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Construction and Analysis of Mutant HSV-1 Viruses Having Mutations in the YY1 And/or Sp1 Binding Sites of the Glycoprotein D (gD) Gene Promoter

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THESIS APPROVAL

The abstract and thesis of Sabine Ludwig for the Master of Arts in Biology were presented January 6, 1998, and accepted by the thesis committee and the department.

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Abstract:

An abstract of the thesis of Sabine Ludwig for the Master of Arts in Biology presented January 6, 1998.

Title: Construction and analysis of mutant HSV-1 viruses having mutations in the YY1 and/or Sp1 binding sites of the glycoprotein D (gD) gene promoter.

The purpose of this study was to construct and analyze four recombinant herpes simplex virus type 1 (HSV-1) viruses having mutations in the YY1 and/or Sp1 binding sites of the glycoprotein D (gD) gene promoter and a control virus containing a wild type (wt) gD promoter. The YY1 and Sp1 binding sites in the HSV-1- gD gene promoter have been shown in earlier *in vitro* experiments to be essential for virus-induced gD gene expression with Sp1 playing the major role. The first part of my project required the construction of four different plasmids containing a HSV-1 sequence that included the gD gene with promoter and additional flanking sequences. The gD promoters in theses constructs were either wt or contained the mutations in the YY1 and/or Sp1 binding sites. The second step was to introduce the mutations into the virus. This was achieved by cotransfection of the linearized plasmids and infectious HSV-1-FgDβ DNA, which contains a β-galactosidase gene in place of the gD gene, into a complementing cell line. The recombinant viruses were selected on the basis of β-galactosidase (blue-white) screening. The structure was verified by PCR with subsequent restriction and

sequence analysis of the PCR product. The growth rates and yields of the recombinant viruses were determined by infection assays and one-step growth curves on Vero cells. The results of this analysis showed that the mutations of the YY1 and Sp1 binding sites of the gD promoter had only a slight effect on the growth yield and growth rate of HSV-1. Analysis of gD mRNA will be needed to determine the effects of the mutations on gD expression.

CONSTRUCTION AND ANALYSIS OF MUTANT HSV-1 VIRUSES HAVING MUTATIONS IN THE YY1 AND/OR SP1 BINDING SITES OF THE GLYCOPROTEIN D (GD) GENE PROMOTER

by

Sabine Ludwig

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Table of Contents:

List of Tables

List of Figures

Part I: General introduction:

Herpesviruses:

I: The family *Herpesviridae*:

The herpesviruses are a family of viruses that infect a wide range of host animals including mammals, fish, amphibia, reptiles, and humans. Some herpesviruses have a fairly variable host range, but most infect only one host or cell-type. Transmission is usually by contact through saliva, urogenital excretions, aerosols, or transplantations of infected tissues.

Herpesviridae are structurally characterized by a central core containing the linear double-stranded DNA molecule surrounded by an icosahedral capsid having a diameter of about 100nm to 110nm and consisting of 162 capsomeres. Around the nucleocapsid (core + capsid) is a lipoprotein envelope with viral glycoprotein spikes on its surface, and located between envelope and nucleocapsid is a tegument consisting of amorphous material. The herpesvirus DNA is packaged in a torus-shaped form and its size ranges from 124-235kbp depending on the virus species (38). The DNA of most herpesviruses has direct terminal repeats and the DNA of some species has internal repeats as well. Sometimes these repeats form two covalently linked components of the DNA strand, 'L' which is larger and 'S' which is shorter (reviewed in 123).

All herpesviruses share some biological features as well (reviewed in 123) . They all encode a large array of enzymes involved in nucleic acid metabolism. Among the proteins that all herpesviruses share are a DNA polymerase, DNA binding protein, protease, primase, and at least three different protein kinases. The second feature is that the synthesis of DNA and the assembly of the nucleocapsids occur in the nucleus. The viral replication cycle involves transcription and translation of viral proteins, regulated in a cascade fashion with three major stages and viral DNA replication. Immediate-early genes are transcribed by nuclear enzymes and the mRNAs are transported across the nuclear membrane to the cytoplasm and translated to the immediate-early proteins. These proteins are transported back into the nucleus where they are involved in various regulatory functions including activation of transcription of early and late mRNAs. The early or delayed early mRNAs are translated in the cytoplasm again, transported back into the nucleus, and there they are involved in the viral DNA replication via a rolling circle mechanism after circularization of the DNA. The late mRNAs, transcribed following DNA replication, are translated mainly into structural proteins. The capsids are assembled in the nucleus and acquire their envelope via budding through the inner lamella of the nuclear membrane. The virions are then released by transport through the cytoplasm in membranous vesicles which eventually fuse with the plasma membrane. Production of progeny virus is always accompanied by the irreversible destruction of infected cells, but the viruses have the ability to remain in a latent stage in certain cells of their natural host. In the latent

stage the DNA takes the form of a closed circular molecule and only a few or no viral genes are expressed (reviewed in 123).

According to differences in biological properties the *herpesviridae* have been grouped into three different subfamilies, the *alphaherpesviruses*, the *betaherpesviruses*, and the *gammaherpesviruses* (reviewed in 121, 123).

The *alphaherpesvirinae* have a variable host range, a relatively short reproductive cycle, they spread rapidly in culture, efficiently destroy infected cells, and are able to establish latent infections primarily in sensory ganglia. *Simplexviruses* (Herpes simplex virus) and *Varicelloviruses* (Varizella zoster virus) are genera of this subfamily.

The *betaherpesvirinae* generally have a restricted host range, a long reproductive cycle, and grow slowly in culture. Cells infected with betaherpesviruses frequently become enlarged (cytomegalia) and form syncytia. Latency can be established in secretory glands, lymphoreticular cells, kidneys, and other tissues. *Human Cytomegalovirus* and *Muromegalovirus* (murine cytomegalovirus) are members of this family, as well as *Roseoloviruses* (human herpesvirus 6 and 7).

Gammaherpesvirinae have a limited host range infecting only members of the family or order to which their natural host belongs. They are specific for T or B lymphocytes, and establish latent infections in lymphoid tissue. All members of this subfamily replicate in lymphoblastoid tissue in cell culture. Some members are able to cause lytic infections in vitro in certain epithelioid and fibroblastic cells. This

subfamily contains the genera *Lymphocryptovirus* (e.g.: Epstein Barr Virus) and *Rhadinovirus* (e.g.: herpesvirus saimiri), and the newly identified Human Herpes virus 8 (HHV8).

II: The Human Herpesviruses:

There are human herpesviruses represented in each of the three subfamilies, *alpha-, beta-, and gamma-herpesviruses*. As of today, eight herpesviruses are known to cause infections in humans: *Herpes simplex virus types 1 and 2* (HSV-1 and 2), *Varicella zoster virus* (VZV), *Epstein-Barr virus* (EBV), *Human Cytomegalovirus* (HCV), and *Human herpesvirus 6, 7, and 8* (HHV6, HHV7, HHV8).

HSV-1, HSV-2, and VZV belong to the *alphaherpesvirinae*. The epidemiology and pathology of HSV-1 and HSV-2 have been reviewed in detail (124). HSV-1 generally causes infections such as cold sores of the oropharyngeal mucosal epithelium, but lately more and more cases of genital herpesvirus infections have been found to be caused by HSV-1 as well (170). HSV-2 causes genital herpes. After a primary infection, both viruses travel down sensory axons to sensory nerve ganglia where they establish a latent infection (HSV-1 the trigeminal ganglia, and HSV-2 the sacral ganglia). They both cause periodically recurrent epithelial infections if activated through stress and other stimuli. Transmission occurs through direct contact with infected tissue or tissue fluids. Symptoms other than skin lesions might be a sore throat, fever, mucosal ulcers, and malaise. The development of disease varies greatly

from person to person. Most individuals are asymptomatic and develop protective immunity, and complications as described below other than the usual more or less harmless lesions occur rarely.

In a productive primary infection HSV-1 usually causes gingivostomatitis (infection of the oral mucosa or oral cavity), and then establishes a latent infection in the trigeminal ganglia. Later the virus usually becomes reactivated periodically causing recurrent vesicular stomatitis (cold sores). HSV-1 sometimes can infect the cornea in a primary infection as well as in a recurrent infection when it spreads to the optic nerve and can cause serious eye-infections (herpes keratitis). HSV-1 caused keratoconjuntivitis is the second cause of corneal blindness in the United States (86). In rare cases, during a primary or recurrent infection with HSV-1, the virus can travel through neurons to the central nervous system and cause multi-organ infection and serious encephalitis. This occurs in immunocompromised people such as AIDS patients, transplant patients receiving immunosuppressive therapy, neonates, and pregnant women (see review 124).

HSV-2 causes recurrent lesions in the genital area during the course of a normal infection, and latently infects sacral ganglia. Complications with HSV-2 can occur if the virus spreads systemically and causes meningitis, which occurs in a limited number of cases only. If immunocompromised individuals (including neonates) get a primary sexually acquired infection with HSV-2 or HSV-1, the virus can spread systemically and lead to fatal disseminated multi-organ infection and/or encephalitis (30). Primary

infection of pregnant women during the first 20 weeks of pregnancy can lead to abortion (107), and morbidity including severe intrauterine growth retardation of the fetus have been observed (10). Another very serious problem is the intrauterine or peripartum transmission of HSV-2 from a productively infected mother to her child which can result in encephalitis and/or disseminated infection in the newborn, whereby the outcome of disease is by far more serious with HSV-2 than with sexually acquired HSV-1. The mortality rate of infected newborns is high and, if they survive, they often have to endure lifelong sequelae (94).

Varizella-zoster virus (VZV) is the causative agent of the children's disease chickenpox, and the recrudescent disease shingles (herpes zoster) (reviewed in 30) . The primary site for infection of VZV is the upper respiratory tract, the oropharynx, or conjunctiva. Via the bloodstream VZV enters endothelial cells of the epidermis where it causes focal cutaneous lesions (the pocks characteristic of Varicella. Latent infections can be established in the sensory ganglia (93), neurons, and/or in satellite cells (156). VZV can later be reactivated by immunosuppression, nerve damage or other stimuli resulting in the very painful lesions characteristic of shingles. In contrast to HSV that causes periodically recurrent epithelial infections, VZV is reactivated for one time only in most cases to cause zoster.

Members of the *betaherpesviruses* that have humans as their hosts are *human cytomegaloviruses* (HCMV) and *roseolovirueses* (HHV6 and HHV7).

Cytomegaloviruses (reviewed in 122) are widespread in nature infecting humans (HCMV), other primates, domestic animals, and rodents, and each host has its own specific CMV. 50-80% of the human population are infected with HCMV and most cases are asymptomatic. HCMV is transmitted by bodily fluids, and therefore spreads easily in a population. Acute infection occurs in salivary glands and kidney, causing characteristic nuclear and cytoplasmic inclusions (167). HCMV is able to establish latent infection in kidney, salivary glands, lymphocytes, monocytes, macrophages, and stromal cells. If HCMV is reactivated, it can cause pneumonia and retinitis, and it may cause a type of mononucleosis (30). In immunosupressed patients such as AIDS patients or transplant-patients receiving immunosuppressive treatment, acute or recurrent infection can lead to virus dissemination resulting in fulminant, life-threatening diseases (e.g.: 59,21,122). Another danger involved in HCMV infection is the possible congenital transmission from an infected mother to her child, resembling a primary infection of an immunocompromised person (122). This can result in virus dissemination causing fulminant, life-threatening disease.

HHV6 (reviewed in 85) was first identified in peripheral blood mononuclear cells of AIDS patient in attempts to isolate human immunodeficiency virus (HIV) (130). A large portion of the human population (>85%) seems to be infected with HHV6. The virus establishes latent infection in the salivary glands constantly shedding virus into the oropharynx, and that could be the route by which it spreads (129). It is also found in vaginal and cervical secretions (112). It causes exanthem subitum roseola

(172) and some febrile illnesses in children. In immunocompromised people as in AIDS or transplant patients reactivation of HHV6 can occur and has been identified as a potential pathogen, but there is no clear evidence. The virus is mainly found in CD4+ cells (158) and has been shown to transactivate other viruses as EBV, CMV, and papilloma virus, and to enhance or inhibit HIV replication, but the exact mechanisms of interaction are not known and there is no proof that this might play a role in disease development.

HHV7 (reviewed in 85) was isolated from peripheral blood cells of a healthy individual, also in an attempt to isolate HIV (35). HHV7 is very similar to HHV6 in many aspects. Probably >85% of the human population is infected, transmission occurs in early childhood, and the route of transmission is probably through saliva (129). As with HHV6, HHV7 also has been found in the cervix (112). HHV7 can be found in the peripheral blood of healthy individuals (1), and like HHV6 it grows in CD4+ cells but more slowly. Other than HHV6, HHV7 utilizes the CD4 molecule as a receptor and downmodulates expression of CD4, and therefore its envelope is considered for a possible anti HIV-therapy (92). With the exception of some cases of exanthem subitum (1), no human disease has been linked to HHV7. It has been suggested, however, that HHV7 might reactivate HHV6 from latency (73).

Human herpesviruses belonging to the *gammaherpesvirinae* are *Epstein-Barr virus* (EBV) and *Human Herpes Virus 8* (HHV8), the most recently identified human herpesvirus.

Epstein Barr virus (reviewed in 125) causes infectious mononucleosis, and acts as a cofactor in the induction of neoplastic diseases of Burkitt's lymphoma, nasopharyngeal carcinoma, posttransplant lymphoproliferative disease, non-Hodkin's lymphoma, and oral hairy leukoplakia. Primary infection occurs by the oral route and might result in a productive infection in oropharyngeal epithelial cells, which can become chronic in some cases (40) . The site of latent infection are B-cells. Reactivation of EBV results in infection of epithelial surfaces including the oropharyngeal region, where it sometimes establishes a chronic infection.

HHV8 (reviewed in 85) is the latest identified human herpesvirus to be identified (17). It is found associated with Kaposi's sarcoma in immunocompromised individuals (84), and in B-cell lymphomas in the abdominal cavity (16). HHV8 is suspected to be involved with other lymphopathogenic diseases. In KS patients HHV8 DNA has been detected in sensory ganglia (19). The route of transmission is not clear, however HHV8 is suspected by some researchers to be present in the seminal fluid of some healthy men, and it has been detected in nasal secretions and saliva of HHV8 infected individuals.

III: Herpes simplex viruses:

a.) Introduction:

Herpes simplex viruses (HSV) were the first of the human herpesviruses to be discovered, and are among the most the most studied of all viruses. Some reasons for their attraction to biologists are their ability to establish latent infections and to become later reactivated, and that they serve as a good model to study protein-translocation, synaptic connections of the nervous system, membrane structure, gene regulation and more (recent review: 31). Also, recently they are being considered as candidates as vectors in gene-therapy.

At the end of the 18th century HSV was identified as a disease causing agent, and 1962 Schneweiss reported that there were two different serotypes, HSV type-1 and HSV type-2 (132). The biology and disease caused by the two different serotypes has been discussed above.

b.) Structure:

The structure of HSV is similar to the typical structure of herpesviruses (Figure 1). It consists of a nucleocapsid containing an electron-opaque core with the DNA and an icosahedral capsid surrounding the core. Around the nucleocapsid is the envelope with glycoprotein spikes on its surface, and between the envelope and nucleocapsid is located an amorphous tegument. The virions contain at least 30 different virion

polypeptides, and no host proteins are known to be part of the virions. At least ten of the virion proteins are glycosylated (designated gB, gC, gD, gE, gG, gH, gI, gK, gL, gJ, and gM), and at least nine of them are projecting on the surface of the virion as spikes and are distributed in a nonrandom way as Stannard *et al* reported (153). For most of these proteins possible functions have been determined including attachment, penetration and cell to cell spread. In addition to the glycoproteins, the virion envelope also contains two (UL20 and UL43) and possibly more nonglycosylated intrinsic membrane proteins. The lipids of the HSV envelope seems to be derived from the host cell, as was shown in a study by Spear *et al* (151). They observed that the buoyant density of virus in a cesium chloride gradient differed after passage in different host cells. Only three years ago, van Genderen *et al* showed that the phospholipid composition of the HSV envelopes differs from those of nuclear membranes but is similar to cytoplasmic membranes (163).

A very important structural protein is the major capsid protein VP5 which is present in ratios of 850 to 1,000 copies per capsid. It is a component of both pentameric and hexameric capsomeres (133), and seems to be linked to another structural protein (VP19C) by disulfide bonds (177). VP5 and VP19C are present in approximately similar ratios per virion. VP19C, also known as Infected Cell Protein (ICP)32 was shown to bind to DNA by Braun *et al*, leading to the conclusion that it might play a role in DNA anchoring (8). Newcomb and Braun demonstrated that VP19 and VP23 are on the surface of the capsid and suggested that they together could form

a network of fibers between the capsomers (intercapsomeric fibers) (109). Further, capsids contain three more proteins from the ICP35 family (VP21, VP22a, and VP24) that have been shown by Braun et.al. to play a vital role in capsid assembly and encapsidation of viral DNA (9).

The rest of the virion proteins is contained in the tegument, the space between the undersurface of the envelope and the nucleocapsid (reviewed in 31). The most important proteins of the tegument are the α-trans inducing factor (αTIF, also called ICP25 or VP16) which function is to induce immediate early genes by interacting with host proteins, VP11-12 and VP13-14 which modulate the activity of αTIF, as well as the virion host shut off protein (VHS). Another important tegument protein is the U_S11-gene product which acts as an antiattenuant, binds to the 60s ribosomal subunit, and localizes in the nucleus. Finally, there is a very large protein, VP1-2, that is associated with a complex which binds to the terminal 'a' sequence of the viral genome.

The HSV DNA is linear and double stranded as with all herpesvirus DNAs, and it has a size of 152 kbp (74). In the virion the DNA is packaged in the form of a toroid (38). The capsid protein VP19 (ICP32) has been found to bind DNA and is believed to play a role in DNA anchoring in the capsid (8). The ends of the DNA are probably held in close proximity, since a small portion of the DNA seems to be circularized, and after infection the DNA circularizes rapidly in the host cell nucleus (117). The DNA molecule (reviewed in 31) consist of two parts that are covalently

linked, the L (long) and S (short) unique sequences (Figure 2). Each of the unique sequences is bracketed by inverted repeats (IR). The IR of the L-sequence are commonly designated as ab and b'a', the ones of the S-sequence as c'a' and ca. (164). The number of the a sequence repeats is variable. The HSV genome can be represented as:

$$a_l a_n b\text{-}U_L\text{-}b'a'_m c'\text{-}U_S\text{-}ca_s,$$

where a_l and a_s are truncated terminal sequences with one overlapping base each that form one complete a-sequence upon circularization, a_n and a'_m are terminal a sequences directly repeated sero or more times (n), or one to many times (m). The 'a' sequence is highly conserved, only the number of copies is variable. The U_L and the U_S sequence can invert relative to each other and yield four different isomers of the genome. They have been designated P (prototype), I_L (inversion of the L component), I_S (inversion of the S component), and I_{SL} (inversion of both S and L components) (53) (summarized in Figure 2).

The functional organization of the HSV genome can be described as follows (reviewed in 31, see Figure 3). The immediate-early (IE) or α genes map near the termini of the L and S component. ICP4 and ICP0 are located in the internal repeats of the L and S component and therefore are present in two copies each. ICP47 partially maps into the terminal repeat of Us. There are three origins of replication, two in the

S repeats and one in the U_L . Almost all β and γ genes are scattered throughout both the U_S and U_L region. An exception are the ORF P genes that are located in the reiterated sequences flanking the L component. Most of the γ genes coding for the envelope glycoproteins map next to each other in the U_S-component. There is little gene overlap, and only few instances of RNA splicing are known, for example the mRNAs of ICP22, ICP0 and ICP47.

c.) Replication Cycle:

The replication cycle of herpes simplex virus involves the following steps (reviewed in 31) and is summarized in Figure 4:

1) Attachment of the virus to the cell surface
2) Fusion of the viral envelope with the plasma membrane
3) Transport of the deenveloped capsid to the nuclear pores
4) Release of DNA into the nucleus
5) Transcription of the viral genes by the host RNA polymerase II in the nucleus, and translation in the cytoplasm, which is coordinately regulated and sequentially ordered in a cascade fashion.
6) Replication of the viral DNA by viral enzymes in the nucleus, an a rolling circle mechanism
7) Assembly of the new capsids in the nucleus

8) Budding of the mature virions through the inner lamella of the nuclear membrane (initial envelopment)

9) Release of infectious virus particles from the host cell by budding through the cytoplasmic membrane or by fusion of membranous transport particles with the cytoplasmic membrane.

In fully permissive tissue culture cells this whole process takes about 18-20 hours.

Attachment to and penetration into the host cells involves binding of viral glycoproteins on the virus surface to a receptor on the host cell. There have been numerous studies trying to find one single pathway of attachment, but it now is clear that HSV can utilize more than one pathway. All glycoprotein genes of HSV have been deleted individually from the viral genome except gK, and all of those viral mutants were able to attach to and infect nonpolarized cells (see review 31). It was also shown that five of the ten glycoproteins of HSV are dispensable for entry into and egress from most tissue culture cells (gC, gE, gG, gI, gM). Interestingly, Sears et.al. demonstrated in studies with polarized MDCK cells and HSV-1 that gC is required for viral attachment on the apical surface of polarized cells, but not for attachment at the basal surface of the same cells (134). This demonstrates that there are different pathways for attachment of HSV. Shieh et.al. showed that heparan sulfate proteoglycans are host cell receptors for HSV (140). Heparin inhibits HSV cell attachment, and cells lacking heparan sulfate reduce the levels of virus attachment to about 85%. In a recent study Spear *et al* demonstrated that gC is important in heparin

dependent virus entry (116). That there is not a complete inhibition of attachment indicates that other receptors are involved in virus attachment as well. The Fibroblast Growth Factor Receptor (FGFR) has been implicated in attachment (71), however there is no final proof.

Attachment of HSV to the host cell surface is quickly followed by fusion of the viral envelope with the plasma membrane and release of the capsid into the cytoplasm. The fusion was shown by electron microscopic studies (105), and is underlined by the fact that some virion glycoproteins can be found on infected cell surfaces (114) and that viruses that enter cells by endocytosis are destroyed by the host cell (14). Penetration is probably a multi-step process and involves more than one viral glycoprotein. It was shown that gB, gD, and gH are involved in envelope fusion and virus entry since mutants lacking these proteins attach to but do not penetrate cells (13,34,63). The transition from attached to penetrated virus is very rapid (56).

After entry by fusion the tegument protein VHS (virion host shut off protein) is set free and degrades cellular mRNA thereby shutting down the host macromolecular synthesis (80). The capsids are transported to the nuclear pores which is probably mediated by the cytosceleton (22). At the nuclear pores the viral DNA is released into the nucleus which seems to require a viral function, as a temperature sensitive mutant has been isolated that allows genome release into the nucleus at the permissive temperature only (3).

Upon release of the viral DNA into the nucleus the viral DNA transcription is started (31, 165). The viral genes are transcribed by the host polymerase II, but involve many viral protein functions as well. Translation of the mRNAs occurs in the host cell cytoplasm utilizing the host translational system. The gene expression is sequentially ordered in a cascade fashion and coordinally regulated (summarized in Figure 5). First, the immediate early (IE,α) proteins are transcribed, then transported to the cytoplasm where they are translated by the host translational system. The tegument protein α-TIF (VP16) enhances IE protein transcription (4), and therefore can be assumed to be transported into the nucleus together with the viral genome. The α proteins are transported back into the nucleus, where they activate the expression of the early genes (E, β) as well as downregulate their own expression. As the IE mRNAs, the E mRNAs are transported into the cytoplasm for translation, and the E proteins are transported back into the nucleus. The E proteins include proteins necessary for viral DNA replication (DNA polymerase, DNA binding proteins, ORI binding proteins, and helicase/primase complex), as well as proteins inducing the late gene (L, γ) expression. L-Genes are activated by DNA replication, E proteins, and IE proteins. They code for structural proteins and proteins important in assembly of the capsids, and downregulate β gene expression. L-Genes can be divided into leaky-late ($\beta\gamma$, γ_1) and strict-late (γ,γ_2) genes. $\beta\gamma$-Genes are expressed at low levels prior to DNA synthesis and reach maximal expression after initiation of DNA replication. γ-Genes are only detected after

DNA replication has been started. It is not clear whether the distinction between βγ-genes and γ-genes has any functional significance. The functions and regulation of the different viral proteins will be discussed in more detail later.

Viral capsid assembly takes place in the nucleus (see reviews: 31, 165). Thompson at al assembled capsids in an in vitro study in insect cells infected with recombinant baculoviruses expressing the HSV capsid proteins (161). The viral DNA is packaged into performed capsids with the aid of the protease of the U_L26 gene and the scaffolding protein VP22a (90). The capsids then bud through the inner lamella of the nuclear membrane whereby they are surrounded by tegument proteins some of which may functionally interact to aid envelopment (144). There is some controversy about the way the virus leaves the host cell. It may be transported to the outside via the Golgi apparatus. Van Genderen et.al. showed that the phospholipid composition of the HSV envelopes differs from those of nuclear membranes but is similar to the Golgi-apparatus membrane or cytoplasmic membranes which supports the hypothesis that the virus particles are transported through the Golgi apparatus (163).

d.) latency:

Herpes simplex viruses have the ability to stay latent in its human host for its lifetime. A latent infection of the sensory neurons involves three stages, establishment of latency, maintenance, and reactivation (Figure 6). The establishment begins with the infection of sensory nerve endings followed by the travel of the virus to the nucleus

(154). In the neuron, the viral genome acquires the form of endless or circular DNA (100). There is no viral gene expression during the latent stage that is required for establishment or maintenance of latency as to our current knowledge (see reviews: 31, 165). Only one transcript is known today that accumulates in latently infected neurons and it is named latency-associated transcript (LAT) (155). LAT is believed to be a stable intron of about 2 kbp length, it is not at all or only poorly adenylated, and predominantly nuclear localized. No protein has been associated with the transcript. A lot of research has been done on the LAT, but to date no specific function of that transcript has been identified. Hill et.al. and later others suggested that LAT might increase the efficiency of induced reactivation of latent HSV (55).

HSV is periodically reactivated from its latent state and carried back by axonal transport to peripheral tissues, usually to cells at or near the initial site of infection. Much research has been done and still is being done, on finding the molecular mechanism of reactivation, but today it is still not clear what molecular changes trigger the conversion from the latent genome to a reactivated one. Stress of all sorts can cause a reactivation of HSV, including injury to tissues innervated by latently infected neurons, physical or emotional stress, menstruation, and hormonal imbalance (see review 31) The course and possible complications of the disease caused by reactivated virus has been discussed in more detail above in the biology and disease section.

e.) Regulation of viral gene expression:

A key feature of a productive viral infection is the timely and quantitative regulation of the different groups of viral proteins as described above (reviewed in: 31, 165). The transcription of the different genes is regulated by both viral and cellular factors. Some cellular response elements often found in the promoter of HSV genes include the TATA-and CAAT-boxes, as well as Sp1 and YY1 binding sites. Interestingly, some promoters have been found to not have a TATA box.

In the following section the most important viral regulatory proteins will be discussed, followed by an overall view over the structure of the promoters of the different gene groups and the way the expression of the different gene-groups is regulated.

