1997

Investigation of Transactivation of the Human C-Myc Gene by Herpes Simplex Virus Type 1

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Recommended Citation
Wu, Xian, "Investigation of Transactivation of the Human C-Myc Gene by Herpes Simplex Virus Type 1" (1997). *Dissertations and Theses.* Paper 6328.
https://doi.org/10.15760/etd.8182

THESIS APPROVAL

The abstract and thesis of Xian Wu for the Master of Science in Biology were presented May 30, 1997, and accepted by the thesis committee and the department.

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ABSTRACT

An abstract of the thesis of Xian Wu for the Master of Science in Biology presented May 30, 1997.

Title: Investigation of Transactivation of the Human c-myc Gene by Herpes Simplex Virus Type 1

The purpose of this investigation was to identify the regulatory elements involved in the transactivation of the human c-myc gene by herpes simplex virus type-1 (HSV-1). This study mainly focused on a major c-myc promoter P_2 (Fig. 1). Transient expression assays were carried out with a reporter plasmid, that contained either a wild type (+66wt c-myc) or mutant (+66 mt E2F, +66mt CT -I_2) c-myc promoter fused to the chloramphenicol acetyltransferase (CAT) gene, cotransfected with effector plasmids that contained the HSV-1 immediate early (IE) genes (encoding IE proteins, ICP4, ICP0, ICP27). The transient expression assays revealed that the HSV-1 IE protein, ICP4, was a major effector in transactivation of the human c-myc P_2 promoter by HSV-1, and ICPO provided a synergistic effect with ICP4. Cis-acting elements E2F and CT-1_2, which were potential binding sites for cellular transcription factors E2F and Sp1 respectively, were shown to be important in HSV-1 induced c-myc transactivation. In addition, these assays indicated that ICPO influences ICP4 mediated activation through these two sites. Electrophoretic mobility shift assays, oligonucleotide competition assays, and footprinting analysis together demonstrated that viral proteins didn't directly interact with P_2 promoter region. Three cellular transcription factors, Sp1, E2F, and YY1, however, specifically interacted with the P_2 promoter region, of which, Sp1 and its related proteins SpX bound to several other sites in addition of CT-I_2 site.

Moreover, SpX also appeared to interact with another cellular factor that bound together with it on the P_2 promoter region, and a second unknown cellular factor was also observed to interact with P_2 promoter region.

INVESTIGATION OF TRANSACTIVATION OF THE HUMAN C-MYC GENE BY HERPES SIMPLEX VIRUS TYPE 1

by

XIAN WU

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

MASTER IN SCIENCE

in

BIOLOGY

Portland State University

1997

DEDICATED TO

My Parents

ACKNOWLEDGMENTS

First and foremost, I wish to express my deepest personal gratitude to my thesis adviser, Professor Robert Millette. His trenchant guidance, continuous support, understanding and encouragement kept me going through my graduate study. His open-mindedness and great personality provided a pleasant working atmosphere in our research group.

My thanks to Dr. Lisa Mills for her helpful ideas and suggestions.

Many thanks to Professor Mary Taylor for her theoretical microbiology advice. I also thank Professor Carol Carter and Professor Dirk Iwata-Reuyl for their helpful ideas and discussions. Special thanks to Professor Leonard Simpson for his support during my stay in the biology department graduate program at Portland State University.

Very special thanks to Professor Jacqueline Piret for her scientific training and guidance during my graduate study at Northeastern University in Boston. My thanks to Dr. Barbara Rutledge for her incredible encouragement and faith.

To my parents, I express my deepest appreciation for their nurturing, continuous and unconditional love, especially to my Mom, who came to Portland in support of my graduate study.

Most especially, I thank my husband and my daughter for their continuous support throughout all my years of study in the United States.

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INTRODUCTION

Section I: The c-myc Gene and Protein

To understand the function of c-Myc, it is first necessary to understand the regulation of the cell cycle and the role of c-Myc in this process.

1. General aspects of the eukaryotic cell cycle

The cell cycle is traditionally divided into several phases, termed gap-1(G_1) phase, synthesis (S) phase, gap-2 (G_2) phase, and mitosis (M) phase. The mitosis phase is the most dramatic, leading to nuclear division (Fig. 1). In mitosis the nuclear envelope breaks down, the contents of the nucleus condense into visible chromosomes, and the cell's microtubules reorganize to form the mitotic spindle which leads to separation of the chromosomes. In most cells the whole of M phase takes only about an hour, which is only a small fraction of the total cycle time. Much longer period elapse in the G_1, S, and G_2 phases. Replication of nuclear DNA occurs only in the S phase of the cell cycle. The G_1 phase is the interval between the completion of mitosis and the beginning of DNA synthesis, and the G_2 phase is the interval between the end of DNA synthesis and the beginning of mitosis. These two phases provide additional time for the cell growth. During the G_1 phase the cell monitors its

environmental situation; when time is ripe, it commits itself to DNA replication. The G_2 phase provides a safety check, ensuring that DNA replication is complete before the cell enters into mitosis. Because cell needs time to grow before it divides, the standard cell cycle is generally quite long varying from 12 hours to years for mammalian cells (embryonic cells are exception). Such great variation in cell cycle occurs most commonly in the duration of G_1 phase. Cells in G_1, if they have not committed to themselves to DNA replication, can pause in their progress around the cycle and enter a specialized resting state, called G_0, where they can remain for days, weeks, or years before resuming proliferation.

