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THESIS APPROVAL

The abstract and thesis of Rosemary Ann Lown for the Master of Science in Biology were presented May 18, 1994, and accepted by the thesis committee and the department.

COMMITTEE APPROVALS:

Robert L. Millette, Chair

Deborah Duffield

Lester Newman

David H. Peyton
Representative of the Office of Graduate Studies

DEPARTMENT APPROVAL

Robert O. Tinnin, Chair
Department of Biology

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ABSTRACT

An abstract of the thesis of Rosemary Ann Lown for the Master of Science in Biology, presented May 18, 1994.

Title: Investigations of Factors Affecting the Transcriptional Regulation of Herpes Simplex Virus Type 1 $\beta\gamma$ (Leaky-Late) Genes.

Herpes simplex virus type 1 (HSV-1) is a virus commonly causing cold sores in humans, however, virulent infections are known to produce debilitating encephalitis and death.

HSV-1 transcription is carried out by the host cell RNA polymerase II in a tightly regulated temporal cascade. The first genes transcribed, the α genes, are activated in the absence of viral DNA synthesis. Transcription of the other temporal classes, the β , $\beta\gamma$, and γ genes is dependent upon the protein products of the α genes for activation.

The purpose of this study was to investigate the factors that contribute to this rigid regulation of HSV-1 transcription. This investigation sought to identify some of the cellular and viral transcription factors that activate transcription of genes of the later kinetic classes. Two separate approaches were utilized in these

investigations. 1) *In vitro* transcription using a soluble, cell free system to study the transcriptional regulation of the VP5 gene, and 2) DNA competition binding assays to identify and characterize the protein-DNA complexes resulting from interaction between the *cis*-acting DNA sequences of the VP5 gene, other viral genes, and the proteins that bind to them.

Attempts at *in vitro* transcription of β , $\beta\gamma$, and γ genes were unsuccessful. Because these genes require α products for activation, it was necessary to prepare nuclear extracts from infected cells. However, HSV-1 contains endogenous RNase activities which are components of the biochemical machinery by which the virus directs host transcription to the synthesis of viral molecules. The uses of virus deficient in the host shut-off function and various drugs were unsuccessful.

Previous work in the Millette laboratory demonstrated a sequence in the VP5 promoter that played a significant role in the up regulation of expression of that gene. DNA binding competition studies using a number of HSV-1 sequences exhibiting partial homology to this sequence demonstrated that these sequences all compete for the binding of the same protein factor. Similarly, a piece of the human immunodeficiency virus (HIV) exhibiting a seven base pair homology also exhibited weak competition.

INVESTIGATIONS OF FACTORS AFFECTING THE TRANSCRIPTIONAL
REGULATION OF HERPES SIMPLEX VIRUS TYPE 1 $\beta\gamma$ (LEAKY-LATE)
GENES

by

ROSEMARY ANN LOWN

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
BIOLOGY

Portland State University
1994

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CHAPTER I

INTRODUCTION

Viruses are simple, submicroscopic structures capable of infecting an enormous variety of organisms. These noncellular genetic elements characteristically have an extracellular form that is the infective phase of the virus. The basic simplicity of viruses makes them obligate parasites, requiring host cells to carry out replicative processes.

Typical viral composition includes a nucleic acid genome and at most a few proteins contained within a proteinaceous capsid. These submicroscopic parasites have the capacity to invade a cell and alter normal processes in a matter of minutes. However, the mechanisms by which viruses commandeer and alter the usual host physiology is at best poorly understood.

Investigating viral systems allows a researcher access to two levels of analysis: In addition to studying viruses themselves, it is possible for a researcher to utilize viruses to perturb the normal cell physiology without causing immediate cell death. Therefore it becomes possible to study normal cellular processes utilizing a viral parasite.

Herpes simplex virus type 1 (HSV-1) is a common pathogen that afflicts humans. Most frequently HSV-1 infections result in cold

sores; however, these viruses are also known to cause severe, debilitating and frequently fatal encephalitis, blindness and disfiguring scarring. Once a productive infection is established, HSV-1 enters the sensory neurons of the infected cells and produces a latent infection in the cell body of the compromised neuron. Once sustained, latent infections persist throughout the lifetime of the host. Latent infections can periodically give rise to a lytic infection in the epithelial cells innervated by the affected nerve.

Virus Structure

In comparison to cells, viruses are structurally relatively simple. All herpesviruses exhibit the same four basic anatomic elements: A membranous envelope, a tegument, a capsid and a core (figure 1). The herpesvirus envelope is derived from the inner lamella of the nuclear membrane in the host cell. This viral envelope is composed of lipids, proteins and the polyamines spermine and spermidine (22). Despite the cellular origin of the viral envelope, it is remarkable for its lack of host proteins. Instead, the membrane-bound proteins found in the envelope are virally encoded glycoproteins. The mechanism by which the host cell nuclear envelope is modified to exclude normal host proteins and incorporate only viral proteins is not clear. The viral envelope glycoproteins are believed to be transported to the nuclear membrane via a process involving the Golgi apparatus. What is clearly understood is that

viral glycoproteins are the means by which a virus attaches itself to and enters a cell.

Figure 1. Structure of herpes simplex virus type 1. The toroidal DNA genome is contained within an icosahedral capsid. The capsid is surrounded by tegument contained within the host derived envelope. Viral receptors protrude from the envelope layer. Legend: A. Envelope. B. Capsid. C. Viral DNA. D. Tegument. E. Glycoproteins.

Separating the viral envelope from the underlying capsid is the amorphous tegument. The components of this structure have not been thoroughly defined. However, it is known that a number of

viral proteins are typically found in the tegument, particularly the transcription regulatory factors α trans-inducing factor (α TIF), ICP4, ICP0, ICP27, and the viral host shutoff (VHS) protein (52).

Beneath the tegument, the proteinaceous capsid houses the genetic material. The capsid is icosahedral in shape, and consists of seven virally-encoded proteins. It has been observed that the actual protein composition of the capsid varies somewhat depending on whether or not the viral DNA has been packaged within it and whether or not it has been enveloped (52). Within the capsid, the DNA is toroidal.

The genome of HSV-1 is a linear, double-stranded molecule of DNA approximately 150 kilobase pairs (kbp) in size. The G+C (guanine plus cytosine) content is high (68% - 69%). Structurally, the DNA appears to consist of two components, the long (L) and short (S) segments, covalently linked end-to-end (figure 2). Each component contains a length of unique DNA sequence wedged between regions of inverted repeats (IRs) designated *ab* and *a'b'* flanking the long sequence, and *a'c'* and *ac* flanking the short sequence. The unique long (U_L) region has a length of approximately 108 kbp. The unique short (U_S) sequence is 13 kbp in length. The long terminal repeats (TR_L) and short terminal repeats (TR_S) are roughly 9.2 kbp and 6.6 kbp respectively. The IRs themselves contain elements that are highly conserved, but they can exhibit some variability with respect to the number of internal repeated elements. The region of covalent linkage between the L and S components is the so called hinge

region. During replication, the L and S components can invert relative to one another at the hinge thus giving rise to four structural isomers (52).

Figure 2. The genome of HSV-1 illustrating the elements of the long (L) and short (S) components. The inverted repeats *ab* and *a'b'* flank the L component, and *a'c'* and *ac* flank the S component. The hinge region is located between the *a'* inverted repeats. Modified from Fields and Knipe (52).

Herpesvirus Replicative Cycle

The HSV-1 replication cycle begins with the attachment of the virion to receptors located in the host cell membrane. Particularly, heparan sulfate proteoglycans are recognized in one of the initial binding events (68). This initial attachment requires either (or both) viral glycoprotein B (gB) or glycoprotein C (gC) (43, 52). The initial stage of penetration is dependent upon the viral glycoproteins gB, gC and gH:L. Penetration of the virus into the cell interior is accomplished by virally-directed fusion of the HSV-1 envelope and the cell membrane, a process that requires the viral glycoproteins gB

and gD. Once fusion between the virion and the cell membrane has occurred, the viral nucleocapsid and tegument enters the cell cytoplasm. In the process of penetration, the viral envelope is removed. (52).