The most important viral regulatory proteins:

α TIF (VP16, ICP25):

In the initial stage of transcription the transcription of IE proteins is enhanced by the viral tegument protein α TIF (α-transinducing factor, =VP16, ICP25) (169). α-TIF acts by both binding to DNA and by recruiting cellular transcription factors to the promoter of IE genes.

The N-terminal half of α-TIF interacts with the cellular factor Oct-1 and accessory proteins (HCF) to form a DNA/protein complex at the consensus sequence 'TAATGARAT' which is found in one or many copies in the upstream enhancers of IE genes (78, 169). The extremely acidic C-terminal half of α-TIF can interact with TFIID and TFIIB and thereby activate α genes in *trans* by recruiting these factors to the promoters (157, 89).

The α4-protein (ICP4):

ICP4 is essential for virus replication and for viral gene expression. It is an activator of early and late genes as well as a downregulator (autoregulator) of immediate early genes ICP4 and ICP0. ICP4 binds strongly to the consensus sequence ATCGTCnnnnCnGnn at or near the cap site of its own promoter and of other promoters containing this consensus sequence (29). It also binds weakly to nonconsensus sequences that are present in promoters downstream of the cap site of early and late genes (102). Binding of the protein to its consensus sequence in IE genes inhibits transcription, and therefore ICP4 autoregulates its own transcription. The ICP0 promoter has a consensus binding site for ICP4 as well and is believed to be downregulated by binding of ICP4 to the promoter as well (77). Lium *et al* supported this theory by showing in an extensive study that destruction of the ICP4 binding site in the ICP0 promoter results in continuous expression of the ICP0 gene (91). They

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Zhu and Schaffer demonstrated in an extensive study that ICP27 is responsible for the nuclear localization of ICP0 and ICP4, concluding that intracellular compartmental constraints may well play a role in the regulation of HSV-1 gene expression (176). Panagiotidis *et al* showed in a following study that ICP27 physically

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ICP27 was also shown to inhibit mRNA splicing of both the host cell mRNAs and the viral mRNAs (50). In the context of this results it is interesting to note that only five viral mRNAs are spliced, and four of them are dispensable for viral growth. In addition, viral mRNAs are in excess in relation to host cell mRNAs during a productive infection and therefore splicing of host cell mRNA is stronger affected than that of viral mRNA. Multiple studies demonstrated that the protein acts at a co-or post-transcriptional level (reviewed in 31, 165). ICP27 is also known to promote efficient transport of some late mRNAs from the nucleus, and a nuclear localization signal (NLS) has been identified in the amino-terminal portion of the protein (98).

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Structure and Function of HSV promoters:

The structure and function of the promoters of the different HSV-1 gene classes has been reviewed in detail recently by Wagner *et al* as well as by Roizman and Sears (31, 165). This is summarized in the following paragraphs

Immediate early (IE, α) promoters:

IE-promoters contain many features reminiscent of most cellular RNA polymerase II promoters. These include TATA and a CAAT boxes as well as other cellular transcription factor binding sites. In addition to that they also contain IE specific control elements such as multiple upstream copies of the α-enhancer that interacts with αTIF. The α-enhancer was shown by Spector *et al* to play an important role in IE-gene expression (152). As discussed above, some of the immediate early promoters also have binding sites for ICP4 at or near the cap site. Binding of ICP4 to those sites represses the transcription of the genes.

ICP27 lacks this specific ICP4 binding site, and this results in expression at high levels throughout the replication cycle (175). This continuous expression may correlate with the importance of the ICP27 protein in late gene expression. The ICP0 promoter contains the ICP4 binding sites but it shows an unusual pattern of gene expression. At first its transcription is repressed, but late in the productive cycle it regains a high level of expression (52). The mechanism of this reactivation is not clear but certainly of much interest.

Early (E, β) promoters:

Promoters controlling early genes as exemplified by the TK gene are typical RNA polymerase II promoters as shown in many studies by Mc Knight *et al* (reviewed

in 165). These results were enhanced by studies by Smiley *et al* who showed that a cellular promoter with similar structure (the rabbit beta-globin gene) recombined into the viral genome was expressed with typical E kinetics (147). Mutational studies by Imbalzano *et al* on the TK gene promoter indicate that two upstream Sp1 binding sites, an upstream CAAT box and a TATA box are required for efficient transcription. However, even though the mRNA level of the TK gene is greatly reduced through these mutations, the timely regulation of expression according to the typical E pattern is not altered (60). Deletion of sequences around the cap site of the TK gene did not have any significant effect on the expression of the gene (48). it is well established that ICP4 is required for trans-activation of the early genes, but the mechanism is not (171). There are no promoter elements known that are specific for only early promoters. In summary, Wagner *et al* concludes that the elements critical for early expression of the TK promoter cluster between the TATA box to about 100 bases upstream (including two Sp1-sites and a CAAT box), and that there are no significant elements downstream of the TATA box in early promoters that affect their expression (165).

The early gene expression is shut following viral DNA replication, but he mechanism of this shut-down still is obscure. Theories are that a turnover of trans-acting factors required for efficient early-gene expression is responsible for the progressive decrease of early-gene expression, or that large quantities of viral DNA could titrate out critical factors. Yet another theory is that relocation of the viral genome into replication compartments at the time of DNA replication leads to an

alteration of the transcriptional environment and thereby downregulates early-gene expression. The last theories are supported by the fact that inhibition of viral DNA synthesis prevents the formation of replication compartments and the expression of γ-genes, but doesn't effect the expression of β-genes (76).

The late (L, γ_2) gene promoters:

Several mutational studies on the viral late promoters have shown that they lack functional cis-acting sites upstream of the TATA box, and that they consist of a TATA box and critical cis-acting elements at or near their cap site (reviewed in 165). The U_L38 gene encoding for the VP19C capsid protein seems to be a good model for late promoters. Mutational analysis of this promoter showed that there are three critical cis-acting elements, a TATA box at -31, an initiation element spanning the transcription start site to +9, and a downstream activation sequence (DAS) in the 5' untranslated leader sequence (33, 45). The TATA box and the initiation element are essential for transcription of the VP19C gene, and deletion of the DAS element reduces transcription levels up to ten-fold. DAS was later shown by Wagner *et al* to be the binding site for a cellular protein, the DAS binding factor (DBF) (46). The structure of the late gene promoters is in strong contrast to the structure of early promoters, as in the late promoters all essential elements are located at or near the cap site downstream of the

TATA box, while all significant elements of the early promoters are located upstream of the TATA box.

In addition to the factors discussed above, the late genes are trans-activated by ICP4 via its interaction with cellular proteins (113, 33). Wagner theorizes that ICP4 may act to activate a basal transcription process potentiated by cellular factors (165). He further suggests that the interaction of TBP (the TATA binding protein of the multi-subunit transcription factor TFIID) with the TATA box is stabilized and/or facilitated by interaction with the DBF with DAS. The γ initiator element might act as additional stabilizer or initiator, possibly through interaction with an as yet unidentified cellular protein.

There are two key-hypothesis to explain why the late genes are significantly only expressed after onset of viral DNA synthesis (reviewed in 31). The first one is that negative control factors specifically repress late gene expression prior to DNA synthesis (99), or that particular DNA secondary structures are formed in the vicinity of the late genes, and these effects could be relieved by DNA replication. The second hypothesis is that during viral DNA replication a trans-acting factor is produced or modified to activate late gene promoters, however there is no evidence for this theory. Studies by Mavromara-Nazos and Roizman showed that late gene expression is tightly linked to DNA synthesis (96). It was also shown however, that late genes are expressed at low levels in the presence of inhibitors of viral DNA synthesis, as well as at nonpermissive temperature with ICP8 temperature sensitive (ts) mutants which are

III: Herpes simplex viruses:

a.) Introduction:

Herpes simplex viruses (HSV) were the first of the human herpesviruses to be discovered, and are among the most the most studied of all viruses. Some reasons for their attraction to biologists are their ability to establish latent infections and to become later reactivated, and that they serve as a good model to study protein-translocation, synaptic connections of the nervous system, membrane structure, gene regulation and more (recent review: 31). Also, recently they are being considered as candidates as vectors in gene-therapy.

At the end of the 18th century HSV was identified as a disease causing agent, and 1962 Schneweiss reported that there were two different serotypes, HSV type-1 and HSV type-2 (132). The biology and disease caused by the two different serotypes has been discussed above.

b.) Structure:

The structure of HSV is similar to the typical structure of herpesviruses (Figure 1). It consists of a nucleocapsid containing an electron-opaque core with the DNA and an icosahedral capsid surrounding the core. Around the nucleocapsid is the envelope with glycoprotein spikes on its surface, and between the envelope and nucleocapsid is located an amorphous tegument. The virions contain at least 30 different virion

polypeptides, and no host proteins are known to be part of the virions. At least ten of the virion proteins are glycosylated (designated gB, gC, gD, gE, gG, gH, gI, gK, gL, gJ, and gM), and at least nine of them are projecting on the surface of the virion as spikes and are distributed in a nonrandom way as Stannard *et al* reported (153). For most of these proteins possible functions have been determined including attachment, penetration and cell to cell spread. In addition to the glycoproteins, the virion envelope also contains two (UL20 and UL43) and possibly more nonglycosylated intrinsic membrane proteins. The lipids of the HSV envelope seems to be derived from the host cell, as was shown in a study by Spear *et al* (151). They observed that the buoyant density of virus in a cesium chloride gradient differed after passage in different host cells. Only three years ago, van Genderen *et al* showed that the phospholipid composition of the HSV envelopes differs from those of nuclear membranes but is similar to cytoplasmic membranes (163).

A very important structural protein is the major capsid protein VP5 which is present in ratios of 850 to 1,000 copies per capsid. It is a component of both pentameric and hexameric capsomeres (133), and seems to be linked to another structural protein (VP19C) by disulfide bonds (177). VP5 and VP19C are present in approximately similar ratios per virion. VP19C, also known as Infected Cell Protein (ICP)32 was shown to bind to DNA by Braun *et al*, leading to the conclusion that it might play a role in DNA anchoring (8). Newcomb and Braun demonstrated that VP19 and VP23 are on the surface of the capsid and suggested that they together could form

a network of fibers between the capsomers (intercapsomeric fibers) (109). Further, capsids contain three more proteins from the ICP35 family (VP21, VP22a, and VP24) that have been shown by Braun et.al. to play a vital role in capsid assembly and encapsidation of viral DNA (9).

The rest of the virion proteins is contained in the tegument, the space between the undersurface of the envelope and the nucleocapsid (reviewed in 31). The most important proteins of the tegument are the α-trans inducing factor (αTIF, also called ICP25 or VP16) which function is to induce immediate early genes by interacting with host proteins, VP11-12 and VP13-14 which modulate the activity of αTIF, as well as the virion host shut off protein (VHS). Another important tegument protein is the U_S11-gene product which acts as an antiattenuant, binds to the 60s ribosomal subunit, and localizes in the nucleus. Finally, there is a very large protein, VP1-2, that is associated with a complex which binds to the terminal 'a' sequence of the viral genome.

The HSV DNA is linear and double stranded as with all herpesvirus DNAs, and it has a size of 152 kbp (74). In the virion the DNA is packaged in the form of a toroid (38). The capsid protein VP19 (ICP32) has been found to bind DNA and is believed to play a role in DNA anchoring in the capsid (8). The ends of the DNA are probably held in close proximity, since a small portion of the DNA seems to be circularized, and after infection the DNA circularizes rapidly in the host cell nucleus (117). The DNA molecule (reviewed in 31) consist of two parts that are covalently

linked, the L (long) and S (short) unique sequences (Figure 2). Each of the unique sequences is bracketed by inverted repeats (IR). The IR of the L-sequence are commonly designated as ab and b'a', the ones of the S-sequence as c'a' and ca. (164). The number of the a sequence repeats is variable. The HSV genome can be represented as:

$$a_l a_n b\text{-}U_L\text{-}b'a'_m c'\text{-}U_S\text{-}ca_s,$$

where a_l and a_s are truncated terminal sequences with one overlapping base each that form one complete a-sequence upon circularization, a_n and a'_m are terminal a sequences directly repeated sero or more times (n), or one to many times (m). The 'a' sequence is highly conserved, only the number of copies is variable. The U_L and the U_S sequence can invert relative to each other and yield four different isomers of the genome. They have been designated P (prototype), I_L (inversion of the L component), I_S (inversion of the S component), and I_{SL} (inversion of both S and L components) (53) (summarized in Figure 2).

The functional organization of the HSV genome can be described as follows (reviewed in 31, see Figure 3). The immediate-early (IE) or α genes map near the termini of the L and S component. ICP4 and ICP0 are located in the internal repeats of the L and S component and therefore are present in two copies each. ICP47 partially maps into the terminal repeat of Us. There are three origins of replication, two in the

S repeats and one in the U_L . Almost all β and γ genes are scattered throughout both the U_S and U_L region. An exception are the ORF P genes that are located in the reiterated sequences flanking the L component. Most of the γ genes coding for the envelope glycoproteins map next to each other in the U_S-component. There is little gene overlap, and only few instances of RNA splicing are known, for example the mRNAs of ICP22, ICP0 and ICP47.

c.) Replication Cycle:

The replication cycle of herpes simplex virus involves the following steps (reviewed in 31) and is summarized in Figure 4:

1) Attachment of the virus to the cell surface
2) Fusion of the viral envelope with the plasma membrane
3) Transport of the deenveloped capsid to the nuclear pores
4) Release of DNA into the nucleus
5) Transcription of the viral genes by the host RNA polymerase II in the nucleus, and translation in the cytoplasm, which is coordinately regulated and sequentially ordered in a cascade fashion.
6) Replication of the viral DNA by viral enzymes in the nucleus, an a rolling circle mechanism
7) Assembly of the new capsids in the nucleus

8) Budding of the mature virions through the inner lamella of the nuclear membrane (initial envelopment)

9) Release of infectious virus particles from the host cell by budding through the cytoplasmic membrane or by fusion of membranous transport particles with the cytoplasmic membrane.

In fully permissive tissue culture cells this whole process takes about 18-20 hours.

Attachment to and penetration into the host cells involves binding of viral glycoproteins on the virus surface to a receptor on the host cell. There have been numerous studies trying to find one single pathway of attachment, but it now is clear that HSV can utilize more than one pathway. All glycoprotein genes of HSV have been deleted individually from the viral genome except gK, and all of those viral mutants were able to attach to and infect nonpolarized cells (see review 31). It was also shown that five of the ten glycoproteins of HSV are dispensable for entry into and egress from most tissue culture cells (gC, gE, gG, gI, gM). Interestingly, Sears et.al. demonstrated in studies with polarized MDCK cells and HSV-1 that gC is required for viral attachment on the apical surface of polarized cells, but not for attachment at the basal surface of the same cells (134). This demonstrates that there are different pathways for attachment of HSV. Shieh et.al. showed that heparan sulfate proteoglycans are host cell receptors for HSV (140). Heparin inhibits HSV cell attachment, and cells lacking heparan sulfate reduce the levels of virus attachment to about 85%. In a recent study Spear *et al* demonstrated that gC is important in heparin

dependent virus entry (116). That there is not a complete inhibition of attachment indicates that other receptors are involved in virus attachment as well. The Fibroblast Growth Factor Receptor (FGFR) has been implicated in attachment (71), however there is no final proof.

Attachment of HSV to the host cell surface is quickly followed by fusion of the viral envelope with the plasma membrane and release of the capsid into the cytoplasm. The fusion was shown by electron microscopic studies (105), and is underlined by the fact that some virion glycoproteins can be found on infected cell surfaces (114) and that viruses that enter cells by endocytosis are destroyed by the host cell (14). Penetration is probably a multi-step process and involves more than one viral glycoprotein. It was shown that gB, gD, and gH are involved in envelope fusion and virus entry since mutants lacking these proteins attach to but do not penetrate cells (13,34,63). The transition from attached to penetrated virus is very rapid (56).

After entry by fusion the tegument protein VHS (virion host shut off protein) is set free and degrades cellular mRNA thereby shutting down the host macromolecular synthesis (80). The capsids are transported to the nuclear pores which is probably mediated by the cytosceleton (22). At the nuclear pores the viral DNA is released into the nucleus which seems to require a viral function, as a temperature sensitive mutant has been isolated that allows genome release into the nucleus at the permissive temperature only (3).

Upon release of the viral DNA into the nucleus the viral DNA transcription is started (31, 165). The viral genes are transcribed by the host polymerase II, but involve many viral protein functions as well. Translation of the mRNAs occurs in the host cell cytoplasm utilizing the host translational system. The gene expression is sequentially ordered in a cascade fashion and coordinally regulated (summarized in Figure 5). First, the immediate early (IE,α) proteins are transcribed, then transported to the cytoplasm where they are translated by the host translational system. The tegument protein α-TIF (VP16) enhances IE protein transcription (4), and therefore can be assumed to be transported into the nucleus together with the viral genome. The α proteins are transported back into the nucleus, where they activate the expression of the early genes (E, β) as well as downregulate their own expression. As the IE mRNAs, the E mRNAs are transported into the cytoplasm for translation, and the E proteins are transported back into the nucleus. The E proteins include proteins necessary for viral DNA replication (DNA polymerase, DNA binding proteins, ORI binding proteins, and helicase/primase complex), as well as proteins inducing the late gene (L, γ) expression. L-Genes are activated by DNA replication, E proteins, and IE proteins. They code for structural proteins and proteins important in assembly of the capsids, and downregulate β gene expression. L-Genes can be divided into leaky-late (βγ, γ_1) and strict-late (γ,γ_2) genes. βγ-Genes are expressed at low levels prior to DNA synthesis and reach maximal expression after initiation of DNA replication. γ-Genes are only detected after

DNA replication has been started. It is not clear whether the distinction between βγ-genes and γ-genes has any functional significance. The functions and regulation of the different viral proteins will be discussed in more detail later.

Viral capsid assembly takes place in the nucleus (see reviews: 31, 165). Thompson at al assembled capsids in an in vitro study in insect cells infected with recombinant baculoviruses expressing the HSV capsid proteins (161). The viral DNA is packaged into performed capsids with the aid of the protease of the U_L26 gene and the scaffolding protein VP22a (90). The capsids then bud through the inner lamella of the nuclear membrane whereby they are surrounded by tegument proteins some of which may functionally interact to aid envelopment (144). There is some controversy about the way the virus leaves the host cell. It may be transported to the outside via the Golgi apparatus. Van Genderen et.al. showed that the phospholipid composition of the HSV envelopes differs from those of nuclear membranes but is similar to the Golgi-apparatus membrane or cytoplasmic membranes which supports the hypothesis that the virus particles are transported through the Golgi apparatus (163).

d.) latency:

Herpes simplex viruses have the ability to stay latent in its human host for its lifetime. A latent infection of the sensory neurons involves three stages, establishment of latency, maintenance, and reactivation (Figure 6). The establishment begins with the infection of sensory nerve endings followed by the travel of the virus to the nucleus

(154). In the neuron, the viral genome acquires the form of endless or circular DNA (100). There is no viral gene expression during the latent stage that is required for establishment or maintenance of latency as to our current knowledge (see reviews: 31, 165). Only one transcript is known today that accumulates in latently infected neurons and it is named latency-associated transcript (LAT) (155). LAT is believed to be a stable intron of about 2 kbp length, it is not at all or only poorly adenylated, and predominantly nuclear localized. No protein has been associated with the transcript. A lot of research has been done on the LAT, but to date no specific function of that transcript has been identified. Hill et.al. and later others suggested that LAT might increase the efficiency of induced reactivation of latent HSV (55).

HSV is periodically reactivated from its latent state and carried back by axonal transport to peripheral tissues, usually to cells at or near the initial site of infection. Much research has been done and still is being done, on finding the molecular mechanism of reactivation, but today it is still not clear what molecular changes trigger the conversion from the latent genome to a reactivated one. Stress of all sorts can cause a reactivation of HSV, including injury to tissues innervated by latently infected neurons, physical or emotional stress, menstruation, and hormonal imbalance (see review 31) The course and possible complications of the disease caused by reactivated virus has been discussed in more detail above in the biology and disease section.

e.) Regulation of viral gene expression:

A key feature of a productive viral infection is the timely and quantitative regulation of the different groups of viral proteins as described above (reviewed in: 31, 165). The transcription of the different genes is regulated by both viral and cellular factors. Some cellular response elements often found in the promoter of HSV genes include the TATA-and CAAT-boxes, as well as Sp1 and YY1 binding sites. Interestingly, some promoters have been found to not have a TATA box.

In the following section the most important viral regulatory proteins will be discussed, followed by an overall view over the structure of the promoters of the different gene groups and the way the expression of the different gene-groups is regulated.

The most important viral regulatory proteins:

α TIF (VP16, ICP25):

In the initial stage of transcription the transcription of IE proteins is enhanced by the viral tegument protein α TIF (α-transinducing factor, =VP16, ICP25) (169). α-TIF acts by both binding to DNA and by recruiting cellular transcription factors to the promoter of IE genes.

The N-terminal half of α-TIF interacts with the cellular factor Oct-1 and accessory proteins (HCF) to form a DNA/protein complex at the consensus sequence 'TAATGARAT' which is found in one or many copies in the upstream enhancers of IE genes (78, 169). The extremely acidic C-terminal half of α-TIF can interact with TFIID and TFIIB and thereby activate α genes in *trans* by recruiting these factors to the promoters (157, 89).

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To trans-activate genes ICP4 interacts with the TATA binding protein (TBP) of TFIID and with TFIIB as was shown by Smith *et al* (148). Gu and DeLuca also demonstrated the involvement of TBP associated factors (TAFs) (43), and later Carrozza and DeLuca reported that gene-activation by ICP4 requires the interaction of ICP4 with TFIID involving TAF250 and that this interaction takes place at the C-terminal region of ICP4 (15).

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Zhu and Schaffer demonstrated in an extensive study that ICP27 is responsible for the nuclear localization of ICP0 and ICP4, concluding that intracellular compartmental constraints may well play a role in the regulation of HSV-1 gene expression (176). Panagiotidis *et al* showed in a following study that ICP27 physically

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ICP0 is an unusual HSV protein in that its mRNA is spliced and contains two introns (166). A small portion of the mRNA is incompletely spliced in that the second intron is not cut out. This yields a product designated as ICP0R, which was found in transient expression assays to be a dominant negative repressor of trans-activation by ICP0 (166). A recent study by Spatz *et al* demonstrated by generating a series of mutant viruses that indeed very little amounts of ICP0R dominantly represses ICP0 trans-activation in vivo (150). These results lead Spatz *et al* to the speculation that ICP0R might act as a downregulator of viral gene expression.

Structure and Function of HSV promoters:

The structure and function of the promoters of the different HSV-1 gene classes has been reviewed in detail recently by Wagner *et al* as well as by Roizman and Sears (31, 165). This is summarized in the following paragraphs

Immediate early (IE, α) promoters:

IE-promoters contain many features reminiscent of most cellular RNA polymerase II promoters. These include TATA and a CAAT boxes as well as other cellular transcription factor binding sites. In addition to that they also contain IE specific control elements such as multiple upstream copies of the α-enhancer that interacts with αTIF. The α-enhancer was shown by Spector *et al* to play an important role in IE-gene expression (152). As discussed above, some of the immediate early promoters also have binding sites for ICP4 at or near the cap site. Binding of ICP4 to those sites represses the transcription of the genes.

ICP27 lacks this specific ICP4 binding site, and this results in expression at high levels throughout the replication cycle (175). This continuous expression may correlate with the importance of the ICP27 protein in late gene expression. The ICP0 promoter contains the ICP4 binding sites but it shows an unusual pattern of gene expression. At first its transcription is repressed, but late in the productive cycle it regains a high level of expression (52). The mechanism of this reactivation is not clear but certainly of much interest.

Early (E, β) promoters:

Promoters controlling early genes as exemplified by the TK gene are typical RNA polymerase II promoters as shown in many studies by Mc Knight *et al* (reviewed

in 165). These results were enhanced by studies by Smiley *et al* who showed that a cellular promoter with similar structure (the rabbit beta-globin gene) recombined into the viral genome was expressed with typical E kinetics (147). Mutational studies by Imbalzano *et al* on the TK gene promoter indicate that two upstream Sp1 binding sites, an upstream CAAT box and a TATA box are required for efficient transcription. However, even though the mRNA level of the TK gene is greatly reduced through these mutations, the timely regulation of expression according to the typical E pattern is not altered (60). Deletion of sequences around the cap site of the TK gene did not have any significant effect on the expression of the gene (48). it is well established that ICP4 is required for trans-activation of the early genes, but the mechanism is not (171). There are no promoter elements known that are specific for only early promoters. In summary, Wagner *et al* concludes that the elements critical for early expression of the TK promoter cluster between the TATA box to about 100 bases upstream (including two Sp1-sites and a CAAT box), and that there are no significant elements downstream of the TATA box in early promoters that affect their expression (165).

The early gene expression is shut following viral DNA replication, but he mechanism of this shut-down still is obscure. Theories are that a turnover of trans-acting factors required for efficient early-gene expression is responsible for the progressive decrease of early-gene expression, or that large quantities of viral DNA could titrate out critical factors. Yet another theory is that relocation of the viral genome into replication compartments at the time of DNA replication leads to an

alteration of the transcriptional environment and thereby downregulates early-gene expression. The last theories are supported by the fact that inhibition of viral DNA synthesis prevents the formation of replication compartments and the expression of γ-genes, but doesn't effect the expression of β-genes (76).

The late (L, γ_2) gene promoters:

Several mutational studies on the viral late promoters have shown that they lack functional cis-acting sites upstream of the TATA box, and that they consist of a TATA box and critical cis-acting elements at or near their cap site (reviewed in 165). The U_L38 gene encoding for the VP19C capsid protein seems to be a good model for late promoters. Mutational analysis of this promoter showed that there are three critical cis-acting elements, a TATA box at -31, an initiation element spanning the transcription start site to +9, and a downstream activation sequence (DAS) in the 5' untranslated leader sequence (33, 45). The TATA box and the initiation element are essential for transcription of the VP19C gene, and deletion of the DAS element reduces transcription levels up to ten-fold. DAS was later shown by Wagner *et al* to be the binding site for a cellular protein, the DAS binding factor (DBF) (46). The structure of the late gene promoters is in strong contrast to the structure of early promoters, as in the late promoters all essential elements are located at or near the cap site downstream of the

TATA box, while all significant elements of the early promoters are located upstream of the TATA box.

In addition to the factors discussed above, the late genes are trans-activated by ICP4 via its interaction with cellular proteins (113, 33). Wagner theorizes that ICP4 may act to activate a basal transcription process potentiated by cellular factors (165). He further suggests that the interaction of TBP (the TATA binding protein of the multi-subunit transcription factor TFIID) with the TATA box is stabilized and/or facilitated by interaction with the DBF with DAS. The γ initiator element might act as additional stabilizer or initiator, possibly through interaction with an as yet unidentified cellular protein.