For unicellular organisms, where each cell division generates a new individual, their proliferation is typically restrained only by the availability of nutrients and sex. In multicellular species, however, the component of cells must follow strict controls that limit their proliferation in order to produce and to maintain their organization of the body. At any instant, most cells in an adult are not growing or dividing, but instead they are in a resting state G_0 and performing their specialized function. In order to grow and divide, a cell has to receive specific signals from other cells. Many of these signals are protein growth factors, which bind to transmembrane receptors at the surface of target cell (Fig. 2). The intracellular portion of the receptor then catalyzes the production of molecules that act as intracellular signals to stimulate other molecules that are required for cell proliferation. This growth-factor-receptor generated intracellular signal transduction results in changes in gene expression.

Genes induced by growth factors fall into two classes: i) early-response genes

that are induced within 15 minutes of growth factor treatment, and whose induction does not require protein synthesis, and ii)delayed-response genes which, by contrast, are not induced until at least 1 hour after growth factor treatment, and their induction requires protein synthesis. The delayed-response genes are usually induced by the products of the early response genes (Fig. 2). Both classes of genes are silent and are not transcribed in the G_0 stage, but they are induced to a high level when growth factors are added to the medium. The early response genes, such as myc, fos, and jun, often encode gene regulatory proteins with oncogenic potential that function as transcription factors (60, 123). These proteins facilitate a cell's progression through the cycle to eventually achieve DNA synthesis in S phase (100). Overexpression of them can cause uncontrolled cell proliferation.

2. The role of human c-Myc in the cell cycle

The human c-myc gene belongs to one of cellular "early response" genes because its expression is independent of protein synthesis, and it is activated by most mitogenic stimuli during the G_0 to G_1 transition (68, 82). The c-myc is strongly induced within the first two hours by most growth stimuli (136) (Fig. 3). Cells expressing constitutively high levels of an exogenous c-myc gene introduced by transfection have a dramatically shortened G_1 phase (58). A study using a chimeric c-Myc polypeptide and the ligand-binding domain of the human estrogen receptor has shown that activation of c-myc is sufficient to stimulate DNA synthesis (33, 34). This tightly linked action of growth factors with the entry into the cell cycle strongly suggest that c-myc gene

expression is a major component in the regulatory networks associated with normal cell proliferation (19).

The c-myc gene is classified in the category of proto-oncogenes that can induce unrestrained cell growth when mutated, or when expressed inappropriately. Abnormal c-myc expression would alter the regulation of cellular genes, rendering cells more susceptible to transformation (2). Numerous studies have uncovered many mechanisms by which the c-myc gene can be activated in tumor cells. These include proviral insertion (51), gene amplification (20), and chromosomal translocation (21). The study with avian bursal lymphomas shows that gene activation by retroviral insertion is associated with levels of c-myc mRNA that are 10-100 fold higher than those in normal cells (51). In this case the inserted viral long-terminal repeat acts as a promoter for c-myc transcription. The similar high level of c-myc mRNA is also observed in human Burkitt's lymphoma in which a c-myc gene is translocated into an immunoglobulin gene. This results in displacement of the untranslated first exon by the immunoglobulin promoter (135). Each of these mechanisms leads to constitutive or elevated levels of c-myc expression. A further study established a consensus model in which "c-myc promotes tumor cell growth via a quantitative increase in protein level, rather than by qualitative changes in the protein" (19).

3. The c-myc gene structure

The c-myc gene is well conserved among mammals. The gene contains three exons (7). The first exon is a long nontranslated leader region which is preceeded by

three promoters, or transcriptional start sites, termed P_0, P_1, and P_2 (Fig. 4). The second and the third exons encode the major product of c-myc, a 65-kD nuclear protein. In normal proliferating cells, transcription from P_2, lying 174 bp downstream from P_1, is predominant, giving rise to about 75-90% of the cytoplasmic c-myc messenger RNAs (mRNAs); the transcription from P_1 accounts for the remaining of 10-20%. The third promoter, P_0, contributes only about 5% of the effective c-myc transcription (82). Both the P_1 and P_2 promoters contain TATA sequences and a GC-rich region that may bind the transcriptional factor Sp1.

4. The c-Myc polypeptides and their properties

The human c-myc gene encodes two polypeptides (Myc-1 and Myc-2) of 453 and 439 amino acids long with approximately 68,000 and 65,000 Dalton respectively (82). The amino acid sequences of Myc-1 and Myc-2 differ at the amino termini by 14 amino acids. Myc-1 is initiated at a CUG codon near the 3' end of the gene's first exon. Myc-2, the predominant protein, starts at a standard AUG initiation codon at the beginning of the second exon (Fig. 4) (46). Protein stability studies have shown that the c-Myc proteins are unstable in cells. Its half life is about 20-30 minutes, which is appropriate for a protein with a potential role in the regulation of cell proliferation (81).

