28), the bovine papilloma virus (35), and the parvovirus minute virus of mice (31). Likewise, YY1 is associated with both activation and repression of adenovirus genes (53, 54), and repression of genes from human papilloma virus genes (36) and Epstein-Barr virus (42). Importantly, mutation of the YY1 site did not result in increased constitutive basal activation of the gD promoter, which indicates that YY1 binding is not repressing HSV-1 gD activation and is in the absence of viral proteins in contrast to what occurs in adenovirus, human papilloma virus, and Epstein-Barr virus.

While mutation of the SpX binding site always reduced gD promoter transactivation, in some cases mutation of the YY1 site led to increased gD promoter transactivation rendering explanation of the physiological role of YY1 binding difficult. Although SpX and YY1 binding may need to occur in a specific sequential order to activate the gD promoter, the data do not support this model. However, the lowered CAT activity from the double mutations and from the YY1 mutation in the truncated promoter, supports a potential role for YY1 binding in specific situations, for example, activation in specific tissues (25).

The significant CAT activity induced by the cotransfection of the ICP0 and ICP4 genes with the gD plasmid containing the YY1 mutation (gDY-392CAT) implies that ICP0 and ICP4 can be sufficient to perform the functions needed to allow SpX-associated transactivation of the gD promoter. Our data showed that both ICP0 and ICP4 are required since neither protein alone was sufficient to induce significant CAT expression. Several possible mechanisms are proposed here for future investigation of the participation of ICP0 and ICP4 in the SpX-associated activation of gD. ICP0 or ICP4 could enhance, directly or indirectly, the phosphorylation of SpX. Alternatively, ICP0 or ICP4 could act to increase the amount of SpX available to transactivate the gD promoter by relieving the inhibition of Sp1 (or SpX) caused by several recently identified proteins (4, 44). The viral proteins could transactivate the SpX gene(s), leading to a stoichiometric increase of SpX molecules as compared to YY1 (20) although our data do not support an extensive change in the levels of SpX. Furthermore, in the assays performed using the gD-392 series promoters which contain two high affinity ICP4 binding sites, the ability of ICP4 to stabilize the preinitiation transcriptions complex of TFIID and TFIIB (16, 56) probably enhances transactivation. Finally, the possibility exists that either ICP0 or ICP4 binds directly to SpX, or a coactivator binding to SpX, in spite of our inability to observe such complexes in our gel mobility shift assays (data not shown). Further experiments will help elucidate the interactions between cellular YY1 and SpX and

viral regulatory proteins such as ICP0 and ICP4 as they participate in gD promoter transactivation.

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CHAPTER 5: DISCUSSION

I. General Results and Conclusions

My long term goal is identifying the molecular mechanisms which activate and regulate the transcription of the HSV γ_1 genes. My initial aim was to identify the cellular factor which bound to the VP5 promoter and to several other γ_1 gene regulatory regions. An unidentified cellular factor, initially called the leaky-late binding factor or LBF, was required for optimally transactivation of the VP5 gene (Article #1). I also established that the LBF bound to several γ_1 gene regulatory regions. Thus, I hypothesized that the LBF might be important to the activation of many γ_1 genes.

The LBF consensus site appeared very similar to the consensus binding site for a cellular transcription factor, YY1, which had been recently cloned at that time (84, 110, 176, 238, 293). This similarity prompted me to evaluate the LBF protein and I established that the LBF protein complexes were composed of YY1 (Article #2).

Having determined that YY1 was the cellular factor important to VP5 activation, my next goal was to examine the role of YY1 in the activation and regulation of another classical γ_1 gene promoter which contained an YY1 consensus binding site, the gD promoter. I first established that YY1 bound the gD promoter DNA. Then I mutated the YY1 binding site and, as a control, the region 5' to the YY1 binding site. I soon realized that one or more additional cellular factors bound to the gD promoter at the 5' region which also played a role in gD promoter activation.

The binding site for these factors overlapped the YY1 site in a region which had previously been called the G1 region by Everett (76).

The additional cellular factors binding the G1 region were identified as Sp1 and/or members of the Sp1 family. The multiple protein complexes were not further identified, instead the complexes collectively were called "SpX".

My initial working hypothesis at this time was that YY1 was required for optimal activation of the gD promoter. In addition, we believed that SpX cooperated with YY1 in activating the gD promoter. My results rapidly proved this prediction wrong. Of the two cellular proteins, SpX was the more important one for gD activation. Moreover, I did not observe cooperation between YY1 and SpX. Indeed, the presence of the YY1 binding site appeared to inhibit the maximum possible transactivation associated with SpX and the HSV-1 α proteins ICP0 and ICP4. However, YY1 was associated with positive activation of the gD promoter because mutations in both the SpX and YY1 sites resulted in lower gD promoter activation than did a mutation of either the YY1 or the SpX site alone.

The increased transcription of the gD construct in which the YY1 site was mutated was not due to a change in the kinetics of expression since both the wild type gD construct and the gD construct containing the YY1 mutation began expressing CAT activity at the same time, but at different rates (Fig. 5-1).

Thus my current data support the following scenario: (i) Both YY1 and SpX play a positive, but non-cooperative role in gD activation. (ii) SpX, rather than YY1, plays the major role in gD activation, and SpX associated activation requires the

presence of ICP0 and ICP4. (iii) YY1 may be required for optimal expression in a cell-specific manner (Article #3).

II. Possible Molecular Models for gD Transactivation

There are several possible molecular models which would explain the data. In the first model, HSV-1 infection of the cell increases the overall amount of SpX available to bind the SpX site in the gD promoter which displaces YY1 and provides an enhanced level of activation. In the second model, interactions between viral proteins (i.e., ICP0 or ICP4) and SpX or YY1 enhance the activation of transcription from an otherwise weak promoter. A possible third model, in which the binding of YY1 and SpX both are required at different times to specifically enhance recruitment or stabilization of the RNA Pol II initiation complex (e.g., 278), is not supported by the data presented here, but is acknowledged as a formal possibility.

In the first model, HSV-1 infection of cells could enhance SpX availability by increasing the transcription of the SpX gene as a result of the presence of viral transactivators ICP0 and ICP4. Increased SpX transcription is supported by the work of Hilton, et al., (115) who found that slight increase in Sp1 levels occurred as a result of wild type HSV-1 infection. My unpublished data also showed increased SpX complex formation, relative to YY1 levels, in some preparations of HSV-1 infected nuclear extract.

SpX availability also could be increased by releasing the SpX protein from an inhibited state i.e., through a change in the phosphorylation state of SpX or through releasing the SpX protein from an inhibitory protein. For example, the ability of Sp1 to bind DNA is increased by increased phosphorylation (137). Although ICP0 or ICP4 have not been shown to be kinases (263), they could have a an indirect affect on SpX phosphorylation. In addition, Sp1 is inhibited by a direct interaction with a protein called Sp1-I which inhibits DNA binding by Sp1. The Sp1 protein is liberated from Sp1-I by pRB (42). Since the identity of the protein(s) affected by ICP0 are unknown, interactions between ICP0 with SpX, Sp1-I, or a host of unidentified regulatory proteins can not be excluded as possible mechanism affecting gD promoter activation.

The ability of ICP0 and ICP4 to interact with other proteins leads to a second model in which these viral proteins directly or indirectly interact with SpX to specifically increase transactivation of the gD promoter in contrast to providing a general increase in SpX availibity. While my assays did not show direct protein binding between viral protein and SpX or YY1, gel assays are not the only, or the best, method of establishing protein interactions.

Although neither SpX in the uninfected cell nor the addition ICP4 alone was sufficient to measurably stimulate activation of any of the tested gD constructs, a joint interaction between ICP4 and SpX on TFIID in the presence of ICP0 might increase transcription from an otherwise poorly utilized promoter. Adhya, et al. (1) propose that a poor promoter could be rescued by altering the architecture of the DNA as a result of the binding of activators such that the RNA Pol II complex is more optimally

placed, or caged, within the promoter DNA framework (Fig. 5-2). The positioning of ICP4 and SpX on the gD promoter together with ICP0 might alter the local geometry of the DNA sufficiently to aid effective transcription.

In addition, ICP4 interacts with TFIIB and one or more TAFs in TFIID to increase the rate of RNA Pol II initiation complex formation (47). Likewise, Sp1 also facilitates transcription by physically binding to one or more TAFs which compose TFIID. Thus, the transcription of the gD promoter could be synergistically increased in the presence of ICP0 with ICP4 and SpX by modifying the structure of the initiation complex.

Some preliminary data suggest that HSV-1 infection specifically transactivates the gD promoter as opposed to generating an overall increase in the number of SpX molecules available to bind the gD promoter. When the gD-75 construct containing the YY1 mutation (gDY-75CAT) was cotransfected with either ICP0+4+27 or ICP0+4, the CAT activity did not exceed 50% of CAT activity produced from the prototypical wild type plasmid wt-gD-392CAT or 74% of the gDwt-75 (Table 4-3). This is in contrast to the longer gD-392 construct containing the YY1 site mutation (gDY-392CAT) which produced CAT activity exceeding 100% of the CAT activity from wt-gD-392CAT in the same context. These data suggest that the sequences upstream from the SpX site exert a strong positive effect on the transactivation of the gD promoter which is associated with SpX, ICP0, and ICP4. Since SpX binds to its site in the truncated promoter as well as it does to the SpX site in the longer promoter, the differences between the transactivation of the short and the long

promoters are not due to changes in SpX binding ability. Furthermore, these data imply that viral transactivation of gD involving SpX is more dependent on promoter context than on a change in the ability of SpX to bind to the DNA.

Further support for specific activation of the gD promoter is offered by the data from another series of preliminary experiments which are described in Article #3. In these experiments the *cis*-acting element containing the SpX and YY1 sites (located at -75 to -53 bp in the gD promoter) was relocated 10 bp upstream. The binding of novel proteins was not observed for the promoter fragment from this gD construct (gDSY-392CAT)in gel mobility shift assays (data not shown). However, the CAT activity expressed from gDSY-392CAT following HSV-1 superinfection averaged twice that of wt-gD-392CAT, even though both the YY1 and SpX sites were present in each plasmid (Fig. 5-1). The CAT activity of gDSY-392CAT is more comparable to the CAT activity level that is transactivated from the YY1 mutation construct gDY-392CAT in contrast to wt-gD-392CAT. This difference in CAT activity suggests that specific protein interactions or the structural elements of the gD promoter are more important to gD transactivation than the relative level of SpX molecules present in the cell. (Presumably, the same number of molecules of SpX, on the average, is available during the HSV-1 superinfection of either the wt-gD-392CAT or the gDSY-392CAT plasmids.)

III. Future Research Directions

While transient expression assays establish the elements needed to activate the gD gene promoter, evaluating the relevance of the SpX and YY1 sites to the regulation of the gD promoter will require examining the SpX and YY1 binding sites in the context of the entire HSV-1 genome. Work has begun on generating viruses containing the previously examined mutations substituted into the natural, wild-type gD promoter in front of the gD gene. These mutated viruses will first be plaque purified on cells which produce complementing HSV-1 gD protein, since gD is an essential viral protein (142, 297). The first step in evaluating the mutated viruses will be determining if the specific mutated virus can grow on non-complementing cells. Poor growth would indicate that the gD protein was not produced in normal amounts. gD production would be evaluated by Northerns to compare overall levels of gD transcription, Westerns to determine overall gD protein production and nuclear run-offs to identify changes in rate of gD transcription. The mutated virus should be evaluated in several cell types including primary epithelial cells and neuronal cells, to explore a potential cell-specific role for either YY1 or SpX binding.

In addition to establishing the biological importance of the SpX and YY1 sites in the virus, determining the actual interactions between the HSV-1 proteins ICP0 and ICP4 and the cellular proteins SpX and YY1 will help to elucidate the functions of the viral proteins in gene activation. First, the identity of each specific SpX complex must

be clarified. The data from Article #3 strongly suggests that complexes SpX-C and SpX-A contain the prototypical Sp1; thus Sp1 can be used as a starting point for the investigations into function while the components of the other SpX complexes are identified. Using GST fusion proteins or immunoprecipitation assays will determine whether or not ICP4 or ICP0 binds directly, or through a protein intermediary, to either Sp1 or YY1. The protein domains important to the activation functions of these proteins can be established by cotransfecting gD promoter constructs with plasmids expressing mutated ICP4 or ICP0 dominant non-functioning YY1 or Sp1 peptides.

Finally, the requirement for these cellular proteins in gD promoter activation can be established definitively by using either an *in vitro* reconstitution transcription system or transient expression assays in cells lacking either Sp1 or YY1. Confirmation of the independent roles of the YY1- and the SpX-associated transactivation requires a system which lacks either cellular proteins, as opposed to inactivating the respective DNA binding sites, because both YY1 and Sp1 regulate gene expression in the absence of known binding sites (71, 218, 284).

The research described in this thesis was the first to identify that YY1 activated HSV-1 genes, and was the first to identify the requirement for a member of the Sp1 family in the transactivation of the gD gene. The finding that these two cellular proteins are involved in γ_1 gene activation has opened up several new avenues of inquiry which will increase our knowledge of how viruses interact with the host cell to preferentially activate viral genes instead of the cellular genes.

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Table 1-1. Differences between *Herpesvirus hominis* types 1 and 2

	Antigenic type	
	Type 1	Type 2
I. Clinical	infects primarily non-genital sites	infects primarily genital sites
II. Epidemiological	transmission primarily via non-genital route	transmission primarily via genital route (venereal or mother-to-new-born)
III. Laboratory host systems[1]		
A. *Tissue culture*		
1. Cytopathic effect	tight adhesion of rounded cells	loose aggregates of rounded cells; syncytia common
2. Plaques	small	large
B. *Chick embryo*		
1. Pocks on CAM[2]	small	large
2. Histological findings on CAM	primarily ectodermal proliferation	involvement of all membrane layers; syncytia more common
C. *Mice*		
Genital or intra-muscular inoculation	less neurotropic	more neurotropic

[1] Refers to fresh virus isolates.
[2] Chorioallantoic membrane.

From Nahmias, 1968

Table 1-2. Herpes Simplex Virus

Infection	Predominant Virus Type	Outcome	Recurrence
Ocular HSV	1	Visual impairment	Yes
Orofacial HSV	1 > 2	Resolution	Yes
Genital HSV	2 > 1	Resolution	Yes
Neonatal HSV	2 > 1	Retardation	No
Disseminated HSV	1 > 2	Resolution or death	No
Encephalitis HSV	1	Neurologic damage or death	No
Meningoencephalitis HSV	2	Resolution	No

HSV = herpes simplex virus.

From Liesegang, 1991

Table 2-1. Competition of VP5 promoter complex formation by unlabeled viral and cellular DNA fragments

Source of fragment	DNA fragments showing:	
	No competition	Competition
HSV-1 DNA	gH, gC (γ)	VP5, gD, gB+136 (βγ), UL37+750 (β or βγ)[a]
	UL14/15, UL24, UL37-137, UL46, VP16, (βγ)[b]	
	TK, UL40 (β)	
	ICP47 (α)	
Adenovirus type 2 DNA	Major late promoter	
	NF-1 site	
Cell DNA	ε-globin promoter	

[a] UL37, see references 17a and 56.