The next event in HSV-1 replication is the transport of the viral nucleocapsids to the nuclear membrane by a mechanism thought to involve the cellular cytoskeleton. Upon arriving at the nuclear pores, the viral DNA is released into the nucleoplasm (52).

Following the initial viral infection, there is a rapid shut off of host macromolecule synthesis and a switch of the cellular machinery to the production of viral proteins. This conversion from cellular macromolecule synthesis to viral macromolecule synthesis occurs in several stages. First, host polypeptide synthesis is suppressed coincident with the disaggregation of the infected cell polyribosomes. This is followed by a subsequent degradation of host cell mRNAs. The onset of viral polypeptide synthesis is associated with the formation of new, viral-specific polyribosomes that have a size distribution that is different from that of the host (32, 61). The initial shut-off, the disaggregation of host polyribosomes, is mediated by a virion structural component that is carried into the host cell in the tegument of the virus, the VHS protein. This *vhs* function enables the virus to remove host mRNA from the pool of translatable message, thus aiding the virus in switching from host protein synthesis to virion protein synthesis (30, 31). Additionally, data

indicates that the *vhs* gene product facilitates the transition between regulatory classes by destroying α and β mRNAs (61).

The complete degradation of host mRNA requires expression of viral genes and is a secondary phenomenon coded for by a viral gene of the β or γ class. It can occur without the prior host shut off events. (The viral host protein shut off is not required for the production of viral progeny.[32]).

The transcription of HSV-1 genes is a coordinately regulated process in which the several kinetic classes of genes are transcribed in a temporal cascade by the host RNA polymerase II (figure 3) (64, 67).

The α genes, the first kinetic class, consist of five genes ($\alpha 0$, $\alpha 4$, $\alpha 22$, $\alpha 27$ and $\alpha 47$). These genes are transcribed in the absence of viral protein synthesis. As a group most of these genes map in the long and short repeat regions of the HSV-1 DNA molecule, with $\alpha 0$ and $\alpha 4$ present in two copies per genome. Typically, the α genes encode transcription regulatory factors and are required for the synthesis of the polypeptide products of subsequent kinetic classes (52).

Transcription of the α genes themselves is induced by the viral *trans*-activating protein, α TIF (VP16) (24, 25). As previously mentioned, α TIF is a constituent protein of the tegument. As such it is released from the virus after penetration into the cell. This protein is transported to the nucleus independently of the viral DNA

and interacts, not with a *cis*-acting regulatory DNA sequence, but with a cellular transcription factor, OTF-1, which in turn binds

Figure 3. The replicative cycle of HSV-1 (49).

to a specific site in the viral α promoter. This sequence, located less than 400 bp upstream from the cap site, is unique to and characteristic of α promoters. OTF-1 binding to this region initiates transcription of α genes (24, 25, 38, 39, 49). Expression of α polypeptides peaks two to four hours post infection.

Viral DNA replication and nucleotide synthesis are the major events associated with β protein expression and require prior expression of α proteins. Some β products appear very early in infection. These have been designated the β_1 genes, and include the large subunit of the viral ribonucleotide reductase and the major DNA binding protein. However, unlike α genes, they require viral transcription factors for activation. The protein products of β genes are generally involved in nucleotide and viral nucleic acid metabolism and viral DNA replication, e.g., thymidine kinase (*tk*), ribonucleotide reductase and the viral DNA polymerase among others (11, 28). Peak polypeptide production of β genes occurs five to seven hours following infection.

Inclusion of viral genes in the third kinetic class, the γ genes, is somewhat arbitrary. Although expression of the entire complement of these genes requires viral DNA synthesis, the $\beta\gamma$ or γ_1 genes of HSV-1 are expressed at low levels before DNA replication, and at high levels following DNA replication (11, 64). In contrast, the true late, γ or γ_2 genes, require prior DNA replication for their expression. The mechanism of activation of both is poorly understood. It is known that α genes are required for initial onset of $\beta\gamma$ and γ transcription (8). However, it has been observed that viral DNA replication is required for maximum $\beta\gamma$ and γ . In cells in which HSV-1 replication has occurred, the mRNAs and proteins for the $\beta\gamma$ and γ genes are among the most numerous. Many of the $\beta\gamma$ and γ genes code for viral

structural proteins, e.g. the major capsid protein VP5, and a number of glycoproteins (gB, gC, and gD) (52, 55).

Following transcription, the viral mRNAs are capped, methylated, polyadenylated and transported to the cytoplasm. Protein synthesis is carried out by the host translation apparatus, apparently on both free and endoplasmic reticulum-bound polyribosomes. Following translation and post-translational processing (splicing, glycosylation, etc.), again carried out by host enzymes, virion proteins are transported back into the nucleus for assembly into mature progeny virus. The mechanism by which the protein constituents of HSV-1 are transported has not been defined, but assembly seems to occur by a spontaneous process akin to that described for the large bacteriophages, e.g., T4 (52).

Viral DNA replication is first detected about three hours post infection. Viral DNA isolated early after the onset of replication yields both linear branched and circular forms of DNA. Indications are, however, that late in replication, HSV-1 is copied via the rolling circle mechanism from three origins of replication. Two origins are contained within the inverted repeats of the S segment, and the third in the approximate center of the unique long (U_L) region. It has been observed that deletion of one of the S origins and the L origin does not adversely affect the ability of the virus to replicate (51).

Production of mature infectious viral particles takes place in the nucleus. Capsids are formed from the proteins previously transported into the nucleus from the cytoplasm. Genome lengths of

DNA are processed and packaged into the empty capsids. It has been observed that, late in infection, the nuclear envelope typically contains areas of thickened, concave or convex patches that contain virion proteins, and that viral envelopment occurs at these patches. The DNA-filled viral capsids attach to the modified areas of the inner lamella of the nuclear envelope and bud through, encasing in the inner membrane (figure 3). Enveloped capsids then pass into the endoplasmic reticulum and are ultimately conveyed into the extracellular space. Production of progeny virus necessarily results in the destruction of the host cell.

Latency

One of the most puzzling aspects of an HSV-1 infection is the phenomenon of latency, that is, the ability of the virus to assume a quiescent state in host sensory neurons. Once latency is established, the virus can remain dormant for years, only to erupt into a fully lytic infection following some physiological trigger.

Like so many aspects of the herpesvirus life cycle, the events of latency are obscure. What is known is that HSV-1 enters the sensory neurons associated with the infected cells. The capsids are transported to the nuclei of the neurons in the trigeminal nerve. Studies on latently infected neurons reveal only one set of viral transcripts, designated LAT RNAs (for latency-associated transcripts), that accumulates abundantly in the nuclei. These are transcribed from the terminal repeat of the unique long region of the herpes

genome and are at least partially complementary to the 3' end of ICP0 (63). The function of these latency RNAs is currently under intense investigation by many laboratories and their role in maintaining latent viral infections and possibly in viral reactivation to productive infection is still obscure.

Once established, the HSV-1 latent infection remains throughout the life of the host. Reactivation of the virus can result either from some local stimulus to the infected nerve, such as injury to the tissue, or a systemic stimulus, e.g., emotional stress, menstruation, etc.

Structure of Eukaryotic Genes

Regulation of transcription in eukaryotic systems is intricate and details are only slowly becoming understood. A generalized pattern of required DNA regulatory sequences has painstakingly been constructed by many researchers over the years. It is known that genes of multicellular organisms contain both upstream and downstream *cis*-acting sequences that bind a variety of *trans*-acting protein factors.

Cis-acting sequences include the TATA box, a TA-rich region located 30 base pairs (bp) upstream (-30) from the transcription start site. Proteins necessary for starting transcription of protein coding genes include transcription factor-II_D (TF_{II}D), now called TBP (for TATA binding protein) and TFAs (for TBP-associated factors).