The c-Myc proteins are phosphorylated at many sites by casein kinase (CK). They contain an 8 amino acid long nuclear location signal (NL) encoded in the third exon that directs the Myc's translocalization to the nucleus (Fig. 5) (73, 46). The carboxy-terminal of c-Myc polypeptides contains characteristic helix-loop-helix (H-L-

H) and leucine zipper (LZ) motifs (95). These are potential DNA sequence-specific binding domains that regulate transcription of genes through RNA polymerase II (57). The H-L-H domain of c-Myc proteins covers about 55 amino acids and is positioned at 30 residues from the protein's carboxy terminus; the remaining 30 amino acids constitute its LZ domain (Fig. 5). This unique structure indicates that the c-Myc proteins not only have a potential to bind to a specific DNA sequence, but they also can interact with other proteins with an appended LZ motif. The putative c-Myc target sequence is CACGTG (103). Screening of c-Myc partner proteins reveals that a protein of 160 amino acids, termed Max for " Myc Associated X ", contains a H-L-H(LZ) domain that forms a stable heterodimer with c-Myc. The Myc/Max complex binds about 100 fold more efficiently to the c-Myc target sequence than c-Myc alone (10, 103). Cells transfected with myc and max displayed a potentiation of the biological properties exhibited by myc alone (103). Recent observations demonstrate that (a) Max is considerably more stable than c-Myc, (b) c-Myc concentration is limiting in cells compared to Max, and (c) Myc/Max heterodimers are present in cell nuclei (78). Other experiments revealed that i) c-Myc transactivates a reporter gene with four tandem copies of its binding site fused upstream of a minimal *HSV*-tk gene promoter, ii)Max represses its basal activity, and iii) Myc/Max only yields a modest level of transactivation in fibroblastic cells (46). Not only able to associate with Max, c-Myc also acts as a homodimer or a heterodimer with two other early response proteins, Fos and Jun, to regulate gene transcription in the mammalian cell.

5. Regulation of cellular genes by c-Myc

As mentioned above, the c-Myc protein has all necessary features for a transcriptional regulatory factor, with protein dimerization, transcriptional activation, and DNA-binding domains (Fig. 5). To study the ability of c-myc to activate gene expression, different portions of the human c-myc gene were fused with the DNA-binding domain of the GAL4 yeast transcriptional activator protein and transfected with a reporter gene linked to GAL4 target sequence in yeast. Results from this experiment showed that there were three independent activation regions between amino acids 1 and 143 in the c-Myc polypeptide (59). Other studies using c-Myc-immortalized rodent fibroblasts have shown that c-Myc can positively regulate the hsp70 promoter and enhance expression of two cellular genes, mr1 and mr2, through a posttranscriptional mechanism (64, 104). However, most evidence indicates that overexpression of c-myc is correlated with the downregulation of a variety of different cellular genes. For example the H1 histone gene is downregulated in MEL cells in which terminal differentiation is blocked by a stably integrated and deregulated c-myc gene (18). A variety of cell surface molecules also have been shown to be downregulated by myc expression, such as the expression of major histocompatibility complex (MHC) class I molecules (70). C-Myc is reported to downregulate three collagen genes, the effect of which is dependent upon an intact LZ domain (142).

6. Regulation of c-myc expression

Determining what regulates the c-myc gene expression is central to

understanding the role of c-myc in cell transformation and in the response of cells to mitogens. Unlike the most other oncogenes, which are activated as the result of mutations within the protein coding regions, no amino acid changes are required for c-myc gene activation. Quantitative differences, however, are found in the levels of c-myc mRNA in tumor vs normal cells. The regulation of c-myc gene expression has been shown to occur at multiple levels, including the initiation of transcription, premature termination of transcription (or attenuation), and postranscriptional control (82, 128).

C-myc is transcribed from multiple, independently regulated transcription initiation sites or promoters (P_1, P_2, and P_0), which gives a extremely complex picture in its transcriptional regulation. P_1 and P_2 are the major principal promoters that contribute to about 95% of total cytoplasmic c-myc mRNA (82). In normal proliferating cells, RNA initiated from P_2 is in excess by 5- to 10-fold of the RNA initiated from P_1 (128). However, a shift in promoter usage, in which P_1 initiated RNA is equal to or greater than P_2 initiated RNA, is observed under certain physiological conditions and in many tumor cell lines (18, 130, 143). Numerous studies demonstrate that the regulation of P_1 and P_2 promoter usage acts in at least two ways. First, activity of both promoters can be increased or decreased in parallel in response to various physiological signals; second, the relative activity of the two promoters may change during differentiation and in response to genetic damage (49). This shift in promoter usage in Burkitt's lymphoma cells has been shown to be mediated by the presence or absence of trans-acting factors in these cells, by the juxtapostition of a heterologous

enhancer element from the immunoglobulin locus to which the c-myc gene is translocated (50, 128).