[b] Although these genes are considered in the βγ class, the UL24 promoter shows little homology and the UL14/15, UL46, and VP15 promoters have only limited homology to the LBS site of VP5. DNA fragments used in competitions not shown in Fig. 2 are the following: gH (glycoprotein H, −176 to +17), gC (glycoprotein C, −350 to +256), UL14/15 (2.7-kb βγ, −179 to +32), ICP47 (−300 to +11), UL40 (ribonucleotide reductase, small subunit, −187 to +156), adenovirus major late promoter (−240 to +175), and ε-globin (−207 to +67), in coordinates relative to cap site, and UL37-275 (−302 and −43), UL37+610 (+573 to +950), and UL46 (−525 to +417), in coordinates relative to ATG.

Table 2-2. Promoter sequences homologous to the VP5 LBS sequence

Gene	DNA sequence	Location (relative to cap site or ATG)	Competition against VP5	Sequence reference
Viral genes				
HSV-1				
VP5	GGGCCATCTTGAATG (LBS core sequence)	−70 (cap)	+	37, 38
gD	GGGCCATtTTacg	−63 (cap)	+	37, 38
gB	tGGCCATCgTcgA	+137 (cap)	+	37, 38
	GGcCCcTCTTtgAT	−249 (cap)	−	37, 38
UL46	GGGCCATCggG	−55 (ATG)	−	37, 38
UL37	GcGCCATCTTGA	+750 (cap)	+	17a, 56
UL37	GGGCCATtTgG	−137 (cap)	−	17a, 56
HSV-1 LBS consensus sequence	GGCCATNTT			
CMVs				
MCMV IE1	GCCAT$^{A}_{C}$TTG$^{A}_{G}$	1st exon, three sites +133 to +180 (cap)		31
	GCCATgTAct	−247 (cap)		
HCMV IE68 and SCMV IE94	GCCATNT	Enhancer/promoter, multiple copies		28
Retroviruses				
Murine leukemia viruses[a]	GGaCCATCT	−180 (cap)		61
SIV_{mac}	GGCCtTCTTaA	−131 (cap)		5
SRV-1	GCCATCTTGAATGC	−140 (TATA)		47
Adeno-associated virus				
P5 promoter	tctCCATtTTGA (YY1 site)	+1 (cap)		57
	GcGaCATtTTGc (YY1 site)	−60 (cap)		
Cellular genes				
Immunoglobulin heavy chain	GGCCATCTTGA (μE1 site)	Enhancer		44, 64
c-*myc* (murine)	GaCCTtTTct (CF1 site)	−260 (cap)		52
Skeletal α-actin	cGCCATgTacc	−90 (cap)		52

[a] For example, Akv, Gross, Scoule, and FBR murine leukemia viruses and murine osteosarcomavirus.

TABLE 3-1. DNA fragments, plasmids, and sources

Gene	DNA fragments (position)[a]	LBS homolog within fragment (position)[a]	Plasmid (source [reference])	Plasmid composition[a]
HSV-1 VP5	*Sal*I-*Hin*dIII (−168 to −4)	GGCCATCTTGAA (−70)	pVP5 (−168) CAT[b] (E. Blair and E. Wagner [2])	HSV-1 VP5 promoter (+4 to −168) linked to CAT
HSV-1 gD	*Pvu*II-*Hin*dIII (−259 to +11)	GGCCATtTTacg (−63)	pP017 (G. Hayward[c])	HSV-1 (MP) 4.9-Kb *Hin*dIII N fragment into pCATB
	*Eag*I-*Hin*dIII (−121 to +11)	GGCCATtTTacg (−63)	pgDCAT (R. Everett [5])	HSV-1 gD promoter (−392 to +11) linked to CAT
Human c-*myc*	*Hin*dIII-*Sma*I (−353 to −101)[d]	TACCATtTTct (−236)[e]	c-*myc*-353 (N. Hay [8])	Human c-*myc* DNA (−353 to +513) linked to CAT

[a] Base pairs are relative to the cap site.
[b] CAT, bacterial chloramphenicol acetyltransferase gene.
[c] Department of Pharmacology and Molecular Sciences, Johns Hopkins School of Medicine, Baltimore, Md.
[d] In this plasmid construct, the *Pvu*II site at position −353 in the c-*myc* promoter has been replaced with *Hin*dIII.
[e] Includes a CF1 binding site (underlined).

TABLE 3-2. Oligonucleotides[a]

Name	Source of Sequence	Sequence
LBS[b]	LBFbinding site of HSV-1 VP5 -70bp[c]	CCAGGATCCAGGGCCATCTTGAATGGATCCTGG
CF1[d]	CF1 binding site of murine c-myc promoter -260bp	GGGCGCGAGAAGAGAAAATGGTCGGGC
C2[d]	μE3 binding site of murine IgH intronic enhancer	CCTTGCCATGACCTGCTTCCT
YY1a[e]	YY1 binding site of AAV[f] P5-60	GATCTCCATGGTTTTGCGACATTTTGCGACA
YY1b[e]	YY1 binding site of AAV P5+1	AGGGTCTCCATTTTGAAGCGGG

[a]All oligonucleotides are double stranded
[b]Vollum Institute, Oregon Health University (4)
[c]bp are relative to cap site
[d]K. Riggs and C. Calame (14)
[e]E. Seto and T. Shenk (17)
[f]AAV = adeno-associated virus

TABLE 4-1 Oligonucleotides[a]

Name	Sequence	Source
YY1[b]	5′ - CCAGGATCCAGGGCCATCTTGAATGGATCCTGG	Vollum Institute, OHSU[c]
Sp1[d]	5′ - ATTCGATCGGGGCGGGGCGAGC	Promega Corp.
C2[e]	5′ - CCTTGCCACATGACCTGCTTCCT	Riggs and Calame[f] or MMI
2m	5′ - CCCCCCAAGGAATTCGGGCTCGAGCCCC[g]	MMI Core Facility, OHSU
gDSY	5′ - GATCTCAAGGGGGAGGGGCCATTTTACGC	Center for Gene Research & Biotechnology, OSU[h]
EgI[i]	5′ - CCCGGCCGTGTGACACTATCGTCCATA	MMI Core Facility, OHSU
HIII[i]	5′ - CGCTGAAGCTTATGACCGAACAATCC	MMI Core Facility, OHSU

[a]All oligonucleotides are double stranded except as indicated.
[b]YY1 binding site from HSV-1 VP5 promoter; YY1 binding site underlined.
[c]OHSU = Oregon Health Sciences University, Portland, Oregon.
[d]Sp1 binding site underlined.
[e]μE3 binding region from immunoglobulin heavy chain enhancer ([Riggs, 1991 #28])
[f]A gift from K. Riggs and C. Calame, Columbia University, ([Riggs, 1991 #28]), also synthesized at MMI Core Facility, OHSU
[g]*Sty*I site overlined. *Eco*RI site underlined. *Xho*I site double underlined.
[h]OSU = Oregon State University, Corvallis, Oregon.
[i]This oligonucleotide is single stranded.

TABLE 4-2. CAT activity summary from HSV-1 superinfection experiments

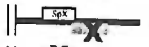
gDCAT Construct		Normalized CAT Activity	
		- HSV	+ HSV-1
	wt-gD-392CAT	1.1 ± 0.2	100.0
	gDY-392CAT	0.5 ± 0.2	182.2 ± 15.2
	gDS-392CAT	0.7 ± 0.6	21.0 ± 4.0
	gD2m-392CAT	0.1 ± 0.1	1.6 ± 0.3
	gDwt-75CAT	4.6 ± 2.4	125.9 ± 9.8
	gDY-75CAT	0.7 ± 0.2	73.9 ± 7.5
	gDS-75CAT	0.5 ± 0.2	7.9 ± 0.9
	gD2m-75CAT	0.1 ± 0.0	5.7 ± 0.8

TABLE 4-3: CAT activity summary from cotransfection experiments

	IE expression plasmids							
Reporter plasmid	ICP0+4+27	ICP0+4	ICP4+27	ICP0+27	ICP0	ICP4	ICP27	none
wt-gD-392CAT	100.0	45.3±4.7	21.1±5.3	11.4±4.3	3.0±0.5	5.4±0.3	1.7±0.2	2.5±0.7
gDY-392CAT	210.9±25.4	129±7.8	15.2±5.2	7.9±1.7	4.6±0.9	5.2±1.2	0.9±0.3	0.6±0.2
gDS-392CAT	17.0±2.0	7.2±2.1	12.0±3.8	0.6±0.4	1.4±0.2	1.0±0.0	1.0±0.5	0.1±0.0
gD2m-392CAT	3.2±0.7	2.3±0.6	3.9±0.2	2.1±0.9	0.2±0.1	0.6±0.1	0.6±0.3	0.3±0.1

CAT activity was measured in pmols [^{3}H]-acetylated chloramphenicol/mg of protein/h. Then CAT activity of plasmids was normalized to the activity induced from wt-gD-392CAT by ICP0+4+27 which was included as a standard in each assay. The CAT activity of wt-gD-392CAT+ICP0+4+27 was defined as being 100% expression (avg= 88,106 ± 28,322 pmols [^{3}H]-acetylated chloramphenicol/mg of protein/h). Data are presented as average relative percentage ± SE. (Number of transfections for each plasmid per effector combination: ICP0+4+27 = 5 to 10; ICP0+4, ICP4+27, or ICP0+27 = 3 to 5; ICP0, ICP4, or ICP27 = 2 to 3)

Figure 1-1. **Herpesvirus**

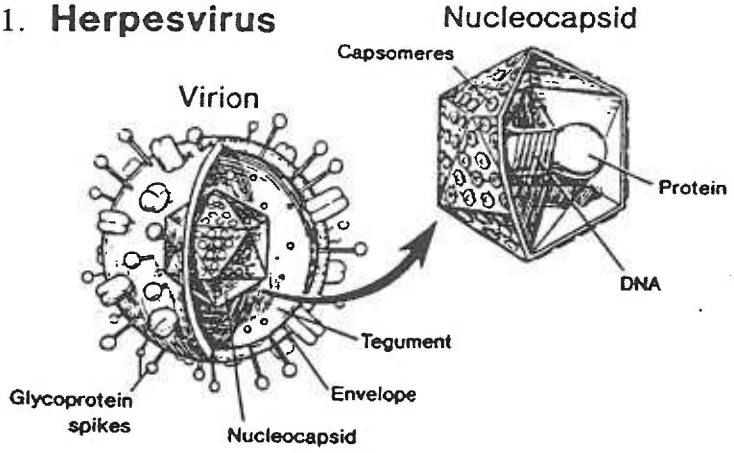


Figure 1. Schematic drawing of a herpesvirus. The protein of the herpesvirus is surrounded by the DNA, much like thread on a spool. A protein structure called the capsid is in the shape of an icosahedron and surrounds this DNA genome core. This combined structure is called a nucleocapsid. An additional phospholipoprotein envelope surrounds the nucleocapsid with glycoprotein spikes projecting from the surface. The tegument is an amorphous protein structure between the nucleocapsid and the envelope. The complete infectious particle is called a virion.

From Liesegang, 1991

Figure 1-2.

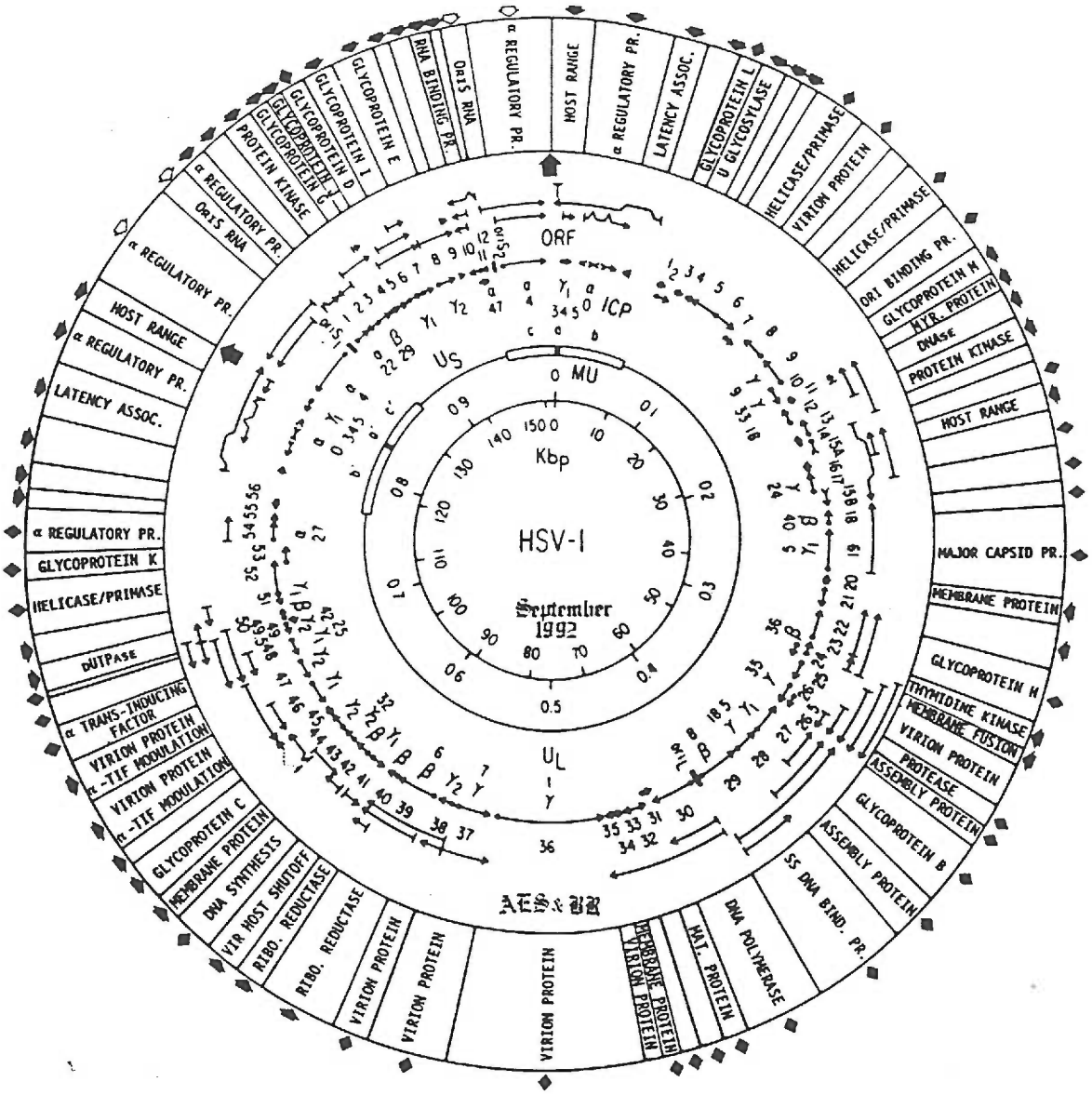


FIG. 8. Functional organization of the HSV-1 genome. The circles are described from inside out. **Circle 1:** Map units and kilobase pairs. **Circle 2:** Sequence arrangement of HSV genome shown as a circularized version of the P arrangement. Cleavage of the circle at 0 map units would yield a linear molecule in the P arrangement. The letters a, b, c, U_L, and U_S identify different domains of the genome. **Circle 3:** Representation of the open reading frames. The letters and numbers indicate the regulatory class to which the gene belongs and the ICP designation of the product. The numbers outside the circle indicate the open reading frames as designated by McGeoch et al. (364,367). **Circle 4:** This represents the direction and approximate size of the transcripts as described by numerous laboratories. **Circle 5:** This lists the known functions of the proteins specified by the open reading frames. **Circle 6:** Essential genes are marked with diamonds, dispensable genes with arrows. Open arrows indicate diploid genes that can be deleted in one but not both copies. The data for circles 3 and 4 are derived from references 11,24,77,94,95,106,112,130,143,172,173,199,223,245,331,364,365,372,378,408,439,442,480,489, 492,561,655. The references for circles 5 and 6 are listed in the text.