RNA polymerase II and various accessory proteins (TFII_B, TFII_E, TFII_F, and TFII_A) are also required (34).

Additionally, activators are required to ensure normal transcription in many genes. These *cis*-acting sequences may include a CCAAT box which is generally found at or near -80 from the transcription start site. However, the CCAAT box can function at distances that vary with respect to the start site and it can function in either orientation. This regulatory sequence is bound by proteins that form heterodimers. Each protein contributing to the heterodimer weakly binds the CCAAT DNA alone, but strongly binds when dimerized.

Other activators include upstream activating sequences (UAS) which are necessary for maximal transcription. Features common to UAS include a location near the gene and a 15 to 20 bp segment that is bound by protein. Additionally, in mammalian genes, these UAS are often present in multiple copies and frequently exhibit dyad symmetry. Examples include the GC boxes, regions containing the consensus sequence GGGCGG, usually found within 200 bp of the transcription start site. These are known to be bound by stimulatory protein 1 (Sp1) (34).

One last category of activators is the set of enhancers. These *cis*-acting sequences differ from other activators in that they can be separated by large distances from the start site and can be located either upstream or downstream. Otherwise, enhancers exhibit characteristics found in sequences in the promoter region (34).

Viral promoters must meet two disparate requirements. They must be similar enough to host promoters to allow the host transcription factors to recognize and transcribe the viral genes. However, the viral promoters must be sufficiently different from the host promoters to allow independent regulation of the viral genes. Both host and viral promoters contain *cis*-acting sequences that bind a number of host *trans*-acting factors: TATA boxes, CCAAT boxes, Sp1 and NF-1 consensus sequences. The *trans*-acting elements include both cellular transcription proteins that interact with these sequences, as well as virally encoded proteins, i.e., $\alpha 4$.

The Problem

VP5 is the major capsid protein of HSV-1. Kinetically, it is a leaky-late ($\beta\gamma$ or γ_1) gene, and consequently requires α gene products for transactivation (10). The VP5 promoter is typical of eukaryotic genes in that many of the *cis*-acting sequences are present, including a TATA box, a CCAAT box, Sp1 boxes, and NF-1/CTF at -75. Additionally, a positive regulatory sequence in the nontranslated region of the VP5 leader is located 50 bp downstream (+50) from the cap site (64). It has been well documented that viral regulatory factors are an absolute requirement for expression of VP5 (14).

The polypeptide product of VP5 is not expressed significantly in the early stages of the HSV-1 replicative cycle, but is absolutely essential in high concentration late in infection to ensure the

production of large numbers of progeny virus. Therefore, the virus must have some mechanism by which this gene is up-regulated.

In this context, the Millette laboratory has been working to elucidate some of the transcriptional controls involved in gene regulation. The goal has been to identify the cellular and viral transcription factors that up-regulate VP5 expression following virus infection

As previously noted, in order to effectively direct the host transcription mechanism away from producing cell proteins toward generating the components required to produce progeny virus, HSV-1 promoters must successfully mimic the promoters native to the cell. In effect, the viral promoters must compete for transcription factors with cell promoters.

A great deal is known about the elements that combine to form eukaryotic promoters, and by implication and direct studies, about HSV-1 promoters as well. However, because a particular binding sequence is present in a viral promoter does not necessarily mean that it fulfills the same role in a virus that it does in a cell. That is, the "on" switch and modulation of viral gene expression may differ subtly from the conditions observed in host promoters. Because a sequence exists that may bind a protein factor does not mean that it plays a regulatory role. An assay to demonstrate regulatory significance is required.

In this thesis research, two separate experimental approaches were utilized to investigate regulation of this gene during the infectious cycle:

1. Cell free *in vitro* transcription was utilized to study the transcriptional regulation of the VP5 gene.
2. DNA binding competition studies were employed to identify and characterize the protein-DNA complexes resulting from interaction between the *cis*-acting sequences of the VP5 gene, other viral genes and the proteins that bind to them.

The goal of this research was to identify *cis*-acting DNA sequences and *trans*-acting protein factors that are involved in the regulation of leaky-late transcription.

CHAPTER II

MATERIALS AND METHODS

Cells and Viruses

HeLa cells (ATCC #CCL2), derived from a human cervical epithelioid carcinoma, (adenocarcinoma) were used for preparation of all nuclear extracts. HEp-2 cells (ATCC #CCL23), a human laryngeal epidermoid carcinoma cell line, were used for preparation of all viral stocks. Herpes simplex virus, type I, (HSV-1) KOS strain (wild type) and *vhs1* (mutant strain deficient in the virus host shut off of protein synthesis) were used in preparation of infected cell extracts.

Preparation of Stock Virus

Stock virus were prepared by infecting nearly confluent (approximately 85% confluent) HEp-2 cell roller bottle cultures in Dulbecco's Modified Eagle Medium (DME) plus 1% inactivated calf serum (Gibco) at low multiplicity (0.02 plaque forming units [pfu] per cell). Viral harvest was done when cells exhibited cytopathic effects indicated by changes in cell morphology (i.e., rounding, loss of confluency), typical of viral infection at approximately three days post infection. Harvest was accomplished by scraping the cells into the medium. The cells were pelleted by centrifugation at 4°C, 2000 x g for 10 minutes. The cells were disrupted on ice with the Bransen

Sonnifier W-350 in 15 second bursts for two minutes with the setting at two. Cell debris was removed by centrifugation for 30 minutes at 2000 x g at 4°C. The supernatant liquid containing the virus particles was stored at -70°C in DME containing 10% heat inactivated fetal calf serum.

The viral titer was determined by plaque assay on Vero cells (ATCC #CCL81), African green monkey kidney cells, grown in monolayer cultures as described by Millette et al. (40).

Preparation of Nuclear Extracts

Nuclear extracts were prepared as described by Dignam, et al. (16) or Shapiro (57) except as follows. HeLa cells were grown in monolayer roller bottle cultures in DME plus 5% inactivated calf serum. Preparation of infected extracts was achieved by infecting monolayer cultures at a confluency of approximately 80% with HSV-1 at a multiplicity of 10 pfu/cell. Cells were harvested at approximately eight hours post infection by rinsing the monolayers twice with ice cold phosphate buffered saline (PBS) and scraping into cold PBS. The cells were pelleted by centrifugation at 2000 x g for ten minutes at 4°C.

Cycloheximide reversed *vhs1* infected extracts were prepared as described by Dignam, et al. (16) or Shapiro (57). The infection protocol utilizing the drugs involved adding cycloheximide (final concentration of 50 µg/ml) was added to the medium one-half hour prior to infection. Virus was introduced at a multiplicity of ten

pfu/cell. One and one-half hours post infection, the infecting suspension was decanted and the cells were overlaid with DME plus 1% inactivated calf serum with cycloheximide, 50 μ g/ml. Five and one-half hours after infection, actinomycin D was added to the medium at a final concentration of 5 μ g/ml. Following a 30 minute incubation, the cycloheximide block was reversed with three washes of medium containing 5 μ g/ml actinomycin D. Harvesting of the cells was carried out one and one-half hours following cycloheximide reversal. Nuclear extracts were prepared as previously described.

DNA and Plasmids

Plasmid DNA was prepared by the alkaline lysis procedure (4) with modifications as described in Sambrook et. al. (54). Briefly, this procedure consists of growing liquid cultures of *E. coli* containing the plasmid of interest with the appropriate antibiotic, i.e., ampicillin at a final concentration of 50 μ g/ml. Plasmids were amplified in late log phase by adding chloramphenicol to a final concentration of 170 μ g/ml. Bacteria were harvested 12 to 16 hours post amplification by centrifugation at 4000 x g. Cells were resuspended in buffer containing 0.1 M NaCl, 10 mM Tris-Cl, and 1 mM EDTA. Cells were lysed with lysozyme (10 mg/ml in 10 mM Tris-Cl) followed by 0.2N NaOH in 10% SDS (sodium dodecyl sulfate). Following the addition 3M potassium in 5M acetic acid to neutralize the alkaline NaOH/SDS, the chromosomal DNA and cell debris were removed by centrifugation. The supernatant liquid containing the plasmid DNA

was filtered through four layers of cheese cloth and plasmids were collected by isopropanol precipitation and purified by precipitation with PEG-8000.