Cis-acting elements that regulate transcriptional initiation from the two promoters (P_1 and P_2) have been studied and characterized by several research groups. Deletion/transfection studies have shown that the nucleotide sequences between -293 and +513 (P_1 at +1) are sufficient for strong activity of P_1 and P_2 (Fig. 6). The region between -293 and -101 has a positive effect on both P_1 and P_2 initiation (49), but the region between -293 and -353 has a negative effect (75). The region between -157 and -101 contains five tandem repeats of the consensus sequence CCCTCCCC called CT elements. In vivo, integrity of this region with all five elements is required for initiating transcription from P_1, and for maximal activity of P_2 in vivo (50). A single copy of this element with inverted orientation, designed as CT-I_2, is positioned at 57 bp upstream of the P_2 transcription start site (Fig. 6). The CT-I_2 element exhibits an inhibitory effect on P_1 transcription and is required for P_2 transcription (Miller, H., 1989; 50). Both the five tandem CT elements and the single element CT-I_2 act additively to activate P_2 transcription. In contrast to their additive effect on P_2 activity, the five tandem CT elements and CT-I_2 have an antagonistic effect on P_1 transcription and thereby maintain high levels of P_2 initiated mRNA and lower levels of P_1 initiated mRNA. The combination of the heterologous transcriptional enhancer, and deletion of both the five tandem CT elements and the CT-I_2 site elicit a shift in promoter usage analogous to the situation observed in Burkitt's lymphoma cells which contain reciprocal translocations involving the human c-myc locus (50). Both the CT and CT-I_2 cis-

elements are able to bind the zinc finger protein Sp1 (50).

Two potential E2F binding sites centered at +100 and +123 (relative to P_1) termed E2F-1 and E2F-2 respectively (Fig. 6) within P_2 promoter have been studied by Hiebert's group. This study demonstrates that E2F is able to bind these two cis-elements which are within the region that is critical for P_2 promoter activity. In addition, the P_2 promoter is transactivated by the adenovirus immediate early protein (E1A), and this transactivation is dependent on these two E2F sites (53).

A cloned 37-kd protein, termed MBP-1, binds upstream of P_2 (Fig. 7). DNase I protection analysis has shown that MBP-1 protects a region from +123 to +153, just at the 5'of the P_2 TATA motif, and a transient expression study indicates that this protein is a negative regulator of c-myc transcription (111).

Initiation of c-myc transcription can either be upregulated or downregulated in response to growth factors and other stimuli. Growth factors such as TGF-α (22), PDGF (41, 122), and IL-2 (61, 52) can potentically upregulate c-myc transcriptional initiation. Elevation of c-myc transcription is also observed in B cells treated with anti-IgG antibody (14). Both TNF-α and TGF-β suppress c-myc expression, with TGF-β acting directly upon initiation of transcription (101). This possibly occurs via the pRB protein (101) since a region in the c-myc gene (+22 to +50) resembles the pRB control element of the c-fos gene.

Section II: Herpes simplex virus type 1 (HSV-1)

Before considering the involvement of herpes simplex virus in c-myc gene regulation, it is relevant to first know some of the features of HSV-1.

1. General background

Herpes simplex viruses (HSV) were the first of the human herpesviruses to be discovered and are among the most intensively investigated of all viruses. Their attraction lies in their biological properties, particularly their abilities to cause a variety of infections, to remain latent for the life time of their host, and to become reactivated and cause lesions at or near the site of initial infection. Herpes simplex virus type 1 (HSV-1) is a member of the Herpesviridae family. All Herpesviridae share common structures which include a double-stranded DNA of approximately 150 kb surrounded by a protein capsid containing 162 capsomeres. The capsomere is composed of either six (hexamers) or 5 (pentamers) subunits. This combined structure is called nucleocapsid. A phospholipoprotein envelope surrounds the nucleocapsid with glycoprotein spikes projecting from the surface (Fig. 8). The amorphous protein structure between the nucleocapsid and the envelop is called the tegument. A complete infectious particle, termed the virion, is composed of the above four components (Fig. 8).

The herpesviridae family is classified into three subfamilies: alphaherpesviruses, betaherpesviruses, and gammaherpesviruses. The alphaherpesviruses are characterized by their ability to grow rapidly in tissue culture in wide variety of cell types. The herpes

simplex viruses belong to the alphaherpesviruses. Herpes simplex viruses are grouped into two closely related types based on their serotypes, herpes simplex virus type-1 (HSV-1), and herpes simplex virus type-2 (HSV-2). The two types share 50% homology at the nucleic acid level, and they can be distinguished from one another, and from the other herpesviruses, by specific biochemical and biological properties. These include differences in gene sequences and protein structure (63). The betaherpesviruses and gammaherpesviruses, by contrast, propagate slowly, have a limited host range, and only infect animals from the original family. The betaherpesviruses include human cytomegalovirus and human herpesvirus 6 and 7. The gammaherpesviruses include Epstein-Barr virus (EBV), and human herpesvirus 8. The betaherpesviruses can infect several cell types, but gammaherpesvirses are usually limited to B and T lymphocytes. Both betaherpesviruses and gammaherpesvirses can be associated with oncogenesis in the host (62).