From Roizman, 1993 and references therein

Figure 1-3.

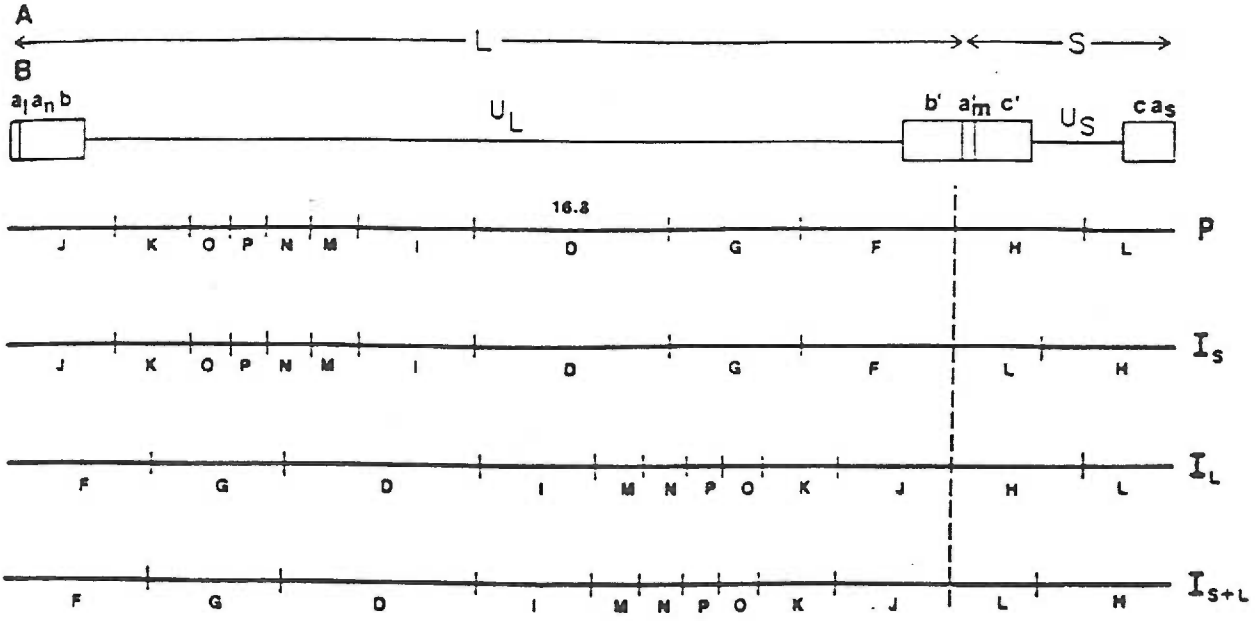


FIG. 2. Schematic representation of the arrangement of DNA sequences in the HSV genome. **A:** The domains of the L and S components are denoted by the arrows. The second line shows the unique sequences (thin lines) flanked by the inverted repeats (boxes). The letters above the second line designates the terminal *a* sequence of the L component (a_L), a variable (n) number of additional *a* sequences, the *b* sequence, the unique sequence of the L component (U_L), the repetition of the *b* sequence and of a variable (m) number of *a* sequences (a_m), the inverted *c* sequence, the unique sequence of the S component (U_S), and finally the terminal *a* sequence (a_S) of the S component. **B:** The HindIII restriction endonuclease map of HSV-1(F) strain for the P, I_S, I_L, and I_{SL} isomers of the DNA. Note that because HindIII does not cleave within the inverted repeat sequences, there are four terminal fragments and four fragments spanning the internal inverted repeats in concentrations of 0.5 and 0.25 M, respectively, relative to the concentration of the viral DNA.

From Roizman, 1993

Figure 1-4.

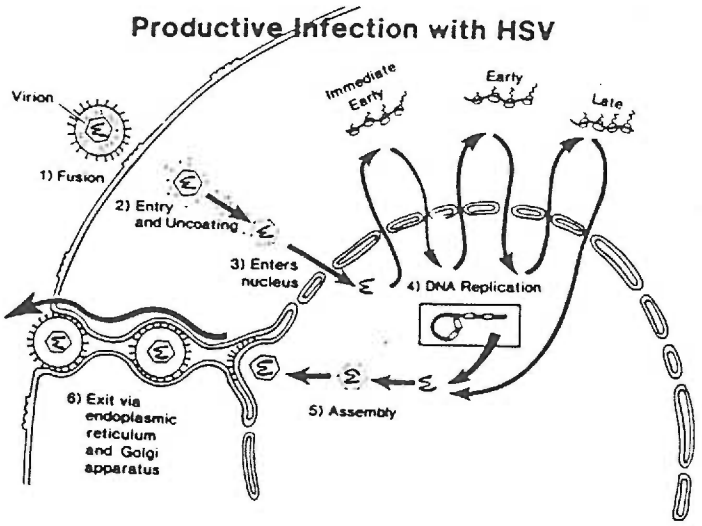


Figure 5. Schematic replication of HSV in a susceptible cell. 1) The virus fuses with the plasma membrane after attachment at specific receptor sites which recognize HSV Type I. The empty envelope is left at the plasma membrane. 2) The nucleocapsid is introduced into the cytoplasm and then transported to the nuclear pore by fast axoplasmic flow. Two proteins are released from the tegument of the virion. One shuts off host protein synthesis and the other is transported to the nucleus as a trans-inducing factor (Vmw65). 3) The viral DNA is released into the nucleus and becomes circularized. The empty capsid coat is left at the nuclear pore. 4) In the presence of the tegument transinducing factor, the transcription of the immediate early genes occurs with transport of mRNA to the cytoplasm and translation to protein products. These products induce the transcription of early genes with transport of mRNA to the cytoplasm and translation to beta proteins which are involved in DNA synthesis. DNA synthesis occurs by a rolling circle mechanism that yields viral DNA. Transcription of gamma genes results in gamma proteins which consist of structural proteins of the virus. 5) The capsid proteins are constructed into complex icosahedron structures, which are packaged with viral DNA cleaved from the rolling DNA concatamers. Viral glycoprotein and tegument protein accumulate and alter the internal nuclear membrane. The nucleocapsid is probably enveloped as it passes through the internal nuclear membrane, de-enveloped between the inner and outer nuclear membrane, and then leaves the nucleus. 6) The virus is transported to the endoplasmic reticulum and Golgi apparatus where it is enveloped and then transported via vesicles to the nerve periphery and released by exocytosis into the extracellular space or to contiguous cells. The glycoprotein spikes of the envelope are matured as the virus travels away from the nucleus (*Modified from the concepts of Roizman B, Sears AE*[9]).

Adapted from Liesegang, 1991

Figure 1-5.

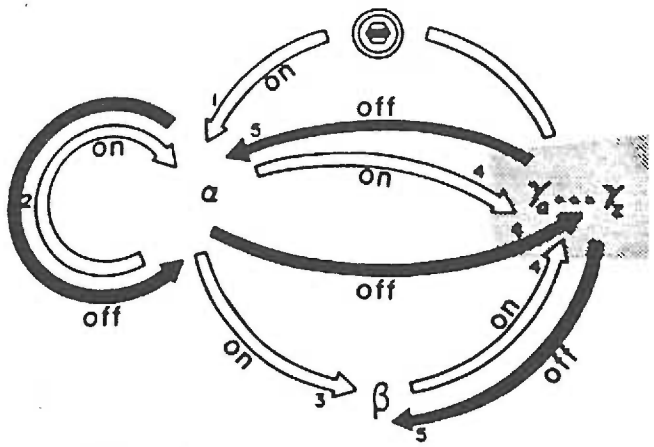
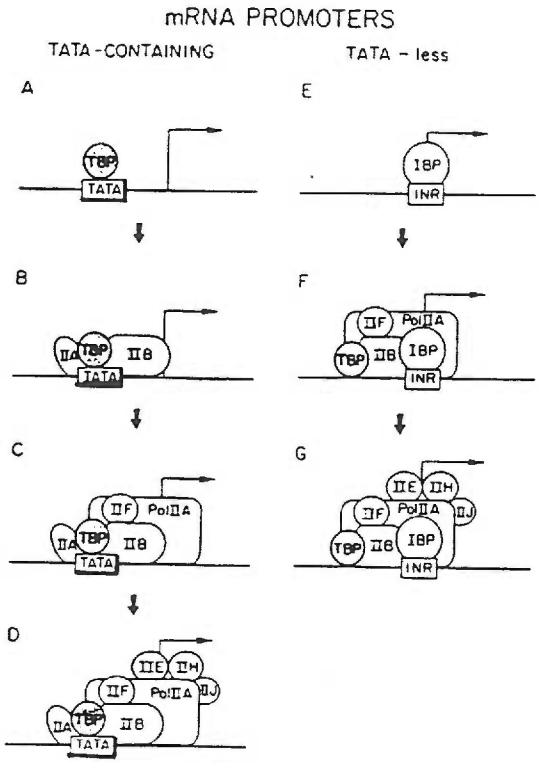


FIG. 5. Schematic representation of the regulation of HSV-1 gene expression. Open and solid arrows represent events in the reproductive cycle that turn gene expression on and off, respectively. **1:** Turning on of α gene transcription by α-TIF, a γ protein packaged in the virion. **2:** Autoregulation of α gene expression. **3:** Turning on of β gene transcription. **4:** Turning on of γ gene transcription by α and β gene products through transactivation of γ genes, release of γ genes from repression, and replication of viral DNA. Note that γ genes differ with respect to the stringency of the requirement for DNA synthesis. The heterogeneity is shown as a continuum in which inhibitors of viral DNA synthesis are shown to have minimal effect on γ_a gene expression but totally preclude the expression of γ_z genes. **5:** Turn off of α and β gene expression by the products of γ genes late in infection.

From Roizman, 1993

Figure 1-6.

Figure 5. Model for the assembly of basal RNA polymerase II initiation complexes on TATA-containing and TATA-less RNA polymerase II mRNA promoters and on RNA polymerase II snRNA promoters. On TATA-containing mRNA promoters, TBP binds the DNA first (*A*), perhaps with TFIIA, followed by TFIIB (*B*) and the RNA polymerase II–TFIIF complex (*C*). TBP interacts directly with these three factors. TFIIE, TFIIH, and TFIIJ then join the complex (*D*). On TATA-less mRNA promoters, the assembly of the initiation complex is thought to start with the binding of an Inr-binding protein (IBP) to the Inr (*E*). This event allows the recruitment of TBP or TFIID, TFIIB, and the RNA polymerase II–TFIIF complex (*F*), most probably followed by TFIIE, TFIIH, and TFIIJ (*G*) as in initiation complexes formed on TATA boxes.



Adapted from
Hernandez, 1993

FIG. 2-1. Gel mobility shift analysis of the VP5 promoter. Various amounts of nuclear extracts from either uninfected (i.e., mock-infected) or HSV-1 infected HeLa cells were reactivated with 0.11 ng of VP5 promoter fragment (-4 to -168 bp relative to the cap site, end labeled with [^{32}P]deoxynucleoside triphosphates) under conditions described in Materials and Methods. The complexes formed were analyzed by polyacrylamide gel electrophoresis. Amounts of nuclear extracts used, in micrograms of protein per reaction, and positions of the two major complexes formed (A and B) and free probe (P) are indicated.

Figure 2-1: Gel mobility shift analysis of the VP5 promoter

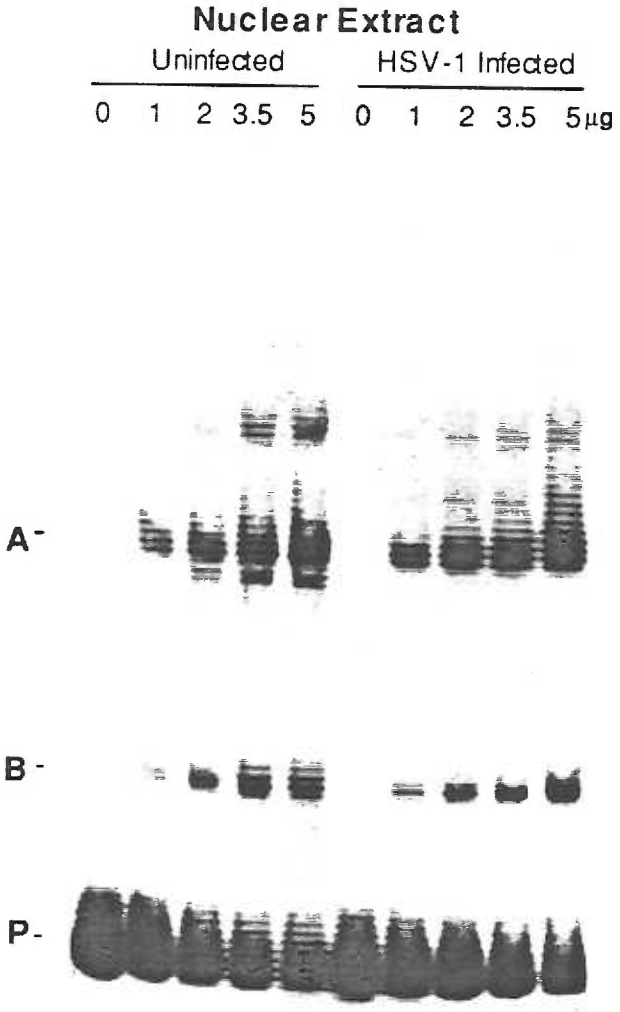


FIG. 2-2. Orthophenanthroline-Cu^+ footprinting of VP5 promoter complexes. (A) Footprints obtained by using an infected-cell nuclear extract and the minus strand of the 164-bp VP5 promoter fragment labeled at the *Sal*I site (-168); (B) footprints obtained by using an uninfected-cell nuclear extract and the plus strand of the VP5 promoter labeled at the *Hind*III site (-4). Lanes A, B, and P, complexes A, B, and free probe, respectively; lanes A+G, A>C, and G, labeled VP5 promoter fragments cleaved by the corresponding Maxam and Gilbert reactions. Regions showing the strongest footprints are designated with brackets and base pairs relative to the mRNA start site are given. (C) Diagram showing protected regions in the VP5 promoter. Regions protected in DNase I footprints are designated with thin-line brackets, those observed in orthophenanthroline-Cu^+ footprints are designated by thick-line brackets. The VP5 TATA box and potential Sp1, CAT box, and NF-1 sites are indicated.