There are two key-hypothesis to explain why the late genes are significantly only expressed after onset of viral DNA synthesis (reviewed in 31). The first one is that negative control factors specifically repress late gene expression prior to DNA synthesis (99), or that particular DNA secondary structures are formed in the vicinity of the late genes, and these effects could be relieved by DNA replication. The second hypothesis is that during viral DNA replication a trans-acting factor is produced or modified to activate late gene promoters, however there is no evidence for this theory. Studies by Mavromara-Nazos and Roizman showed that late gene expression is tightly linked to DNA synthesis (96). It was also shown however, that late genes are expressed at low levels in the presence of inhibitors of viral DNA synthesis, as well as at nonpermissive temperature with ICP8 temperature sensitive (ts) mutants which are

not able to replicate DNA at the nonpermissive temperature (41). Also, ICP27 and ICP22 have been implicated in the regulation of late gene expression as certain mutants in these genes downregulate L-gene expression (31). Viral DNA synthesis is therefore necessary but not sufficient for proper expression of true late genes.

The leaky late ($\beta\gamma$, γ_1) gene promoters:

Leaky-late genes are expressed at low levels prior to DNA synthesis, and with the peak expression following DNA synthesis (late kinetics). There is a widely believed hypothesis that leaky late genes have 'chimeric promoters' containing elements upstream of the TATA box like early gene promoters that allow expression of the genes prior to DNA synthesis, as well as late promoter elements around the cap site that activate gene expression following DNA replication. This hypothesis is the result of analysis of recombinant viruses with engineered promoters containing both, E, and L response elements (97). Indeed, in many leaky-late promoters early gene regulatory elements have been found upstream of the TATA box. However, studies on the VP5 promoter, a model for a leaky-late gene promoter, could not identify any other element except the TATA box as being important for the early expression of the gene (58, 165).

The VP5 promoter contains a number of elements: a TATA box at -30, 4 consensus binding sites for Sp1, a CAAT box, a YY1 binding site, as well as two binding sites for ICP4. The VP5 promoter can be dissected into three functional domains as suggested by transient expression assays (6, 18, 103). Sequences

downstream of -83 are responsible for a basal promoter activity (they include a TATA box, Sp1 and YY1 sites), and a domain between -83 and -125 that negatively regulates basal activity. A domain upstream of -125 that contains ICP4 binding sites and three additional Sp1 binding sites and allows activation of expression by ICP4. Analysis of recombinant viruses by Wagner and co-workers, however, revealed that in vivo the situation is quite different (58, 57) These studies showed that removing the three Sp1 sites, the CAAT box, two ICP4 sites, and the YY1 site upstream of -48 have no significant effect on the level or the kinetics of the expression driven by the VP5 promoter. Their results lead to the conclusion that the critical part of the VP5 promoter consists of a 60 bp spanning region starting at -48 with a critical Sp1 site and extending down to +10. The elements upstream of the critical Sp1 site at -48 do not seem to play a role in the expression of VP5 in the normal productive replication cycle of HSV, but they might play a role in or during reactivation of the virus form the latent stage. Interestingly, deletion of the critical Sp1 binding site or mutations near the transcript start site drastically reduces mRNA levels at late times but does not affect the VP5 transcription at early times (57). In fact, the transcriptional activation observed with these mutations closely resembles that of an early promoter. This *in vitro* and *in vivo* analysis of the VP5 promoter accentuate the importance of *in vivo* studies and suggest that conclusions drawn from *in vitro* studies might not always resemble the *in vivo* situation.

In summary all HSV promoters resemble cellular promoters in some way, but in addition to the requirements of a cellular promoter for full activity, HSV promoters also require trans-activating viral proteins. The tight timely regulation of the expression of the different gene-groups can in part be explained by structural differences in the different promoters, ii) by the availability of trans-acting viral factors at different times post infection and iii) structural characteristics of the DNA during certain stages of the replication cycle.

IV: YYI and SP1:

The cellular transcription factors Yin-Yang 1 (YY1) and Sp1 are known to play regulatory roles in gene expression of some viruses including HSV. YY1 was discovered only 6 years ago (1991), whereas Sp1 has been known since 1983 (24). Both factors have been studied extensively. Much information about the mechanism and roles of YY1 and Sp1 as transcription factors in cellular as well as in viral genes has accumulated over the last years.

a.) YYI

YY1 was cloned and described 1991 by Shi *et al* (139) as a zinc-finger protein related to the *Drosophila Krüppel* protein and having four zinc fingers. The *Drosophila* Krüppel protein has been shown to be able to both activate and repress transcription (126).The YY1 gene was also cloned in other laboratories under different names: NF-E1 acts as a repressor or activator on a immunoglobulin gene (115), factor δ positively regulates the ribosomal protein L30 and L32 genes (51), and the negative transcription factor UCRBP binds in the long terminal repeat sequences of Moloney murine leukemia virus (32).

Shi *et al* analyzed the adeno-associated virus P5 promoter and found that YY1 binds to a promoter element located at -50 to -70 bps as well as to one near to the initiation region, and that binding to either of those elements represses transcription (139). When placed upstream of a synthetic promoter or the SV40 early promoter/enhancer, the YY1 binding site of the P5 promoter mediated repression of transcription. The adenovirus E1A protein was found to relieve the repression of the P5 promoter by YY1 and even stimulate the transcription through the YY1 binding site. Later it was found that this relief of repression by E1A is mediated by the E1A-associated protein p300 (81). The results of the study lead to the conclusion that a YY1/p300 complex is targeted by E1A to relieve the repression by YY1. Lee *et al* further speculates that YY1 might interact with proteins other than p300 that are responsible for the repression of activity of YY1. Since both YY1 and p300 both have

been implicated in cell proliferation (141, 143), these results might be very helpful in understanding more about deregulated cell-growth and tumorigenesis.

Seto *et al* reported that the YY1 binding site at the initiator region of the P5 promoter is necessary and sufficient for accurate basal transcription, and that YY1 binding is responsible for that effect (138). Purified YY1 could restore basal transcription levels from a truncated P5 promoter construct, containing only the P5 promoter sequences from -7 to +11 (the YY1 binding site, but no TATA box or Sp1 binding site), in a HeLa cell extract depleted for YY1, or in a *Drosophila* embryo extract devoid of YY1 activity. Anti-YY1 antibodies blocked this reactivation. These results lead to the conclusion that YY1 is able to activate transcription when present alone or downstream from the TATA or Sp1 site in the P5 promoter (138).

Taken together, the above studies by Shi *et al* and by Seto *et al* indicated that YY1 is able to repress transcription through binding to a site upstream of a promoter initiation site, however, when present alone or downstream from the TATA or Sp1 sites the same element can activate transcription.

Bauknecht *et al* later identified a switch region in the YY1 protein when studying early gene activation of Human Papillomavirus type 18 (HPV-18) by a common upstream regulatory region (URR) (5). A region upstream of the YY1 binding site was identified that had a significant impact on the action of YY1 (switch region). Deletion of the switch region caused YY1 to act as a repressor, while in the presence of this switch region YY1 is a strong activator, and this effect was host-cell

dependent. *In vivo* footprinting and *in vitro* electrophoretic mobility shift assays (EMSA) revealed that proteins, as to now unknown, bind to this switch region. Site directed mutagenesis studies indicated that YY1 and the switch region binding protein work in concert to activate the promoter. Bauknecht *et al* proposed a model for transcriptional activation by YY1 and the switch region binding factor, whereby a switch factor-YY1 complex is formed by a DNA bend, and this complex acts to recruit TBP of TFIID to the TATA box.

The binding sites reported so far for YY1 display considerable heterogeneity, but they all contained a conserved 5'-CAT-3' core. The results of studies by Robin *et al* (119) indicate a 12 bp binding site with some flexibility around the common core-sequence. The consensus binding site can be modeled as

5'-(C/g/a)(G/t)(C/t/a/)CATN(T/a)(T/g/c)-3',

whereby the upper case letters represent preferred bases. This binding sequence fits into a scheme in which all of the four zinc fingers of YY1 bind to the DNA to form a stable DNA/protein complex. In the same analysis, a computer search revealed a wide variety of cellular and viral promoters that contain YY1 binding sites, many of which overlap with sites for known transcription factors (119).

Bushmeyer *et al* characterized the functional domains of YY1 (11). A bipartite activation region containing typical activation domains of regulatory proteins is located near the amino terminus. One domain between amino acids (aa) 16-29 may form an acidic amphipathic helix, and the other domain is rich in proline and glutamine (aa 80-

100). The repression domain seems unlike other repression domains and lies near the carboxy terminus where there are the four zinc fingers (aa370-397). Deletion of the repression domain in YY1 resulted in strong activation mediated by the protein rather then repression. The above mentioned E1A-mediated relief of YY1 repression of the P5 promoter of the adenovirus associated virus is caused by an interaction of E1A with the carboxy terminal region of YY1 including this repression domain (139). However, other proteins such as with Sp1 (82, 136), c-myc (142) or B23 (61) interact with other regions YY1. Bushmeyer at al concluded that the repression domain they identified is only one of several possible repression domains.

Another theory first presented by Natesan and Gilman concerning the mechanism of transcriptional activation or repression by YY1, is that YY1 induces DNA bending (108). Switching the orientation of the repressing YY1 binding site in the mouse *c-fos* promoter resulted in significant activation of the promoter, as did some changes of flanking protein binding sites. They proposed that binding of YY1 to the reversed YY1 binding site in the *c-fos* promoter bent DNA in a way where necessary transcription factors are brought into close contact to each other. However, YY1-binding to the YY1 binding site in its normal orientation results in bending of the DNA so that the transcription factors are physically separated and cannot activate transcription. They concluded that the activation or repression function of YY1 depends on the position and orientation of its binding sites as well as the position of flanking protein binding sites.

The hypothesis of transcriptional regulation by YY1 through DNA bending was further supported by Kim and Shapiro (75). They produced and analyzed synthetic promoters with a TATA box, an NF1 recognition sequence, and one or more YY1 binding sites in different orientations to each other, and with different spacing of the transcription elements to each other. The results indicated that at least for simple synthetic TATA box promoters, YY1 induced DNA bending is important for activation. An artificial intrinsic DNA bending sequence mimicked the effect of YY1 activation. The bending might change the architecture of the DNA in the promoter and thereby facilitate or impair the ability of upstream bound activators to interact with the basal transcription complex bound at the TATA box. The direction of the DNA bend seemed to be dependent upon the orientation of the different transcription factor binding sites to each other. However, this model might be true only for some promoters and cannot be generalized.

Another way of YY1 mediated gene repression by antagonistic protein functions was described by Gualberto *et al* (44). In this study it was shown that the *c-fos* promoter can be activated *in vitro* by binding of the serum response factor (SRF) to the serum response element (SRE) in the promoter. This binding can be inhibited by YY1 binding to an overlapping binding site in a competitive fashion.

Usheva and Shenk recently suggested with *in vitro* studies that YY1 might be able to act as part of the transcription initiation complex on TATA-less promoters (162). They proposed that YY1 can act like TBP in recruiting TFIID to the initiation

site so that pol II can interact with TFIID and start transcription. This is a very plausible model for transcription of genes with TATA-less promoters.

A recent analysis of the different ways in which YY1 can act to suppress activator dependent transcription was presented by Galvin and Shi (39). They showed that zinc finger 2 of YY1 plays the main role in both DNA binding and repression of transcription. They further argue that YY1 can repress transcription through direct physical interaction with certain activator proteins without binding DNA, or through DNA binding without apparent interaction with an activator protein. This emphasizes the widely believed hypothesis that YY1 can act in multiple ways to repress activator-specific transcription.

b.) Sp1:

Sp1 was first detected and identified as a potential cellular transcription factor in 1983 by Dynan *et al* (24). Kadonaga *et al* cloned Sp1 in 1986 and demonstrated that it is indeed a cellular transcription factor, and that it is required for efficient SV40 gene expression. It stimulates transcription by binding to a GC box , 5'-(G/T)GGGCGG(G/A)(G/A)(C/T)-3', which is embedded in a wide variety of cellular and viral promoters (69, 68). An Sp1 trans-activation domain was characterized and subdivided into two subdomains, the A and B boxes which are regions rich in serine, threonine and glutamine residues, and the C Box is composed largely of charged amino acids. In addition it has a D box that enhances activation by the A and B box (20).

Emami *et al* recently demonstrated that Sp1 activation of a promoter containing an initiator (Inr) element or an Inr element and a TATA box only required the glutamine-rich activation boxes A and B, while activation of a promoter containing only a TATA box required the whole Sp1 protein (25). Like YY1, Sp1 also is a zinc finger protein, containing three zinc fingers. Footprint analysis showed that the three zinc fingers bind to the GC box, with the zinc fingers 1 and 2 being most important (79). This analysis also revealed that the binding sequence 5'-GGGCG-3' is critical for GC box recognition by Sp1.

In their analysis of the uteroglobin gene promoter, Hagen *et al* identified two Sp1-related proteins with very similar structural features to Sp1 (47). All three proteins bind to the same binding sites, the GC box as well as a GT box that was identified as 5'-GGGGTGTGG-3'. The factors were designated SPR-1 and SPR-2.

Sp1 is a phosphoprotein, and it was shown by Jackson *et al* in *in vitro* as well as *in vivo* studies that its phosphorylation is dependent upon binding to its cognate DNA recognition sequence, and upon its transcriptional activation domain (62). The protein kinase phosphorylating Sp1 was identified as a ds DNA dependent kinase that phosphorylates only some trans-activators. There was no evidence that the phosphorylation affects transcription, but the timely occurrence of the events suggested this. Jackson concluded that phosphorylation might induce a conformational change in Sp1 that exposes or regulates the function of the glutamine rich activation regions. It

also could provide a mechanism to ensure that the transcriptional properties of Sp1 are only expressed in the appropriate subcellular environment.

A recent study by Rohlff *et al* indicated that Sp1 is stimulated by phosphorylation through a cAMP-dependent signaling pathway (120). The DNA binding activity and transcriptional activation by Sp1 was shown *in vitro* to be activated by cAMP protein kinase (PKA), as well as in transient expression assays in insect cells transiently expressing Sp1 and PKA.

Sp1 was shown to be negatively controlled by a ubiquitously expressed and evolutionarily conserved nuclear protein p74 (106). P74 was shown *in vitro* and *in vivo* to specifically bind to Sp1 and inhibit transcription. Since the abundance of Sp1 is believed to be invariant in most mammalian cell types there very likely is a control mechanism regulating the level of active Sp1. Stable overexpression of Sp1 in mammalian cells was found to be incompatible with viability of cells (174). Therefore, p74 might present a mechanism employed by mammalian cells to prevent a lethal accumulation of Sp1 (106)

Sp1 is also known to interact with other transcription factors. Two papers were published about the physical and functional interaction of Sp1 with YY1 (83, 137). Both groups analyzed the adeno-associated virus P5 promoter that contains a YY1 binding site at the transcription initiation site, and multiple Sp1 sites upstream. Extensive *in vitro* and *in vivo* analysis performed by both groups suggested that Sp1 and YY1 act synergistically to activate transcription of the P5 promoter, and that there

is direct physical interaction between the two proteins. Lee *et al* identified the Sp1 interaction domain with YY1 (83) as the C-terminal 158 aa of Sp1 including the D box and two of the three zinc fingers. More recent studies revealed a competitive interaction between YY1 and Sp1 (173, 2). Sp1 and Ap1 have been shown to activate the human granulocyte-macrophage colony-stimulating factor (GM-CSF) gene, and this activation is repressed by YY1 binding to a DNA recognition site in the GM-CSF promoter (173). YY1 and Sp1 also have been found to have binding sites in the human transferrin gene promoter, and Adrian *et al* suggest that Sp1 activates the promoter while YY1 exhibits a repressory function (2). This is a very interesting theory considering that lower transferrin levels have been associated with the aging process, and that transferrin promoter binding levels of YY1 seem to increase, while those of Sp1 decrease with aging.

Two other protein interactions of Sp1 are worth mentioning. First, the human immunodeficiency virus (HIV) Tat trans-activator protein seems to act together with Sp1 to synergistically activate the HIV-1 promoter (70). Second, cooperative interaction to synergistically activate a promoter has also been observed between Ap1 and Sp1 in the leukocyte integrine gene promoter in myeloid cells (111).

Lately, more and more studies reveal that Sp1 is an important factor of cell growth regulation. Two recent *in vitro* and *in vivo* studies demonstrated a physical and functional interaction of Sp1 with E2F (88, 72), and another study indicated that Sp1 interacts with p107, a regulator of E2F (23). E2F is a transcription factor that is

intimately involved with cell growth control and the regulation of genes involved in transformation, cell growth and DNA replication. This finding implicates Sp1 in cell cycle regulation and possibly in transformation of cells. Indeed, a recent study demonstrated that Sp1 is essential for early embryonic development in mice (95). In addition to these characters of Sp1, the protein was recently identified as the first RNA polymerase II transcription factor that can become an autoantigen in some people (149). Autoantibodies against Sp1 were found in a eight white woman suffering from fatigue, arthritis, Raynaud's phenomenon, malar rash, and photosensitivity. The presence of Sp1 autoantibodies was associated with undifferentiated connective tissue disease (149).

Part II: Research project:

Introduction to the research project:

I: The gD gene and protein:

The glycoprotein D (gD) gene of Herpes simplex virus type-1 (HSV-1) is one of the essential glycoproteins of the virus. Studies using anti-gD antibodies (36, 37, 54, 110) and analysis of liposomes containing viral glycoproteins by Johnson *et al* (64) suggested that gD is important for virus attachment or virus entry into cells, and plays a role in virion-cell fusion. Ligas and Johnson constructed a HSV-1 mutant virus with the gD gene replaced by a β-galactosidase gene (HSV-1-F-gDβ) (87). This virus could be grown and plaque-assayed on a complementing cell line derived from Vero cells and containing the HSV-1 gD gene (VD60 cells). On Vero cells, however, HSV-1-FgDβ was unable to form plaques but was able to infect the cells at a high multiplicity of infection and produce progeny virus. This progeny virus was lacking gD in the viral envelope and was unable to initiate a second round of infection. Marker rescue experiments confirmed that the above effect was due to the lack of the gD gene in HSV-1-FgDβ. Further analysis of HSV-1-FgDβ showed that gD is essential for penetration of HSV-1 into cells but not for attachment of the virus to Vero cells. In

addition, HSV-1-FgDβ induced rapid cell-to-cell fusion of VD60 cells but not of Vero cells suggesting that gD plays an essential role in cell-cell fusion. However, later this cell-to-cell fusion turned out to be the result of a mutation other than in the gD gene (146). Ligas and Johnson concluded that gD plays a crucial role in the penetration of HSV-1 into cells, but only a limited role in cell-attachment (at least with the cells tested).

In a subsequent analysis (63) using HSV-1-FgDβ, Johnson and Ligas reported that cells pretreated with UV-inactivated HSV-1 wild-type were resistant to infection with HSV-1 or 2. However, cells pretreated with UV-inactivated HSV-1-FgDβ virus lacking gD (after passage on Vero cells) were unable to inhibit infection by HSV-1 or 2. This observation implied that the cells have a cell-surface receptor specific for gD that is very limited in number, and that non-infectious virus particles containing gD in their envelope block these receptors. Johnson and Ligas concluded that gD is a receptor binding polypeptide of HSV essential for virus infection. This hypothesis was further supported by other studies that showed that gD of HSV-1 or 2 interferes with virus entry into cells if it is expressed on the surface of the host cell (14, 66). It turned out that this interference is virus-specific and it varies with minor differences of the gD protein of different virus strains. Also, certain amino acid deletions of gD were found to render HSV completely resistant to this interference (49). The mechanism of the interference, however, is not well understood.

A recent study identified a new host cell receptor for HSV, designated as the herpesvirus entry mediator (HVEM), which is a member of the tumor necrosis factor receptor superfamily (104). The gD protein of HSV-1 or 2 was shown to play a role in HVEM mediated cell-entry, as either anti HVEM antibodies, or HSV viruses containing mutations in the gD protein significantly reduced HVEM mediated infection. A later analysis demonstrated a direct physical interaction between the two proteins (168). This suggests that interaction between HVEM and gD is a step in HSV entry into HVEM-expressing cells.

II: Regulation of gD-gene expression:

Glycoprotein D (gD) of HSV is one of the leaky late (LL, $\beta\gamma$, or γ_1) proteins and it is therefore expressed at low levels prior to viral DNA synthesis and at high levels after the onset of DNA synthesis. Deletional analysis of the gD promoter driving a rabbit beta-globin gene showed that sequences that lie within 83 bp of the RNA cap site were essential for the low-level gene expression at early times (27, 28). In a subsequent analysis the gD promoter was truncated to 31 bp from the RNA cap site, and transient expression assays showed that this truncated promoter sequence is sufficient and essential for true late expression of gD (i.e. high level expression following DNA replication) (65). In summary, sequences upstream from -31 (essentially only a TATA box and the RNA cap site) are sufficient for true late gene

expression, and sequences located between -31 to -83 are essential for the leaky-late (γ_1) type of expression (27, 28, 65). A diagram of the gD promoter is shown in Figure 7.

The IE protein ICP4 was also shown to play an important role in trans-activation of gD-gene expression in *in vitro* studies (160, 159). Three ICP4 binding sites were identified in the gD promoter, one non-consensus site at -300 (5'-CTTCTCCGCATGGGTGATGTCGGGT-3'), one consensus site (the consensus sequence is print bold) at -100 (5'-ACT**ATCGTC**CATACCGACCACACCGACGAATCCCC-3')and another nonconsensus site within the coding region, around +130 (5'-TGTTTG**TCGTC**ATAGTGGGCCTCCATGGGGTCCGCGGCAA-3'). *In vitro* transcription studies using gD gene promoter constructs, containing the whole gD promoter and the first part of the gD gene (AvaI subfragment) driving a rabbit beta-globin gene, indicated that all three ICP4 binding sites are important for efficient gD-expression (159). These results were consistent with the finding that recombinant gD promoter constructs containing three or five tandem repeats of the consensus ICP4 binding site at -100 resulted in higher expression, and a construct having a deletional mutation in this ICP4 binding site was activated only minimally (160). However, subsequent *in vivo* analysis using a recombinant virus having mutations in all three ICP4 binding sites revealed that they do not play any major role in gD expression (146). Since many studies have shown that ICP4 is required for trans-activation of

other viral genes including those lacking ICP4 binding sites (as described above), it seemed unlikely that ICP4 is totally dispensable for gD gene expression. Therefore, Smiley *et al* suggested that ICP4 might be able to activate the gD gene by a second mechanism that does not require sequence specific DNA binding (146). More support for such a mechanism was described later by Xiao *et al* (171) using a GAL4-ICP4 fusion protein in transfection assays. Their results suggested that ICP4 can act via a transactivation domain (TAD) which is located near the N-terminus of ICP4, even if it does not bind to DNA (see above). Deletion of the TAD of the GAL4-ICP4 fusion protein abolished transactivation of the gD gene while transactivation of the TK gene that contains no ICP4 binding sites was not affected.

In addition to the ICP4 binding sites and the TATA box, two G-rich sequences between -63 to -73 and -42 to -52 were identified as important regions in the gD promoter (28). A later study performed in this lab revealed that a sequence in the VP5 promoter, another leaky-late promoter, that is similar to the sequence between -55 to -66 of the gD promoter, interacts with a cellular protein (18) . Furthermore, sequence comparison studies showed that the promoter sequences in the VP5, gD and, gB promoters are similar to binding sites for the cellular transcription factor YY1. Indeed, YY1 was subsequently identified as the cellular protein binding to this region in the gD, gB and VP5 promoter, and mutational studies indicated that it might play a role in trans-activation of leaky-late genes (103). Further analysis of the gD gene promoter revealed that a second cis-acting site which corresponds to the GC-rich site at -63 to -

73, upstream from and overlapping the YY1 binding site, also binds a cellular factor (Lisa K Mills and Robert L Millette, unpublished data). Electrophoretic gel mobility shift analysis (EMSA) showed that the factor binding to this site is the cellular transcription factor Sp1 or Sp1-related proteins (SpX). SpX and YY1 were found to bind in an independent and mutually exclusive fashion to their binding sites. *In vitro* analyses using a promoter construct containing the gD promoter sequence from -392 to +11 driving a CAT (Chloramphenicyl-transferase) reporter gene (Figure 7) showed that YY1 and SpX greatly influence gD expression following viral induction, with SpX playing the major role (Figure 8). Mutations were inserted into the YY1 binding site (gDY-CAT), the SpX binding site (gDS-CAT), or both (gD2m-CAT), and a wild type promoter construct (gDwt-CAT) was used as a control (Figure 7). Basal activation of the wt-gD promoter in the absence of any viral proteins was very low, only 3 to 4 times the value of the CAT assay background control. Mutation of the YY1 or SpX binding sites reduced this expression level slightly, indicating that both of these proteins play a role activating the gD promoter.

Cotransfection experiments using the gD promoter-CAT constructs as reporters and plasmids containing the IE genes encoding regulatory proteins ICP0, ICP4, and ICP27 demonstrated that ICP0, ICP4, and ICP27 are required for efficient activation of expression driven by the gD promoter (Figure 9). None of the viral IE genes by itself had any significant effect, but ICP0 and ICP4 together were sufficient to promote a high level of CAT expression from the gD promoter in the presence of a functional

SpX binding site. ICP27 was found to enhance this activation. An intact YY1 binding site alone (in the gDS-CAT construct with the Sp1 site mutated) resulted in a lower level of transactivation of the gD promoter by the viral IE proteins as compared to the wt-CAT construct. The promoter construct having mutations in both the YY1 and SpX binding sites (gD2m-CAT) was not activated at all. In summary, these results suggest that the Sp1 and YY1 binding sites play important roles in the gD gene regulation in the context of a viral infection, with the Sp1 site providing the major activation effect and YY1 providing a lower level of activation. It was speculated that the early proteins ICP4, 0 and 27 might interact with Sp1 in some way to activate the gD promoter.

III: Goal of this study:

The goal of this study was to construct four mutant viruses having the above mutations in the gD promoter, and to test the effect of these mutations on virus infectivity and gD expression. Since gD is an essential gene for virus replication, the infectivity of the mutant viruses should correlate with the production of gD protein. To accomplish this goal, four plasmids were constructed containing the gD gene and considerable flanking sequences with the above mutations inserted in the gD promoter. The linearized plasmid was co-transfected with infectious HSV-1-FgDβ DNA (an HSV-1 F strain mutant containing a β-galactosidase gene inserted in the gD gene and part of the adjacent gI gene) on a gD-complementing cell line (VD60 cells). The resulting virus was plated on VD60 cells (a monkey Vero cell line expressing the HSV-1 gD gene) and overlayed with an agarose-overlay containing X-Gal to discriminate between β-galactosidase plus or minus viruses. Virus clones isolated from single colorless plaques (β-gal minus) were screened for the presence of the mutations by PCR and subsequent restriction enzyme digestion and sequence-analysis of the PCR fragment. The viral mutants obtained by this procedure were designated HSV-1-gDY (with a mutation in the YY1 site), HSV-1-gDS (with a mutation in the Sp1 site), HSV-1-gD2m (with mutations in both sites), and HSV-1-wt (with no mutations). To analyze the infectivity of the mutant viruses, each of the four mutants and a HSV-1 F strain control virus were titered on Vero and VD60 cells in parallel. The growth rate was determined

by infecting Vero cells with the four recombinant viruses and HSV-1-F in parallel, and harvesting and titering the viruses at different time points post infection.

The results of this project will provide more insight into the regulation of HSV-1 gene-expression, as well as into the role that YY1 and Sp1 might play in that regulation. Furthermore, it might help to better understand the mechanisms by which YY1 and Sp1 can act to regulate gene transcription.