2. Biological effect: Fate of the infected cell, disease and latency

Cells productively infected with herpesviruses do not survive. A characteristic of herpesvirus-infected cells is the rapid shutoff of host macromolecular metabolism early in the infection. Thus host DNA synthesis is shut off and protein synthesis declines very rapidly. Almost from the beginning of the reproductive cycle, the infected cells undergo major structural and biochemical alterations that ultimately result in their destruction. The disease induced by HSV-1 includes primary and recurrent epithelial lesions as well as a disseminated disease and encephalitis. This ultimately leads

to destruction of central nerve system (CNS) tissue.

Infection of humans by HSV-1 is quite straightforward. The virus is usually and naturally transmitted by directly contacting between infected and uninfected individuals. The viruses multiply in epithelial cells at the portal of entry, and then spread to, and infect sensory nerve endings. The nucleocapsid is transported by axonal flow to the nuclei of the sensory neurons where it establishes latency (Fig. 9). In latently infected neurons, the viral genome circularizes and expresses no functions. The viruses harbored in latent state can periodically reactivate, and infectious viruses are transported back from the sensory neuron to a cell at or near the site of initial infection (Fig. 9) (38). Although HSV-1 can sometimes cause encephalitis and destruction of CNS, this occurs rarely in healthy, immunocompetent humans because the immune system blocks further virus spread. The clinical symptoms which do occur in the immunocompetent host usually include sore throat, fever, mucosal ulcers and lesions (140).

3. HSV-1 genome structure

The DNA of HSV-1 is a linear double stranded molecule of approximately 152 kbp, with a G+C content of 68%. It consists of two covalently linked components, designated as L (long) and S (short) (Fig. 10A). The L component consists of 107 kb of unique sequence (U_L) bracketed by two 9.3 kb inverted repeats designed as **ab** and **b'a'**. The S component consists of 13 kb of unique sequence (U_S) bracketed by two 6.6 kb inverted repeats designed as **a'c'** and **ca** (Fig.10B). The L and S components of

HSV-1 can invert relative to one another during DNA replication, yielding four linear isomers (Fig. 10B). Therefore the genome of HSV-1 present in the nucleocapsids exists as one of four possible isomers. The explanation of the genome invertion is that recombination taken place between the pac homology sequence which are generated during the synthesis of HSV-1 DNA concatamers (24).

Once the viral DNA enters host nucleus it circularizes and replicates from the original template via a rolling circle process (42). The viral contatamers are then cleaved into monomeric genomes following recombination and then packaged into preformed capsids (4). The whole genome of HSV-1 contains at least 72 open reading frames depending on the particular viral strain (85, 86).

4. Initiation of HSV infection

To initiate infection, the virus must attach to the cell surface receptors. The attachment to the different cell surface receptors is mediated by different viral membrane glycoproteins. Twelve HSV membrane glycoproteins have been indentified (gB, gC, gD,gE, gF, gG, gH, gI, gJ, gK, gL and gM). Removal of one glycoprotein at a time does not completely eliminate attachment because HSV can utilize more than one attachment pathway (15, 72, 40, 55, 117, 23). Attachment to the cell surface mediated by viral surface glycoproteins causes the fusion of the viral envelope with the cell plasma membrane. This fusion process involves at least three viral glycoproteins (gB, gH, gK) (11). Fusion of the envelope with the plasma membrane rapidly follows the initial attachment.

Upon fusion with plasma membrane, the nucleocapsids are released into the cytoplasm and transported to nuclear pores (Fig. 11). As shown by studies with a temperature sensitive (ts) mutant, release of viral DNA with the nucleoplasm requires a viral function. The nulceocapsids of the temperature sensitive mutant HSV-1 tsB7 were found to accumulate at nuclear pores at the nonpermissive temperature and release viral DNA only after a shift down from nonpermissive to permissive temperature (6). The cellular cytoskeleton mediates the translocation of capsids to nuclear pores since translocation of nucleocapsids is inhibited by drugs like nocadazale which disruptes the microtubular structure of cytoskeleton (65). The viral DNA and tegument proteins are released into nucleus from nucleocapsids through the nuclear pore, and then the empty capsids are then degraded in proteasomes of cytoplasm.

The tegument proteins play an indispensable role in viral replication. Two well defined tegument proteins are the virion host shut-off protein (VHS), which is involved in early shut off of host macromolecular synthesis (65, 97, 98), and VP16 (also called α gene trans-induce factor referred as α-TIF) that acts in trans induce α gene (more detail in HSV gene expression and regulation).