Figure 2-2: Orthophenanthroline-Cu^{+} footprinting of VP5 promoter complexes

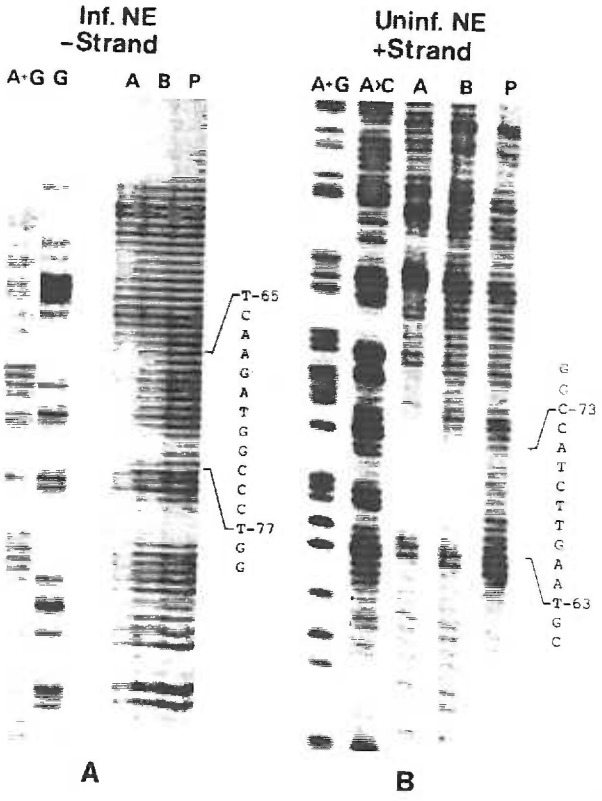


FIG. 2-3. Competition of VP5 promoter complex formation by unlabeled DNA fragments. Gel mobility shift assays were performed as described in the legend to Fig. 1 and Materials and Methods. All reactions contained ^{32}P -labeled 164-bp VP5 promoter probe. 3.6 μg of uninfected HeLa nuclear extract (except in lanes 1), and 100- and 300-fold excesses of various unlabeled DNA fragments as indicated. (A)(Lane 1, VP5 probe alone; lane 2, probe without competitor DNA; lanes 3 and 4, 100- and 300-fold molar excess, respectively, of unlabeled, homologous VP5 promoter DNA (-4 to -268); lanes 5 and 6, unlabeled glycoprotein D (gD) DNA (+13 to -389); lane 7, 300-fold excess of glycoprotein B (gB-137) DNA (+17 to +187); lanes 8 and 9, glycoprotein B (gB -249) DNA (-151 TO -264). (B) Lane 1, VP5 probe alone; lane 2, probe without competitor; lanes 3 and 4, 1090- and 300-fold molar excess of unlabeled VP5 DNA (-4 to 168); lanes 5 and 6, VP16 coordinates, 0 to 189). All competitors except gB-137 were used in 100- and 300-fold molar excesses. HSV-1 gene coordinates are given in base pairs relative to mRNA start sites.

Figure 2-3: Competition of VP5 promoter complex formation by unlabeled DNA fragments

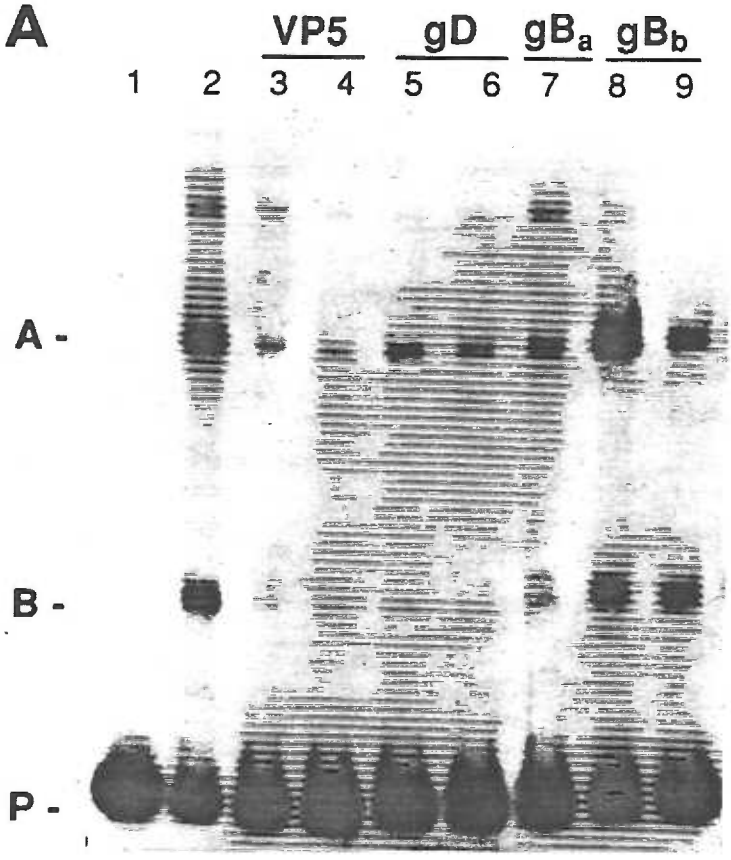


Figure 2-3: Competition of VP5 promoter complex formation by unlabeled DNA fragments

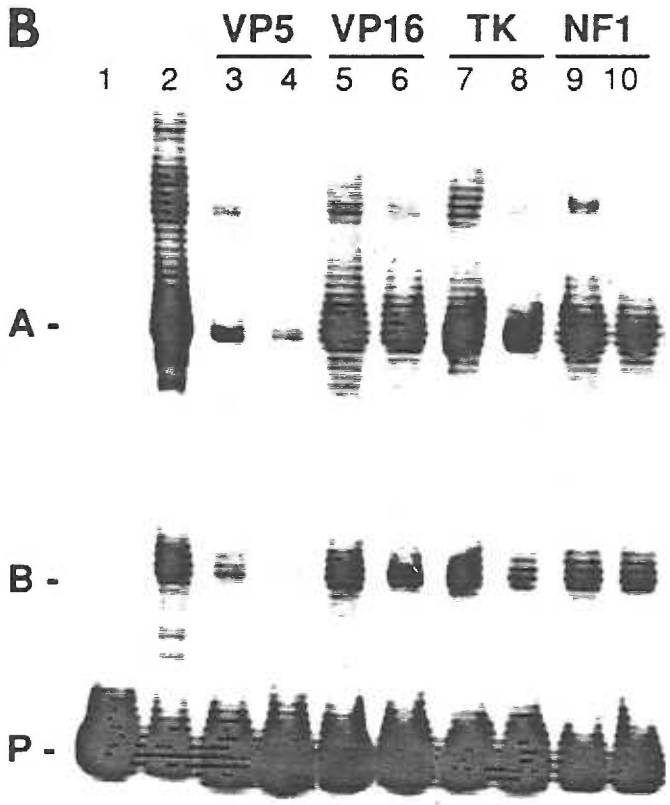


FIG. 2-4. Competition binding of labeled VP5, gD, and gB promoter fragments by oligonucleotides. Gel shift binding reactions were performed as described in the legend to Fig. 1 and in Materials and Methods by using labeled DNA fragments from either the VP5 (0.76 ng; -4 to -168), gD (0.58 ng; +13 to -2609), or gB genes (0.7 ng; +17 5o 187) and excess unlabeled oligonucleotide competitors. Lanes 1, 6, and 11 probe without nuclear extract; lanes 2, 7, and 12 probes with 2.34 μg of uninfected HeLa cell nuclear extract; lanes 3, and 4, 8, and 9, and 13 and 14 contained, in addition, 4, and 8 ng, respectively, of a 33-bp oligonucleotide containing the VP5 LBS; lanes 5, 10, and 15, 7.1 ng of a 23-bp control oligonucleotide, C2, lacking an LBS. See Materials and Methods for oligonucleotide sequences.

Figure 2-4: Competition binding of labeled VP5, gD, and gB promoter fragments by oligonucleotides

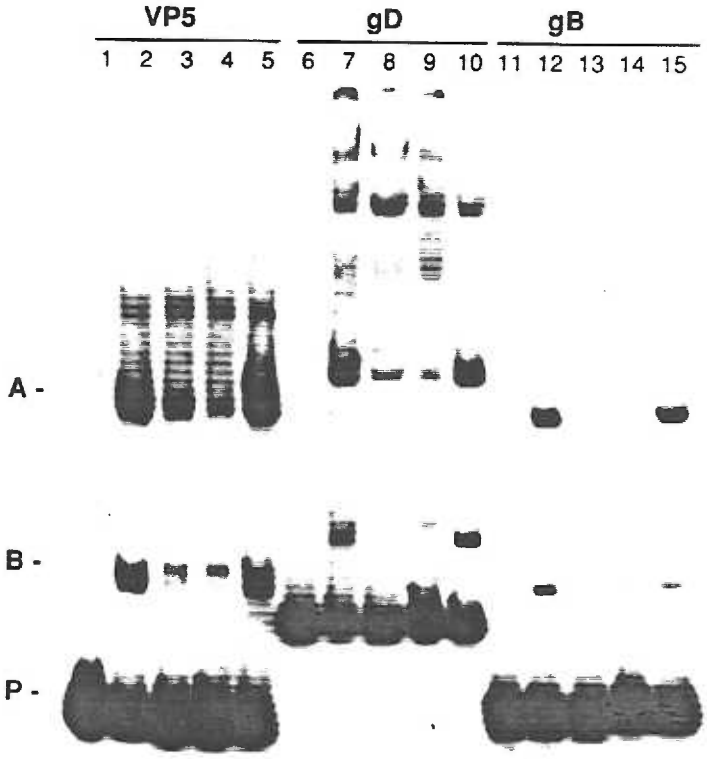
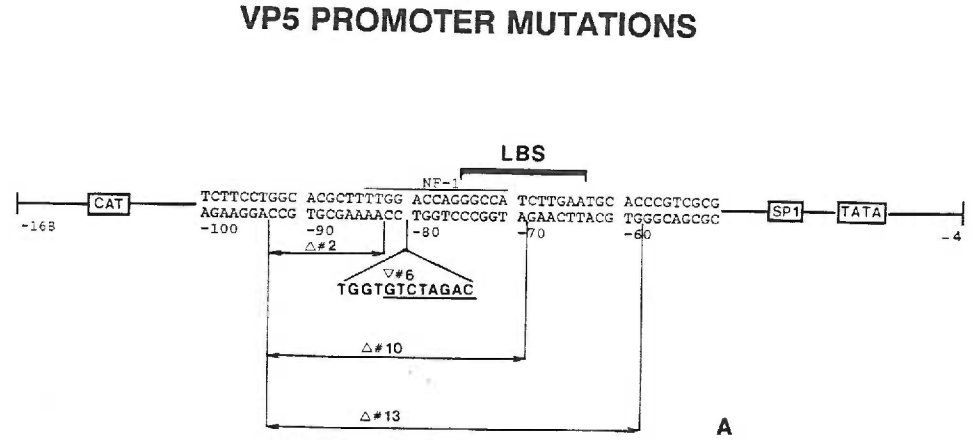


FIG. 2-5. (A) mapping of deletion and insertion constructs in the VP5 promoter. All insertion and deletion constructs contain an inserted *BglII* linker (shown for insertion construct 6). The core LBS sequence bound by the cell factor, LBF, is designated by brackets. (B) Competition binding of labeled VP5 promoter versus unlabeled mutated VP5 promoter fragments. Gel shift assay was carried out with 3.7 μg of protein of infected HeLa nuclear extract as described. A, B, and P, complexes A and B and free probe, respectively; lane 1, free probe; lanes 2 and 9, ^{32}P-VP5-168 without competitor. For the remaining lanes, 20- and 40-fold molar excesses of the indicated wild-type or mutated VP5 promoter fragments (-4 to 168 bp) were used as competitors.

Figure 2-5: Mapping of deletion and insertion constructs in the VP5 promoter



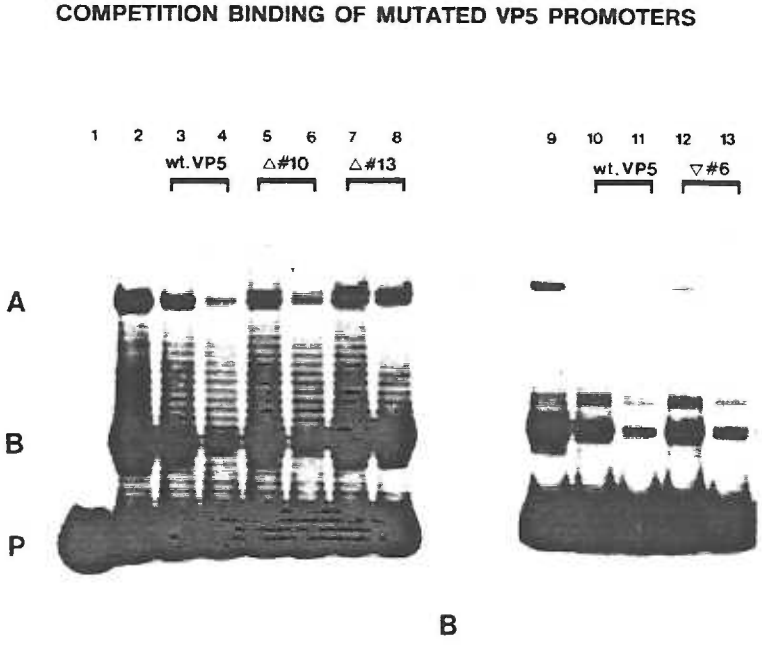


FIG. 2-6. Induction of transfected VP5 promoter-CAT constructs by superinfected HSV-1 or cotransfected IE genes. Amount of acetylated chloramphenicol produced is expressed as picomoles of the monoacetylated form produced in 2 h at 37°C with 25 γl of cell extract. U represents uninduced levels, and I represents induced levels. (A) Induction by HSV-1 (*vhs-1*) superinfection. Numbers on the bar graph indicate fold-induction over uninduced levels. (B) Induction by cotransfected plasmid, pGR150B, containing HSV IE genes for ICP0, -4, and -27. Average uninduced CAT levels for both experiments were 5.6 pmol of acetylated chloramphenicol. The data represent averages of a minimum of three separate transfection assays.