The results of this project are summarized in the following. There was no significant difference in the yields of the four mutant viruses as determined by the ratio of the titer on Vero cells to the titer on VD60 cells. However, HSV-1-gDY and HSV-1-wt grew a little faster than did HSV-1-gDS and HSV-1-gD2m. Since gD is an essential protein, HSV can not form plaques if it doesn't produce the gD protein. Therefore, in terms of virus plaquing efficiency, these results suggest that neither the YY1 nor the Sp1 binding sites are essential. However, the slight difference in the growth rate of the four mutant viruses suggests that the YY1 and Sp1 binding sites have an impact on gD gene expression. This leads to the speculation that the YY1 and Sp1 binding sites alter the timely regulation of expression of the gD gene but are not essential for the overall expression of the gene. To ascertain the effects of these mutations on gD mRNA synthesis, primer extension experiments are planned.

Materials and Methods:

Cells and viruses:

VD60 cells and HSV-1-FgDβ virus (Figure 10) were a gift from Dr. Johnson (see ref. 87). VD60 cells (monkey Vero cells containing a stably integrated HSV-1-gD gene and a histidinol dehydrogenase gene as selectable marker) were maintained on αMEM (GIBCO) containing 7% inactivated fetal bovine serum (IFBS) (Hyclone). On at least every fourth passage the calls were placed on αMEM lacking histidine and containing 0.15mM histidinol (SIGMA) and 7%IFBS to maintain selection for the integrated gene. Prior to virus infection VD60 cells were always passaged at least once on nonselective αMEM. During viral infection cells were always grown in media containing 100 Units/ml penicillin and 0.1 mg/ml streptomycin (pen/strep) (BIO✦Whitaker). HSV-1-FgDβ was propagated and assayed on VD60 cells. Plaque assays were performed in αMEM with 7%IFBS and pen/strep; 0.2% (v/v) inactivated human serum (IHS) containing anti-HSV-1 antibodies was added at 10 to 14 h.p.i.. At 36 to 48 h.p.i., the cells were overlayed with a 0.5% agarose overlay containing 30 μg/ml X-gal (bioWORLD) derived from a 30 mg/ml stock in DMSO, and 24 to 36 hours later HSV-1-FgDβ-plaques were stained blue. The recombinant viruses, having colorless plaques, HSV-1-gDY, HSV-1-gDS, HSV-1-gD2m, and HSV-1-wt, were propagated and assayed on VD60 cells as well. Vero cells were propagated on DMEM

(Hyclone) containing 10% FBS. HSV-1-F was propagated and assayed on Vero cells. All cells were maintained under a 5% CO_2-95%air atmosphere.

Plasmids:

The plasmid pSG25, containing the HSV-1 KOS strain 7.5 kbp EcoR1 'H' fragment cloned into pBR322, originated from this laboratory. Plasmids gDY-CAT, gDS-CAT, gD2m-CAT, and gDwtCAT-were constructed in this laboratory by Lisa K Mills and kindly provided for this project. They contain an HSV-1 strain 17 gD promoter sequence from +11 to -392 driving a Chloramphenicyl-transferase (CAT) gene, and inserted in a pUC9 vector (Figure 7).

Enzymes and buffers:

Restriction enzymes were purchased from New England Biolabs or GIBCO. The enzymes were used in the buffers supplied and suggested by the company, except for the Eag1/HindIII digestion. To provide conditions in which both of these two enzymes work well, a 5×KGB buffer was prepared at pH 8.0 from 10×KGB buffer (131). Proteinase K was purchased from SIGMA. Taq-DNA polymerase for PCR was purchased either from Promega or at SIGMA, and the reaction buffer and $MgCl_2$ were provided by Promega. The dNTPs were purchased from Stratagene.

Plasmid Preparation:

Plasmid pUC18 derivatives were grown *in Escherichia coli* DH5α cells. Growth and purification of plasmids by TENS miniprep-method for screening were essentially as described by Sambrook *et al* (131). All plasmids that were used for further cloning or for marker transfer were purified by Qiagen Maxi-prep. DNA fragments were isolated from an agarose gel containing 0.5 mg/ml ethidium bromide (EthBr) on DEAE-paper as, and ligation and transformation was performed according to the protocols by Sambrook *et al* (131). If a plasmid was linearized with one enzyme only for cloning purposes or had blunt ends, a phosphatase (alkaline phosphatase, Boehringer Mannheim) treatment (131) was performed to avoid recircularization.

I: Construction of plasmids containing the HSV-1 gD gene and flanking sequences with mutations in the gD promoter:

The following protocol describes the construction of plasmids containing a 5.2 kbp sequence of the U_S region of HSV-1 (KOS) surrounding the gD gene, and either wt or mutant gD promoters of HSV-1 (strain 17). Starting with the earlier constructed plasmids gDY-CAT, gDS-CAT, gD2m-CAT, and wt-CAT (by Lisa K Mills), a small piece (132bp) of each gD promoter were excised using Hind III and Eag1 digestion and

were isolated. Then recombinant plasmids were constructed comprised of a pUC18 vector in which the Hind III site was deleted (pUC18/HindIII$^-$), and that contained the BamH1 'j' fragment of HSV-1 KOS strain from the upstream BamH1 site in the U_S3 gene to the Sph1 site in the U_S8 (gE) gene located 5.8kbp downstream from the BamH1 site (Figure 11 shows the HSV-1 DNA fragment in the viral genome).

The Hind III site of pUC18 was deleted by cutting pUC18 with HindIII, filling in the overhanging ends with Klenow fragment, and blunt-end ligating the vector (131). The BamH1 'j' fragment of HSV-1 KOS was derived from the plasmid pSG-25 by digestion with BamH1. The 6.5kbp BamH1 fragment was isolated and cloned into the pUC18/ HIII$^-$ vector. This plasmid was designated pBJ65 (Figure 12). Since Eag1 cut at many sites in the BamH1 'j' fragment of HSV-1, a few substeps were needed to be able to clone the Eag1-Hind III piece of the mutant CAT clones into the pBJ65 vector. The construction of the recombinant plasmids starting with pBJ65 and the gD-CAT series is summarized in Figure 13.

Digestion of pBJ65 with Sph1 yielded three fragments, a 3.8 kbp fragment containing the pUC18/HIII$^-$ vector together with a 1kbp HSV-1 sequence from the upstream BamH1 site to an Sph1 site, a 3.9 kbp fragment containing the gD gene and some 5' and 3' flanking sequences, and a 1.4 kbp fragment containing HSV-1 sequences downstream from the gD gene and part of the pUC18 polylinker. The 3.8kbp and the 3.9 kbp fragments were isolated. The 3.8 kbp fragment was

recircularized, and amplified and purified by transformation in *E.coli* DH5α cells and Qiagen maxiprep. The purpose of this purification step was to avoid contamination with the 3.9kbp fragment which does not contain a ampicillin resistance gene. The correct plasmid was identified by restriction enzyme analysis. The new plasmid was designated pSJ38-vector.

The 3.9 kbp fragment mentioned above was ligated into a pUC18/HIII$^-$ vector and screened by restriction enzyme-digestion for the correct insert. The correct plasmid was designated pSL39, and it contained only one unique Eag1 site in the gD gene promoter. The pSL39 plasmid was digested with Eag1 and HindIII, and the large 3.9 kbp-vector piece was isolated. Into this vector, each of the four 132bp Eag1/HindIII fragments derived from the mutant gD-CAT clones (gDY-CAT, gDS-CAT, gD2m-CAT, and gDwt-CAT) were inserted. This resulted in four different plasmids containing a 3.9kbp HSV-1 sequence with three different mutations and a wt control in the gD gene promoter. These plasmids were designated pSL39-gDY, pSL39-gDS, pSL39-gD2m, and pSL39-wt. The HSV-1 sequence in those was excised with Sph1 and ligated back into the pSJ38-vector after linearization of the vector with Sph1.

The end result of this molecular cloning was four different plasmids derived from the pUC18 vector and containing 5.2 kbp of the HSV-1 U_S sequence including the gD gene with the same mutations in the gD promoter as the gD-CAT constructs. These plasmids were designated pSJgDY, pSJgDS, pSJgD2m, and pSJwt (Figure 14).

Extensive restriction analysis was performed to confirm the plasmid structures. A sequence analysis of the gD promoter region was performed by the Core facilities of the Molecular Microbiology and Immunology department at Oregon Health Science University (OHSU, Portland, OR) to ascertain that the correct mutations were present in the gD promoter. The primer for sequencing was chosen so that sequencing would start in the gD gene at +131 bp relative to the RNA cap site and cover the entire gD promoter and sequences further downstream (5'-GCC CAC TAT GAC GAC AAA CAA-3').

II: Marker transfer:

To construct mutant viruses, the plasmids pSJgDY, pSJgDS, pSJgD2m, and pSJwt were linearized with ScaI and cotransfected together with infectious HSV-1-FgDβ DNA into VD60 cells (Figure 15). Cotransfection was performed essentially according to the Ca_2PO_4 co-precipitation method described by Graham and van Der Eb (42). Infectious HSV-1-FgDβ DNA was generated as described by Smiley *et al* (145) with slight modifications according to kindly provided protocols from Kim Goldsmith (OHSU). A crude viral DNA extract was produced by harvesting VD60 cells 24 hours post infection with 5 pfu (plaque forming units) per cell of HSV-1-FgDβ (six $150cm^2$ flasks were used). After centrifugation, the cells were resuspended at 2.5×10^7 cells/ml in 0.2 mM EDTA pH 8.0, 0.5% SDS, and 100μg/ml of proteinase K. The lysate was

incubated over night at 37°C. The lysate was gently extracted twice with phenol/chloroform/isoamylalcohol (25:24:1) and once with chloroform/isoamylalcohol (24:1) (SIGMA), then dialyzed over a period of 2-3 days in 1 liter of 0.1×SSC (131) with 4 changes of buffer. The lysate was tested for infectivity by transfection into VD60 cells (~5 pfu/μl). For marker transfer, 120 μl of the lysate together with 1 μg of linearized plasmid DNA per one 9.6 cm^2 well of a six-well cluster dish were used to co-transfect VD60 cells at a confluency of about 40%. The co-transfection was performed in 5ml of αMEM supplemented with 1%FBS and pen/strep, and the cells were incubated at 37°C. At 16 hours post transfection the precipitate was thoroughly washed off the cells and the cells were maintained under αMEM supplemented with 1%FBS and pen/strep. Four to five days post transfection, 100% of the cells showed viral cytopathic effects (infection was complete), and the cells were harvested by centrifugation, and sonicated to release the virus.

III: Screening and isolation of recombinant viruses:

The viral lysate from the cotransfections was plated onto 60 mm dishes containing 80 to 90 % confluent VD60 cells in αMEM with 7% IFBS and pen/strep. At 36 to 48 hours post infection (h.p.i.) the cells were overlayed with 0.5% agarose in αMEM with 7% IFBS and pen/strep containing 30 μg/ml of X-Gal (5-bromo-4 chloro-3 indolyl-galactopyranoside) (87). Thirty hours later colorless plaques were selected,

picked with P20 micro-pipette-tips with cut-off tips, and transferred into 24 well dishes onto 50% confluent VD60 cells. The infected cells were incubated for four to five days until infection was complete. The cells were harvested by scraping, and one half of each of the virus clone preparation was quick frozen in a dry-ice ethanol bath and stored at -90°C. The other half was used for screening by PCR as follows, according to a protocol provided by Kim Goldsmith (OHSU). The cells were harvested by centrifugation, washed with PBS-A (phosphate buffered saline) and suspended in a lysis buffer containing 10mM Tris pH 8.0, 10 mM EDTA pH8.0, 0.5% SDS, and 100μg/ml of proteinase K. The lysates were incubated over night at 37°C, and 0.2 μl of each lysate was used for screening by PCR in a 50 μl reaction volume. For primers, the oligonucleotides 5'-CTG GAT CCG CCC CGG CCC CCA ACA AAA ATC-3' and 5'-CGA ATT CTG TCC ACC GTC ACC CCC TGG TG-3' were chosen and synthesized by the Core Facilities of the Molecular and Microbiology Department of OHSU (Oregon Health Science Universities, Portland, OR). The primers covered a 958 bp DNA sequence of the gD gene from -152 to +806 (see Figure 16). PCR was performed at 2 mM $MgCl_2$ to provide very stringent conditions so that the HSV-1-F BamH1 fragment contained in the genome of the VD60 cells would not be amplified. Negative controls were run using an uninfected cell-lysate, HSV-1-F and HSV-1-FgDβ virus lysates, as well as no DNA at all. Positive controls were done using the plasmid pSJgDY and the virus HSV-1-KOS grown on VD60 cells as the recombinant viruses. Positive PCR products were analyzed by restriction analysis (Xho1 for HSV-1-gDY,

BglII for HSV-1-gDS, and both Xho1 and EcoRI for HSV-1-gD2m and SacII, which cuts twice in the sequence, for all recombinant viruses). The positively identified viral clones were plaque-purified a second time and retested by PCR and restriction analysis as described above. Finally, the PCR products of the plaque-purified viral clones were purified by Microcon-100 treatment as described by the distributor (Amicon), and sequenced (performed by the Core Facilities of the Molecular Microbiology Department of OHSU, Portland, OR) around the gD promoter sequence to assure that only the correct mutations were present in the promoters. A large stock of each of the four recombinant viruses was propagated on VD60 cells.

IV: Infectivity of the mutant viruses:

To test the infectivity of the four mutant viruses (HSV-1-gDY, HSV-1-gDS, HSV-1-gD2m, HSV-1-wt), the four viruses and an HSV-1-F virus control were titered on VD60 cells and on Vero cells in parallel (from the stock produced on VD60 cells). Two times three different dilutions in a range easy to count (between 50 and 500 plaques per 9.8cm^2 well) were plated onto VD60 and Vero cells. When plaques were visible, the cells were fixed for 30 minutes with methanol and stained with Giemsa (SIGMA), and counted. The ratio of the average numbers of plaques on Vero cells to VD60 cells was determined to compare the infectivity for each of the five viruses.

V: Growth rate of the mutant viruses:

To determine the growth rates of the four different mutant viruses and HSV-1-F, a one-step growth experiment was performed. Vero cells in 9.8cm^2 cultures and at 95% confluency were infected with the viruses HSV-1-gDY, HSV-1-gDS, HSV-1-gD2m, HSV-1-wt, and HSV-1-F, respectively, at an m.o.i. (multiplicity of infection) of 2. At the time points 0, 4, 8, 12, 16.5, 20, and 24 h.p.i. (hours post infection), the infected cells were harvested by scraping and virus was released by sonication. The volume of each virus lysate was adjusted to 4 ml. Serial dilutions of each virus lysate were plated onto Vero cells in a double infection assay (two samples per dilution), and when plaques had formed, the cells were fixed, stained, and the plaques were counted. The titers of each of the five viruses at each time point was determined, and all five growth curves were plotted.

Results:

I: Construction of four plasmids containing the gD gene with mutations in the gD promoter and flanking sequences:

To be able to insert mutations in the YY1 and Sp1 binding sites in the gD promoter of HSV-1 by marker transfer, four different plasmids, pSJgDY, pSJgDS, pSJgD2m, and pSJwt were constructed (Figure 14). The plasmids contained a 5.2 kbp HSV-1 DNA sequence including the gD gene and promoter and considerable 5' and 3' flanking sequences inserted into a pUC18 vector. The plasmid pSJgDY contained a mutation in the YY1 binding site that has a restriction site for Xho1, pSJgDS contained a mutation in the Sp1 binding site that has a restriction site for BglII, pSJgD2m contained mutation in both YY1 and Sp1 binding sites that have restriction sites for Xho1 and EcoRI respectively, and pSJwt contained no mutation. These mutations were shown previously to block binding of the respective transcription factors. The detailed construction of the plasmids is described in Material and Methods, and is summarized in Figure 13. In short, the HSV-1 BamH1 'j' fragment was excised from the plasmid pSJ-25 and cloned into a pUC18/HIII^{-} vector (pUC18 in which the HindIII site had been eliminated). The resulting plasmid (pBJ65) was digested with Sph1 and yielded three fragments (Figure 13). The fragment containing the pUC18-vector and about 1.3 kbp of the HSV-1 sequence was recircularized and isolated (pSJ38 vector). The

fragment containing about 3.9 kbp of HSV-1 sequence including the gD promoter was isolated and cloned into a second pUC18/HIII$^-$ vector. This produced the plasmid pSL39. Into the pSL39 plasmid, four different 132bp Eag1/HindIII fragments, previously excised from the gD-CAT series of plasmids and containing the different mutations in the gD promoter, were inserted (pSL39 mutants). The four pSL39 mutants were digested with Sph1 to excise the HSV-1 sequence containing the different mutations, and this piece was ligated into the pSJ38 vector. This resulted in the production of four different plasmids, pSJgDY, pSJgDS, pSJgD2m, and pSJwt. Following transformation at each cloning step, five bacterial clones that grew on selective LB medium (containing ampicillin), were picked and screened by plasmid miniprep analysis. Each plasmid was analyzed by extensive restriction analysis for the correct insert. Plasmids containing the mutations were analyzed with the respective restriction enzymes cutting in the mutations (as described above) to confirm that the mutations were present. One positively identified clone of each was purified by the Qiagen maxiprep procedure and used for further cloning steps.

The final plasmids (pSJgDY, pSJgDS, pSJgD2m, pSJwt) were identified by extensive restriction analysis. An example of one such restriction analysis is shown in Figure 17. Restriction of the plasmids by MluI yielded a double band running at about 3.8 kbp (Figure 17, lanes 3,6,9, and 13). Subsequent analysis showed that this large band in was comprised of two bands of 3.6 and 3.8 kbp that run very close together. The determined fragment sizes agree well with the predicted sizes (3.87 kbp and 4.01

kbp). Digestion with SacI resulted in bands of 3.4, 2.8, and 1.7 kbp for each plasmid (lanes 4, 7, 10, 14 in Figure 17) which is very close to the expected fragments of the sizes 3.3, 2.75, and 1.75 kbp. Digestion of each plasmid with the enzymes specific for the gD promoter mutations demonstrated the presence of the desired or the wild-type promoter sites. Thus, both pSJgDY and pSJgD2m were linearized by XhoI (lanes 5 and 11), and pSJgDS was linearized by BglII (lane 8). EcoRI (which cuts once in the wild-type plasmid and once in the gD2m mutation) correctly linearized pSJwt (lane 15) and cut pSJgD2m into two fragments close to the expected sizes of 5.75 and 1.95 kbp (lane 12, and Table 1). As expected, XhoI did not cut pSJwt and pSJgDS, BglII did not cut pSJgDY and pSJgD2m, and EcoRI linearized pSJgDY and pSJgDS (data not shown in Figure 17, but see Table 1). The results of these and additional restriction analyses are summarized in Table 1. Excellent agreement was observed between the expected and observed fragment sizes, thus confirming that the constructed plasmids have the predicted structure and contain the correct gD promoter mutations.

To confirm that the gD promoter does contain the desired mutations and no other mutations, sequence analysis of the gD promoter region was performed. The primer for sequencing was chosen so that sequencing would start in the gD gene at +131 bp relative to the RNA cap site, and cover the whole gD promoter and sequences further upstream. Sequencing was performed by the dideoxymethod using fluorescent-labeled dideoxynucleotides. The sequence was read from the resulting colored curves by a computer and compared to a sequence obtained from the GeneBank (access-#:

J02217 for HSV-1-KOS, and X02138 for HSV-1 strain 17). The results are shown in Figure 18. As can be seen in that figure, each plasmid contained the desired mutations (printed in bold) in the gD promoter (compare to Figure 7b). All bases other than the mutation sites matched with the predicted HSV-1 sequence for each of the recombinant plasmids. The bases depicted by 'N' could not be resolved by the computer, but could be confirmed by manually reading the sequencing curves. This analysis demonstrated that the plasmids pSJgDY, pSJgDS, pSJgD2m, pSJwt contained the correct HSV-1 sequence and the desired mutations in the gD promoter. A map of the plasmids showing the contained HSV-1 sequence and the mutation sites in the gD promoter is shown in Figure 14.

II: Construction of the four mutant viruses:

To construct mutant viruses having mutations in the YY1 and Sp1 binding sites in the gD promoter, marker transfer was performed using infectious HSV-1-FgDβ DNA and the linearized plasmids pSJgDY, pSJgDS, pSJgD2m, and pSJwt (as described in Materials and Methods). Crude viral DNA prepared by lysis of HSV-1-FgDβ infected VD60 cells, was tested for infectivity by transfection into VD60 cells (~5 pfu/μl). The viral lysate and the linearized plasmids were cotransfected into VD60 cells by the calcium phosphate coprecipitation method as described in the Materials and Methods section.

Following incubation for four to five days at 37°C, the virus resulting from the cotransfection was plated out onto VD60 cells, and the cells were overlayed with an agarose-overlay containing X-Gal. Colorless plaques were picked, and the viruses from each were propagated on VD60 cells in 24 well dishes. After cell lysis and proteinase K digestion, the resulting DNA was screened by PCR for the presence of the gD gene and promoter (described in Materials and Methods). The PCR analysis was expected to yield a 958 bp product (Figure 16). Analysis of the PCR products be gel electrophoresis is shown in Figure 19. Screening of 10 viral clones resulting from cotransfection using the plasmid pSJgDY is shown in Figure 19A, lanes 3 to 12. The DNA from one viral clone yielded the expected 958 bp PCR product and therefore was considered a candidate for a positive HSV-1-gDY clone (lane 6). All negative PCR controls did not produce any band, and all positive controls yielded the correct 958 bp band. The PCR was specific for HSV-1-KOS strain DNA, but was not supposed to recognize HSV-1-F strain DNA because the DNA used for recombination was HSV-1-KOS, but the VD60 cells contained the gD gene of HSV-1-F to complement for the deficiency. The 958 bp band was produced by positive controls using HSV-1-KOS strain DNA (lanes 13 and 14), but not by negative controls using HSV-1-F strain DNA (lanes 15 and 16). HSV-1-F strain DNA amplified under less stringent conditions produced the 958 bp band (lane 17), as did a positive control using the plasmid pSJgDY as template (lane 18). Lane 19 contains a negative control containing all PCR ingredients but no DNA. Other negative PCR controls that were performed but are

controls run with cell-lysate lacking any virus, and a lysate from HSV-1-FgDβ infected cells. As expected, both yielded no product (data not shown).

Figure 19B shows the results of PCR analysis of viral clones resulting from cotransfections with pSJgDY (lanes 3 to 6) pSJgD2m (lanes 7 to 10), pSJgDS (lanes 11 to 14), and pSJwt (lanes 15-18). Possible positive viral clones as evidenced by a 958 bp band can be seen in lane 7 (for HSV-1-gD2m), lane 12 (for HSV-1-gDS), and lane 18 (for HSV-1-wt). In this PCR analysis the same controls as above were run and confirmed on an agarose gel, but only a HSV-1-F negative control (lane 19) and a no DNA control in lane 20 are shown on this gel.

Table 2 shows the number of screened and positively identified for each virus. On average, 1.5 out of 14.5 clones was positive (10.3%) indicating an average frequency of obtained recombinant viruses of about 0.34%.

To confirm that the desired mutations were inserted in the gD promoter of the virus (and therefore were present in the PCR products), each of the PCR products was analyzed by restriction analysis with the specific restriction enzymes that cut in the mutation sites (Figure 20). Digestion with the respective enzyme (XhoI for gDY, BglII for gDS, and XhoI and EcoRI for gD2m) was expected to truncate the 958 bp PCR product to a 880 bp fragment (see Figure 16). Digestion with another enzyme, SacII, was expected to yield three fragments of the PCR products, 401 bp, 308 bp, and 249 bp in size. Figure 20Table 3 shows the results of this restriction analysis. Lanes 4, 6, 8, and 11 show uncut PCR products from the recombinant viruses. Lane 13 contains

the uncut PCR product that resulted from HSV-1-F DNA under low stringency conditions, and lane 15 shows the results of PCR on HSV-1-F under the conditions used for the PCR screening. Lane 16 shows the uncut PCR product from the plasmid pSJgDY. A 'no DNA'-PCR control is represented in lane 17. Digestion with the specific enzyme that cuts in the mutation site (XhoI for HSV-1-gDY, BglII for HSV-1-gDS, and XhoI or EcoRI for HSV-1-gD2m) truncated the PCR product and produced bands having close to the expected 880 bp size (lanes 5, 7, 9, and 10). Digestion of the PCR product from HSV-1-wt with SacII (lane 12) yielded three fragments running close to the expected sizes of 0.42 kbp, 0.31 kbp, and 0.257 kbp (Table 3). The PCR products of the other three recombinant viruses were also digested with SacII and yielded the same size fragments (data not shown). Digestion of the PCR product derived from HSV-1-F DNA yielded the same three fragments (lane 14), confirming that the PCR products contain the desired DNA sequence. The PCR products from all viral clones were also digested with the enzymes specific for the mutations not inserted in the gD promoter, and as expected none of the PCR products was truncated (data not shown).

Results from the restriction analysis of PCR products from screening the recombinant virus clones is summarized in Table 3. These results confirmed that the gD promoter of the by PCR positively identified viral clones contained the correct mutations. All other clones that had been identified as positive by PCR also contained the correct restriction sites in the PCR piece.

One clone for each mutant was selected and plaque purified, and three single plaques of each virus clone were picked propagated, and retested by PCR. All of the screened plaques after plaque purification were found to be positive. A restriction analysis of the PCR product of one selected clone of each mutant is shown in Figure 21. Lanes 4, 8, 12, and 13 show the truncated PCR product after digestion with the enzyme specific for the desired mutation as described above. The fragment ran close to the expected size of 880 bp. Lanes 5, 6, 9, 10, 14, 16, 17, and 18 contain the PCR products of the recombinant viruses after digestion with the enzymes specific for the mutation sites not inserted into the gD promoter of the respective viral clone. All of these fragments were not cut and run at the expected 958 bp size. Digestion of the PCR product of each viral clone with SacII resulted in three fragments of the predicted sizes of 0.42, 0.31, and 0.257 kbp (lanes 7, 11, 15, and 19). Table 4 summarizes the results of these analyses. These results indicate that the PCR products and thereby the viral clones isolated have the correct structure and the desired mutations in the gD promoter.

To confirm that the viruses have the correct DNA sequence in the gD promoter region, the PCR product from each viral clone was purified, and subjected to sequence analysis as described above for the plasmids, using the same primer. The sequences obtained were compared to those obtained earlier from the plasmids pSJgDY, pSJgDS, pSJgD2m and pSJwt and to the HSV-1 sequence derived from the GeneBank. The results from this analysis are shown in Figure 22. This comparison chart shows the

DNA sequence of the gD promoter from -151 bp to +11 bp relative to the mRNA cap site, covering all parts of the gD promoter that are known to play a role in gene regulation. The mutated bases in the YY1 and/or Sp1 binding sites are printed in bold, and match to the mutations of the gD-CAT plasmids (Figure 7b). All other bases match to the sequence derived from the gene bank as well as to the earlier determined sequence of the recombinant plasmids. These results verified that the gD promoter in the viruses contained the correct mutations and no other base changes were detected in the region analyzed (Figure 22).