Fragments were isolated from restricted plasmids by the DEAE paper electroelution method (19). DNA fragments were labeled at the 3' ends by Klenow fill-in (54) using [α - 32 P]dCTP.

Plasmid pKSBBO was made in this lab by the insertion of the 2.24 kB BstEII-BamHI fragment of the BamZ fragment of HSV-1 into pBR322. This fragment contains the ICP0 gene. Plasmids pSG22 (23), pHSV106 (39), pVP5(-168)CAT (6), pVP5(-168)CAT Δ 2 (10) and pJB3 (29), pRB122 (66), pHKg (1), pIM10 and pGTSa2 (33), pU3RIII and pU3RIII-167 (53) were either previously prepared in this laboratory or obtained from others. They are described in Table I.

Enzymes

Restriction endonucleases were purchased from Bethesda Research Labs, Inc. (BRL) or New England Biolabs, Inc. Restriction of DNA was carried out using the 10x reaction buffers provided by the manufacturers or the appropriate concentration of KGB buffers (54W). DNA was digested for two to four hours at 37° with three to four units enzyme per μ g DNA.

In Vitro Transcriptions

In vitro transcriptions were performed as described in Dignam, et al. (16) and Shapiro (56) except [α - 32 P]CTP was used. Briefly, nuclear extracts were prepared by one of the above methods.

TABLE I
PLASMIDS

PLASMID	VIRAL INSERT	VECTOR	GENES
pGTSa2	Sall subfragment of BamHI J fragment	pBR322	gB
pHKg	HindIII K fragment map units 0.527-0.592	pBR322	UL37
pHSV106	BamHI Q	pBR322	Thymidine Kinase gH:L
pIM10	1.7 kB Sall fragment	pBR322	VP5
pJB3	SmaI subfragment of BamHI J fragment	pACYC177	gD
pKSBB0	2.24 BstEII-BamHI fragment of IR _L , residues 1201-5520	pBR322	ICP0
pRB122	BamHI Z fragment	pBR322	IE 4/5 (ICP4/ α 47)
pSG22	EcoRI I fragment	pBR325	UL47
pU3RIII	-453 - +80 of 3' LTR of HIV	pSVIXCAT	<i>trans</i> -acting factor
pU3RIII-167	-167 - +80 of 3' LTR of HIV	pSVIXCAT	<i>trans</i> -acting factor
pVP5(-168)CAT	VP5 promoter -4 to -168	pSVOd-CAT	VP5
pVP5(-168)CAT Δ 2	VP5 promoter	pSVOd-CAT	VP5

Transcriptions were carried out in 50mM Tris HCl, pH 8, 10 mM to 12 mM MgCl₂, 0.6 mM ATP, GTP and UTP, 1 mM CTP, 80 mM KCl, 200 mM DTT (dithiothreitol), 50 μ M [α -³²P]CTP (10 μ Ci), 15 to 29 ng/ μ l DNA, and nuclear extract (75 μ g/ml protein). The transcription mixture was incubated for 60 minutes at 30°C. RNA was purified by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. The RNA pellets were dried under vacuum for 15 minutes and denatured with glyoxal. Analysis of RNA was accomplished by gel electrophoresis for 600 volt-hours in a 1.6%

agarose gel in phosphate buffer with recirculation as described by Beck and Millette (2). The gels were dried under vacuum and exposed to Fuji RX film for autoradiography at -80°C for 24 to 48 hours.

Gel Mobility Shift Assays

Gel mobility shift reaction mixtures contained 0.5 ng to 1.5 ng (5000 to 8000 cpm) DNA probe, 2.8 μg poly(dI-dC):poly(dI-dC) (Pharmacia), and nuclear extract (1.8 μg protein) mixed in a reaction buffer providing final concentrations of 6 mM Tris-HCl, pH 7.9, 40 mM KCl, 2 mM EDTA, 0.2 mM DTT (dithiothreitol), 8% (v/v) glycerol in a total volume of 25 μl . Following a 30 minute incubation at room temperature, 2 μl of 0.25% bromphenol blue was added and the samples were electrophoresed at 4°C through polyacrylamide gels made with 4% acrylamide, 0.13% bisacrylamide in a Tris-borate buffer that was 25 mM Tris base, 25 mM boric acid, and 1 mM EDTA. Gels were vacuum dried and exposed to Fuji RX films with intensifying screens.

DNA Competition Binding Assays

The DNA competition binding assays were carried out under the same conditions specified for gel mobility shift assays except for the following. A 40-100 molar excess of unlabelled competitor DNA added to the reaction mix which was then incubated at room temperature for ten minutes prior to adding the radiolabelled probe.

Then 0.2 to 0.5 ng of DNA probe was mixed into the reaction mixture, and room temperature incubation continued for an additional 20 minutes. The samples were then electrophoresed as described above for gel mobility shift assays.

CHAPTER III

RESULTS

I. USE OF AN *IN VITRO* TRANSCRIPTION SYSTEM TO STUDY HSV-1 $\beta\gamma$ (γ_1) GENE TRANSCRIPTION

It is possible to generate RNA from an isolated promoter sequence via *in vitro* transcription in a soluble cell free system (16, 57). Using extracts prepared from cells not infected with HSV-1, it is expected that promoters from HSV-1 α genes would be readily transcribed. Because β and $\beta\gamma$ genes require α products for expression, transcription from these promoters is not predicted under the same experimental conditions.

A. Immediate Early (α) Genes, But Not Later Classes, Are Transcribed by a Nuclear Extract from Infected Cells.

Before the regulation of VP5 transcription could be studied by *in vitro* assay, it was necessary to demonstrate that it is possible to generate RNA from HSV-1 promoter templates in soluble cell-free systems. This was accomplished by choosing promoters from the various kinetic classes and subjecting them to transcription with extracts prepared from uninfected HeLa cells by the Dignam protocol as described. The ICP0 promoter (α gene) transcribed readily in the soluble cell-free extracts yielding a specific transcript of 1.4 kbp

(figure 4, lane 4). However, promoters from the later kinetic classes transcribed poorly and, at most, yielded only end-to-end transcripts on a consistent basis. Thus the *tk* (β_1) and gB ($\beta\gamma$) templates gave no discernible transcripts (figure 4, lanes 3 and 5 respectively). The VP5 ($\beta\gamma$) template gave only low yields of the 1.1 kbp RNA (figure 4, lane 6).

Repeating this experiment with a new preparation of extracts from uninfected cells made by the Dignam method yielded results consistent with the previous experiment (figure 5). The α promoter ICP0 once again yielded a prominent specific transcript of 1.4 kbp. (figure 5, lanes 2 and 7). Promoters of all other kinetic classes failed to yield any specific RNA products (figure 5, lanes 3, 4, 6, 8 and 9), including the VP5 promoter (figure 5, lane 5).

As demonstrated from the preceding experiments illustrated in figures 4 and 5, promoters of the β and $\beta\gamma$ classes supported little or no transcription in *in vitro* systems in the absence of HSV-1 α products. This is consistent with what is known about the requirements for herpesvirus replication *in vivo*. Logically, the next step would be to prepare nuclear extracts which contained α proteins.