Figure 2-6: Induction of transfected VP5 promoter-CAT constructs by superinfected HSV-1 or cotransfected IE genes

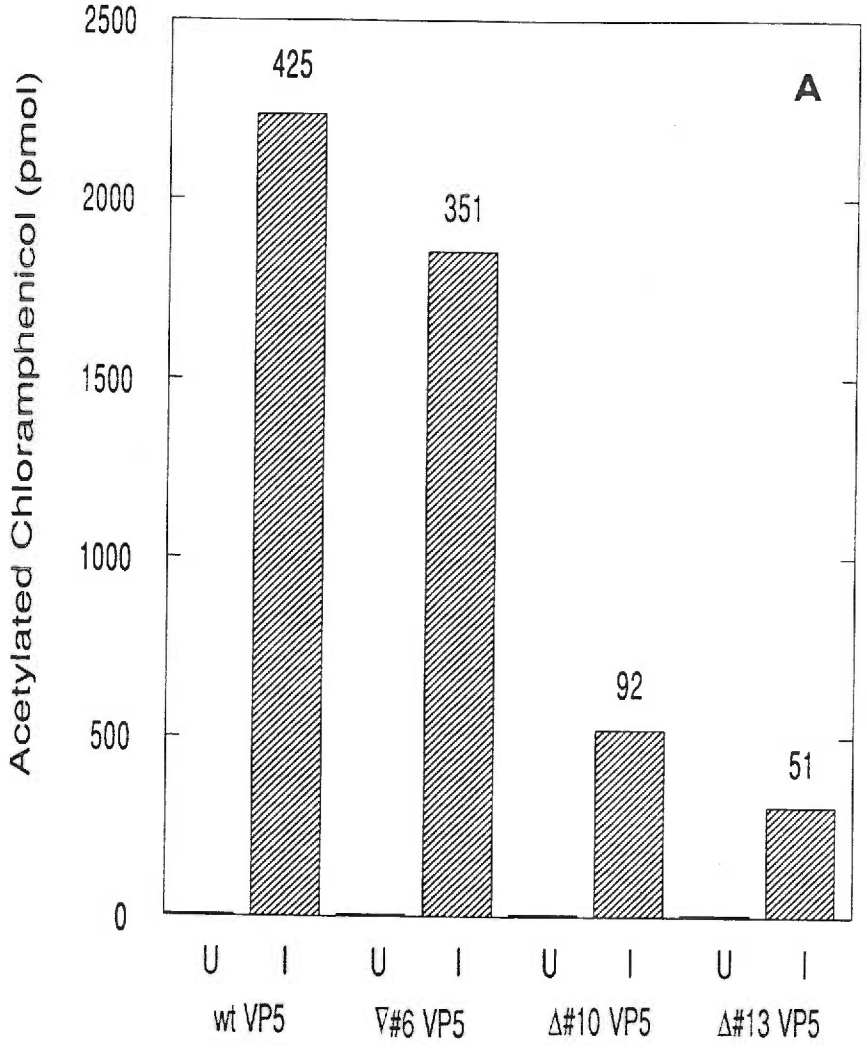


Figure 2-6: Induction of transfected VP5 promoter-CAT constructs by superinfected HSV-1 or cotransfected IE genes

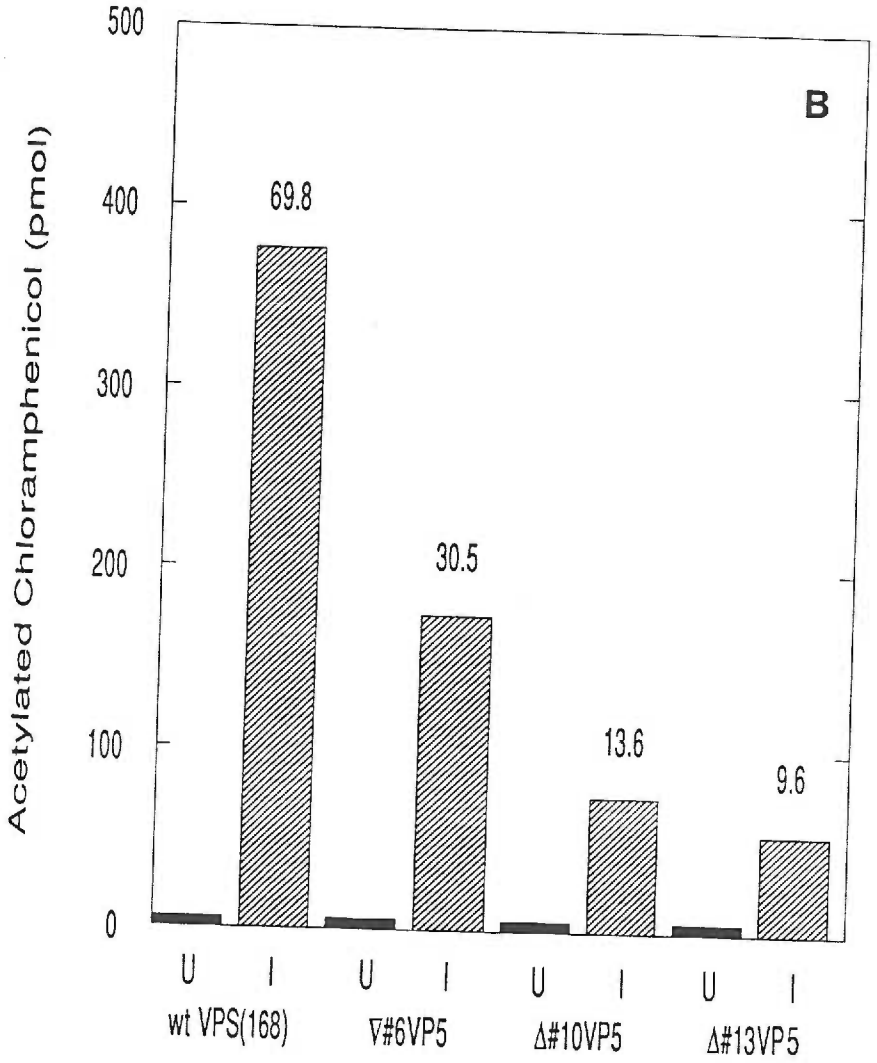


FIG. 3-1. Inhibition of VP5, gD, and c-*myc* promoter DNA complexes by LBS or CF1 oligonucleotides. Uninfected nuclear extract (1.5 μg protein) was incubated with ^{32}P-labeled promoter fragments from HSV-1 VP5 (-4 to -168), gD (-259 to +11), or human c-*myc*-353 (-353 to -101) genes (bp relative to cap site). Competing unlabeled double-stranded oligonucleotides were included in the reactions as indicated: VP5 LBS was added at 150-fold (lane 3) or 300-fold (lanes 4, 9, and 14) molar excess of probe. Both CF1 (lanes 5, 10, and 15), and C2 (lanes 6, 11, and 16) were at 300-fold excess of probe. Complexes (A and B) and free probe (P) are indicated. Polyacrylamide gels (4%) were run at 250 volts for 2-1/4 hours in 1x nonrecirculating buffer, DBB-1 (3).

Figure 3-1: Inhibition of VP5, gD, and *c-myc* promoter DNA complexes by LBS or CF1 oligonucleotides

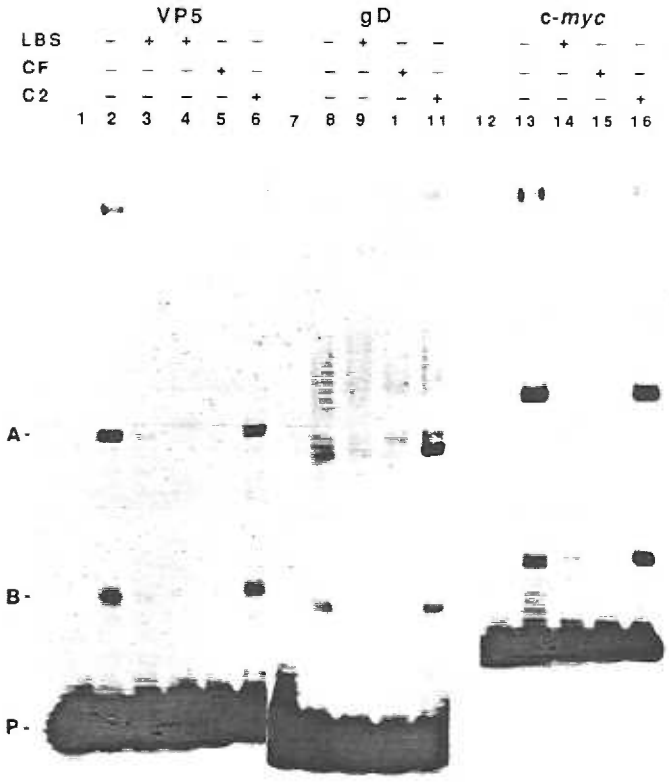


FIG. 3-2. Inhibition of VP5 promoter complex formation by oligonucleotides containing YY1 binding sites. Electrophoretic gel shift and competition assays were performed as described in Fig. 1 using uninfected nuclear extract reaction with ^{32}P-labeled VP5 promoter DNA (lanes 2-6). The following unlabeled oligonucleotides (described in Table 3-2) were added at 300-fold excess of labeled probe: LBS (lane 3), YY1a (lane 4), and YY1b (lane 5) containing adeno-associated virus (AAV) YY1 binding sites located in the P5 promoter at -60 and +1, respectively, (lane 5), and C2 (lane 6), a control oligonucleotide lacking YY1 sites.

Figure 3-2: Inhibition of VP5 promoter DNA complexes by oligonucleotides containing YY1 binding sites

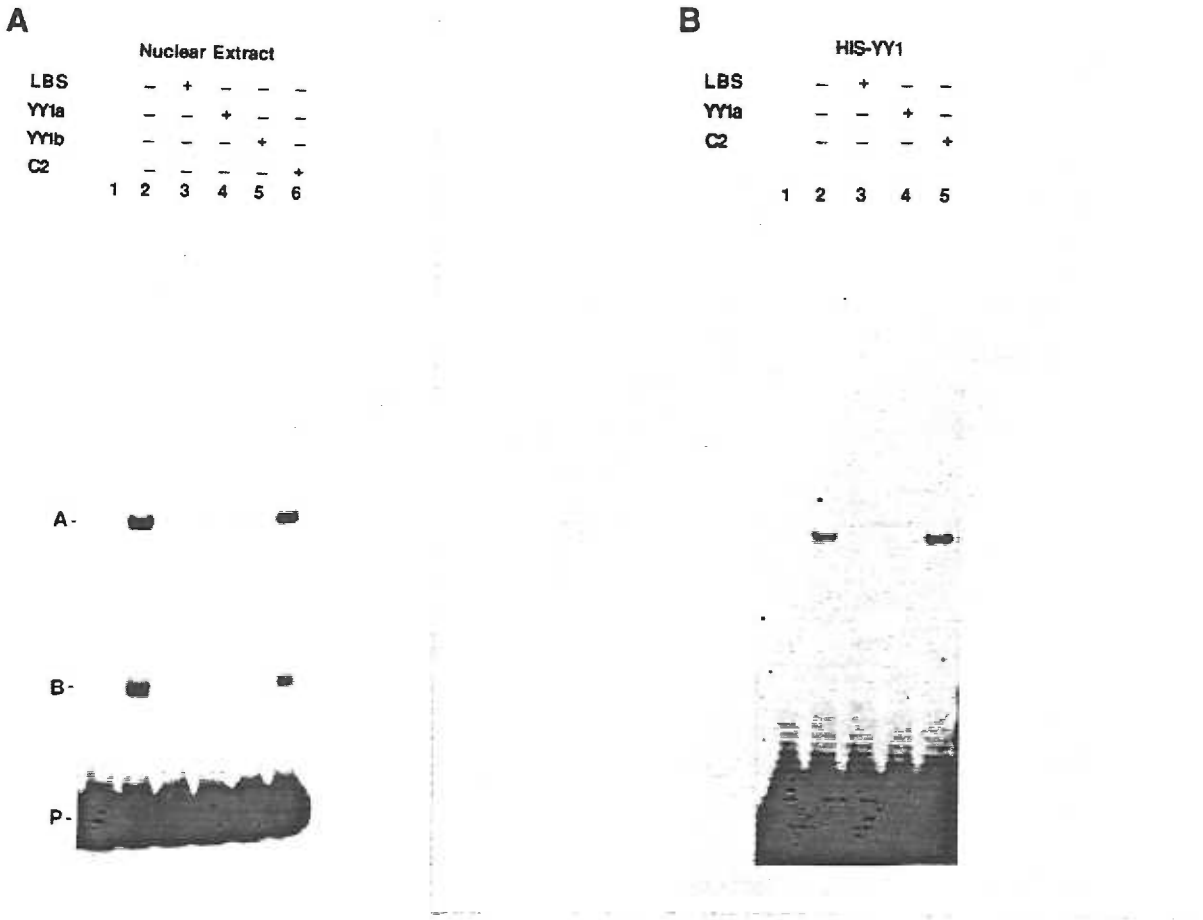


FIG. 3-3. Partial proteolytic "band-clipping" analysis by V8 endoprotease. **(A)** 2.4 μg of uninfected nuclear extract was incubated with either ^{32}P-labeled VP5, gD, or c-*myc* promoter DNA fragments for 10 minutes. V8 endoprotease, in the amounts indicated above each lane, was added to each reaction mixture and digestion was allowed to proceed for 20 minutes at room temperature. Gel shift analysis was performed as described for Fig. 1. Locations of the major complexes A and B and free probe, P, are indicated for the VP5 run. Dots indicate protein fragments retaining DNA binding capability which are formed with increasing endoprotease digestion. **(B)** ^{32}P-labeled VP5 DNA was incubated with either 125 ng HIS-YY1 or 2.4 μg uninfected nuclear extract. V8 endoprotease was added in the amounts indicated and digestion was carried out as described previously.

Figure 3-3: Partial proteolytic band-clipping analysis by V8 endoprotease

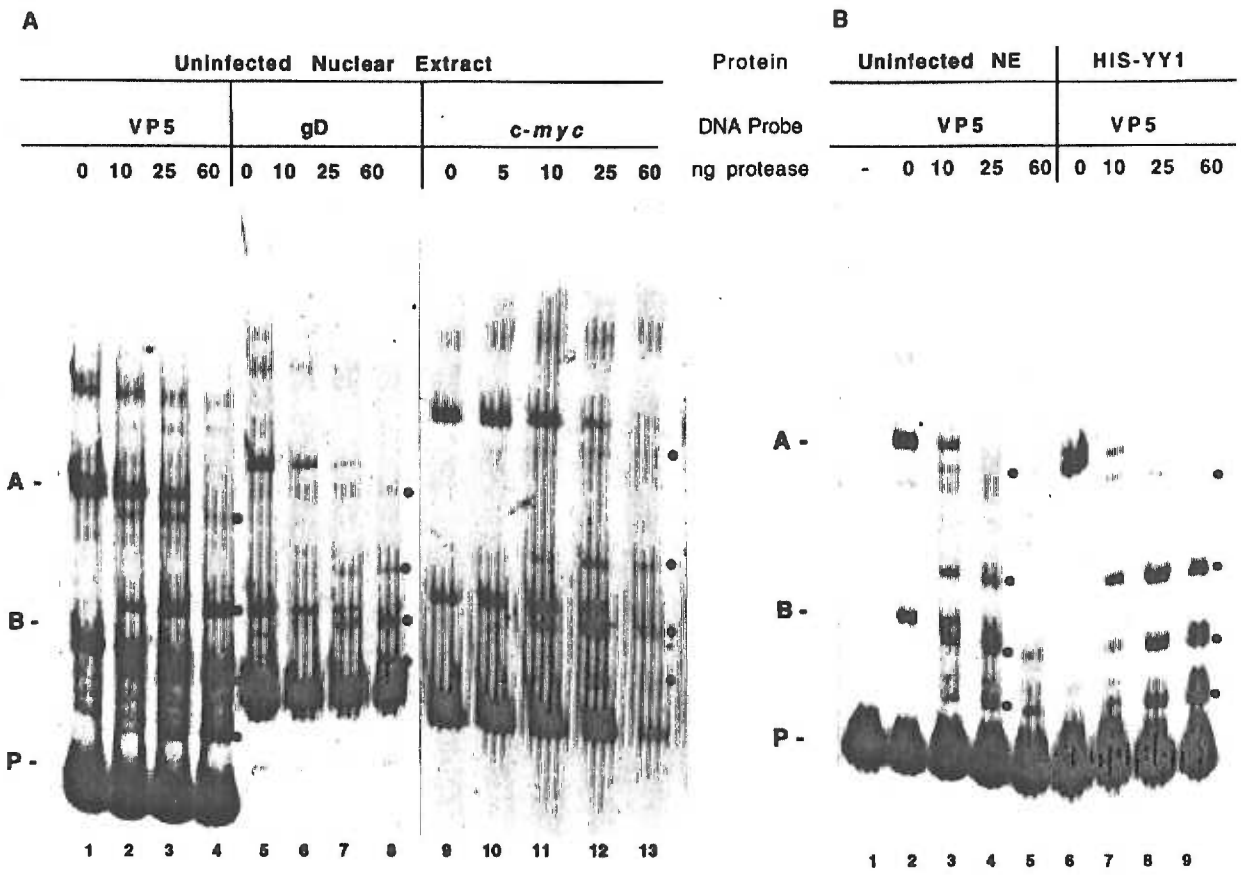


FIG. 3-4. Inhibition of VP5 complex formation by a YY1 monoclonal antibody. ^{32}P-labeled VP5 promoter DNA was incubated with either uninfected cell nuclear extract (1.5 μg) (lanes 2 through 6) or HIS-YY1 (250 ng) (lanes 7 through 11). Anti-YY1 was added at 1.2 μg (lanes 3 and 8) or 2.4 μg (lanes 4 and 9). A control antibody (anti-ICP4, provided by W. C. McClements) was added at 1.2 μg (lanes 5 and 10) or 2.4 μg (lanes 6 and 11). Gel shift assays were performed as described for Fig. 1.