III: Infectivity of the four mutant viruses:

In order to test the infectivity of the four recombinant viruses compared to each other and to the HSV-1-F strain virus, the viruses were titered on VD60 and Vero cells in double assays, respectively (Table 5). To compare the infectivity of the different viruses to each other, the ratio of the titer on Vero cells to the titer on VD60 cells was determined. This ratio for the wild-type virus (HSV-1-wt) was set at 100%, and the percentage of infectivity of the other four viruses in relation to HSV-1-wt was calculated (Table 6 and Figure 23). The statistical significance of the differences in the infectivity of the five viruses was analyzed by the t-test.

All viruses formed syncytia on Vero as well as on VD60 cells. This is the result of a mutation present in the HSV-1-FgDβ virus other than in the gD gene (146). There was no significant difference in the relative titer of the four recombinant viruses

on Vero cells (Table 5 and Table 6, and Figure 23). The infectivity of HSV-1-gDY was somewhat lower than that of HSV-1-wt (about 85% as compared to 100%), whereas the infectivity of HSV-1-gDS and HSV-1-gD2m was slightly higher (113% and 131 %). The infectivity of HSV-1-F was about 124% as compared to HSV-1-wt. A t-test analysis showed that the differences for HSV-1-gDY, HSV-1-gD2m, and HSV-1-F as compared to HSV-1-wt are statistically significant with 99% probability. The difference between the growth yield of HSV-1-gDS and HSV-1-wt are statistically significant with only 90% probability.

Since gD is a protein essential for HSV-1 replication, a drastic reduction in the quantity of gD expression would be expected to reduce virus infectivity and growth rates which would be apparent in this analysis. The above results indicate that this is not the case. The differences in the infectivity are very little, even though the statistical analysis showed that they are statistically significant. Therefore, the YY1 and Sp1 binding sites in the gD promoter are not essential for virus replication under the conditions tested. However, it is interesting to note, that the plaques of all four recombinant viruses grew to a large size within 24 to 30 hours on Vero cells, whereas the plaques of HSV-1-F on Vero cells required at least 48 hours to reach the same size. This is probably due to syncytia formation by the recombinant viruses but not by HSV-1 F. HSV-1-gDY and HSV-1-wt had formed large plaques on Vero cells after 24 h.p.i., with the plaques of HSV-1-gDY being somewhat larger than the plaques of HSV-1-wt. HSV-1-gDS and HSV-1-gD2m appeared to replicate more slowly,

requiring about 30 hours to reach the same plaque size as HSV-1-wt after 24 hours. The virus growth on VD60 cells was considerably slower for all viruses, requiring two to three days until the plaques had reached the same size as they had on Vero cells after only one day. The titer of all five viruses was higher on Vero cells than on VD60 cells (Table 5). This could be explained by gD-mediated interference (49). Since low levels of gD might be expressed on the surface of VD60 cells, this might inhibit infection of the cells by HSV-1.

IV: One-step growth curve:

The above mentioned observation that the plaques of the viruses HSV-1-gDS and HSV-1-gD2m appeared to require more time to reach the same size as plaques of HSV-1-wt and HSV-1-gDY in less time suggested that the growth rate of the four recombinant viruses might differ. To test this, a one-step growth curve was performed as described in materials and methods. Vero cells were infected with the four recombinant viruses and HSV-1-F, and were harvested at 0, 4, 8, 12, 16.5, 20, and 24 h.p.i.. The resulting viral lysates were titered on Vero cells. The average titers and standard deviations are shown in Table 7. The titers of the five viruses were plotted against the different time points at which the viruses were harvested, respectively (Figure 24). The growth curves show that HSV-1-F, HSV-1-wt replicate with similar growth rates with the main growth starting at 4 h.p.i. HSV-1-gDY seems to replicate slightly, but not significantly, slower than HSV-1-wt and HSV-1-F. Both HSV-1-gDS

and HSV-1-gD2m appear to grow with an even slower growth rate, with the major growth starting at about 8 h.p.i, and slower growth before that. Since the virus solution that was used to infect the cells was not removed after absorption time, the results of this study might be shifted a little by later absorption of some viruses. However, all virus stocks had been grown on complementing VD60 cells and therefore should not absorb with different rates. Also, all viruses were treated exactly the same way, and thus a comparison of the growth rates should be possible using my data. In the plaque assays for determining the titers of the different virus yields at the different time points, all dishes were overlayed with medium supplemented with inactivated human serum containing anti HSV-1 antibodies after absorption, and therefore a slower absorption rate would be apparent in the plaque assays.

Discussion:

1.) Construction plasmids:

Four plasmids were constructed containing a 5.2 kbp HSV-1 sequence surrounding the gD gene and having mutations in the YY1 and/or Sp1 binding sites of the gD gene promoter. These plasmids were constructed in order to introduce the mutations in the gD promoter into the viral genome of HSV-1 by marker transfer. The mutations were derived from the gD-CAT series plasmids that have been constructed in this lab previously by Lisa K Mills (Figure 7). A small fragment of the gD promoter containing the mutations was excised from the gD-CAT plasmids, and inserted into a larger HSV-1 DNA sequence. The gD-CAT series contained the gD gene promoters of HSV-1 strain 17, while the larger DNA sequence surrounding the gD gene was derived from HSV-1 strain KOS. There are some minor but no major differences in the gD gene and promoter of the different strains (49) , and therefore the results of this study should not be affected by using different HSV-1 strains to construct mutant viruses. However, a wild type (wt) control plasmid (and later virus) was constructed using the same methods as for the mutants. This enabled us to directly compare the effect of the mutations in the gD promoter to exactly the same virus having no mutations, and to see whether the use of different strains has any effect on the virus.

The four constructed plasmids, pSJgDY, pSJgDS, pSJgD2m, and pSJwt, were analyzed extensively by restriction analysis. The sizes of the fragments were compared to expected sizes as determined from an analysis of the DNA sequence obtained from the gene-bank (Internet address: http://www.ncbi.nlm.nih.gov) on the Webcutter (Internet address: http://www-biol.univ-mrs.fr/english/logline.html). All restriction digestions yielded the expected fragments, indicating that the four plasmids contained the correct HSV-1 DNA sequence. Restriction analysis using the enzymes for which sites had been inserted into the YY1 and Sp1 binding sites of the gD promoter linearized the plasmids or resulted in the expected fragments (see Figure 17). This confirmed that the gD promoter in the plasmids contained the mutations of the YY1 binding site (pSJgDY), the Sp1 binding site (pSJgDS), both the YY1 and the Sp1 binding sites (pSJgD2m), and none (pSJwt). Sequence analysis of the gD promoter validated that the gD promoter contained the desired mutations but no other mutations. Therefore, the four constructed plasmids were suitable to use for marker transfer in order to insert the mutations into the HSV-1 genome.

2.) Marker Transfer:

Recombinant HSV-1 viruses having mutations in the YY1 and/or Sp1 binding sites of the gD promoter were constructed by cotransfecting infectious HSV-1-FgDβ DNA and the four linearized plasmids pSJgDY, pSJgDS, pSJgD2m, pSJwt into VD60 cells. The plasmids were linearized to avoid incorporation of the whole plasmids

including the pUC18 sequence into the viral genome by a single cross-over event. The use of linearized plasmids for cotransfection allows incorporation of only the HSV-1 DNA sequence into the viral genome by a double cross-over event (as shown in Figure 15). The resulting recombinant viruses would be devoid of the β-galactosidase gene of HSV-1-FgDβ and therefore form colorless plaques under an agarose-overlay containing X-gal. This characteristic was used to identify recombinant viruses. However, since the VD60 cells contain the 6.5 kbp BamH1 'j' fragment of HSV-1 strain F which includes the gD gene and surrounding sequences, homologous recombination might also occur between the cellular F-strain HSV-1 sequence and the HSV-1-FgDβ genome. This also would result in colorless plaques. Thus, selecting colorless plaques by itself is not sufficient to identify recombinant viruses containing the desired mutations in the gD promoter. To accomplish this, PCR was performed on the recombinant viruses using primers that would produce a 958 bp product that included most of the gD promoter (see Figure 16). The primers were chosen to be specific to HSV-1 strain KOS, and not to anneal to HSV-1 strain F. Multiple tests using HSV-1-F virus, HSV-1-FgDβ virus, or cellular DNA only, confirmed that these sequences are not recognized by the primers if used under very stringent conditions (low Mg_2^+ concentration). Therefore, a virus clone giving a positive PCR result could be considered positive. However, to confirm correct promoter structures, the PCR products were analyzed by both restriction analysis (Figure 21) and sequence analysis (Figure 22). Sequence analysis covered the gD promoter region from -142bp to +11bp

which includes all regions known to be required for gD expression (see Figure 7.a). The correct sequence of the gD promoter was confirmed for each of the recombinant viruses. Therefore, there are no other mutations in the gD promoter that might influence gD-gene expression.

However, in the unlikely case that the gD gene and promoter were incorporated into an area other than the correct place in the viral genome, and the β-galactosidase gene was disrupted as well, the viral plaque would appear colorless and the PCR would be positive, but the recombinant virus would not be the desired virus. In order to exclude this possibility, a southern blot of the viral DNA could be performed using a probe specific for the β-galactosidase gene. Would the Southern blot analysis show that the viral DNA does not contain the β-galactosidase gene any more, it would be proven that the gD gene and promoter are inserted in that locus. To get proof that the phenotypes of the constructed viruses are the result of only the gD promoter mutations, the recombinant virus would have to be rescued by co-transfection with a wt-gD-promoter. If this resulted in conversion of the mutant phenotype to a wt-phenotype it would prove that the mutations in the gD-promoter cause the alterations of the virus.

3.) Infectivity of the four mutant viruses:

In order to determine how the infectivities of the four recombinant viruses compare to each other and to the HSV-1 F strain virus, the viruses were titered on VD60 and Vero cells in parallel in duplicate assays, respectively (Table 5). The virus

infectivity on Vero cells was determined by calculating the ratio of the titer on Vero cells to the titer on VD60 cells (Table 5). For an easier comparison, the ratio of the titers of HSV-1-wt was set as 100% . The percentage of infectivity of the virus having mutations in the gD gene promoter, and of HSV-1-F, was calculated in relation to HSV-1-wt and is shown in Table 6 and Figure 23. A t-test analysis showed that the infectivity for HSV-1-gDY, HSV-1-gD2m, and HSV-1-F are statistically different from HSV-1-wt with a probability of 99%, and HSV-1-gDS is statistically different from HSV-1-wt with a probability of 90%. Therefore, the results of the t-test support that there are slight differences in virus infectivity between the different recombinant viruses and HSV-1-F. However, a t-test assumes that the standard-deviation is the same for each value taken into consideration, whereas the standard deviations obtained from my data differ somewhat from each other. In any case, the difference between the infectivity of the four recombinant viruses to each other and to HSV-1-F were very small. This indicates that the YY1 and Sp1 binding sites in the gD promoter affect the virus infectivity very slightly.

The relative titer on Vero cells of the virus containing a mutation in the YY1 binding site (HSV-1-gDY) was consistently lower than that of the HSV-1-wt (85.1 ± 9.8% as compared to 100 ± 1.4% for HSV-1-wt). An intact YY1 binding site has been shown in CAT-assays performed earlier (Figure 8 and Figure 9) to lower virus induced gD-gene expression *in vitro*, and mutation of the YY1 binding site resulted in a higher level of gD-expression than the wt promoter *in vitro*. Therefore it was unexpected that

the virus containing a mutation in the YY1 binding site of the gD promoter would show a lower infectivity than the virus containing a wt promoter. We expected its plaquing efficiency to be higher than that of the wt-virus, if different at all.

The titer of HSV-1-gDS was a little higher than that of the wt virus (113.1 ± 12.15% in relation to HSV-1-wt. Again, the opposite was expected, since Sp1 binding has been shown to play an important role in virus induced gD gene activation *in vitro* (Figure 8 and Figure 9). Therefore, mutation of the Sp1 binding site was expected to result in a lower infectivity of the virus, if there would be any difference at all.

The infectivity of HSV-1-gD2m was even higher than that of HSV-1-gDS. Destruction of both the YY1 and Sp1 binding sites in the gD promoter was expected to result in the lowest virus infectivity corresponding to the lowest *in vitro* CAT-expression from that same promoter (Figure 8 and Figure 9). In that *in vitro* analysis both YY1 and Sp1 seemed to activate the gD promoter, and a promoter containing a double mutation resulted in no activation of gD-gene expression at all. In this study, the virus containing the same double mutation seemed to have the highest virus infectivity.

Taken together, these data suggest that mutation of the YY1 binding site in the gD promoter of HSV-1 results in a slightly lower virus infectivity, whereas mutation of the Sp1 binding site in the gD promoter results in a slightly higher virus infectivity. Mutation of both sites results in an even higher infectivity than mutation in only the Sp1 site. Possible reasons for these results can be speculated as follows.

First, the difference is not very large and might just reflect an inaccuracy in the count of plaques or in the dilution. However, a total of six samples were counted in a statistically significant range, and they consistently gave the same result, and t-test analysis supported that the difference is statistically significant. Second, differences in the level of gD gene expression (as were suggested by the *in vitro* analysis) would not necessarily correspond to the infectivity rate of the virus. Too much gD could somehow interfere with virus growth. Third, the CAT assays were performed in Hela cells, but the infection assays were performed in Vero cells. The two different kinds of cells might contain different amounts of YY1 and Sp1, and this could influence the effect that the YY1 and Sp1 binding sites have on gD gene expression. Also, binding of YY1 and Sp1 to their binding sites in the gD promoter might have a different function in Vero cells than it does in HeLa cells. It has been suggested and demonstrated in several studies, that YY1 binding to its promoter element might cause a different outcome in a different cellular environment (e.g.: 5, 11, 39, 44). Finally, the mutation in the YY1 binding site could cause gD to be expressed with different kinetics than usual, and that could result in a lower virus infectivity. This hypothesis is supported by analysis of another HSV-1 leaky-late promoter, the VP5 promoter (57). Those experiments demonstrated that a critical Sp1 site at -48bp to the RNA cap site is responsible for the late gene expression of the VP5 gene, and destruction of this binding site resulted in a conversion of that promoter into an early promoter. Therefore it is likely that the Sp1 and YY1 binding sites in the gD promoter likewise

regulate the timely expression of the gD gene. In this scenario mutation of the Sp1 binding site would result in mainly early expression of the gD gene and some low level expression caused by the intact YY1 binding site. Mutation of the YY1 binding site would result in a higher than usual level of gD gene-expression at late times, because YY1 could not bind to its binding site any more and thereby downregulate gD gene expression. Mutation of both sites would result in early but no late gD gene expression. This might cause only slight differences in the growth infectivity of the mutant viruses. However, the above are speculations only, and an analysis of gD-mRNA expression will have to be performed before any conclusions can be drawn.

The virus infectivity of HSV-1-F was higher than that of the recombinant wild type virus HSV-1-wt (124.3 $\pm$ 11.21%). This may be due to strain specific differences in gD-mediated interference of HSV-1 infection (49). Since the VD60 cells contain the viral gD gene, low levels of gD might be expressed on the cell surface and could interfere with HSV-1 infection of the cells. Hansi *et al* (49) showed that different HSV strains exhibit different sensitivity to gD-mediated interference, and that slight differences in the gD protein are responsible for that. They showed that HSV-1 strain KOS is about ten times more sensitive to gD interference than is HSV-1 strain F. However, the results obtained in this analysis suggest that HSV-1-F is slightly more sensitive to gD interference than is HSV-1-wt (which contains the KOS strain gD gene in the context of the HSV-1-F genome). This would be in contrast to the results of Hansi *et al.* However, these results could be explained by the fact, that the four

recombinant viruses constructed in this project contain a mutation outside the gD gene that causes them to form syncytia (146). Syncytia formation results in a direct cell-to-cell spread of the virus rather than release of progeny virus and infection of adjacent cells. In this case gD interference might not play a role. Also, VD60 cells are not designed to express the gD gene without viral infection, and therefore they would express only low levels of gD if at all prior to infection. An alternative explanation is that there is no significant difference in gD-interference between HSV-1-F and HSV-1-wt, since other viral glycoproteins are involved in gD mediated entry into the host cells (e.g. gB, and gH) (7), and these other proteins are derived from the HSV-1 F strain in both the HSV-1-F and HSV-1-wt viruses.

4.) One step growth curve of the four recombinant viruses:

In the above analysis the viruses HSV-1-gDY and HSV-1-wt seemed to grow faster than the viruses HSV-1-gD2m and HSV-1-gDS since the plaques appeared to be larger at earlier times. To test the growth rate of the mutant viruses compared to each other and to HSV-1-F a one-step growth curve was performed. Vero cells were infected with a multiplicity of infection of 2, and at 0, 4, 8, 12, 16.5, 20, and 24 hours post infection the infected cells were harvested and virus was released by sonication. The titers of each sample were determined by assaying the viruses on Vero cells and counting the plaques resulting from that (Table 7). A plot of the results (Figure 24) shows that the virus containing a mutation in the YY1 binding site of the gD promoter

(HSV-1-gDY) grows with approximately the same rate or very slightly slower as the wild type virus HSV-1-wt and HSV-1-F. The viruses containing the mutations in the Sp1 binding site (HSV-1-gDS) and in both the YY1 and Sp1 binding sites (HSV-1-gD2m) both exhibit a noticeable even though not markedly slower growth rate. These differences were expected, but we expected the differences in the growth rates to be much more prominent.

These data support the above results that mutation of the YY1 and/or Sp1 binding sites in the gD promoter of HSV-1- have a slight but not significant affect on the viral replication, with mutation of the Sp1 binding site playing the main role. Therefore, mutation of the Sp1 binding site in the gD promoter somewhat slows down the replication of HSV-1 even though not very significantly, and mutation of the YY1 binding site of the gD promoter has only a slight or no affect on the HSV-1 replication.

Considering the results of the *in vitro* CAT assays with the gD promoter, it can be speculated that mutation of the YY1 binding site might result in overexpression of the gD gene and that this overexpression has only a slight effect only on viral replication. Mutation of the Sp1 site or both the Sp1 site and the YY1 site, however, might result in lower gD expression and this would lead to a somewhat slower viral replication. However, these are pure speculations and no final conclusions can be drawn before a quantitative mRNA analysis was performed. In this analysis, mRNA levels of the four recombinant viruses and HSV-1-F should be determined at different times during the replication cycle.

In summary, the results of my study show that the Sp1 and YY1 binding sites in the gD promoter are not essential for HSV-1 growth *in vivo*, and do not significantly alter the yield or growth rate of viruses containing those mutations. Mutation of the YY1 binding site (HSV-1-gDY) resulted in a very slightly lower yield and had little impact on the growth rate. Mutation of the Sp1 binding site (HSV-1-gDS) resulted in a slightly higher yield and a noticeable but little slower growth rate of HSV-1. A virus containing a double mutation (HSV-1-gD2m) seemed to behave similar to the virus containing a mutation in the Sp1 binding site only, but it exhibited an even slightly higher yield and growth rate as compared with the Sp1 mutation only. A complete repression of gD gene expression by the mutations would be apparent in these analyses, since a HSV-1 virus lacking gD is not able to form plaques on Vero cells at all (87). Therefore, the mutation introduced into the virus might alter but do not completely shut down gD gene expression.

Mutations in the Sp1 and YY1 binding sites of the gD promoter might, however, alter the kinetics and/or quantity of gD gene expression without having a significant effect on viral growth yield or growth rate. This remains to be tested by an mRNA analysis. Such an analysis would include the mRNA isolation from the four recombinant viruses at different times post infection, covering the time of early gene expression (~ 8 h.p.i.) and late gene expression (~12 h.p.i.). This mRNA could be quantitated by either RNAse protection assay or primer extension analysis. The results of this analysis would demonstrate the effect that the YY1 and Sp1 binding sites have

on the level of gD gene expression at early and at late times during infection. It also is important to analyze the mutant and wt viruses on other cells such as HeLa cells, since the YY1 and Sp1 levels might differ in different cell lines, and this as well as other cell specific factors might affect the impact of the mutations. Finally, it remains to be proven that the phenotype of the recombinant viruses really is caused by the mutations in the YY1 and/or Sp1 binding sites of the gD promoter and no mutations other than those. In order to do so, the mutant viruses would have to be rescued by cotransfection with a wt (strain KOS) gD gene and promoter, and if that would convert the mutant phenotype to wt phenotype, the proof would be complete. However, since the differences we found so far were very small, this would not be a very significant analysis.

A following project using the here constructed recombinant viruses could be the analysis of the Sp1 and/or YY1 binding site mutations in concert with mutations in the 3 ICP4 binding sites of the gD gene promoter, or with a virus containing mutations in the transactivation domain (TAD) or other domains of the ICP4 protein. The presence of the viral proteins ICP4 and 0 are critical for gD gene expression (Figure 9), and the ICP4 protein binds to three sites in the wt gD promoter and thereby activates *in vitro* transcription from this promoter (159). It is possible that YY1 or Sp1 interact with ICP4 to regulate transcription, or that YY1 and Sp1 act differently in the presence or absence of ICP4. In any case, results of such an analysis would be interesting and

might help to better understand the regulation of the gD gene in the context of the viral genome.

Plasmid	Restriction enzyme	Expected fragments [kbp]	Obtained fragments [kbp]
pSJgDY, pSJgDS, pSJgD2m, pSJwt	MluI	4.01 3.87	} 3.75 3.6
	SacI	3.374 2.843 1.67	3.3 2.75 1.75
	AccI	3.817 2.876 1.189	3.62 2.02 1.18
	ScaI/HindIII	4.073 3.809	3.85 3.6
	BamHI	7.88	7.6
	SphI/ScaI	3.89 3.10 0.926	3.95 3.13 0.91
pSJgDY	Xho1	7.88	7.882
	BglII	uncut	uncut
	EcoRI	7.88	7.88
pSJgDS	BglII	7.88	7.882
	XhoI	uncut	uncut
	EcoRI	uncut	uncut
pSJgD2m	Xho1	7.88	7.882
	EcoRI	5.89 1.989	5.75 1.95
	BglII	uncut	uncut
pSJwt	XhoI	uncut	uncut
	BglII	uncut	uncut
	EcoRI	7.88	7.882

Table 1. Fragment sizes of restriction digest of recombinant plasmids (Figure 17)

This table shows the obtained and expected fragment sizes after restriction analysis of the constructed recombinant plasmids pSJgDY, pSJgDS, pSJgD2m, and pSJwt. The first part of the table shows the results of restriction digestion with enzymes cutting the four plasmids the same way. The second part shows results of restriction analysis using enzymes specific for each plasmid.

mutant virus being isolated	total number of plaques	number of colorless appearing plaques	percent colorless plaques [%]	number of colorless plaques screened by PCR and restriction analysis	number of positive virus clones	percent of colorless plaques positive for desired virus [%]	frequency of virus recombination [%]
HSV-1-	~600	24	4%	16	2	12.5%	0.5%
HSV-1-	~600	24	4%	14	1	7.14%	0.3%
HSV-1-	~600	24	4%	14	2	14.0%	0.6%
HSV-1-wt	~600	23	3.8%	14	1	7.14%	0.3%

Table 2. Recombination frequency after co-transfection:

HSV-1-FgDβ DNA and the linearized plasmids pSJgDY, pSJgDS, pSJgD2m, and pSJwt were cotransfected into VD60 cells (Figure 15). Colorless plaques were picked and screened by PCR and restriction analysis of the PCR product. The frequency of colorless appearing plaques and of positively identified colorless plaques from the ones screened by PCR and restriction analysis was calculated. The approximate frequency of virus recombination was determined from the percent of colorless appearing plaques and the percent of positive virus clones after PCR and restriction analysis screening. It reflects the percentage from the 600 plaques plated of viral recombinants containing the desired mutation.

Virus clones	Restriction enzyme	Expected fragments [kbp]	Obtained fragments [kbp]
HSV-1-gDY	none	0.958	0.96
	XhoI	0.880	0.90
HSV-1-gDS	none	0.958	0.96
	BglII	0.880	0.90
HSV-1-gD2m	none	0.958	0.96
	XhoI	0.880	0.90
	EcoRI	0.880	0.90
HSV-1-wt/ HSV-1-F	none	0.958	0.96
	SacII	0.401 0.308 0.249	0.42 0.31 0.257

Table 3. Restriction fragments of PCR products (Figure 20)

This table shows the results of restriction digestion of the PCR products after screening the viral clones for recombinants. The expected fragment sizes and the obtained fragment sizes are shown. Figure 20 shows an agarose gel of these results.

Virus clones	Restriction enzyme	Expected fragments [kbp]	Obtained fragments [kbp]
HSV-1-gDY	XhoI	0.888	0.90
	BglII	0.958	0.96
	EcoRI	0.958	0.96
	SacII	0.401 0.308 0.249	0.42 0.31 0.257
HSV-1-gDS	BglII	0.888	0.90
	XhoI	0.958	0.96
	EcoRI	0.958	0.96
	SacII	0.401 0.308 0.249	0.42 0.31 0.257
HSV-1-gD2m	XhoI	0.888	0.90
	EcoRI	0.888	0.90
	BglII	0.958	0.96
	SacII	0.401 0.308 0.249	0.42 0.31 0.257
HSV-1-wt	XhoI	0.958	0.96
	EcoRI	0.958	0.96
	BglII	0.958	0.96
	SacII	0.401 0.308 0.249	0.42 0.31 0.257

Table 4. Restriction fragments of PCR products (Figure 21)

The results of restriction analysis of the PCR products obtained from screening the plaque-purified viral clones are shown for each recombinant virus. The expected fragment sizes as well as the obtained fragment sizes are depicted. Figure 21 shows these results on an agarose gel.

Titer (pfu/ml$\pm\sigma_{n-1}$)	VD60 cells	Vero cells	Vero/VD60 -ratio
HSV-1-gDY	$5.96\pm0.62\times10^8$	$10.8\pm0.56\times10^8$	1.81 ± 0.21
HSV-1-gDS	$3.87\pm0.49\times10^8$	$9.45\pm0.53\times10^8$	2.44 ± 0.34
HSV-1-gD2m	$3.1\pm0.27\times10^8$	$8.76\pm0.26\times10^8$	2.26 ± 0.26
HSV-1-wt	$12.2\pm0.12\times10^8$	$26.1\pm0.18\times10^8$	2.14 ± 0.03
HSV-1-F	$1.2\pm0.10\times10^8$	$3.2\pm0.10\times10^8$	2.68 ± 0.24

Table 5. Titer of the four recombinant viruses and HSV-1 F strain on VD60 and Vero cells:

The four recombinant viruses and HSV-1 F strain were titered on VD60 and Vero cells. Three different dilutions of each virus were plated out in duplicate, so as to provide 50 to 500 plaques per 9.8 cm^2 well. The plaques were counted and the titers and standard deviations (σ_{n-1}) were calculated. The ratio of the titer on Vero cells to the titer on VD60 cells provides a measure of any replication impairment of the mutant viruses.

relative infectivity	Vero/VD60 ratio	Vero/VD60 [%±σ_{n-1}]
HSV-1-gDY	1.8±0.21%	84.6±9.8%
HSV-1-gDS	2.44±0.34%	114±15.8%
HSV-1-gD2m	2.26±0.26%	105.6±12.15%
HSV-1-wt	2.14±0.03%	100±1.4%
HSV-1-F	2.68±0.24%	125.2±11.21%

Table 6. Percentage of infectivity of the three mutant viruses and HSV-1-F as compared to the recombinant wt (wild type) virus:

To better compare the virus yields of the four recombinant viruses and HSV-1 F strain to each other, the ratio of the titer on Vero cells to the titer on VD60 cells (as shown in the first column) for HSV-1-wt was set as 100% infectivity. The percentage of infectivity for the mutant viruses and HSV-1-F was calculated from the ratios of the titers on the two cell lines (first column) in relation to HSV-1-wt. The standard deviation (σ_{n-1}) was adjusted to percent as well.