B. Transcription With Infected Cell Extracts

The simplest and most direct method of enriching a nuclear extract with early HSV-1 proteins is to prepare the extracts from cells that have been infected with the virus. Such extracts were

Figure 4. *In vitro* transcription from various HSV-1 promoters using a cell free soluble nuclear extract prepared by the Dignam protocol. Lanes 1 and 7, bacteriophage λ DNA cut with HindIII as markers. Lanes 2 and 8, pBR322 DNA cut with A1uI as markers. HSV-1 DNAs used as templates for *in vitro* transcription and the promoters they contained are as follows: Lane 3, gB promoter (2.7 kb fragment SalI/KpnI fragment of pGTSa2); Lane 4, ICP0 promoter (2.9 kb EcoRI/SalI fragment of pKSBB0); Lane 5, tk promoter (2.04 kb PvuII fragment of pHSV106); Lane 6, VP5 promoter (1.7 kb SalI fragment of pIM10). End-to-end transcripts are indicated by • and specific transcripts by >.

Figure 5. *In vitro* transcription of HSV-1 promoters from various kinetic classes testing two soluble cell free nuclear extracts separately prepared via the Dignam protocol. Lanes 2 through 5 are the results of transcriptions performed with an extract at 3.1 μg per μl protein concentration. Lanes 6 through 8 are the results of transcriptions performed with an extract with a concentration of 1.3 μg per μl protein. Lanes 1 and 10, bacteriophage λ DNA cut with HindIII as a marker. Lanes 2 and 7, ICP0 promoter (2.9 kb EcoRI/SalI fragment of pKSBB0). Lanes 3 and 8, *tk* promoter (2.05 kb PvuII fragment of pHSV106). Lanes 4 and 9, gB promoter (2.7 kb SalI/KpnI fragment of pGTSa2). Lane 5, VP5 promoter (1.7 kb SalI fragment of pIM10). Lane 6, gH:L promoter (1.21 kb KpnI/PvuII fragment of pHSV106).

prepared and tested for *in vitro* transcription. They uniformly failed to yield detectable specific RNA product.

Multiple attempts were made to optimize conditions for transcription, including substituting other divalent cations (Zn^{++} and Ca^{++}) for magnesium, as well as changing the Mg^{++} concentration. All trials were without success. Representative results of such trials are seen in figure 6, lanes 3 and 4. It was interesting to note that there was a slight difference in RNA produced under conditions of 10mM versus 12mM Mg^{++} (figure 6, all lanes). Despite the lack of readable transcripts from transcriptions run using extracts made from infected cells, there appeared to be materially greater yield of nonspecific RNA (figure 6, lanes 3 and 4). I therefore elected to conduct subsequent experiments with β and $\beta\gamma$ promoters with 12 mM Mg^{++} .

C. *In Vitro* Transcriptions With Shapiro Extracts

While this work was in progress, a new method for making HeLa nuclear extracts for cell free transcription systems was published (57). The authors asserted extracts produced by this protocol yield an increased efficiency of transcription initiation ranging from 2 fold to 20 fold over Dignam extracts. The major difference between the Shapiro system and the Dignam system involved replacing the divalent cations normally found in cell extraction buffers, principally Ca^{++} and Mg^{++} with a mixture of the polyamines spermine and spermidine. The authors maintained that

Figure 6. *In vitro* transcription of HSV-1 promoters testing magnesium ion concentrations in extracts prepared via the Dignam protocol. Extracts from mock infected cells were used in lanes 1, 2, 5, 6, 7, 8). Extracts from HSV-1 infected cells were used in lanes 3 and 4. Lanes 1, 3, 5, and 7 were run at 10 mM magnesium. Lanes 2, 4, 6, and 8 were run at 12 mM magnesium. End-to-end transcripts are indicated by ■ and specific transcripts by >.

the presence of polyamines may more nearly approximate the intranuclear environment than do buffers containing divalent cations. Additionally, the authors proposed that the substitution of polyamines for divalent cations might eliminate the possible activation of endogenous DNases within the cell by Mg^{++} and Ca^{++} . To determine if Shapiro extracts might provide better transcription of the later gene classes, I prepared uninfected and infected extracts according to the published protocol. These were then tested for their ability to transcribe from various HSV-1 promoter templates.

The uninfected Shapiro extracts revealed some evidence of discrete RNA bands from the α promoters (figure 7, lanes 2 and 6). However, there were no detectable transcripts from promoters of the later HSV-1 kinetic classes (figure 7, lanes 1, 3, 4 and 5). A transcription performed with nuclear extracts made from HSV-1 infected cells yielded no detectable product (figure 7, lane 7).

Despite the lack of clearly distinguishable RNA bands, I was optimistic that it might still be possible to produce a cell free, soluble system capable of transcribing β and $\beta\gamma$ promoters. The transcription done with the VP5 and *tk* promoters yielded more product RNA (albeit degraded) than had been detected on previous assays. I thought it was conceivable that a signal from discrete bands might be obscured by the heavy degradation, and that it might be possible to modify transcription conditions to enhance the likelihood of readable transcripts.

Figure 7. *In vitro* transcription using uninfected (lanes 1-6) and infected (lane 9) nuclear extracts prepared by the Shapiro protocol. Transcription templates are: Lane 1, gB promoter (2.7 kb SalI/KpnI fragment of pGTSa1); Lane 2, ICP0 promoter (2.9 kb EcoRI/SalI fragment of pKSBB0); Lane 3, VP5 promoter (1.7 kb SalI fragment of pIM10); Lane 4, *tk* promoter (2.05 kb PvuII fragment of pHSV106); Lane 5, gD promoter (SmaI subfragment of BamHI J); Lane 6, IE4/5 promoter (1.89 BamHI fragment of pRB122); Lane 7, gH:L promoter (1.21 kb KpnI/pVulI fragment of pHSV106); Lane 8, bacteriophage λ DNA digested with HindIII used as a marker. End-to-end transcripts indicated by • and specific transcripts by >.

Subsequently, transcription conditions were varied experimentally in an attempt to optimize discrete bands of product and minimize RNA fragmentation. Additionally, attempts were made to isolate any possible sources of exogenous RNase activity. These trials included eliminating the RNase inhibitor (Inhibit-Ace) to determine if it was effective in decreasing enzymatic RNA degradation, and eliminating the creatine phosphokinase and creatine phosphate from the reaction mix as possible origins of RNase. Because I was specifically interested in transcription from the later promoters, gB (β) was used as the template.

These attempts were all to no avail. Despite the presence of prominent end-to-end transcripts with uninfected extracts, no specific RNA products were detected (figure 8, lanes 1, 4 and 6). Attempts at transcription using infected cell extracts yielded no detectable RNA bands (figure 8, lanes 2, 3, 5 and 7).

D. In Vitro Transcriptions using *vhs-1* Infected Nuclear Extracts

One hypothesis explaining the failure of infected cell extracts to yield discrete RNAs is that these extracts contain a virus-induced nuclease (RNase). It is known that RNase activity is utilized by the virus in the process of switching protein synthesis from host to viral products (30, 31, 32, 61). As previously noted, one of the mechanisms utilized by the virus to redirect the host biochemistry away from its own macromolecule synthesis toward viral protein

Figure 8. *In vitro* transcription testing various reaction conditions with uninfected and infected nuclear extracts prepared by the Shapiro protocol. The transcription template in all trials was the gB promoter (1.7 kb SalI/KpnI fragment of pGTSa2). Lanes 1, 3, 4, and 6 were run with uninfected extracts. Lanes 2, 5 and 7 were run with HSV-1 (KOS strain) infected extracts. Lanes 1 and 2, Shapiro reaction conditions. Lane 3, uninfected Dignam extract run under Shapiro reaction conditions. Lanes 4 and 5, Shapiro conditions minus the RNase inhibitor Inhibit-Ace. Lanes 6 and 7, minus creatine phosphokinase and creatine phosphate.

production is the disaggregation of host polyribosomes and degradation of host mRNAs. This process is mediated by a virion-specific protein, the VHS protein that is present in the tegument of the infecting virion, and therefore is introduced into the host cell with initial penetration of the particle.