Figure 3-4: Inhibition of VP5 complex formation by a YY1 monoclonal antibody

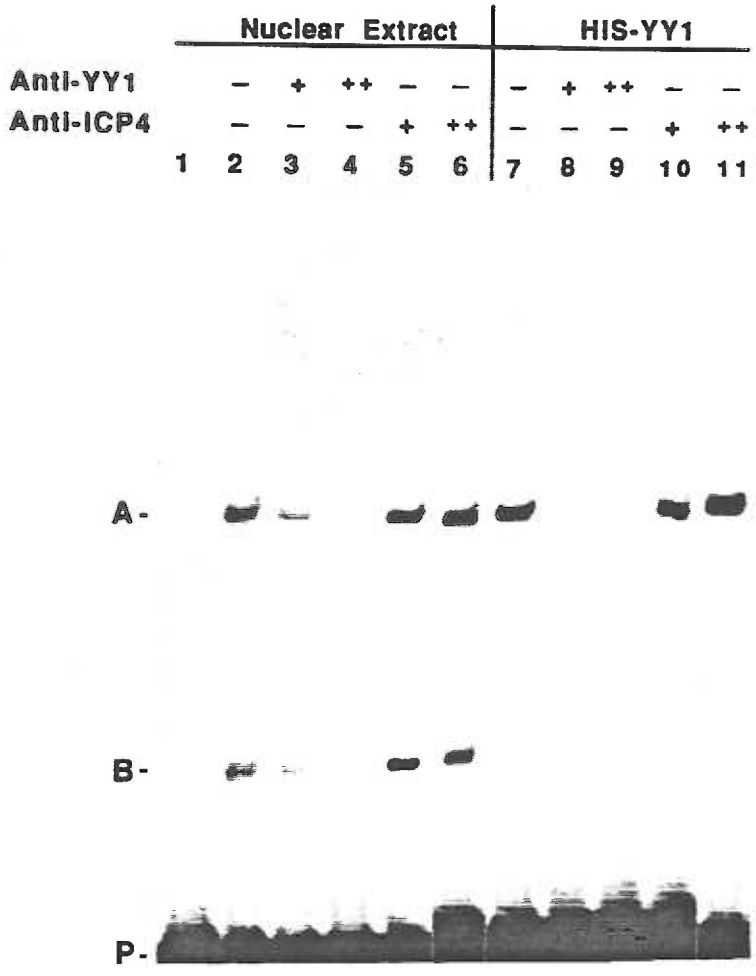


FIG. 4-1 Structure of gD-392 series plasmids. (A) Plasmid maps of wt-gD-392CAT, gD-67/56CAT, and a generic mutated gD-392 promoter CAT construct, (not drawn to scale). The gD promoter region is indicated by the open box, the CAT gene by the shaded box. The restriction enzyme digestion sites are indicated as follows: H = *Hin*dIII, C = *Cla*I, R = *Eco*RI, X = *Xho*I, E = *Eag*I, S = *Sty*I, B = *Bam*HI, L = *Sal*I, O = *Nco*I, P = *Pvu*I, *XL* = *Xho*I linker. "N" = the gD promoter region in which mutations with new restriction enzyme site were introduced. Numbering within the gD promoter is in bp relative to the gD cap site. The pUC9 vector sequences are indicated by the thin line. (B) Detailed map of protein binding sites in the wt-gD-392 promoter, (not drawn to scale). Numbering and restriction enzyme sites are indicated as in Figure 4-1A. Open boxes labeled "G1" and "G1/SpX" both indicate the SpX binding site. The shaded box labeled "YY1" indicates the YY1 binding site. The other boxes indicate binding sites or regions of interest and are labeled accordingly. Ovals indicate consensus protein binding sequences not yet tested for specific binding by the appropriate protein. (C) Sequences of the site-directed mutations constructed in the gD-392 promoter series. (i) Wild-type gD promoter sequence from -80 bp to -41 bp. The SpX binding site is boxed, the YY1 site is bold, and the G2 region is single-underlined. (ii) Sequences of the indicated gD-392 series mutations showing only the bases changed in the mutations. (D) Comparison of gD-75 series mutations. (i) Sequence of gDwt-75 promoter from -75 bp to -41 bp and including the 5' vector-promoter junction. The vector sequences are in italics and the *Eco*RI site is overlined. (ii) Sequences of mutations and 5' vector-promoter junction of the gD-75 series mutation. The new restriction sites generated by the mutations are listed for each promoter series.

Figure 4-1: Structure of the gD-392 series and the gD-75 series plasmids

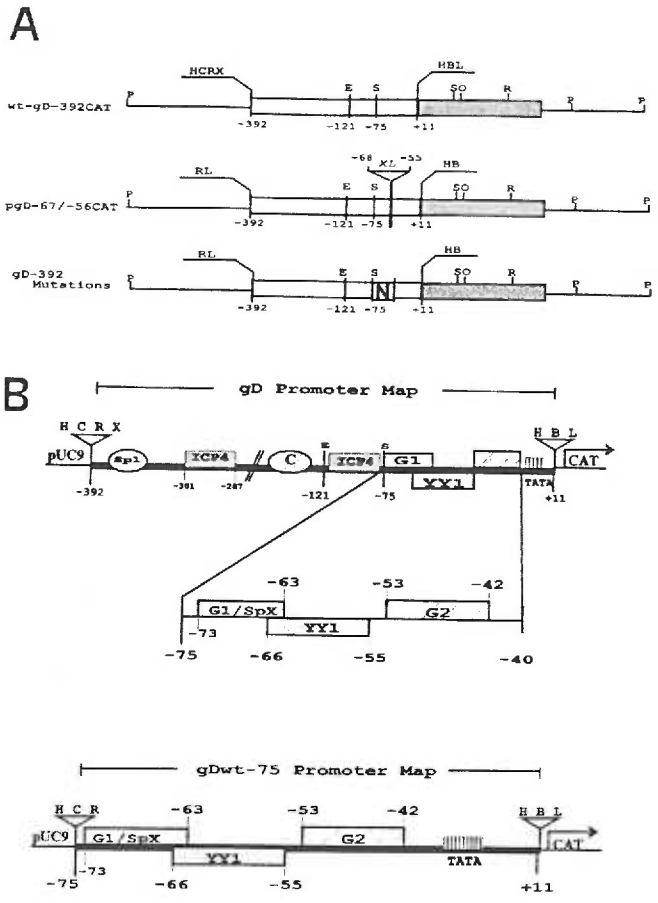
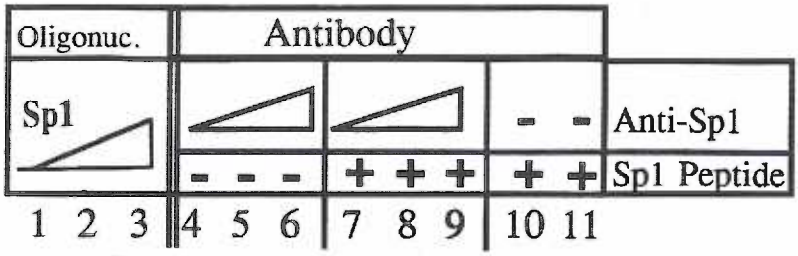


FIG. 4-2. Identification of Sp1 or Sp1-related proteins binding the gD promoter. Infected nuclear extract (1 μg per reaction, prepared by method of Dignam 9])) was incubated with ^{32}P-labeled wt-gD DNA fragment (-121 bp to +11 bp). Reaction buffer contained 1.2 mM $MgCl_2$. Sp1 oligonucleotide was added at 25-fold (lane 2) or 150-fold (lane 3) molar excess (lane 2 and 3). Anti-Sp1 antibody was added at the μg indicated above the lane (lane 4 to 9). Sp1 control peptide was present at 200 ng (lane 7 to 11). Sp1 control peptide alone (lane 10) and Sp1 control peptide plus nuclear extract (lane 11) were included as controls. Complexes are labeled as follows: A = SpX-A, B = SpX-B, C = SpX-C, D = SpX-D, Y = YY1 complex, bd = YY1 and SpX breakdown products, P = free probe. Arrow indicates the postion of the complex containing both HSV-1 ICP4 plus SpX discribed in the text. Dot indicates supershifted Sp1 complex.

Figure 4-2: Identification of Sp1 or Sp1-related protèins binding to the gD promoter



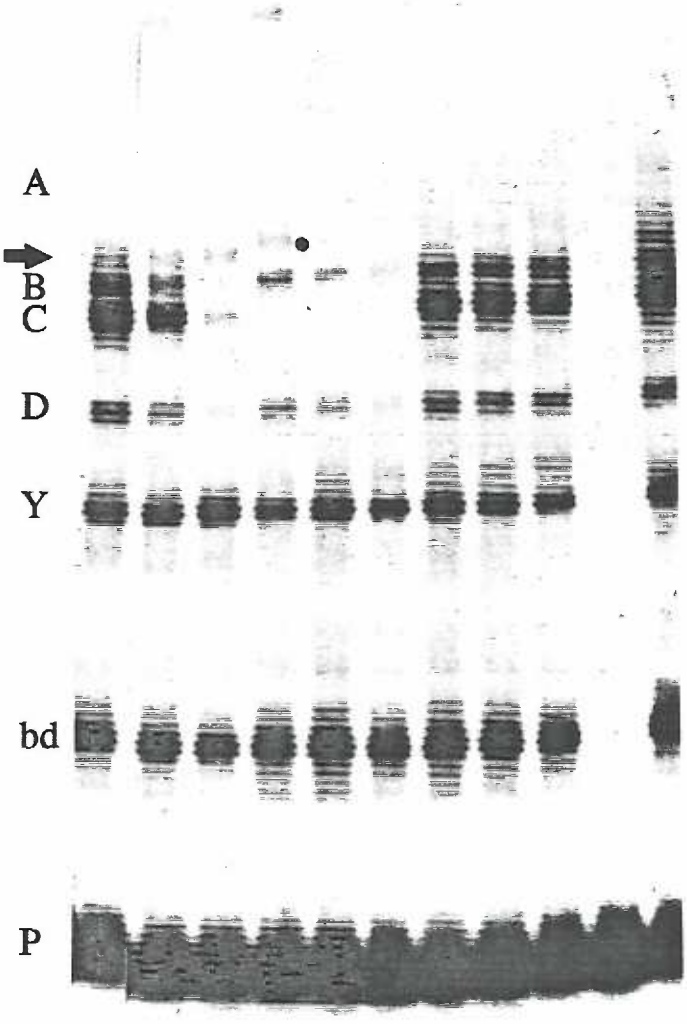


FIG. 4-3A. YY1 and SpX bind the gD promoter independently and mutually exclusively. (A) YY1 and SpX complexes form independently. Infected nuclear extract (1.95 μg per reaction, prepared by method of Dignam 9])) was incubated with ^{32}P-labeled wt-gD DNA fragment (-121 bp to +11 bp). Reaction buffer contained 1.2 mM $MgCl_2$ and was run with limiting concentrations of DNA. Lane 1 was probe alone. Unlabeled oligonucleotides were added at 25-fold (lanes 3, 7, and 11), 150-fold (lanes 4, 8, and 12), or 500-fold (5, 9, and 13) molar excess as indicated. Complexes are labeled as follows: A = SpX-A, B = SpX-B, C = SpX-C, D = SpX-D, Y = YY1 complex, bd = YY1 and SpX breakdown products, P = free probe. Arrow indicates the position of the complex containing both HSV-1 ICP4 plus SpX.

Figure 4-3A: YY1 and SpX bind the gD promoter independently

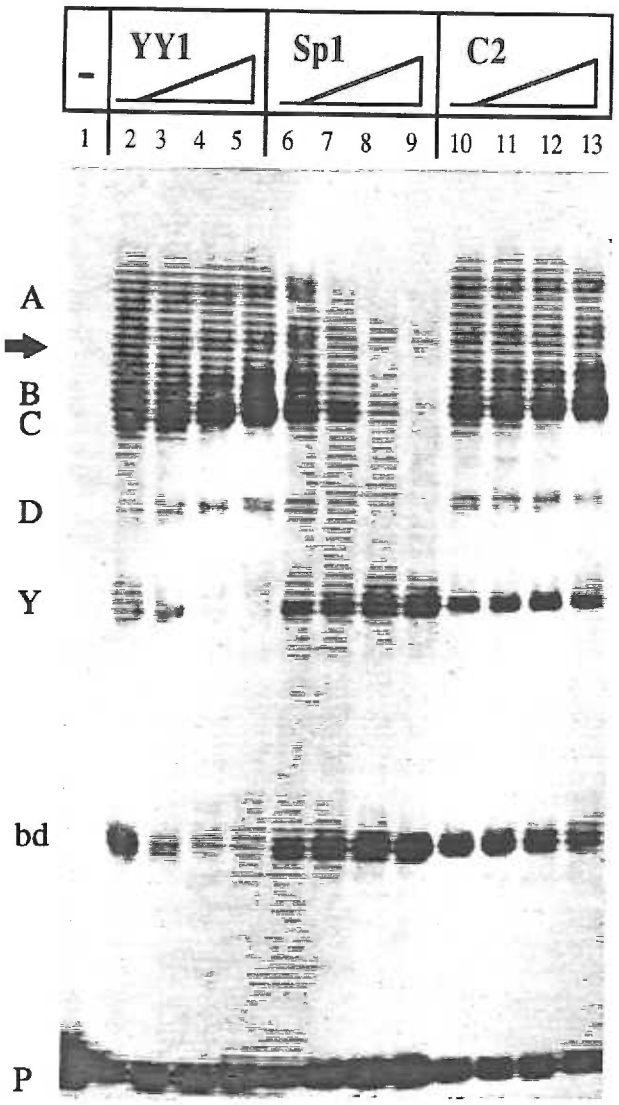


FIG. 4-3B. YY1 and SpX bind the gD promoter independently and mutually exclusively. (B) Displacement of prebound YY1 and enhancement of SpX complex formation by purified rhSp1. Uninfected nuclear extract (U.NE, 3.0 μg per reaction, prepared by the NP40 method 40]) was incubated for 16 min. at R.T. with ^{32}P-labeled wt-gD DNA fragment (-121 bp to +11 bp). At that time the following was added: rhSp1 at 4 ng (lane 2), 8 ng (lane 3), 16 ng (lane 4), or 32 ng (lane 5); HSV-1 infected nuclear extract (H.NE) at 250 ng (lane 7), 500 ng (lane 8), or 1000 ng (lane 9); or BSA at 2.5 μg (lane 11), 5 μg (lane 12), 10 μg (lane 13), or 20 μg (lane 14). Then the reactions were allowed to incubate 15 minutes longer at R.T. prior to being loaded on the gel. Lane 1 is U.NE incubated with DNA for 15 min. Lanes 6 and 10 are U.NE incubated with DNA for 30 min. Complexes labeled as discribed previously for figure 4-3A.