Table 7. Titers and standard deviations (σ_{n-1}) of the one-step growth curve (growth rates):

Vero cells were infected at an m.o.i.=2 with the four recombinant viruses and HSV-1-F. At 0, 4, 8, 12, 16.5, 20, and 24 h.p.i. the cells were harvested and virus was released by sonication. The resulting virus lysates were assayed on Vero cells, and the titer in plaque forming units per milliliter [pfu/ml]and standard deviation (σ_{n-1}) were determined and are shown in the Table 7. The data are plotted in Figure 24.

Table 7. Titers and standard deviations (σ_{n-1}) of the one-step growth curve (growth rates):

recombinant virus	**0 h.p.i.** [pfu/ml±σ_{n-1}]	**4.0 h.p.i.** [pfu/ml±σ_{n-1}]	**8.0 h.p.i.** [pfu/ml±σ_{n-1}]	**12.0 h.p.i.** [pfu/ml±σ_{n-1}]	**16.5 h.p.i.** [pfu/ml±σ_{n-1}]	**20.0 h.p.i.** [pfu/ml±σ_{n-1}]	**24.0 h.p.i.** [pfu/ml±σ_{n-1}]
HSV-1-gDY	2.0±0.14 x 10^6	6.9±0.08 x 10^5	4.84±0.45 x 10^6	5.38±0.08 x 10^7	1.64±0.61 x 10^8	1.58±0.22 x 10^8	1.48±0.46 x 10^8
HSV-1-gDS	2.63±0.39 x 10^6	1.23±0.17 x 10^6	5.16±0.3 x 10^6	6.16±0.02 x 10^7	1.27±0.24 x 10^8	1.26±0.23 x 10^8	1.13±0.34 x 10^8
HSV-1-gD2m	3.77±0.43 x 10^6	1.62±0.07 x 10^6	8.54±0.18 x 10^6	8.38±0.55 x 10^7	1.30±0.12 x 10^8	1.40±0.09 x 10^8	1.47±0.47 x 10^8
HSV-1-wt	3.03±0.15 x 10^6	1.24±0.01 x 10^6	2.1±0.17 x 10^7	1.36±0.06 x 10^8	2.21±0.42 x 10^8	2.28±0.5 x 10^8	1.46±0.33 x 10^8
HSV-1-F	5.02±0.14 x 10^6	2.93±0.17 x 10^6	4.01±0.36 x 10^7	2.27±0.78 x 10^8	4.36±0.14 x 10^8	4.01±2.18 x 10^8	1.08±0.7 x 10^8

Figure 1. Structure of herpesviruses

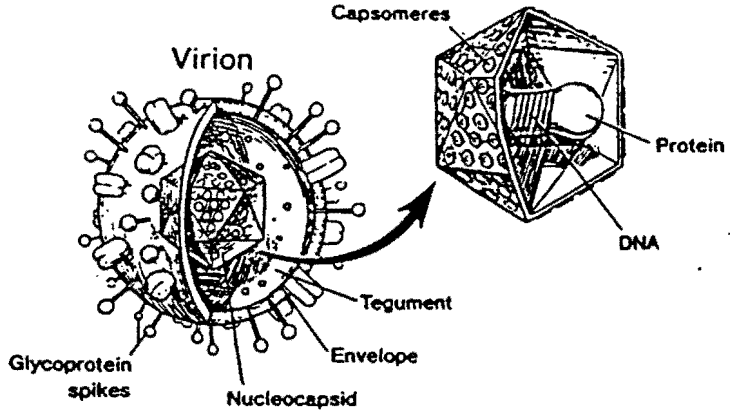


Figure 1. Schematic drawing of a herpesvirus. The protein of the herpesvirus is surrounded by the DNA, much like thread on a spool. A protein structure called the capsid is in the shape of an icosahedron and surrounds this DNA genome core. This combined structure is called a nucleocapsid. An additional 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 2. Schematic representation of the arrangements of DNA sequences of HSV

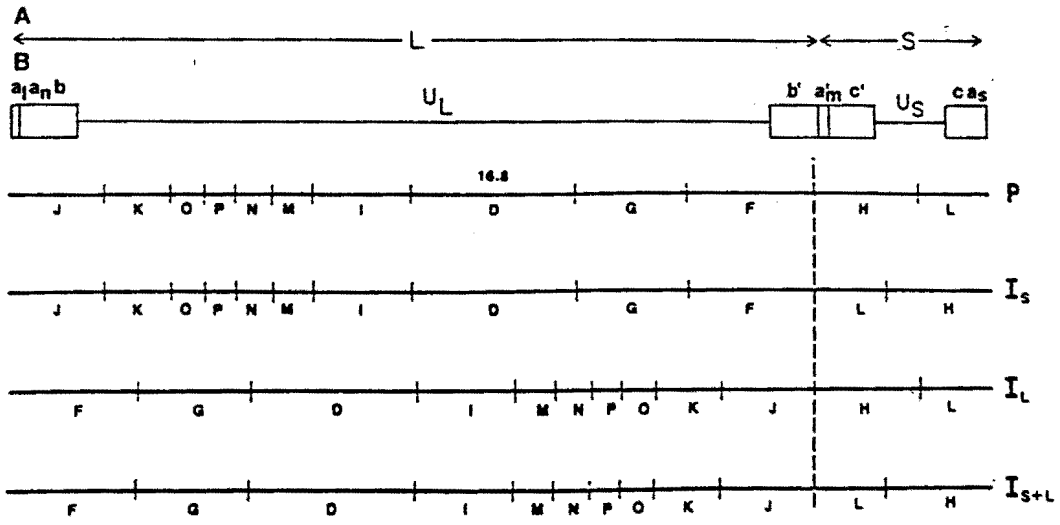


FIG. 2. Schematic representation of the arrangement of DNA sequences in the HSV genome. **A:** The domains of the L and S components are denoted by the *arrows*. The second line shows the unique sequences (*thin lines*) flanked by the inverted repeats (*boxes*). The letters above the second line designate the terminal *a* sequence of the L component (a_1), a variable (n) number of additional *a* sequences, the *b* sequence, the unique sequence of the L component (U_L), the repetitions 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 *et al.* 1996

Figure 3. Functional organization of the HSV genome

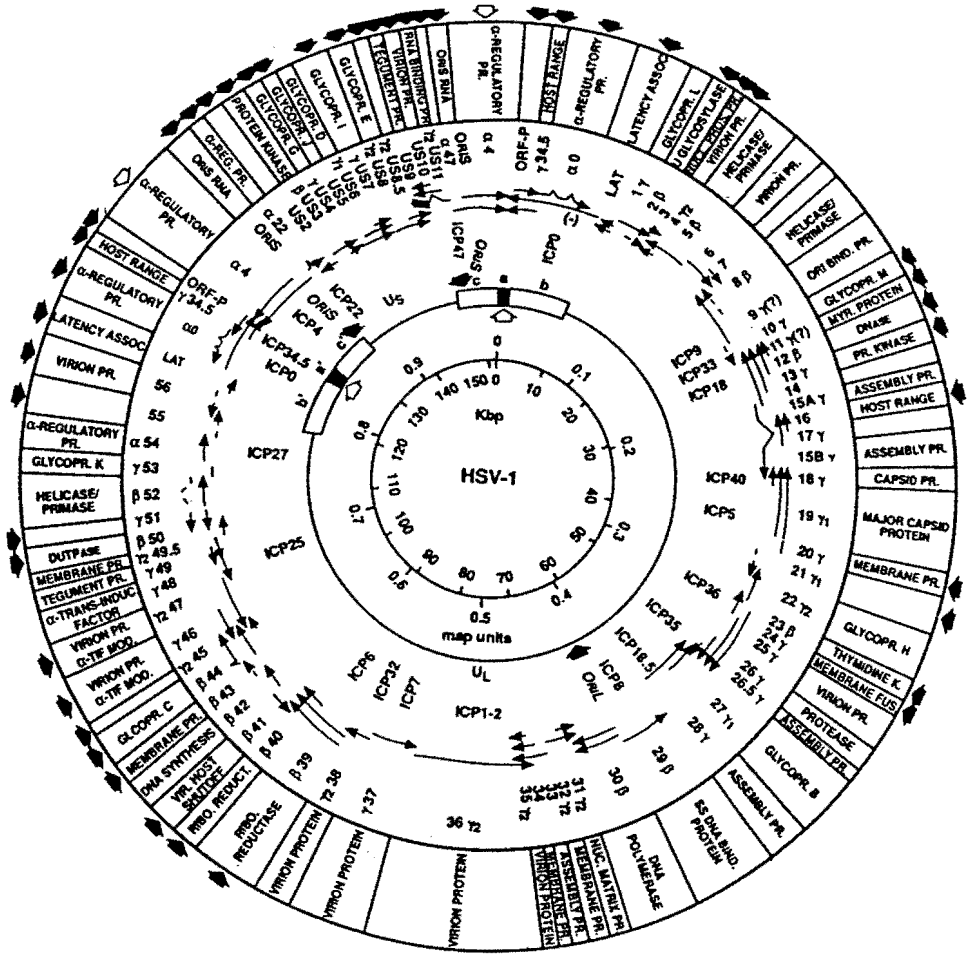


FIG. 8. Functional organization of the HSV-1 genome. The circles are described from inside out. Circle 1: Map units and kilobase pairs. Circle 2: Sequence arrangement of HSV genome. The letters *ab*, U_L, and *b´a´* identify the long (L) component consisting of the unique sequences U_L flanked by the inverted repeats. The letters *a´c´*, U_S, and *ca* identify the corresponding sequences of the short (S) component. The *open arrow* shows the sites of cleavage of concatemeric or circular DNA to yield linear DNA. Since the L and S components can invert relative to each other, the arrangement shown is that which would yield the "prototype arrangement" if linearization were to occur by cleavage of the DNA between map units 0 and 100. The *filled arrows* represent the three origins of viral DNA synthesis, one in the middle of U_L (OriL) and two (both designated OriS) within the inverted repeats flanking the S component. Circle 3: The transcriptional map of the HSV-1 genome. The map serves the purpose of identifying the direction of transcription, the approximate initiation and termination sites, and the families of 3´ coterminal transcripts. *Dashed lines* identify transcripts mapped imprecisely. The designation between the second and third ring identify proteins encoded by the transcripts according to their ICP number. The designations outside this ring identify the open reading frame (those mapping in U_S have the prefix US) number and the kinetic class (α, β, or γ) to which they belong. Circle 4: The known functions of the proteins specified by the open reading frames. The *filled arrows* identify open reading frames which can be deleted without affecting the ability of the virus to multiply in cells in culture. The *open arrows* identify the two copies of the α4 gene; only one copy of this gene can be deleted without affecting the capacity of the virus to multiply. The data for the circles 3 and 4 are derived from refs. 11, 28, 53, 81, 97, 113, 125, 150, 168, 200, 201, 230, 255, 280, 346, 371, 402, 402, 411, 416, 448, 481, 484, 523, 537, 540, 614, 707, and 716.

from Roizman *et al.* 1996 (31, and references within)

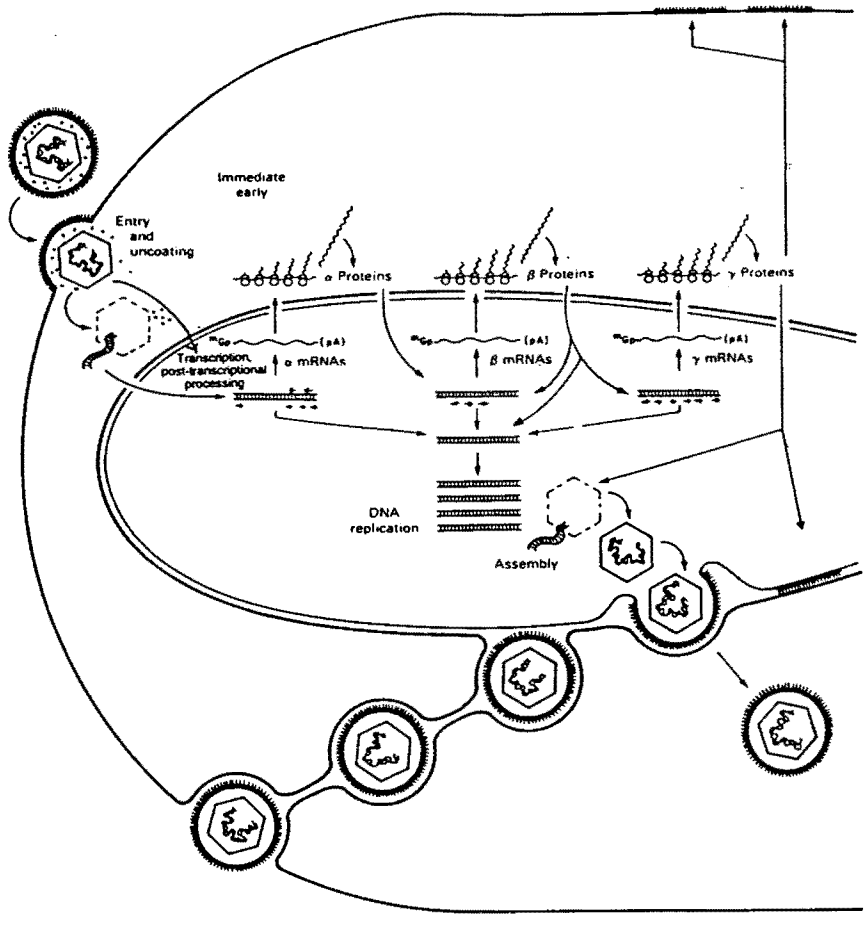


Figure 4. HSV replication

Sequence of events in the multiplication of herpes simplex virus from entry of virus into cell by fusion of the virion envelope with the cell plasma membrane to assembly of virions and their exit from the cell through the endoplasmatic reticulum. Also illustrated are transcription and coordinadet sequential processing of mRNAs and synthesis of sets of proteins ($\alpha \rightarrow \beta \rightarrow \gamma$) required for DNA replication and virion structure. (Modified from diagram kindly supplied by B. Roizman, University of Chicago). modified from R.Dulbecco *et al.* 1988

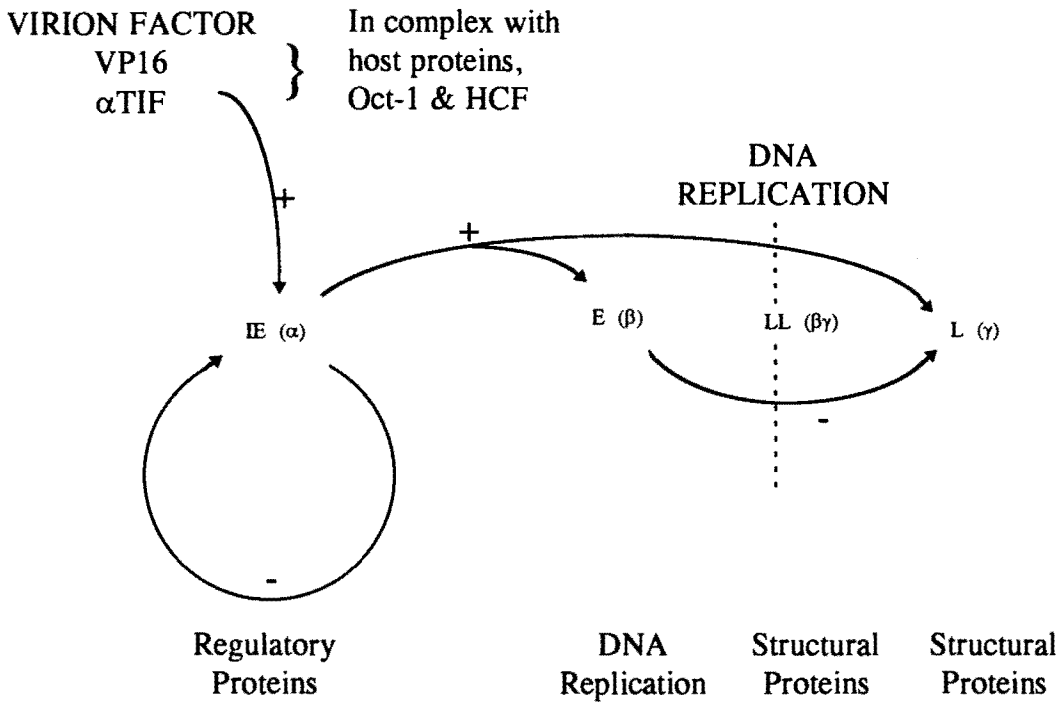


Figure 5. Regulation of HSV gene expression

A schematic picture of the sequentially ordered and coordinally regulated gene expression of HSV. The immediate-early (IE, α) proteins are transcribed first, and the tegument protein VP16 (α-TIF) enhances IE-transcription. The IE proteins autoregulate their own transcription, and at the same time activate early or delayed-early (E, EL, β) gene expression. E proteins turn on DNA replication and activate late (L, γ) gene expression, and downregulate IE gene transcription. True-late (L, γ) genes start being transcribed only after onset of DNA replication, but leaky late (LL, βγ) genes are transcribed at low levels prior to DNA synthesis and have their peak expression with L-kinetics. Late and leaky-late gene expression is activated by IE proteins and repressed by DE proteins.

Figure 6. HSV latency

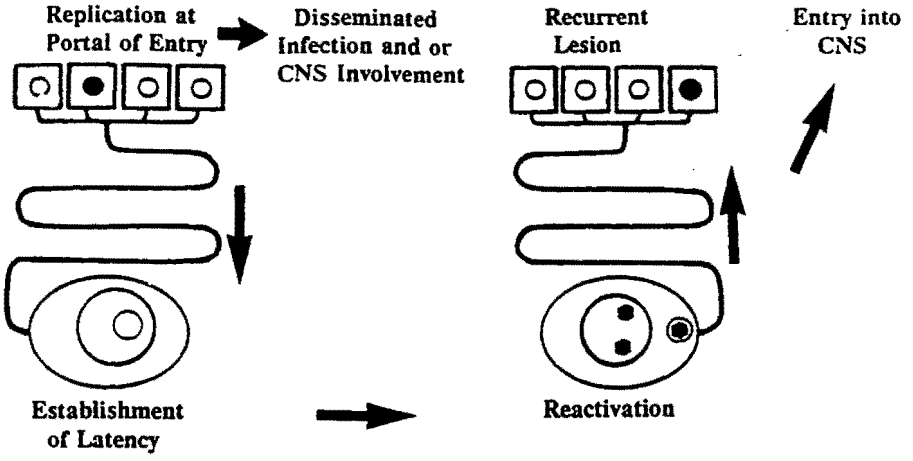


FIG. 12. Natural course of HSV infection *in vivo*. Virus first replicates in epithelial cells (*squares*) at the portal of entry and then moves through neurites (*curved lines*) to establish latent infections in neurons (*ovals*).

from Roizman *et al.* 1996

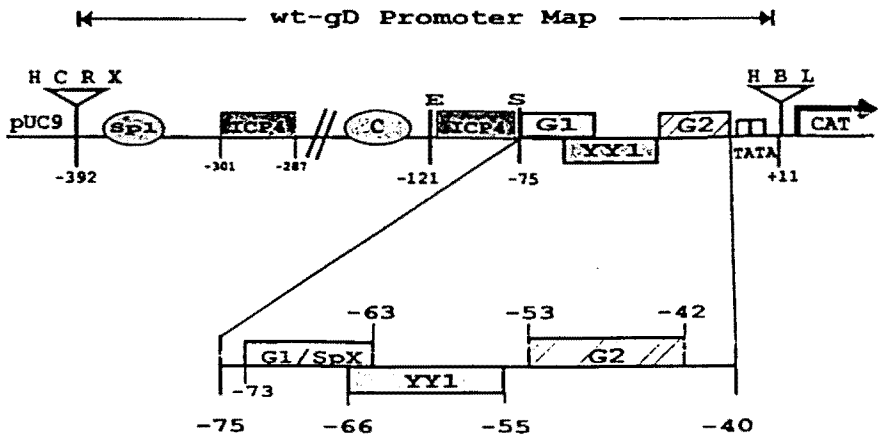
Figure 7. gD-CAT plasmid series

a.) Detailed map of protein binding sites in the wt-gD-392 promoter, (not drawn to scale) in the gD-CAT plasmid series. 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. Restriction enzyme sites are indicated as follows: H=HindIII, C=ClaI, R=EcoRI, X=XhoI, E=EagI, S=StyI, B=BamHI, L=SalI. (by Lisa K. Mills, 1994)

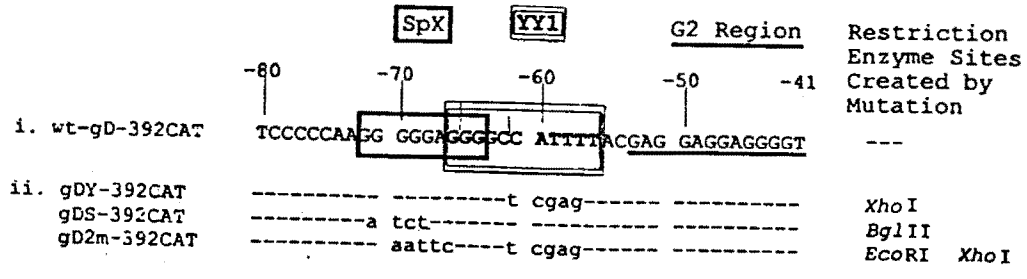
b.) Sequences of the site-directed mutations constructed in the gD-392-CAT promoter series. (i) Wild-type gD promoter sequence from -80 bp to -41 bp. The SpX site is boxed, the YY1 site is boxed in bold, and the G2 region is single underlined. (ii) Sequences of the indicated gD-392 CAT series mutations showing only the bases changed in the mutations. (by Lisa K. Mills, 1994).

Figure 7:

a.): Model of the gD promoter (by Lisa K. Mills):



b.) Sequences of the mutations in the YY1 and Sp1 binding sites of the gD-promoter (by Lisa K. Mills):



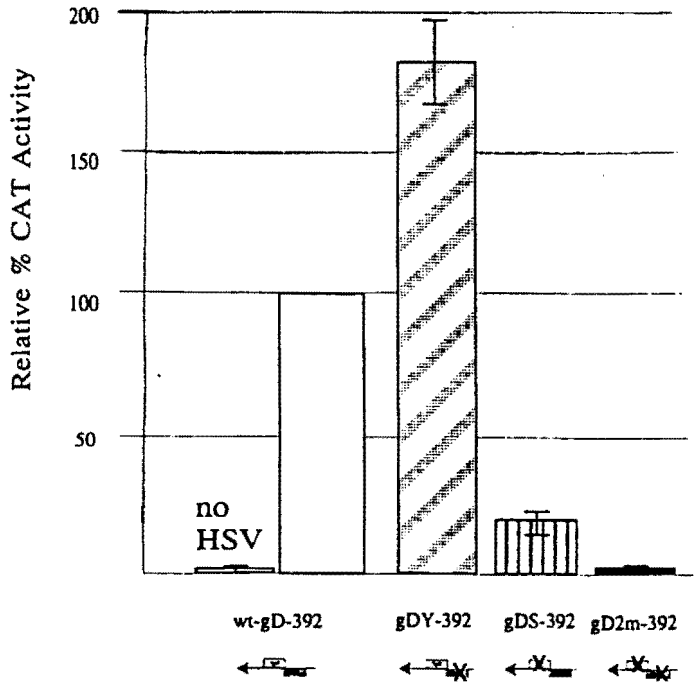


Figure 8. CAT activity of the gD-CAT plasmid series following HSV-1 superinfection

Comparison of CAT activity induced by HSV-1 superinfection after transfection of wt-392CAT, gDY-392CAT, gDS-392CAT, and gD2m-392CAT into HeLa cells. Plasmid and promoter descriptions are indicated below the appropriate bars.

By Lisa K. Mills, 1994

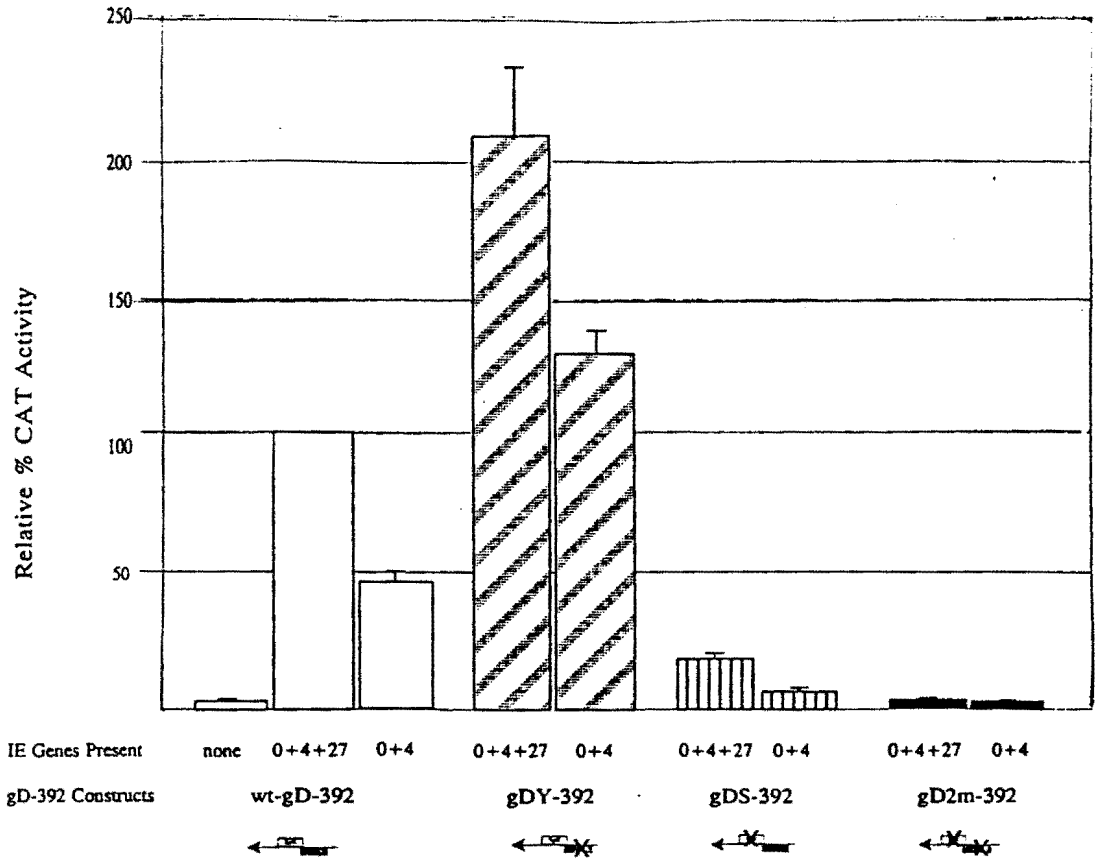


Figure 9. Relative CAT activity from the gD-392 CAT series following cotransfection with ICP0+2+27 and ICP0+4

Comparison of CAT activity from wt-gD-392CAT, gDY-392CAT, gDS-392CAT, and gD2m-392CAT, induced by ICP0+4+27 or ICP0+4 cotransfection into HeLa cells. The IE gene-expressing plasmids included in the cotransfection are specified below the appropriate bars. Plasmids and promoter descriptions are indicated below the effector plasmids.