The first effort to eliminate the nuclease activity involved the use of a *vhs1* mutant. The *vhs* mutant is derived from the parental HSV-1 KOS strain and is defective in the virion-associated host protein synthesis shut-off. Cells infected with the *vhs1* mutant in particular exhibit prolonged mRNA half-life compared to cells infected with the parental KOS strain (61).

I decided to attempt to exploit the characteristics of the *vhs1* mutant and produce an extract from infected cells that might be capable of transcribing from β or γ class promoters. The initial trial was the production of Dignam extracts from HeLa cells infected with *vhs1* virus for eight hours. Transcriptions run with the infected HeLa extracts revealed no transcription from the infected system.

As previously noted, the VHS protein is a γ product, but it is packaged in the tegument of an infecting virion. It is released into an infected cell with initial penetration of the virus. However, it is known that HSV-1 has a secondary host shut-off function that is coded for by a γ gene. I thought it possible that the degradation observed on transcriptions might be due to this late viral RNase rather than the defective VHS protein. I attempted to exploit this separation of RNase activities to produce HSV-1 infected extracts

capable of *in vitro* transcription.

I therefore prepared a Dignam extract with cycloheximide reversal and actinomycin D block using *vhs1* mutant virus. The antifungal agent cycloheximide is a protein synthesis inhibitor, and the antineoplastic drug actinomycin D blocks transcription. The protocol developed involved infecting HeLa monolayers with *vhs1* virus in the presence of cycloheximide. Because the β and γ genes require α products for protein synthesis (and the secondary *vhs* function is mediated by a γ gene), this treatment enriches the cell culture in α mRNAs (32). Subsequent reversal of the cycloheximide block in the presence of actinomycin D would result in translation of α mRNAs but prevent transcription of the β or γ genes and thereby prevent expression of all delayed early and late gene expression, including the RNase activity.

Transcriptions were run utilizing a variety of promoters from the various HSV-1 kinetic classes with both uninfected and infected, cycloheximide reversed, actinomycin D blocked nuclear extracts. Unfortunately, attempts at transcription with these infected nuclear extracts did not yield any detectable transcription products. Even ICP0 and IE 4/5, promoters that had consistently produced specific transcripts from uninfected extracts, did not reveal any RNA bands (figure 9, lanes 1 through 4). As expected, the gH:L promoter did not generate discrete RNA (figure 9, lanes 5 and 6). These results can be explained by the possibility that the extracts prepared from uninfected cells still contained RNase activity.

Figure 9. *In vitro* transcription utilizing uninfected and vhs1 infected nuclear extracts prepared via the Dignam protocol. All extracts were prepared from cycloheximide-reversed, actinomycin D blocked cells. Transcription templates were: Lanes 1 and 2, ICP0 promoter (2.9 kb EcoRI/SalI fragment of pKSBB0); lanes 3 and 4, IE 4/5 promoter (1.89 kb BamHI fragment of pRB122); lanes 5 and 6, gH:L promoter (1.21 kb KpnI/PvuII fragment of pHSV106). Lane 7, λ DNA cut with HindIII and used as a marker.

An alternative explanation for the failure of *in vitro* transcription to generate distinct RNA is that the infected cell extracts contain DNase activity that destroyed the templates. It is known that HSV-1 codes for at least one alkaline exonuclease (17) and there may possibly be others. This led to the question of whether, in addition to the documented RNase activity present in infected cells, there might also be some DNase activity that destroys the templates before detectable products were present. Therefore, a series of experiments was devised to address this question.

The basic approach of these experiments was to incubate DNA under transcription reaction conditions for varying increments of time from zero to 60 minutes with extracts made of infected and mock infected cell nuclei. To differentiate between exonuclease and endonuclease activity, intact, covalently closed circular plasmid DNA (pBR322) was used in half the trials (figure 10, lanes 4, 6, 8, 10, 12, 14, 16, and 18). Linear DNA, pBR322 opened at the EcoRI site was used in the other half (figure 10, lanes 5, 7, 9, 11, 13, 15, 17, 19). The possibility of DNase activity in the nucleic acid purification portion of the experiment was ruled out by incubating intact and linear pBR322 for 60 minutes in Tris-EDTA buffer and extracting with phenol-chloroform-isoamyl alcohol (PCIA) by the usual technique. Finally, to test for the presence of nuclease activity in the transcription buffer itself, an additional 60 minute incubation of linear and uncut DNA in transcription buffer alone was done. This

Figure 10. Linear (L) and circular (U) pBR322 incubated with Dignam nuclear extracts from HSV-1 (KOS strain) infected (H) or uninfected (M) HeLa cells. Plasmid DNA was either left uncut or linearized at the EcoRI site and incubated under transcription conditions for the indicated time periods. Following incubation, the DNA was extracted with phenol-chloroform-isoamyl alcohol (PCIA), then CIA, and purified by ethanol precipitation. The DNA was redissolved in Tris-EDTA, pH 7.4, electrophoresed on an agarose gel, and visualized by ethidium bromide staining. Lanes 1 and 20, bacteriophage λ DNA restricted with HindIII. Lane 2, uncut pBR322. Lane 3, pBR322 linearized at the EcoRI site. Lanes 4 through 7, DNA incubated approximately 10 seconds. Lanes 8 through 11, DNA incubated 20 minutes. Lanes 12 through 15, DNA incubated 60 minutes. Lanes 16 and 17, DNA plus transcription buffer without added nuclear extracts. Lanes 18 and 19, DNA plus transcription buffer without added nuclear extracts and PCIA extraction.

last trial was not extracted with PCIA. The fate of the DNA was then determined by agarose gel electrophoresis (figure 10).

Interestingly, these tests revealed rapid degeneration of circular DNA in all nuclear extract incubations (figure 10, lanes 4, 6, 8, 10, 12, 14). The linearized plasmid DNA survived degradation to some extent in all extracts tested (figure 10, lanes 5, 7, 9, 13, 15).

While the preceding experiment did not confirm our suspicions about a DNase activity destroying linear templates in the transcription mix, it effectively closed the door on pursuing another alternative that had been under consideration, the use of a circular construct as a transcription template.

Despite repeated attempts at manipulating conditions, virus and extracts, I was unsuccessful in achieving the goal of this avenue of inquiry--successful *in vitro* transcription from cell free soluble extracts of the VP5 promoter. It was decided, therefore, that further elucidation of the viral transcription using nuclear extracts was beyond the scope of the current question, and I elected to pursue another course of inquiry.

2. USE OF COMPETITION BINDING ASSAYS TO STUDY VP5 LEAKY-LATE-DNA BINDING PROTEIN INTERACTIONS

Shin Chen, working in the Millette laboratory, identified a sequence located between -64 and -75 relative to the VP5 cap site that is important in transcriptional regulation (figure 11). It was found that this site bound a cellular factor (LBF) which, by DNaseI

footprint analysis was found to bind to the sequence GGGCCATCTTGAA. This sequence was provisionally designated the leaky-late binding sequence (LBS). Deletion or mutation of this sequence resulted in decreased expression from the VP5 promoter in

Figure 11. VP5 promoter identifying the putative leaky late binding sequence.

transient expression assay studies with HSV-1 superinfection (10). DNA binding studies carried out in the Millette laboratory revealed that this VP5 sequence forms similar complexes with both infected or uninfected cell nuclear extracts, yielding two major complexes: Complex A, the slower moving band, and Complex B, the faster (10).

Computer analysis of the HSV-1 DNA sequence identified similar sequences in the promoters of the genes for glycoprotein D (36), and in the unique long 37 and unique long 46 (UL37 and UL46) genes (37). Additionally, a sequence exhibiting high homology to the LBS was identified in the coding (internal) portion of the UL37 gene. Similar sequences are also found in the human immunodeficiency

virus (HIV) long terminal repeats (LTRs). However, none of these sequences are identical to the identified putative LBS (table II).