5. Viral Gene Expression and regulation

(1). Introduction of HSV-1 gene classes (39)

The HSV-1 genome contains at least 70 unique genes that are divided into three classes: immediate early (Alpha), delayed early (Beta), and late (Gamma) genes based on the temporal order of their expression during the productive infection cycle.

Alpha-genes (α-genes) are defined as those that are expressed in the absence of viral protein synthesis. Functional α-proteins are required for the synthesis of the subsequent polypeptide (β, γ) groups. There are five known α genes. Their products are termed infected cell polypeptides (ICPs), and they are refered to as ICP0, ICP4, ICP22, ICP27, and ICP47 respectively. The synthesis of α polypeptides reaches peak rates at approximately 2-4 hours postinfection, but α proteins continue to accumulate until late in infection. All of the α proteins, with the exception of α47 (ICP47), have been shown to have regulatory functions in gene expression. ICP4 and ICP27 are essential for viral replication (119, 35). ICP4 is required for activation of transcription of genes of β and γ classes (26, 35), and ICP27 plays an important role in the processing of mRNAs and enhancing viral DNA replication (48). ICP0 and ICP22 synergistically effect ICP4's action and also activate many cellular and viral genes independently (35, 124, 134). ICP47 does not show any gene regulatory function but it acts at an early stage of infection to block major histocompatibility I protein presenting viral peptides. This results in inhibition of infected cell lysis by $CD8^+$ CTLs (56).

Beta-genes are not expressed in the absence of competent α proteins. The β groups of polypeptides reach peak rates of synthesis at about 5-7 hours postinfection. Their products demonstrate three major functions: i)inhibiting host macromolecule synthesis, ii)processing nucleotides for viral replication, and iii) synthesizing viral DNA, such as viral thyminide kinase (TK), viral DNA polymerase, enzymes involved in viral nucleic acid metabolism, helicase-primase, as well as DNA binding proteins. The

appearance of β gene products signals the onset of viral DNA synthesis (115).

Gamma-genes mainly encode viral structure proteins. These include envelope glycoproteins, capsid proteins and tegument proteins such as VP16. They are differentiated from the β group in that they require viral DNA replication in addition of the presence of α proteins for normal expression.

(2). Viral gene expression and regulation

The genes of herpes simplex virus type 1 are coordinately and sequentially expressed during productive infection. The manner in regulatory cascade is orchestrated by the immediate proteins (Fig. 12).

Upon entry of viral DNA into the nucleus, α-genes are transcribed and translated by cellular machinery. Expression of the α-proteins is activated by the α-gene trans-inducer factor (α-TIF, or VP16). The α-TIF is a viral tegument protein encoded by one of the γ genes. It is packaged within the virion envelope during assembly. Virion fusion with host membrane releases tegument proteins and the nuleocapsid from the virion (Fig. 11, from 1 to 2). The α-TIF and nuleocapsid are then transported into the nuclear pore where they can enter into the nucleus. α-TIF has a unique affinity for a cellular protein Oct-1 with which it form an active complex, which is able to strongly bind to TATGARAT sequences in the α promoters. The complex of α-TIF with Oct-1 then interact with TFIIB or another adaptor protein to recruit general transcription factor and activate transcription of α-genes (80, 105).

The production of α gene transcription is tightly regulated at the α gene mRNA level. Transient expression analysis and studies with viral mutants that are unable to express the IE proteins demonstrate that the four products of 5 α genes, ICP0, ICP4, ICP27, ICP22, participate in regulation of transcription (16, 27, 87). ICP4 is a major transcriptional regulatory protein in HSV gene expression (35). It is required to activate transcription of β and γ genes, and it represses its own transcription as well as that of other α genes (25). The recent studies suggest that ICP4 can modulate any promoter that contains a TATA homology cis-acting element by interacting with general transcription factors at the TATA box. No specific binding is required for transactivation by ICP4, specific located binding site, however, is required for ICP4 repression of IE genes (26). Although it is not a classic transcriptional activator, ICP0 has the ability to further enhance the expression of any viral gene that exhibits a basal level of transcription (146). The transient expression studies suggest that it acts as a trans-activator of the α4 gene, but no cis-acting site has been detected. ICP27 by itself appears to have little effect on the expression of HSV genes (37, 124); however, it is able to further enhance or repress the transactivation activities of ICP4 and ICP0. Transient expression studies demonstrate that ICP27 represses α gene expression induced by ICP0, and β gene expression induced by ICP0 and ICP4, whereas it further enhances γ gene expression induced by ICP0 and ICP4 (87, 113, 134). The studies of ICP27 mutant-infected cells and cells transfected with ICP27 expression plasmids indicate that ICP27 is responsible for the posttranslational modification of ICP4 (87,

113). ICP27 has been demonstrated that it plays a role in gene regulation at several post-transcriptional level. They include selection of transcription termination sites, inhibition of RNA splicing, stimulation of DNA synthesis, and posttranscriptional destabilization of α mRNA (87, 113). Studies of viral mutants that lack the genes encoding ICP0, ICP4, and ICP27 have shown that ICP4 and ICP27, but not ICP0 are essential for virus replication (26, 84, 120). Although the ICP0 is not absolutely required for replication, it is necessary for efficient expression of β and γ genes, especially at low multiplicities of infection (16). Immunofluorescence studies reveal that both ICP4 and ICP27 affect the intracellular localization of ICP0. ICP4 promotes and ICP27 inhibits the nuclear localization of ICP0 (147, 148). Several lines of evidences suggest that ICP0, ICP4, and ICP27 act cooperatively in HSV-1 gene expression. ICP22 also plays a regulatory role and it is associated with incomplete phosphorylation of the C-terminal repeat domain of the RNA polymerase II enzyme which in some cell types it correlates with enhanced γ gene transcription (108).