By Lisa K. Mills, 1994

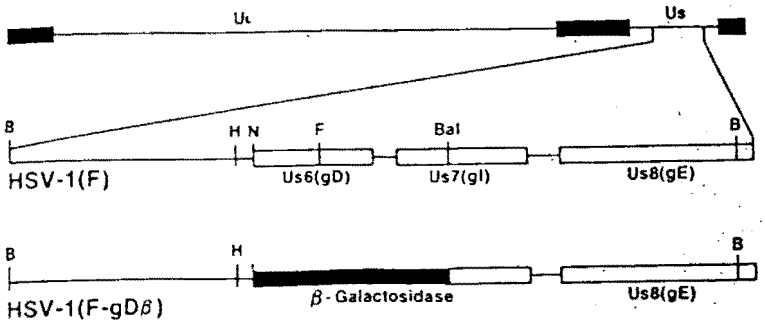


Figure 10. Schematic representation of the DNA sequence arrangements of the HSV-1-FgDβ virus

The region of HSV-1 DNA containing the US6 (gD), US7 (gI), and US8 (gE) genes is depicted for HSV-1 F and the recombinant F-gDβ. In F-gDβ β-galactosidase sequences replace gD structural sequences and part of the gI gene from an NcoI site near the initiation codon of gD to a BalI site in the gI gene. Abbreviations: B=BamHI, Bal=BalI, H=HindIII, F=FspI, N=NcoI, U_L=long unique segment, U_S=short unique segment.

by M.W.Ligas and D.C.Johnson, 1988 (87, and reference within)

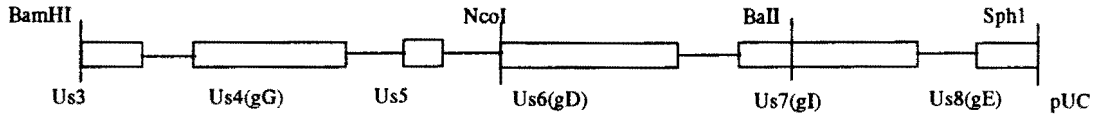


Figure 11. Schematic representation of the DNA sequence arrangements in the HSV-1 BamHI-Sph1 fragment containing the gD gene and surrounding sequences

The region of HSV-1 DNA containing the gD gene and surrounding sequences as it was used for construction of recombinant plasmids is depicted. The region was derived from the BamHI 'j' fragment of the unique short segment (Us) of the HSV-1 KOS genome. BamHI cuts at the end o the Us3 gene, and Sph1 cuts in the first part of the Us8 (gE) gene. Genes are shown by open boxes, and the name of the gene is specified below the appropriate box. The most important restriction enzyme sites are shown. In the HSV-1-FgDβ virus the region from the NcoI site to the BalI site is replaced by the β-galactosidase gene (see Figure 10). Abbreviations: B=BamHI, Bal=BalI, N=NcoI, S=Sph1, Us=short unique segment.

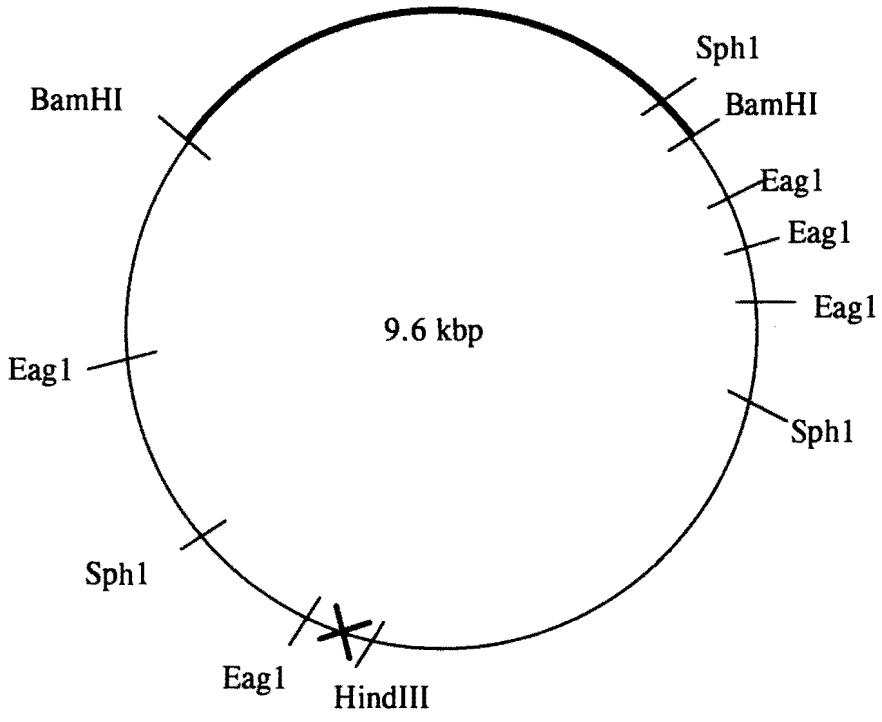


Figure 12. Structure of the plasmid pBJ65:

The structure and the for my purposes important restriction enzyme sites of the plasmid pBJ65 are shown. This plasmid contains the 6.5kbp BamHI 'j' fragment in the pUC18/HIII$^-$ vector, and was used as the starting plasmid for the construction of the four recombinant plasmids (pSJgDY, pSJgDS, pSJgD2m, pSJwt) comprised of the gD gene containing mutations in the YY1 and Sp1 binding sites of its promoter, and flanking sequences. The pUC18/HIII$^-$ vector is drawn in bold, and the HSV-1 sequence is drawn as a simple line. The sites that were to be mutated are indicated by the '×'.

Figure 13. Construction of recombinant plasmids

This schematic figure shows the construction of the recombinant plasmids pSJgDY, psJgDS, psSJgD2m, and pSJwt starting from the plasmid pBJ65 that contains the BamHI 'j' fragment of HSV-1. The thick lines represent DNA sequences of the pUC18 vectors, and the thin lines indicate HSV-1 sequences. The mutation sites (both YY1 and Sp1) are indicated by a cross.

The HSV-1 BamH1 'j' fragment was excised form the plasmid pSJ-25 and cloned into a pUC18/HIII$^-$ vector (not shown in the figure). This resulting plasmid (pBJ65) was digested with Sph1, yielding three fragments. The fragment containing the pUC18-vector and about 1 kbp of the HSV-1 sequence was recircularized and isolated (pSJ38 vector). The fragment containing about 3.9 kbp of HSV-1 sequence including the gD promoter was isolated and cloned into a second pUC18/HIII$^-$ vector (pSL39). Into this plasmid, four different 132bp Eag1/HindIII fragments, that had been excised from the gD-CAT series of plasmids and that contained the different mutations in the gD promoter, were inserted (pSL39 mutants). The four pSL39 mutants were digested with Sph1 to excise the HSV-1 sequence containing the different mutations, and this piece was cloned into the pSJ38 vector.

Figure. 13:

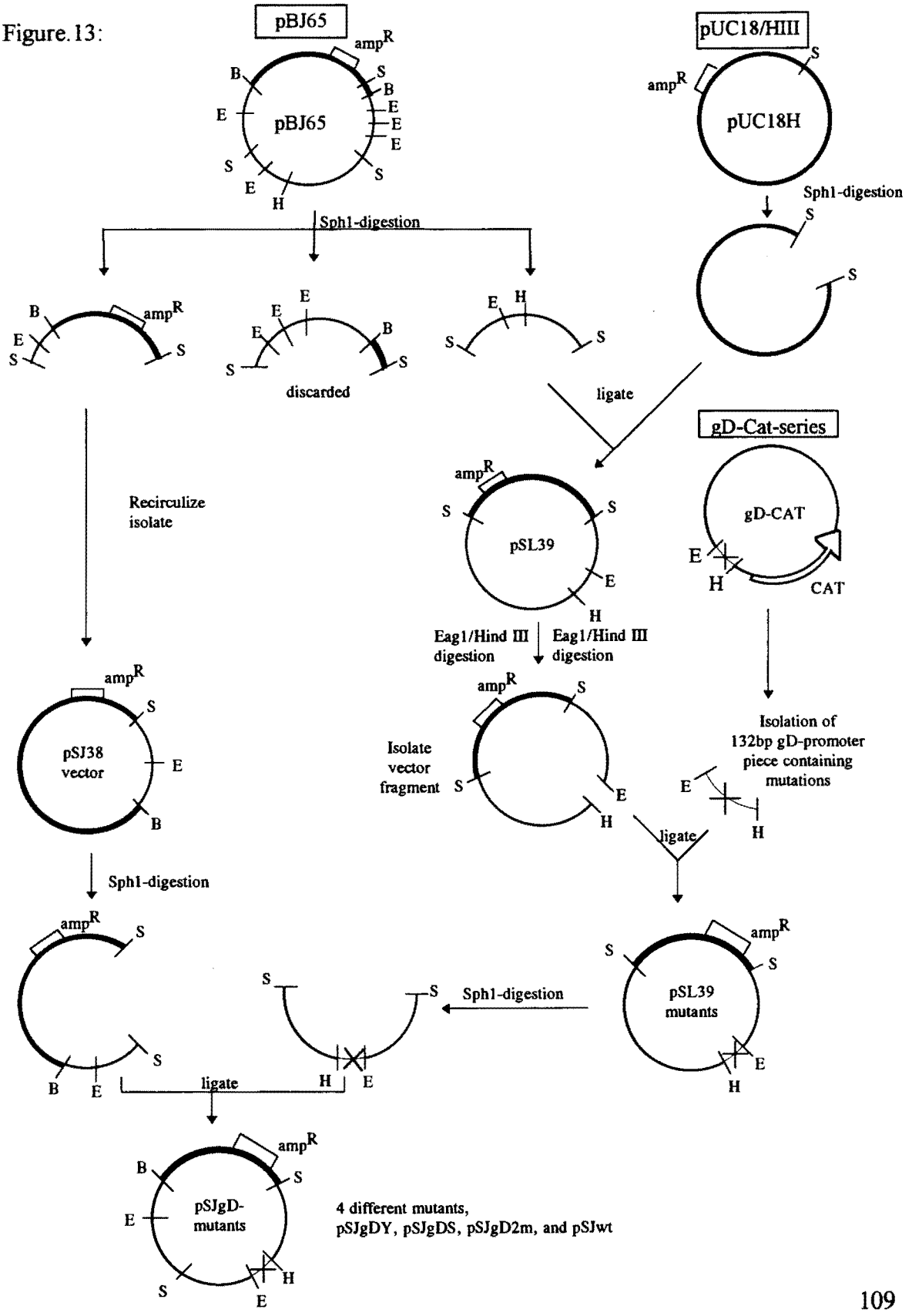

pBJ65
pBJ65
pUC18/HIII
pUC18H
Sph1-digestion
Sph1-digestion
discarded
ligate
gD-Cat-series
gD-CAT
CAT
pSL39
Recirculize isolate
Eag1/Hind III digestion
Eag1/Hind III digestion
pSJ38 vector
Isolate vector fragment
Isolation of 132bp gD-promoter piece containing mutations
ligate
Sph1-digestion
pSL39 mutants
Sph1-digestion
ligate
pSJgD-mutants
4 different mutants,
pSJgDY, pSJgDS, pSJgD2m, and pSJwt

Figure 14. Structure, DNA-sequence arrangement, and mutation sites of the recombinant plasmids pSJgDY, pSJgDS, psJgD2m, and pSJwt

A: A map of the plasmids is shown, with the most important restriction sites indicated. The pUC18/HIII^{-} sequence is shown in bold, and the HSV-1 sequence in a simple line. The site of mutation is indicated by the cross.

B: The DNA sequence arrangement of the 5.2 kbp HSV-1 sequence contained in the plasmids is shown. Genes are drawn as open boxes, with the name of the gene under the appropriate box. Restriction enzyme sites and the names of the enzymes are shown, and the mutation site is indicated by the cross.

C: The sequence of the gD promoter from -80 to -41 as contained in the four recombinant plasmids (pSJgDY, pSJgDS, pSJgD2m, and pSJwt) is shown. The name of the plasmid is depicted at the left side of the sequences, and the restriction enzymes cutting in the mutated sites are shown at the right. In the wt gD promoter (pSJwt) the G2 region is indicated by underlining, and the YY1 and SpX binding sites are indicated by a box, with the SpX binding site in bold. The sequences of the recombinant gD promoters is shown only at the mutated sites. (the picture was derived and modified from a picture by Lisa K. Mills, 1994).

Figure 14. Structure and DNA sequence arrangement of the recombinant plasmids pSJgDY, pSJgDS, pSJgD2m, and pSJwt:

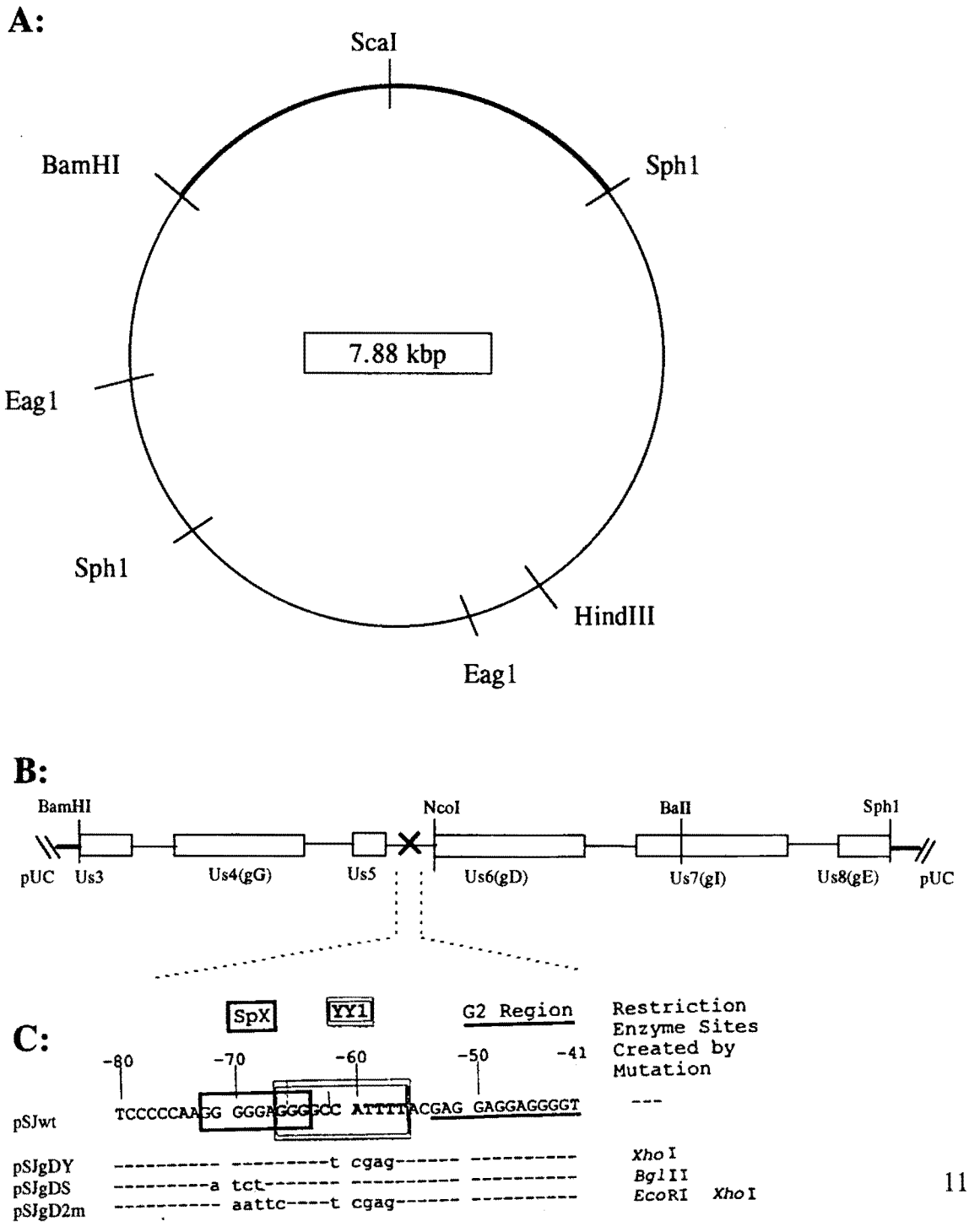
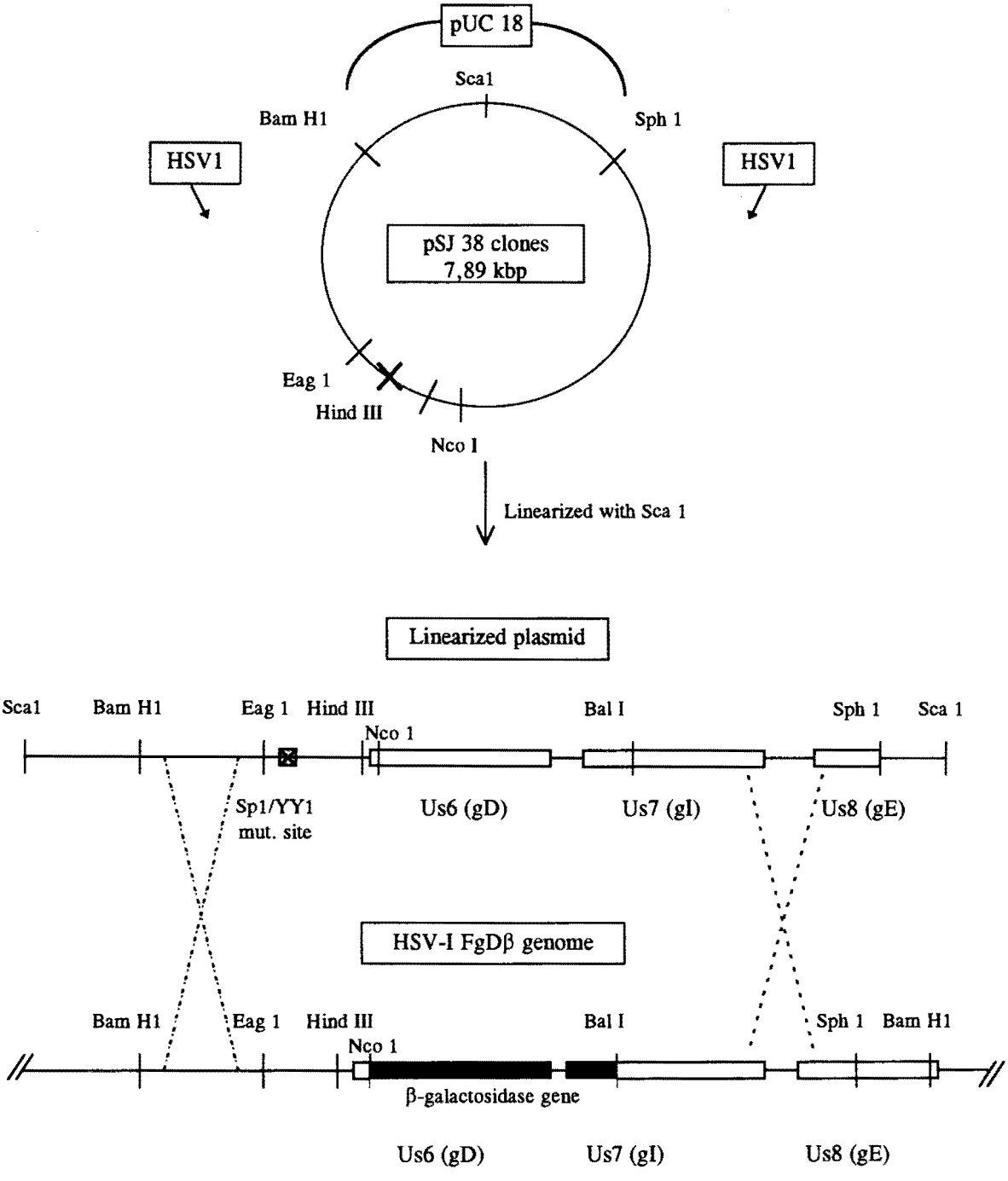


Figure 15. Marker Transfer

A schematic representation of the construction of the four recombinant viruses by marker transfer is shown. Important restriction enzyme sites are depicted, and genes are shown as open boxes or as a black box in the case of the β-galactosidase gene, and labeled below with the appropriate names. The regions in which cross over can occur are indicated by the dotted lines forming crosses. The site containing the mutations in the gD promoter is specified by a cross.

The plasmids pSJgDY, pSJgDS, pSJgD2m, and pSJwt were linearized with ScaI. The linearized plasmids were cotransfected by the calcium phosphate cotransfection method into the complementing VD60 cell line together with infectious HSV-1-FgDβ DNA.

Figure. 15 Market transfer:



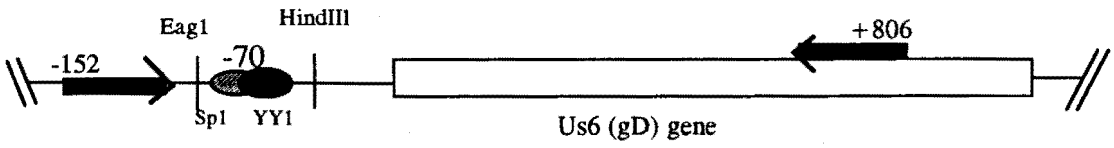


Figure 16. HSV-1 DNA-sequence screened by PCR analysis:

The gD gene and part of the promoter, and the PCR priming sites are shown. The primers are depicted by thick black arrows pointing in the direction of DNA-synthesis. The YY1 and Sp1 binding sites are indicated by a dark gray and a light gray oval, respectively. The figure is drawn to approximate scale, and the restriction sites for Eag1 and HindIII are indicated. The map locations of the primers and the mutation sites are indicated by base pairs (bp) relative to the RNA cap site. The complete PCR product resulting from this PCR is expected to be 958 bp, and restriction digestion with enzymes specific for the mutations is expected to result in a 880 bp and a 80 bp fragment.

Figure 17. Restriction analysis of the recombinant plasmids

A photograph of an agarose gel showing restriction analyses of the four recombinant plasmids pSJgDY, pSJgDS, psJgD2m, and pSJwt is depicted. Lane 1 and 16 contain a λ-HindIII DNA marker with the fragment sizes: 23.1 kbp, 9.4 kbp, 6.557 kbp, 4.36 kbp, 2.32 kbp, and 2.02 kbp. Lanes 2 and 17 contain a pBR322-Alu1 DNA marker with the fragment sizes 0.91 kbp, and 0.659 kpb. Lanes 3 to 15 show restriction fragments from the digestion analysis of the recombinant plasmids pSJgDY, pSJgDS, pSJgD2m , and pSJwt. In lanes 3, 6, 9, and 13 restriction digestion of the plasmids with MluI is shown. Two fragments were expected with the sizes 4.01 kbp and 3.87 kbp. The two fragments run so close to each other that they appear as one fragment. In lanes 4, 7, 10, and 14 restriction digestion with SacI is shown. Expected were three fragments of sizes 3.374 kbp, 2.843 kbp, and 1.67 kbp. Lane 5 shows a restriction digestion of pSJgDY with XhoI which cuts in the mutated YY1 site and is expected to linearize the plasmid (7.88 kbp). Lane 7 shows a restriction digestion of pSJgDS with BglII which cuts in the mutated Sp1 binding site and would therefore linearize the plasmid. Lanes 11 and 12 show restriction digestion of pSJgD2m with XhoI (cuts in mutated YY1 binding site), and EcoRI (cuts in mutated Sp1 binding site), respectively. XhoI linearized the plasmid as was expected, and EcoRI yielded two fragments (5.89 kbp and 1.99 kbp) also as expected. The plasmid pSJwt was cut with EcoRI as control which was expected to linearize the plasmid (7.88 kbp). More restriction analysis not shown in this picture is listed in Table 1.

Figure 17. Restriction analysis of the recombinant plasmids pSJgDY, pSJgDS, pSJgD2m, and pSJwt:

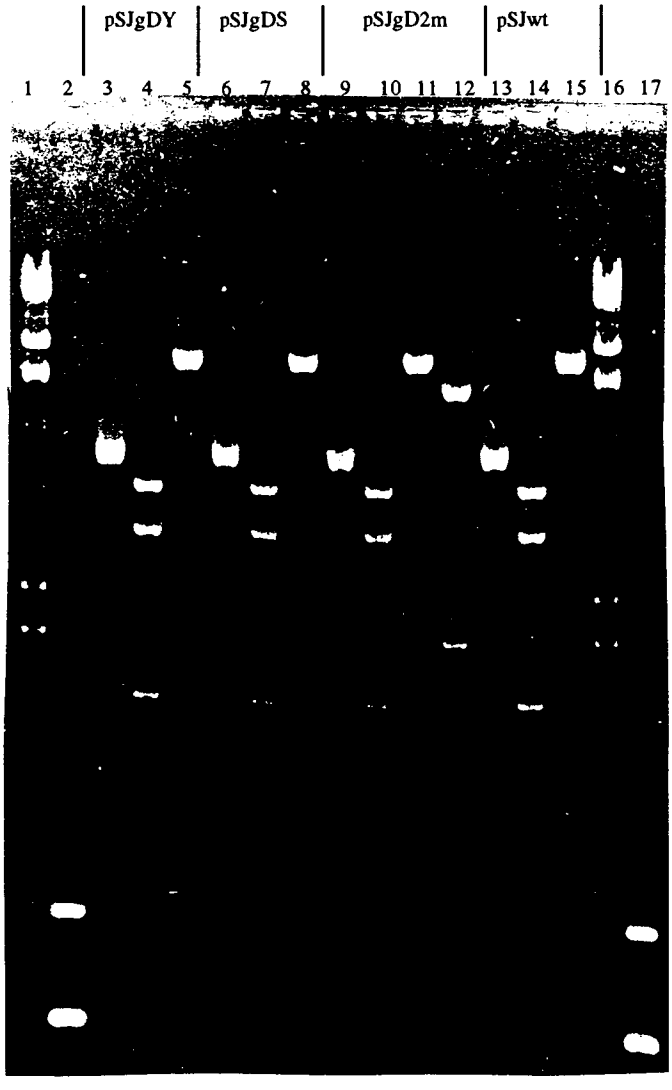


Figure 18. Sequencing data from the recombinant plasmids

Sequencing data from the four recombinant plasmids pSJgDY, pSJgDS, pSJgD2m, and pSJwt are shown from -325 to +50 relative to the gD mRNA cap site. This covers all known and suspected promoter elements (see Figure 7.a). The obtained sequences were compared to published sequences of HSV-1 strain 17 and strain KOS accessed from the GenBank, as described in the results section (as in the recombinant plasmids, the 132 bp Eag1-HindIII fragment is shown from strain 17, and the rest from strain KOS). The YY1 binding site is indicated by a noncolored box, and the Sp1 binding site is indicated by a lightly shaded box. Mutated bases are printed in bold. The mRNA start site is indicated by a line and by the symbol +1. 'N' indicates nucleotides that could not be identified by the computer analysis of the sequencing results.