I chose to investigate the following question: Does the cellular protein LBF bind to any of the above sites and therefore possibly

TABLE II

VIRAL GENE PROMOTER	DNA SEQUENCE	LOCATION RELATIVE TO CAP SITE
HSV-1 SEQUENCES		
VP5	LBS Core Sequence GGGCCATCTTGAATGCAC	-70 (Cap)
gD	GGGCCATtTTacg	-63 (Cap)
gB	GGCCATCgTcgA GcCCgTCTTtgAT	+134 (Cap) -249 (Cap)
UL46	GGGCCATggG	-55 (ATG)
UL37	GGGCCATtTgGg	-279 (Cap)
UL37 (internal coding sequence)	GcGCCATCTTGA	+610 (ATG)
HIV SEQUENCE		
HIV (-168)	gtTCTTGAA	-134 (Cap)

play a role in the expression of the corresponding genes? With this question answered, it would then be possible to do a sequence comparison of the promoters that exhibited competition and deduce a consensus sequence for the binding of this protein. In addition, this analysis would provide clues as to whether similar binding sites are involved in the regulation of other viral genes.

A series of DNA binding competition experiments was designed in which the promoters and other DNA fragments exhibiting sequence similarities to the VP5 LBS would be incubated with DNA containing the VP5 sequence and nuclear extracts under DNA binding conditions. In binding studies, DNA containing the sequence of interest is labeled with radioactive phosphorus (^{32}P) by Klenow fill-in and subsequently incubated with nuclear extracts under conditions that allow DNA binding proteins to adhere to the labeled probe. The reaction mix is then electrophoresed on an acrylamide gel. In this assay, the DNA that is bound by protein will migrate through the acrylamide at a rate that is slower than unbound probe DNA. After the gel is dried, it is exposed to x-ray film and the radioactively labeled DNA probe will cause a darkening of the film at a point corresponding to the location of the DNA or DNA-protein complex in the gel.

In competition experiments unlabeled DNA, the competitor, is included in large molar excess over the probe in the reaction mix with limited amounts of protein. The basic idea is that sequences exhibiting homology to the DNA that is bound by a particular protein will themselves bind that same protein, i.e., they will compete with the probe for the limited DNA binding protein. If competition occurs, then a decreased signal from the labeled DNA-protein complex results on the autoradiogram. The stronger the competition, the weaker the signal from the bound probe, with the strongest competitor possible being the unlabeled homologue of the probe

DNA.

The first problem was to design the experiment so that the detection of weak competition is possible. Even under optimal competitive conditions, i.e., large molar excess of homologous DNA, there is often an appreciable signal from the protein-bound probe. It therefore can be difficult to detect competition at some level less than what would be expected from a labeled homologue. That is, the sensitivity of the assay needed to be maximized. It is imperative to avoid an excess of protein, or competition will not cause a decreased signal from the probe.

To accomplish this, a series of experiments was carried out in which a potential competitor was utilized to define conditions for subsequent studies. The glycoprotein D promoter fragment contains a sequence homology to eight out of ten bases with the VP5 promoter sequence of interest. If this segment of the gD promoter was bound by the putative leaky-late binding factor, I thought it likely that competition would occur, but at some degree less than that of an unlabeled VP5 homologue. Therefore, the gD promoter was used to define conditions for the competition studies. Detectable competition was represented as a slightly fainter signal in complex A, and a somewhat greater difference in signal strength at complex B. This competition occurred between gD and VP5 under the conditions of approximately 0.75 μ g protein with a 40-fold molar excess of gD DNA. An example of such competition with the gD promoter can be seen in figure 12, lane 6 and 7.

Figure 12. Competition binding of labeled VP5 promoter versus unlabeled DNA fragments. DNA binding competition was carried out with 1.8 μ g of protein of infected nuclear extracts as described. A, B, and P, complexes A, B and free probe, respectively. Lanes 1 and 2, free probe. Lane 3, labeled VP5 promoter without competitor. Lanes 4 and 5, 40-fold and 60-fold molar excesses of unlabeled VP5 promoter. Lanes 6 and 7, 40-fold and 60-fold molar excesses of glycoprotein D promoter. Lane 8, 40-fold molar excess of HIV promoter (+1 to -423). Lanes 9 and 10, 40-fold and 60-fold molar excesses of HIV promoter (+1 to -167).

Competition studies were subsequently performed with 0.75 μ g protein in which all the above-mentioned sequences were tested against the VP5 LBS. As expected, the degree of competition declined with decreasing homology between the LBS and the competing DNA. In an attempt to detect weak competition, the quantity of competitor excess was titrated against the LBS and DNA binding protein in the competition mix.

The initial experiment involved running competition studies with labeled VP5 probe versus unlabeled homologue as a positive control. Additionally, DNA containing a promoter for a gene coding for a *trans*-acting factor located in the 3' long terminal repeat (LTR) of the human immunodeficiency virus (HIV) was included. This HIV promoter exhibited a region of homology with only the terminal 7 of 12 bases in the proposed LBS (table II).

Competition was detectable in all cases, although it varied with respect to the strength of competition and with respect to which of the two major complexes, A or B, was competed against more strongly. As expected, the unlabeled VP5 homologue exhibited the strongest competition, (figure 12, lanes 4 and 5) but with the stronger competition occurring against the upper band, complex A. The glycoprotein D promoter competed as expected against the VP5 fragment, but with a stronger signal reduction occurring in the faster moving complex B (figure 12, lanes 6 and 7).

The HIV fragments also exhibited competition, although it was somewhat weaker than the competition exhibited by the other

fragments. This was not unexpected because the HIV sequence exhibits homology with only the terminal seven of 12 bases of the putative LBS. Of interest is the difference in competition exhibited by the two HIV fragments. Both of these pieces of DNA originate from the same portion of the viral genome, the only difference between them is that the HIV promoter fragment in figure 12, lane 8 contains 256 bases upstream from the cap site that are not present in the other HIV fragment run in lanes 9 and 10 in figure 12, i.e., +1 to -423 versus +1 to -167.

To verify the competition between the VP5 fragment and the HIV fragment, another test was run utilizing a 100-fold excess of the small (-167 to +1) HIV promoter (figure 13, lane 4) against an 80-fold excess of the unlabeled VP5 competitor (figure 13, lane 3). As demonstrated previously, there was stronger competition against complex A from the VP5 fragment. The same phenomenon is exhibited with respect to the HIV fragment (figure 13, lanes 3 and 4).

Computer search had revealed the presence of potential homologues of the VP5 LBS in other HSV-1 genes. To determine if these too bound LBF, further competition binding studies were performed to assess whether these sequences bound the same cellular factor bound by VP5 LBS.

As seen in previous experiments, the HIV sequences competed with the VP5 fragment, although weakly (figure 14, lanes 4 and 5). With respect to the herpes sequences, it was interesting to note that

Figure 13. Competition binding of labeled VP5 promoter versus unlabeled DNA fragments. DNA binding competitions were carried out with 1.8 μ g of protein infected nuclear extracts as described. A, B and P indicate complexes A, B and free probe respectively. Lane 1, free probe. Lane 2, labeled VP5 promoter without competitor. Lane 3, 80-fold molar excess of unlabeled VP5 promoter. Lane 4, 100-fold excess of HIV promoter (+1 to -167).

Figure 14. Competition binding of labeled VP5 promoter versus unlabeled DNA fragments. DNA binding competition was carried out with 1.8 μ g of protein of infected nuclear extracts as described. A, B and P indicate complexes A, B and free probe respectively. Lane 1, free probe. Lane 2, labeled VP5 promoter without competitor. Lane 3, 40-fold molar excess of VP5 homolog. Lane 4, 80-fold molar excess of HIV (+1 to -423). Lane 5, 80-fold molar excess of HIV (+1 to -167). Lane 6, 100-fold molar excess of UL37 (+203 to -415). Lane 7, 100-fold molar excess of UL37 coding region (+333 to +1019). Lane 8, 100-fold molar excess of UL46 promoter (+118 to -161). Lane 9, 100-fold molar excess gB promoter (+134 to -249).

the internal UL37 sequence exhibiting homology to the VP5 coding sequence exhibited stronger competition than did the promoter sequence (figure 14, lanes 6 and 7). Both UL46 and glycoprotein B exhibited some weak competition, more remarkably against complex B than against complex A (figure 14, lanes 8 and 9).