6. Assembly and Maturation

After empty capsids are assembled in the nucleus, newly synthesized viral DNA is processed and packaged into these preformed empty capsids. The processing involves amplification of **a** sequences, cleavage of viral DNA lacking free ends. The cleavage and packaging of DNA are closely linked processes (76). The capsids become modified during the packaging of the DNA, and only modified capsids are able to bind the modified patches at inner lamellae of the nuclear membrane. The capsids become

enveloped at the inner lamellae, and de-enveloped at the outer lamellae, reenveloped by the endoplasmic reticulum, and released in the extracellular environment either by enveloped at the plasma membrane or by fusion of vesicles carrying enveloped virus at the plasma membrane (39). In fully permissive tissue culture cells, the entire process takes approximately 18 to 20 hours.

Section III: History of c-myc study in this lab

Three years ago this lab found that HSV-1 infected Hela cells could strongly transactivate (about 75-105-fold) the expression from the human c-myc promoter in transient expression assays. UV irradiated viruses failed to produce this effect (Table 1) indicating that HSV-1 viral gene expression was required in this transactivation (R. Millette, J. Pauson, unpublished results). The c-myc promoters containing 101 bp and 353 bp nucleotides upstream from P_1 (referred to -101 c-myc and -353 c-myc respectively) were transactivated by HSV-1 superinfection in transient expression assay (Table 1). This suggested that the upstream binding sites for Fos/Jun octamer complex at -343/-318 and YY1 at -236/-242 (Fig. 7) were not involved in this activation. Furthermore, this lab found that HSV-1 infection could also activate an endogenous human +66 wild type c-myc promoter. Either deletion at CT-I_2 or mutation at E2F-1 site gave apparently reduction in c-myc activation level (Fig. 13). This experiment was done through that HSV-1 infected the mouse L cells which had stably integrated +66 human c-myc-CAT plasmids (cell lines was obtained from N. Hay lab). The results indicated that activation of c-myc expression by HSV-1 proteins

might play an important role in HSV-1 infection. The viral proteins might stimulate cellular proteins and result in activation of c-myc expression. CT-I_2 and E2F sites might be an indispensable cis-elements in this transactivation.

Various c-myc promote-CAT constructs superinfected with HSV-1 were used in transient expression assays to delineate the promoter sequences involved in the HSV-1 induced c-myc promoter activation. Results, presented in Table 1, 2, and 3, demonstrated respectively that +66 c-myc promoter (relative to P_1, and +108 bp upstream from P_2) exhibited almost the same degree of transactivation as -101, -157, and -353 promoter in HVS-1 superinfection. This suggested that the +66 c-myc promoter region suffice for activation by HSV-1 proteins. Both deletion/superinfection study (J. Pauson, R. Millette unpublished results) and mutation/superinfection study (H. Li, R. Millette unpublished results) showed that changing either CT-I_2 or E2F-1 site would decrease the HSV-1 induced c-myc transactivation level.

Section IV: Significant of this Study

Previous studies have showed that HSV-1 can activate both an exogenous c-myc promoter in transient expression assays, and an endogenous cellular c-myc promoter during cell infection. However, the molecular mechanism of this transactivation has not been elucidated. The purpose of this study is to identify possible regulatory elements involved in the transactivation of human c-myc gene by HSV-1. Because of the important role of c-myc in controlling cell proliferation,

differentiation, and enabling regulation of cellular gene expression, activation of c-myc expression by HSV-1 may be crucial in establishing a cellular environment that supports maximum virus replication. Moreover, because of the regulatory ability of c-myc in transcription, and the ability of interaction with other regulatory proteins (such as pRB, Max etc.), and the presence of putative c-Myc binding sites in a number of other HSV-1 promoters (R. Millette, computer search), the activation of cellular c-myc gene may be important in regulating expression of viral genes during the infectious cycle. Therefore, studying the mechanism of transactivation of human c-myc gene by HSV-1 will not only provide a better understanding of the subtle interactions of herpes simplex virus and its proteins with host cellular genes and proteins, but also will shed a light on the function of major HSV-1 regulatory proteins (ICP4, ICP0 and ICP27), and providing a new insight to the mechanisms of HSV-1 pathogenesis.

Section V: Specific objective of this study

Since previous studies had demonstrated that a +66 c-myc promoter suffices in HSV-1 induced c-myc activation, my study focused exclusively on this promoter (the 108 bp immediately 5' to the P_2 transcription start site).