Abbreviations: gb=GenBank (HSV-1 sequence derived from the GenBank), Y=pSJgDY sequence, S=pSJgDS sequence, m=pSJgD2m sequence, wt=pSJwt sequence.

Figure 18: Sequencing data of the recombinant plasmids:

```
gene-bank: 5'-TTCTCCGCGT GGGTGATGTC GGGTCCAAAC TCCCGACACC ACCAGCTGGC ATGGTATAAA TCACCGGTGC GCCCCCCAAA
pSJgDY:    5'-TTCTCCGCAT GGGTGATGTC GGGTCCAAAN TCCNGACACC ACCAGNTGGC ATGGTATAAA TCACCGGTGC GCCCCCCAAA
pSJgDS:    5'-TTNTCCGCAT GGGTGATGTC GGGTCCAAAN TCCNGACACC ACCAGNTGGC ATGGTATAAA TCACCGGTGC GCCCCCCAAA
pSJgD2m:   5'-TTCTCCGCAT GGGTGANGTC GGTTCCANAC TCCCGACACN ACCAGCTGGC ANGGTATAAA TCACTGGTGN GCCCCCCAAA
pSJwt:     5'-TTNTCCGCAT GGGTGATGTN GGGTCCAAAN TCCNGACACC ACCAGNTGGC ATGGTATAAA TNACCGGTGC GCCCCCCAAA

gb: CCATGTCCGG CAGGGGGATG GGGGGGCAAT GCGGAGGGCA CCCAACAACA CCGGGCTAAC CAGGAAATCC GTGGCCCCGG CCCCCAACAA
Y : CCATGTCCGG CAGGGGGATG GGGGGGCAAT GCGGAGGGCA CCCAACAACA CCGGGCTAAC CAGGAAATCC GTGGCCCCGG CCCCCAACAA
S:  CCATGTCCGG CAGGGGGATG GGGGGCGAAT GCGGAGGGNA CCCAACAACA CCGGGCTAAC CAGGAAATCC GTGGCCCCGG CCCCCAACAA
m:  CNANGTCCGG CAGGGGGATG GGGGGCGAAT GNGGAGGGCA CCCAACAANA CNGGGCTAAC CAGGAAATCA GTGGCCCCGG CCCCCAACAA
wt: CCATGTCCGG CAGGGGGATG GGGGGCGAAT GCGGAGGGNA CCCAACAACA CCGGGCTAAC CAGGAAATCN GTGGCCCCGG CCCCCAACAA

gb: AAATCACGGT AGCCCGGCCG TGTGACACTA TCGTCCATAC CGACCACACC GACGAATCCC CCAAGGGGGA GGGGCCATTT TACGAGGAGG
Y:  AGATCGCGGT AGCCCGGCCG TGTGACACTA TCGTCCATAC CGACCACACC GACGAATCCC CCAAGGGGGA GGGGCTCGAG TACGAGGAGG
S:  AGATCGCGGT AGCCCGGCCG TGNGACACTA TCGTCCATAC CGACCACACN GACGAATCCC CCAAGATCTA GGGGCCATTT TACGAGGAGG
m:  AGATCGCGGT AGCCCGGCCG TGTGACACTA TCGTCCATAC CGACCACACC GACGAATCCC CCAAGGAATT CGGGCTCGAG TACGAGGAGG
wt: AGATCGCGGT AGCCCGGCCG TGTGACANTN TNGTCCATAC CGACCACACC GANGAATCCC CCAAGGGGGA GGGGCCATTT TACGAGGAGG

                                                                                    +1
gb: AGGGGTATAA CAAAGTCTGT CTTTAAAAAG CAGGGGTTAG GGAGTTGTTC GGTCATAAGC TTCAGCGCGA ACGACCAACT ACCCCGATCA TCAGT
Y:  AGGGGTATAA CAAAGTCTGT CTTTAAAAAG CAGGGGTTAG GGAGTTGTTC GGTCATAAGC TTCAGTGCGA ACGACCAACT ACCCCGATCA TCAGT
S:  AGGGGTATAA CAAAGTCTGT CTTTAAAAAG CAGGGGTTAG GGAGTTGTTC GGTCATAAGC TTCAGTGCGA ACGACCAACT ACCCCGATCA TCAGT
m:  AGGGGTATAA CAAAGTCTGT CTTTAAAAAG CAGGGGTTAG GGAGTTGTTC GGTCATAAGC TTCAGTGCGA ACGACCAACT ACCCCGATCA TCAGT
wt: AGGGGTATAA CAAAGTCTGT CTTTAAAAAG CAGGGGTTAG GGAGTTGTTC GGTCATAAGC TTCAGCGCGA ACGACCAACT ACCCCGATCA TCAGT
```

Figure 19. PCR screening

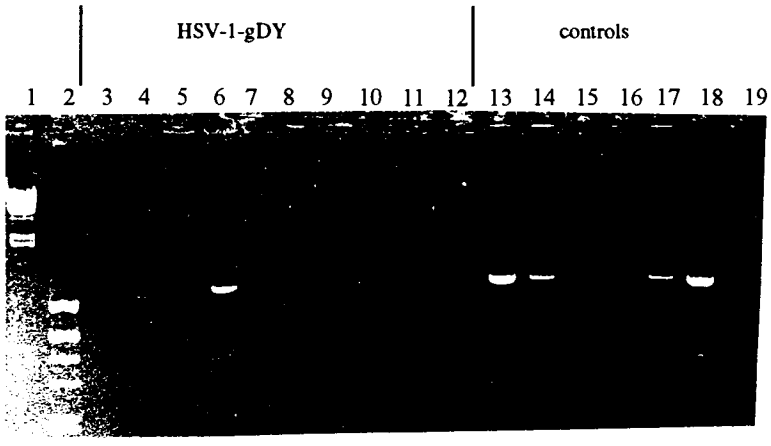
A: A photograph of a 1.2% agarose electrophoresis gel of the PCR products of the mutant viruses is shown. Only one of several pictures is shown. Lane 1 and 2 contain standard size DNA markers, the λ-HindIII marker in lane 1 and the pBR322-AluI marker in lane 2. The first four fragments of the λ-HindIII marker can not be distinguished on this gel, and the 5^{th} and 6^{th} fragment are 2.32 kbp and 2.02 kbp. The smaller lower fragment is 0.57 kbp. The fragments of the pBR322-AluI marker are 0.91 kbp, 0.659 kbp, 0.521 kbp, 0.403 kbp etc.. The PCR fragment was expected to be 0.958 kbp and therefore to run slightly above the first fragment of the pBR322-AluI marker. Lanes 3 to 12 show 10 samples of viral clones screened for HSV-1-gDY (YY1 binding site mutation). The identification of one positive HSV-1-gDY clone is shown in lane 6. Lane 13 to 16 show the PCR products of viral DNA controls: HSV-1-KOS DNA prepared by different methods in lane 13 and 14 (the DNA was usually prepared as in the sample shown in lane 13), and HSV-1 F strain DNA (lane 15 and 16). Lane 17 shows HSV-1 F strain DNA screened by PCR under less stringent conditions with 2mM $MgCl_2$. HSV-1-KOS DNA is amplified under stringent conditions as expected, whereas HSV-1-F DNA is not amplified under the same conditions, but it is amplified under less stringent conditions. Lane 18 shows the PCR product of the plasmid pSJgDY, and in lane 19 is a sample containing all PCR ingredients but no DNA.

B: This photograph shows the identification of a HSV-1-gDS, a HSV-1-gD2m, and a HSV-1-wt viral clone. Markers are as in gel A, and lanes 3 to 6 contain gDY clones,

lanes 7 to 10 contain gD2m clones, lanes 11-14 contain gDS clones, and lanes 15-18 contain wt clones. Lane 19 contains the HSV-1-F control as described above (under stringent conditions), and lane 20 contains the no DNA control as described above. The other controls as above were run together with these PCR samples too, but are not shown on the gel (because they didn't fit on the gel). Lane 7 shows a positive HSV-1-gD2m clone, lane 12 a positive HSV-1-gDS clone, and lane 18 a positive HSV-1-wt clone.

Figure 19. PCR screening:

A:



B:

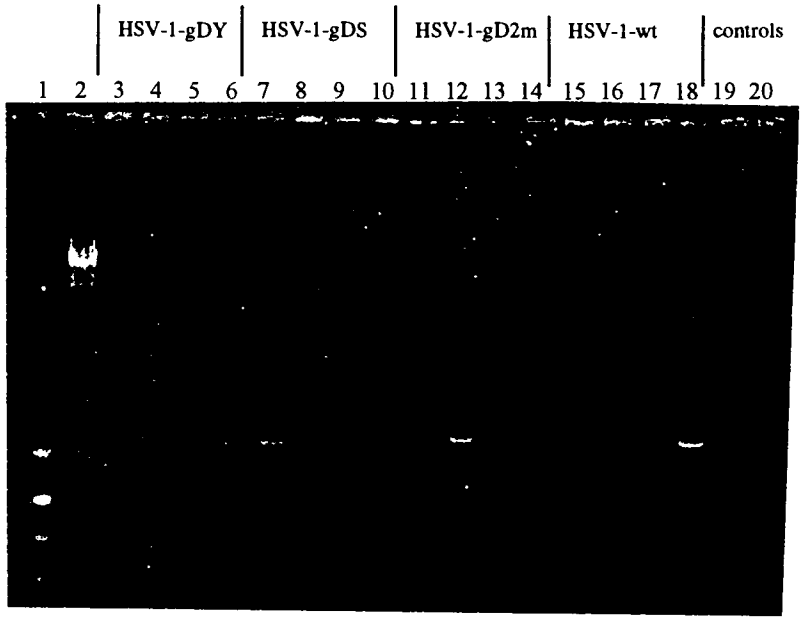


Figure 20. Restriction analysis of the PCR products.

This photograph shows a 2% agarose gel with restriction analysis samples of positive PCR products of the putative HSV-1-gDY, HSV-1-gDS, HSV-1-gD2m, and HSV-1-wt clones. The PCR products were cut with the enzyme cutting in the mutated YY1 (Xho1) or Sp1 (BglII or EcoRI) sites, respectively. This was expected to truncate the 958 bp PCR product to 880 bp. The wt sample and an HSV-1 Fstrain PCR product produced under low stringency conditions were cut with SacII to yield three characteristic fragments of 401 bp, 308 bp, and 249 bp. More restriction analysis was performed as described in the results (data not shown, but see Figure 21).

Lane 1 in the picture shows the fragments of the λ-HindIII marker (first 4 fragments are fused, then 2.32, 2.02, and 0.57). Lane 2 and 3 contain different amounts of pBR322-AluI DNA marker (0.91, 0.659, 0.521, 0.403, 0.281, 0.257, and 0.226 kbp). Lanes 4, 6, 8, 11, and 13 contain the uncut PCR products of gDY, gDS, gD2m, wt, and HSV-1 Fstrain, respectively. Lane 5, 7, 9 and 10, contain the PCR products cut by the respective enzymes recognizing the mutated site (gDY by XhoI, gDS by BglII, and gD2m by EcoRI and XhoI). Lane 12 and 14 show the results of SacII digestion of the PCR products of the recombinant HSV-1-wt and the HSV-1 F virus. Lane 15 contains the PCR sample of HSV-1 F under the usual screening conditions; and lane 16 contains the PCR product of the plasmid pSJgDY. Lane 17 contains the no DNA PCR control.

Table 3 shows the fragment sizes.

Figure 20. Restriction analysis of the PCR products for identification of mutated viruses:

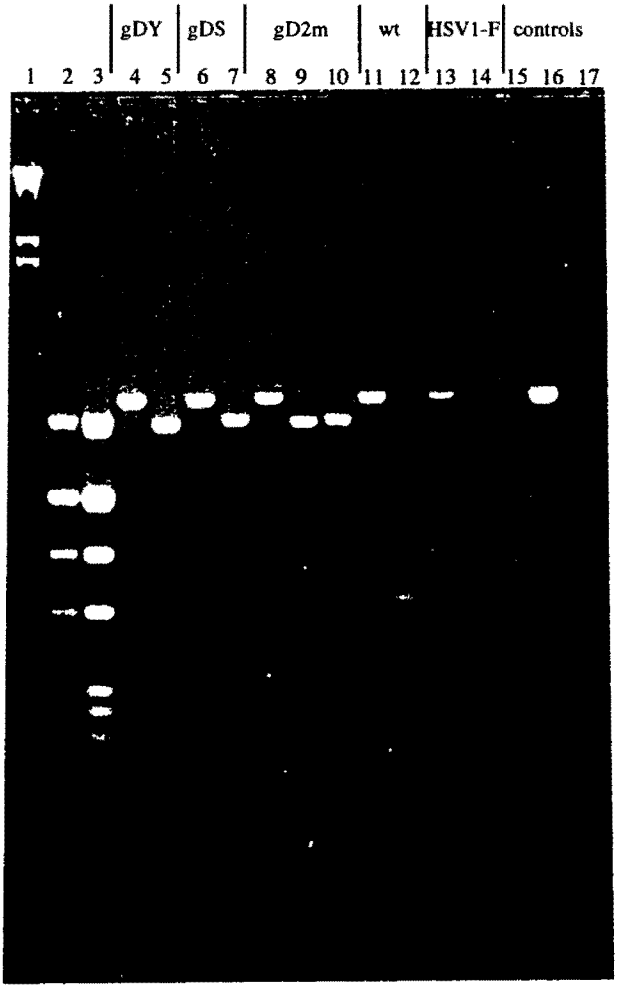


Figure 21. Restriction analysis of the PCR products of plaque purified viral clones

An agarose gel showing the PCR products from DNA from plaque purified viral clones and their restriction analysis. Lane 1 contains the λ-HindIII size marker, and lanes 2 and 3 contain pBR322-AluI size markers. The fragmented sizes of the markers are described in Figure 20. Lanes 4 to 7 contain the HSV-1-gDY PCR product, lanes 8 to 11 the HSV-1-gDS PCR product. Lanes 12 to 15 the HSV-1-gD2m PCR product, and lanes 16 to 19 the HSV-1-wt PCR product. Lane 20 contains the uncut PCR product of the template plasmid pSJgDY. Lanes 4, 8, 12, and 13 show restriction digest of the PCR products with the enzymes cutting in the mutation sites (in order: Xho1, BglII, Xho1, EcoRI), respectively, and truncating the PCR product from 958 kbp to 880 bp. Lanes 5, 6, 9, 10, 14, 16, 17, and 18 show control restriction digestions with the enzymes specific for the mutation not present in the respective PCR product. This is for HSV-1-gDY BglII (lane 5) and EcoRI (lane 6), for HSV-1-gDS XhoI (lane 9) and EcoRI (lane 10), for HSV-1-gD2m BglII (lane 14), and for HSV-1-wt XhoI, BglII, and EcoRI (lanes 16, 17 18). The enzymes did not cut the PCR products supporting the positive results. Lanes 7, 11, 15, and 19 depict the results of a restriction analysis of the PCR products with SacII which is expected to yield three fragments of the sizes: 401 bp, 308 bp, and 249 bp. The fragment sizes are summarized in Table 4.

Figure 21. Restriction analysis of the PCR products of plaque purified viral clones:

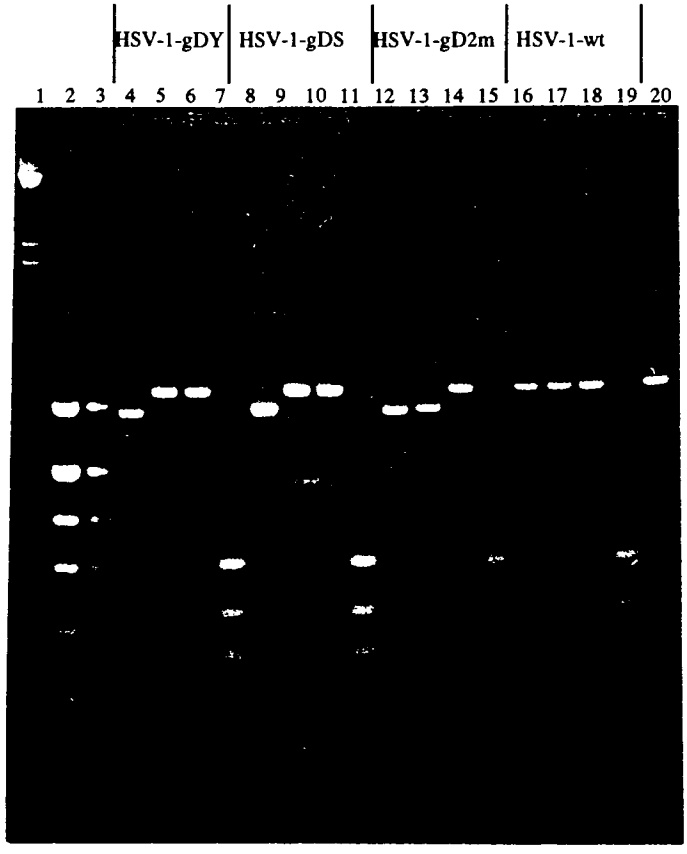


Figure 22. DNA sequence comparison of the sequence obtained from the PCR products

A DNA sequence comparison of the PCR products from the isolated recombinant viruses the four recombinant plasmids used to construct the viruses. The published DNA sequence as expected from the GenBank is shown in the first line (as in Figure 18). The sequence data obtained by sequence analysis of the plasmids (pSJgDY, pSJgDS, psJgD2m, pSJwt) and of the PCR products from viral DNAs (HSV-1-gDY, HSV-1-gDS, HSV-1-gD2m, HSV-1-wt) are shown alternately. The Sp1 binding site is highlighted by a light gray box, and the YY1 binding site by a white box. Mutated bases are printed bold. The sequences are shown in 5' to 3' direction, from -151 to +11 relative to the RNA cap site.

Figure 22

DNA sequence comparison of the sequence obtained from the PCR products to the expected sequence according to a gene-bank derived sequence and to the earlier determined sequence:

Figure 23. Comparison of the virus infectivity

In order to test the infectivity of the four recombinant viruses compared to each other and to the HSV-1-F strain virus, the viruses were titered on VD60 and Vero cells (also see Table 5 and Table 6). To be able to compare the virus infectivity of the different viruses to each other, the ratio of the titer on Vero cells to the titer on VD60 cells was determined. The ratio of the titer on Vero cells to VD60 cells of HSV-1-wt was set as 100 % infectivity, and the percentage of infectivity of the other four viruses in relation to HSV-1-wt was calculated. The standard deviations were determined and are indicated in the Figure. A t-test showed that the values for HSV-1-gDY, HSV-1-gD2m, and HSV-1-F are different from the one of HSV-1-wt with a probability of 99%, and the value determined for HSV-1-gDS is different from the value of HSV-1-wt with a probability of 90%.

Figure 23. Comparison of the virus infectivity

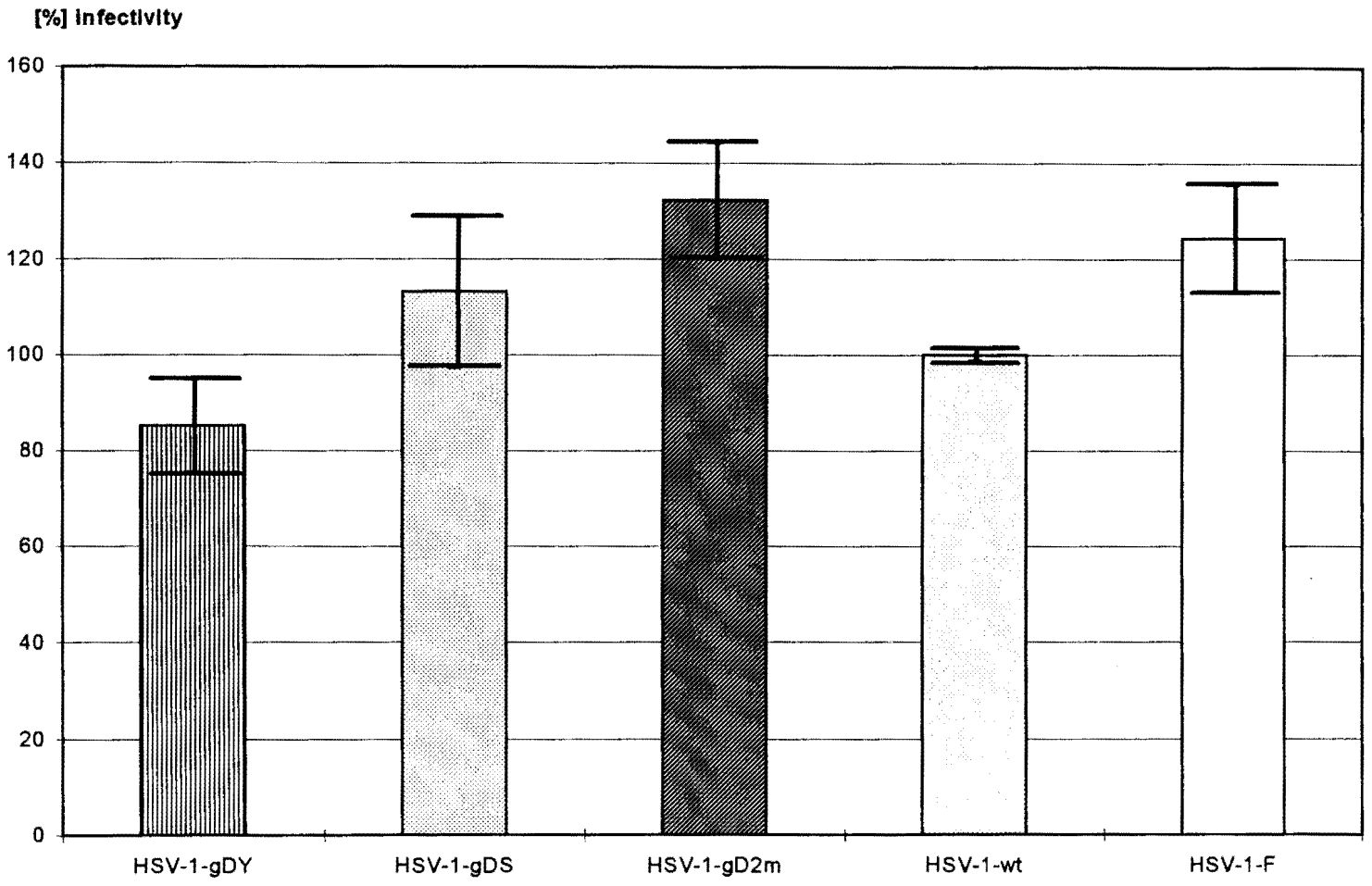
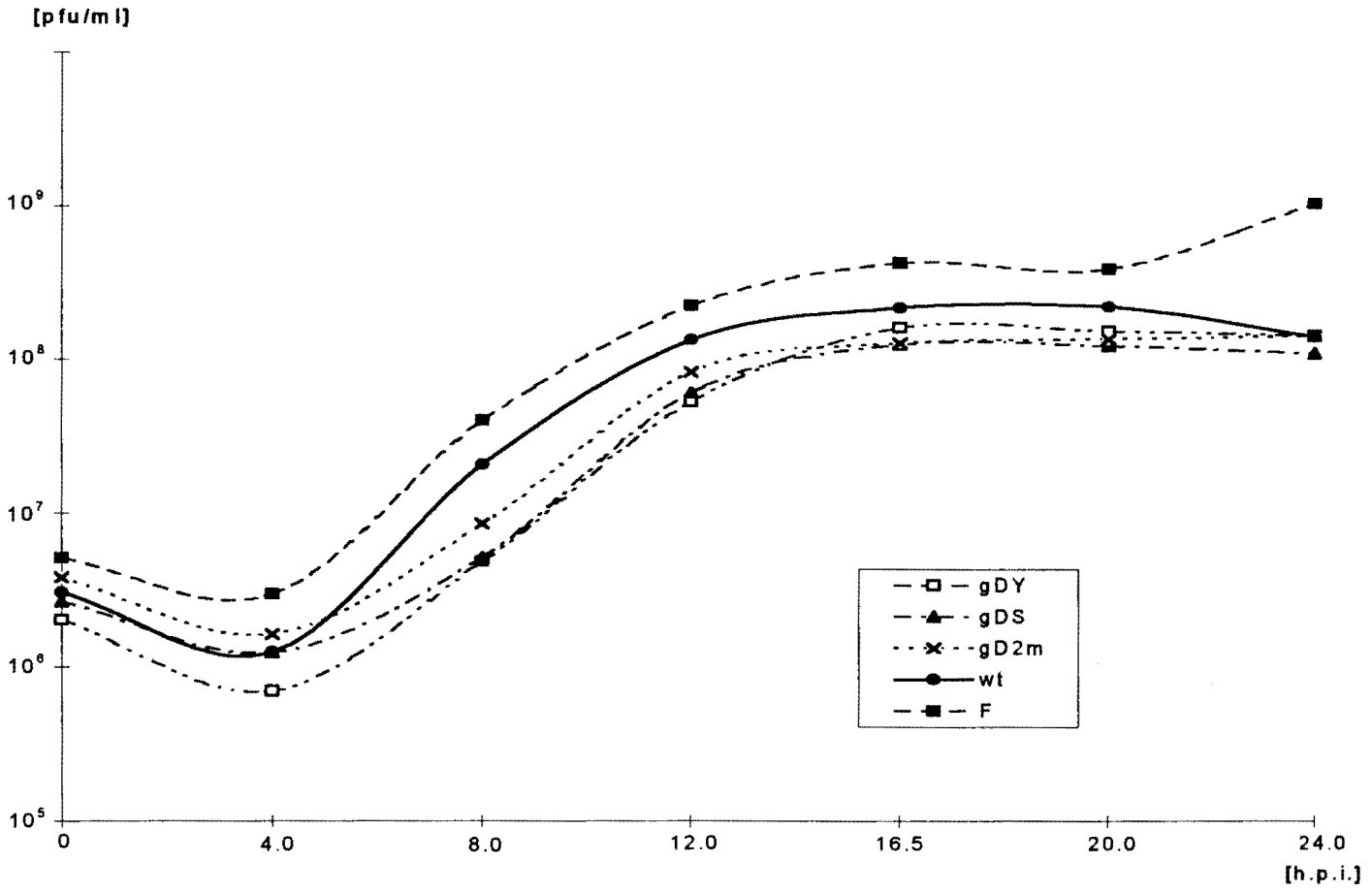


Figure 24. One step growth experiment

Vero cells were infected with the four recombinant viruses and HSV-1-F, and were harvested at 0, 4, 8, 12, 16.5, 20, and 24 h.p.i.. The resulting viral lysates were titered on Vero cells. The titers and standard deviations are shown in Table 7. The titers of the five viruses were plotted against the different time points at which the viruses were harvested, respectively. The growth curves show that HSV-1-F, HSV-1-wt replicate with similar growth rates with the main growth starting at 4 h.p.i. HSV-1-gDY seems to replicate slightly, but not significantly, slower than HSV-1-wt and HSV-1-F. Both HSV-1-gDS and HSV-1-gD2m appear to grow with an even slower growth rate, with the major growth starting at about 8 h.p.i, and slower growth before that.

Figure 24. Growth rates of the recombinant viruses and HSV-1-F



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