Figure 4-3B: YY1 and SpX bind the gD promoter independently

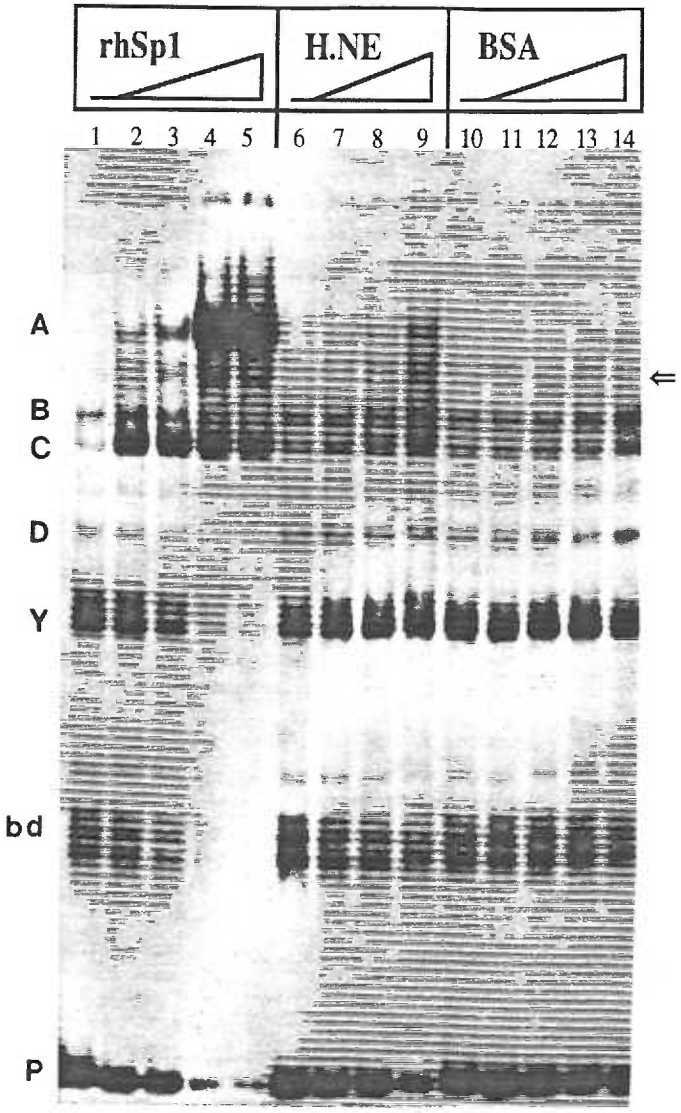


FIG. 4-4 Identification of the complexes forming on the gD promoter sequence located between -75 bp and -53 bp. Infected nuclear extract (2.0 μg per reaction, prepared by the NP40 method 40])) was incubated with ^{32}P-labeled DNA oligonucleotide: CAAGGGGGAGGGGCCATTTTACG. Reaction buffer contained 0.75 mM $MgCl_2$. Unlabeled oligonucleotides were added at 25-fold (+) or 100-fold (++) molar excess as indicated (lanes 2-8). Monoclonal anti-YY1 antibody (Yang Shi, Harvard) was used at 2 μg (+, lane 9). Polyclonal anti-Sp1 antibody (Santa Cruz Biotechnologies) was used at 10μg (+, lane 10) or 100 μg (++, lane 11 and 12). Sp1 control peptide (Santa Cruz Biotechnologies) was used at 200 ng (lane 12). Complexes are labeled as follows: A = SpX-A, B = SpX-B, C = SpX-C, D = SpX-D, Y = YY1 complex, bd = YY1 and SpX breakdown products, P = free probe, * = nonspecific protein.

Figure 4-4: Identification of the complexes forming on the gD promoter sequence located between -75 bp and -53 bp

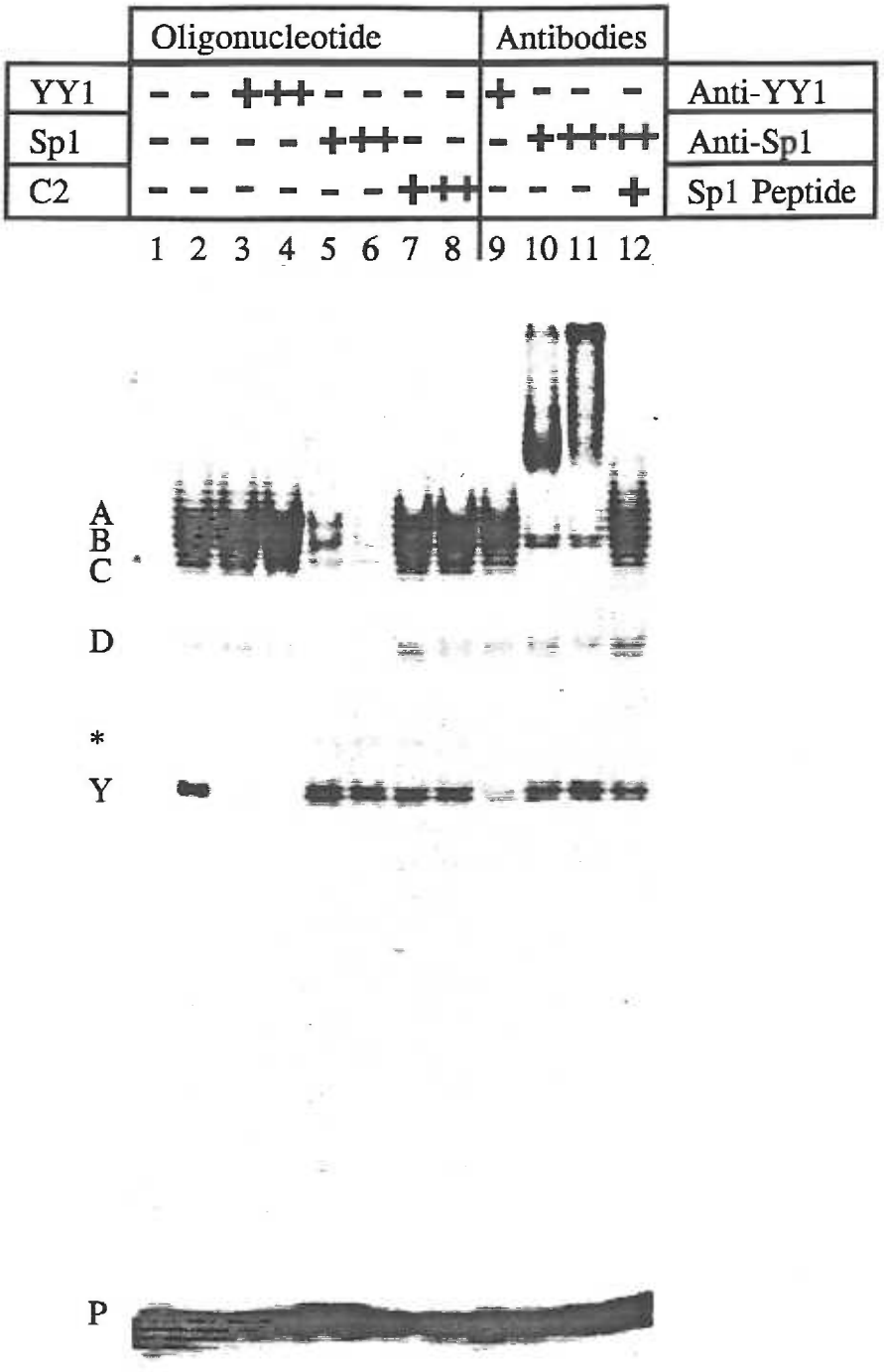


FIG. 4-5. Mutation of SpX or YY1 site results in loss of complex formation. The plasmids from which the mutated promoter DNA fragments (-121 bp to +11 bp) were isolated are indicated above the lanes. A symbol graphically depicting the status of the SpX and YY1 sites is above the plasmid name. The open box indicates the wt SpX binding site. The shaded box indicates the wt YY1 site. The presence of the "X" on the box denotes a mutation in that site. (A) Comparison ofcomplex formation on wt-gD-392CAT and gDY-392CAT promoter fragments. Infected nuclear extract (1.0 μg per reaction, prepared by the NP40 method, (40)) was incubated with ^{32}P-labeled DNA fragment. Unlabeled oligonucleotides were added as specified above the lanes at 150-fold molar excess. Complexes are labeled as for previous figures. (B) Comparison of complex formation on wt-gD-392CAT, gDS-392CAT and gD2m-392CAT promoter fragments. Nuclear extract and unlabeled oligonucleotides are the same as figure 4-5A. Complexes are labeled as for previous figures.