In general, the HSV-1 promoters appeared to compete more strongly against complex B, while the HIV, though weakly competitive, exhibited more striking results against the larger complex A..

CHAPTER IV

CONCLUSIONS

1. *IN VITRO* TRANSCRIPTION

The herpes simplex virus VP5 promoter has, so far, been difficult to transcribe in *in vitro* transcription systems. Repeated attempts at manipulating experimental conditions and trials with different strains of virus were unsuccessful. While it is not possible to definitely ascribe this lack of success to any one attribute of viral physiology, it is possible to hypothesize about the negative results.

Herpes simplex virus has a well documented, though poorly understood system by which host protein synthesis is redirected to the production of viral progeny (30, 31, 32, 50, 60). This system is known to degrade host mRNA, and it has been hypothesized that this RNA destruction is necessary for the efficient switching of transcription between the temporal classes. The persistent presence of what is construed to be RNase activity in the transcription mixture prepared from infected extracts is the most obvious reason for the lack of readable transcripts. Multiple attempts that were made to circumvent this problem and all proved unsuccessful.

Another cause for lack of readable transcripts originating from late promoters may be the failure of the template itself to survive long in the transcription mix from infected cells. It is interesting that

the surviving templates in the incubation experiments (figure 10) were linear, while the circular plasmids were degraded. An endonuclease activity is the obvious explanation for the failure of intact circular DNA to persist in the transcription mix. However, the possible reasons for the preferential degradation of the formerly circular template over a linearized template incubated under the same circumstances remains elusive.

The presence of high molecular weight DNA at the origin in figure 10, lanes 4 and 6 is interesting. This could possibly indicate the presence of a DNA binding protein in the nuclear extract. Incubation with nuclear extract for a few seconds conceivably could permit protein binding.

The reasons for the failure of the Shapiro extracts to produce stable transcripts is not understood. There was one major difference between our protocol and the one published by the author. Because it was necessary to make infected extracts, our cells were grown in a monolayer culture in roller bottles. The published protocol specified the use of cells grown in suspension in spinner cultures. It may be that the physical and biochemical differences between the two HeLa cell lines were significant contributors to the failure to produce stable mRNAs by *in vitro* transcription.

2. DNA BINDING COMPETITION STUDIES

The gel mobility assays were somewhat more successful. As demonstrated in figures 13, 14, and 15, competition occurred in all

cases, although at different strengths. In general, the herpes promoters exhibited a greater level of competition than did the HIV-1 promoter, although the gB promoter was competitive only at a very great (100 fold) molar excess.

It was interesting to note that there was a difference in competition between the HIV preparations. In some cases the HIV DNA competed only weakly (figure 12, lanes 8, 9 and 10; figure 14, lanes 4 and 5). However, in cases in which competition was observed, the promoter from pU3RIII competed more strongly than pU3RIII-167. It is notable that the larger plasmid, pU3RIII, contains 256 bases upstream that are not present in pU3RIII-167. Analysis of this sequence reveals three areas of partial homology to the putative LBS located at -351, -398 and -411 that are not carried by the smaller plasmid (figure 16). Until it is demonstrated otherwise, these regions of possible LBF binding must be considered potentially responsible for the different levels of competition exhibited by the HIV fragments.

It is interesting to note the differences in competition between the UL37 internal coding sequence and the UL37 promoter sequence. The internal sequence exhibits ten base pairs of homology with the VP5 LBS. In contrast, the promoter sequence exhibits three base pairs that are not homologous at positions #8, #10 and #12 of the VP5 LBS (table 3). The difference in competition between these two fragments of UL37 suggests that these three bases are important in binding. It should be noted that the two fragments of HSV-1 UL37

GAACT AGATGG
 GACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTACTTCCCTGATTAGCAGA
 -411 -398

 CCCGGT
 ACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTTGGATGGTGCTACAAGCTAG
 -351

 TACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTTAC
 ACCCTGTGAGCCTGCATGGATGGATGACCCGGAGAGAGAAGTGTTAGAGTGGAGGTTTGA

 ACCGGG AAGTT
 CAGCCGCCTAGCATTTTCATCACGTGGCCCGAGAGCTGCATCGGGAGTACTTCAAGAACTG
 -159 LBS homology

 CTGATATCGAGCTTGCTACAAGGGACTTTCGCTGGGACTTTCAGGGAGGCGTGGCCTG
 GCGGGACTGGGGAGTGGCGAGCCCTCAGATCCTGCATATAAGCAGCTGCTTTTTGCCTGTACT

Figure 15. HIV sequence from -423 to +1 of the 3' long terminal repeat.

DNA are very close to the same size (686 base pairs for the internal sequence versus 616 base pairs for the coding sequence). Thus the possibility of nonspecific DNA binding contributing to the difference in competition is not great.

Based on the above data, the following consensus is proposed for the LBS: GGGCCATNTTGA (table III).

After this work was completed, several authors reported successful isolation of a protein that binds to a similar sequences in several different genes (45, 50, 56, 58).. This protein, alternately named YY1, NF-E1, and NF- δ among others, has been reported to function as a transcription activator in the context of ribosomal protein promoters (56), and as a transcriptional repressor when binding the human immunoglobulin κ 3' enhancer and the human

TABLE III

VP5	G	G	G	C	C	A	T	C	T	T	G	A	A
gD	G	G	G	C	C	A	T	t	T	T	a		
gB				C	C	A	T	g	a	T	G	A	A
UL46	G	G	G	C	C	A	T	g	g	g			
UL37 - Promoter			G	C	C	A	T	t	T	g	G	g	
UL37 - Coding			G	C	C	A	T	C	T	T	G	A	
HIV							T	C	T	T	G	A	A

Table 3. The proposed binding sequence of fragments with homology to the LBS. Upper case letters and shading indicate base pair homology. Lower case letters and unshaded areas indicate lack of homology.

immunoglobulin heavy chain μ E1 site (45). Subsequent work carried out by Lisa Mills in the Millette lab has demonstrated that the cellular protein LBF is the same molecule as transcription factor YY1 (41) and that this protein plays a role in the HSV-1 $\beta\gamma$ genes by viral factors.

3. SUMMARY

In summary, I have presented several lines of experimental investigation that have indicated that genes of the β , $\beta\gamma$, and γ kinetic classes of herpes simplex virus type 1 are resistant to analysis by *in vitro* transcription. HSV-1 genes of the α class require only the viral

protein α TIF in addition to cellular transcription factors for activation. Because α TIF is packaged in the tegument of the infecting virion, it is released into the host cell with initial viral penetration. Therefore, it is present at the onset of the transcription of α genes. However, promoters of the later genes require α gene products. Attempts at preparing nuclear extracts capable of transcribing these later promoters in general, and from $\beta\gamma$ promoters in particular, were uniformly unsuccessful. This failure to generate specific RNA may be attributable to persistent RNases in the nuclear extracts which are coded for by the viral DNA and are therefore endogenous to the virus. Further attempts at *in vitro* transcription will necessarily depend upon the successful repression of these virus specific enzymatic activities.

DNA binding competition studies revealed that an assortment of herpes simplex virus type 1 genes exhibiting partial homology to the LBS of the HSV-1 VP5 promoter competed for the same protein factor. Additionally, a short fragment of human immunodeficiency virus from the 3' long terminal repeat containing a seven base pair homology exhibited weak competition. Based on this work a consensus sequence was proposed. Subsequent work in the Millette laboratory demonstrated that this sequence binds a ubiquitous cellular transcription factor, alternately named YY1.

CHAPTER V

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