Figure 4-5A: Mutation of SpX or YY1 site results in loss of complex formation

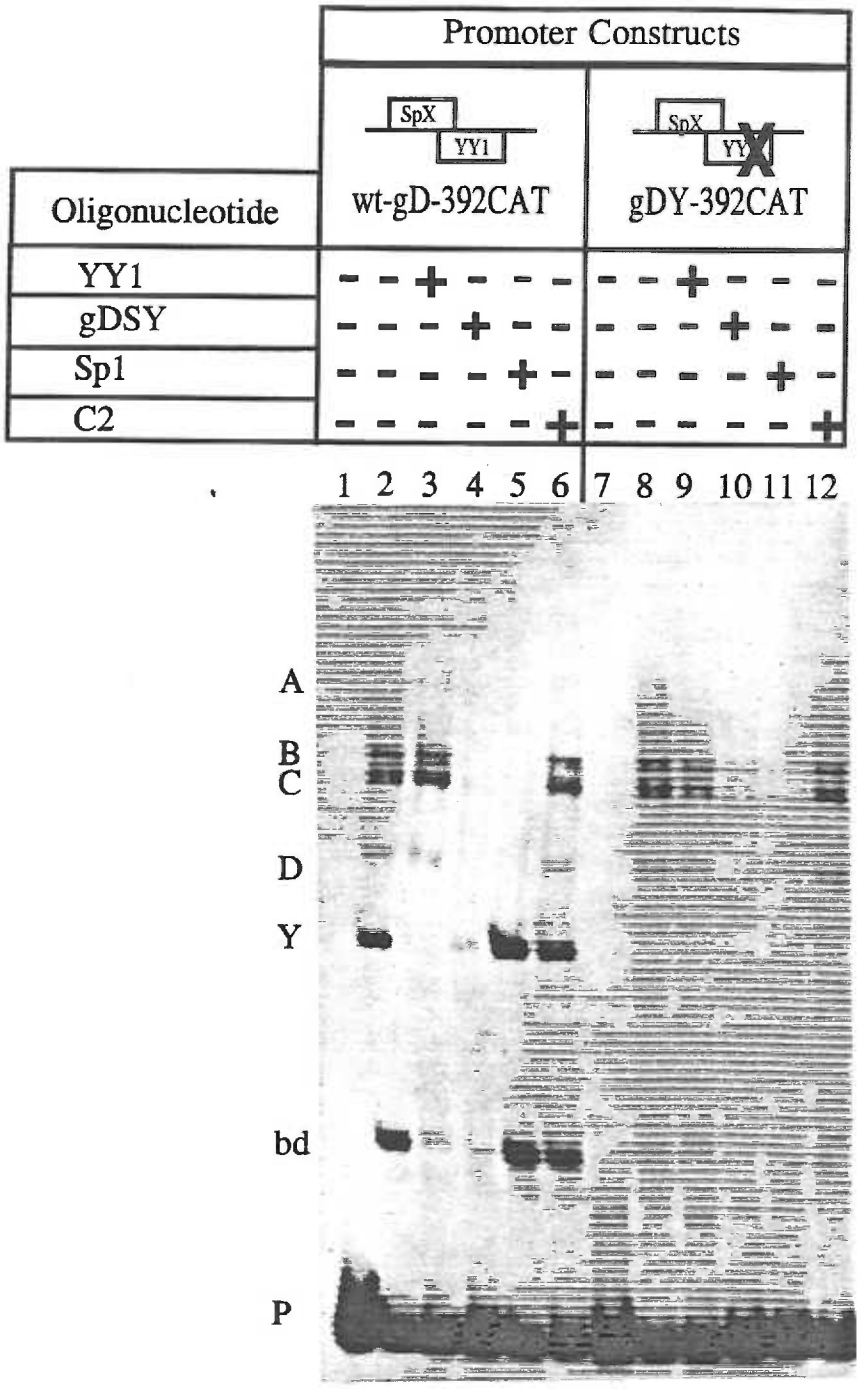


Figure 4-5B: Mutation of SpX or YY1 site results in loss of complex formation

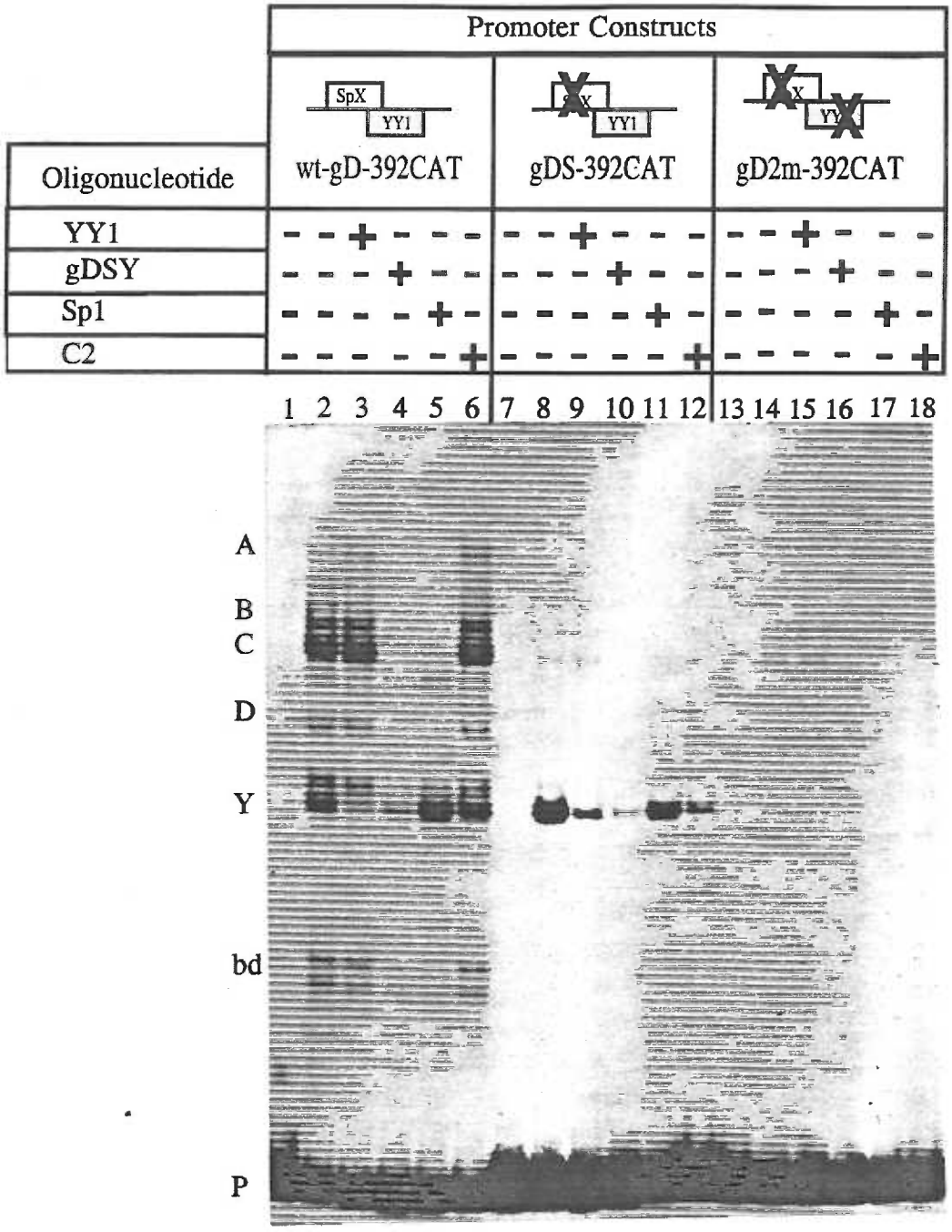


FIG. 4-6. Relative CAT activity from the gD-392 series and the gD-75 series of plasmids following HSV-1 superinfection. CAT activity was measured in pmols [^{3}H]-acetylated chloramphenicol/mg of protein/h. The CAT activity following HSV-1 superinfection of wt-gD-392CAT was defined as being 100% expression (avg. = 41,309 $\pm$ 5,633 pmols [^{3}H]-acetylated chloramphenicol/mg of protein/h). Data are presented as average relative percentages $\pm$ SE from at least 4 separate experiments. Plasmid and promoter descriptions are indicated below bars. The gD-392 series promoter symbols have an arrow, while the gD-75 series promoter symbols are truncated with vertical lines. The presence (+) or absence (-) of HSV-1 is specified under the individual bar.

Figure 4-6: Relative CAT activity from the gD-392 series and the gD-75 series following HSV-1 superinfection

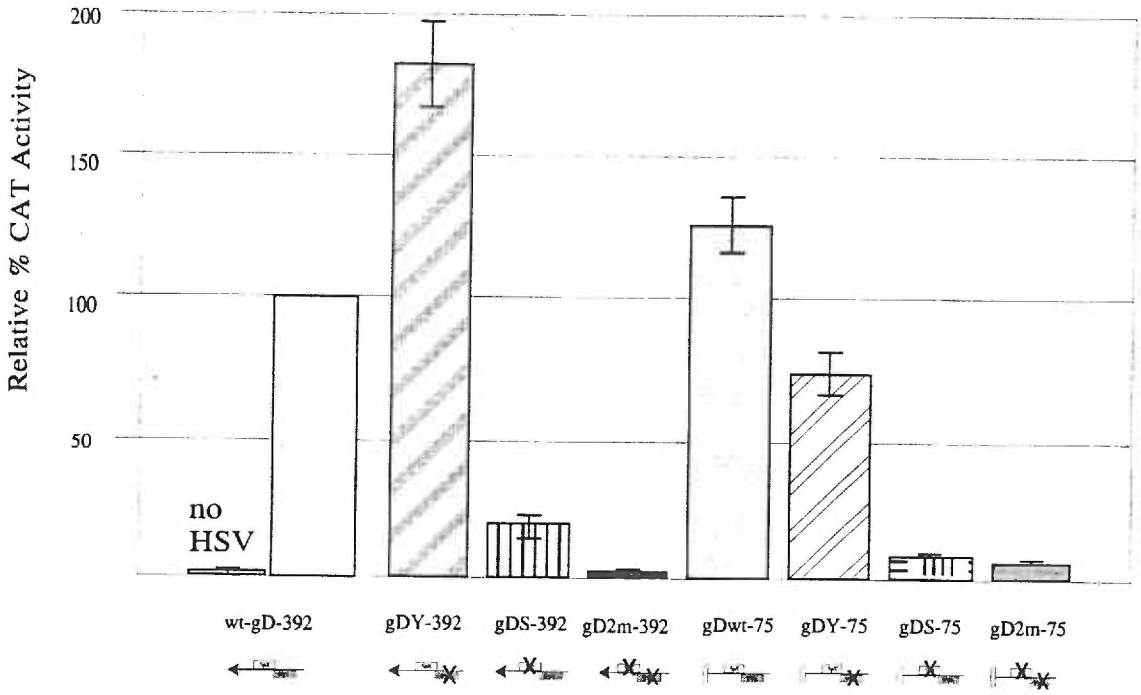


FIG. 4-7. Comparison of CAT activity induced by ICP0+4+27 or ICP0+24 cotransfections from wt-gD-392CAT, gDY-392CAT, gDS-392CAT, and gD2m-392CAT. Assays performed and data presented as in Table 4-3. The IE gene-expressing plasmids included in the cotransfection are specified below the appropriate bars. Plasmid and promoter descriptions are indicated below the effector plasmids.

Figure 4-7: Relative CAT activity from the gD-392 series following cotransfection with ICP0+4+27 and ICP0+4

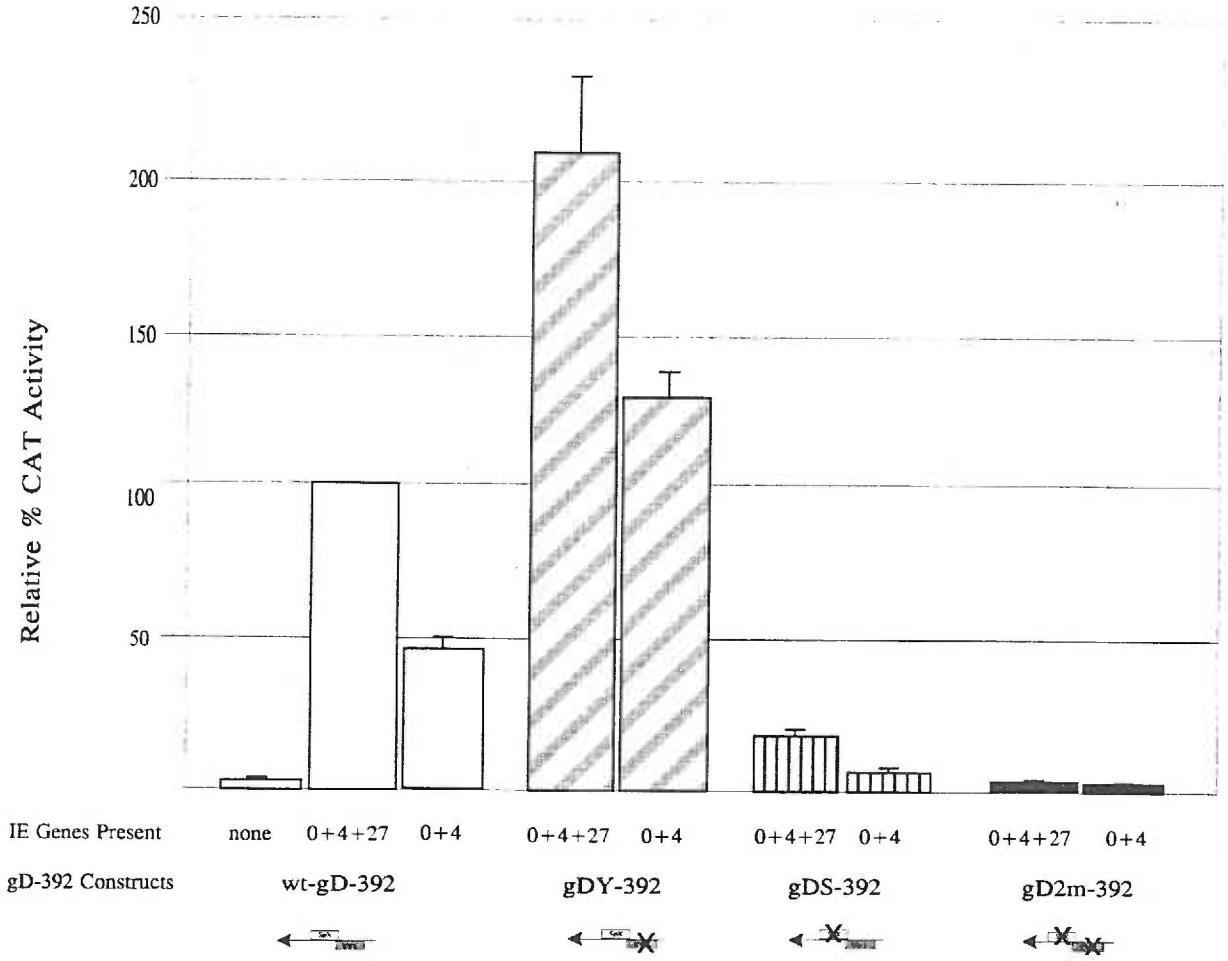


FIG. 5-1. Initiation of CAT activity following HSV-1 superinfection. The wt-gD-392CAT and gDY-392CAT plasmids were transfected into HeLa cells as described in Article #3. The cells were superinfected after transfection with HSV-1 (*vhs*) at an moi = 3. Cells were harvested at the indicated time points, lysed immediately following harvest, quick-frozen, and stored at -70° C until the performance of the assay. CAT activity was assayed as described in Article #3.

Figure 5-1. Initiation of CAT Activity by wt-gD-392CAT and gDY-392CAT

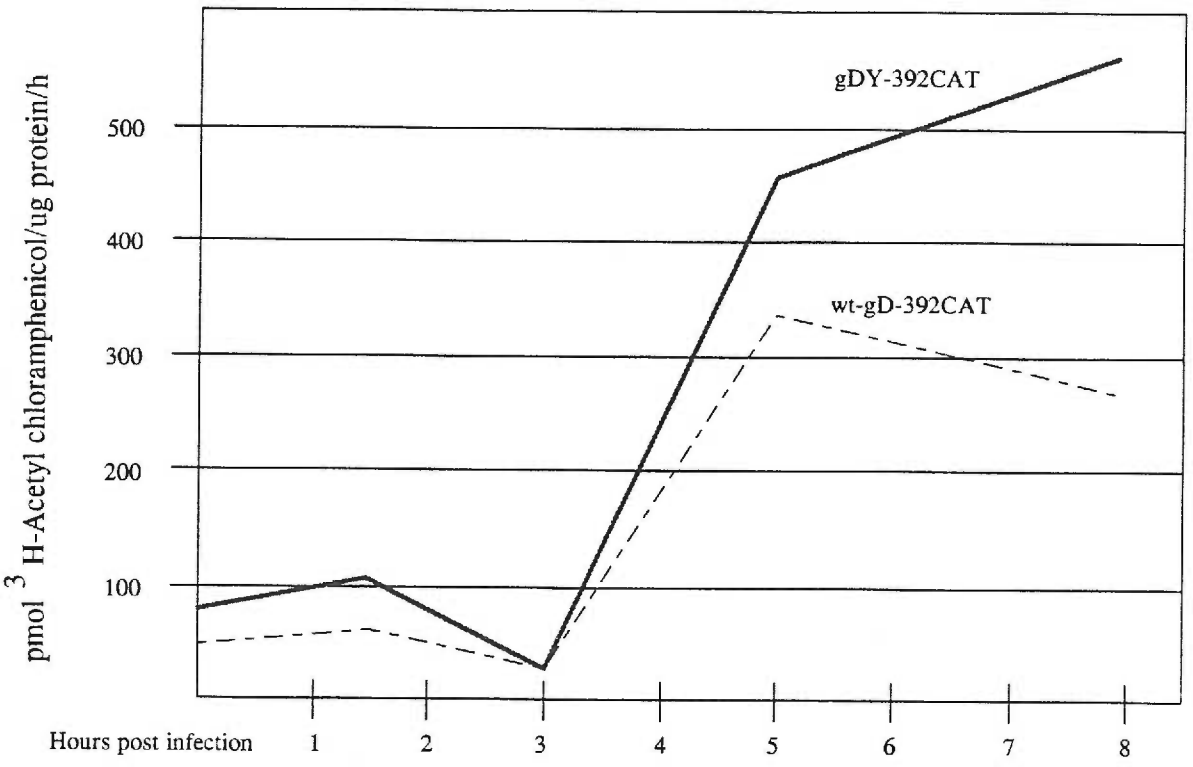


Figure 5-2.

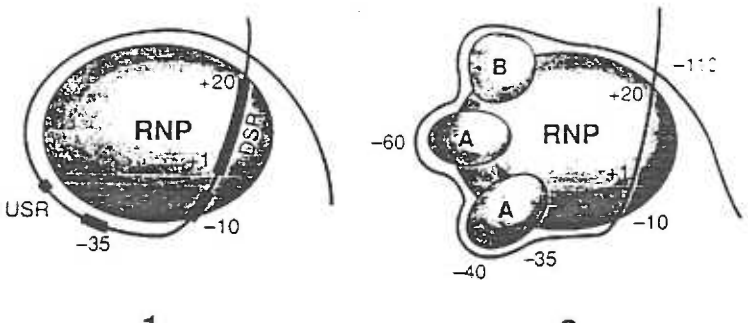


Fig. 2. Model 1: Proposed structure of an optimal promoter. RNP contacts the −10 and −35 regions of the promoter and is caged in because of intrinsic DNA bending. Portions of upstream and downstream DNA, including the *USR* and *DSR* elements mentioned in the text, contact RNP. Model 2: Examples of sites within the promoter region where aid can be given to an inadequate promoter. Activator A (or bender B) can bind at sites around −40, −60, −85, or −110 and help cage RNP to optimize the promoter. For example, CRP binds around nt −40 in *galP1*, −60 in *lacP1*, and IHF binds around nt −85 of p_L as discussed in text. The figure is not drawn to scale.

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FIG. 5-3. CAT activity following superinfection expressed from wt-gD-392CAT and gDY-392CAT as compared with gDSY-392CAT. HeLa cells were transfected with plasmids and superinfected as described in Article #3. The CAT activity analyses were performed as described in Article #3. Results presented are the average data $\pm$ S.E. from 3 experiments.

Figure 5-3: Comparison of relative CAT activity from wt-gD-392CAT and gDY-392CAT with gDSY-392CAT following HSV-1 superinfection