1. To determine the effect and the distinct role of the three major HSV-1 IE proteins ICP4, ICP0, and ICP27, in human c-myc gene transactivation

The discovery that herpes simplex virus type 1 could activate both an exogenous c-myc promoter in transient expression assays and an endogenous c-myc

gene following HSV-1 infection, prompted me to examine whether the major immediate early (IE) proteins of herpes simplex virus type 1 were involved in c-myc transactivation by virus. Three of the five IE proteins (ICP0, ICP4, and ICP27) of HSV-1 had demonstrated major regulatory functions either individually or cooperatively in both viral and cellular gene expression. In order to precisely determine their role, transient expression assays were carried out as described in the following text. Cotransfection assays using c-myc-CAT plasmids, containing the +66 c-myc promoter coupled to the CAT (Chloramphenicol acetyltransferase) reporter gene, and plasmids containing genes that encode HSV-1 IE proteins as effectors were used in this investigation (details see Materials and Methods).

2. To determine the role of cis-acting regulatory elements in HSV-1 induced c-myc activation

The sequence analysis by computer reveals that several potential Sp1 and E2F cis-elements exist in the P_2 promoter region (Fig. 18). There, an Sp1-binding site called CT-I_2 at +117 relative to P_1 and an E2F-1 site at +100 have been shown to be essential elements for P_2 promoter transcription (50, 53). In addition, two potential E2F sites were shown to be required for transactivation by the adenovirus E1A protein (53). In order to evaluate the role of these two sites in HVS-1 induced c-myc transactivation, mutations in the c-myc promoter either at the CT-I_2 or E2F-1 site (Fig. 15) were examined by transient expression assays.

3. To identify possible viral and cellular regulatory factors and their binding sites that are involved in c-myc promoter activation by HSV-1

To completely understand the mechanisms of controlling gene regulation, it is essential to identify the nucleic acid sequence elements in the promoter region and the protein factors that interact with these sequences. Electrophoretic mobility shift analysis (EMSA) and oligonucleotide competition assays were used to identify trans-acting elements through protein-DNA binding complexes formed on the +66 wild type (wt) c-myc promoter region. In addition, DNA footprinting analysis with the orthrophenanthroline-copper ion method was used to identify these binding sites.

Chapter 2

Materials and Methods

Bacteria and Cells

Escherichia coli DH5α strain was used as the host for propagating all chimeric c-myc-CAT plasmids. Hela cells, obtained from the American Type Culture Collection, were maintained at 37°C under 5% CO_2 atmosphere in Dulbecco's modified Eagle medium (DMEM) (GIBCO Laboratories, Inc) containing 10% of heat-inactivated calf serum (Hyclone Labs, Inc.). DMEM containing penicillin (100U/ml), streptomycin (100ug/ml) and 10%(v/v) heat inactivated fetal bovine serum (DMEM-10% IFBS-P/S) was used during cell propagation for transient expression assays.

Plasmids and DNA

Plasmid c-myc +66 wt-CAT contains a wild type (wt) c-myc P_2 promoter region, from +66 bp to +513 bp relative to P_1, fused to the bacterial chloramphenicol acetyltransferase (CAT) gene in a pBR vector (pBR-CAT, 43, 139). Plasmid c-myc +66mt E2F-CAT and c-myc +66mt CT-I_2-CAT were similar to c-myc +66 wt-CAT, but with each having three base pair mutations (mt) either in a potential E2F-1 binding site at +100 bp relative to P_1 or in a CT-I_2 site at +117 bp respectively (Fig. 8). The plasmid c-myc -157 wt-CAT contained a wild type P_2 promoter region and a partial P_1

promoter region from -157 bp to +513 bp relative to P_1, fused with the CAT gene in the pBR vector (pBR-CAT). All of the above plasmids were kindly gifted by Nissim Hay of the Ben Hay Institute, the University of Chicago. Plasmids pGH78, pGH94 and pGR215 contain HSV-2 genes encoding herpes simplex virus immediate early proteins ICP4, ICP0, and ICP27 respectively were kindly provided by G. Hayward, John Hopkins University. The wt-gD-392 CAT plasmid, containing the wild type (wt) glycoprotein D (gD) promoter of HSV-1 (from -392 bp to +11 bp relative to cap site) coupled to the CAT gene in pBR vector was a gift by R. Everett, Glasgow. The wild type pDHF-210 CAT plasmid, containing the chinese hamster dehydrofolate reductase (DHFR) gene promoter region (-210 bp to -23 bp relative to translation start site of DHFR gene) ligated into pUC18-CAT, was a gift by J. Azizkhan, University of North Carolina.

All plasmids were propagated in *Escherichia coli* DH5α for experiment usage. Plasmid DNA were prepared by the alkaline lysis procedure (9). The DNA fragments from restricted plasmids were isolated by the DEAE paper electroelution method (31). The fragments were labeled at either 5' ends or 3' ends by Klenow fill in with α-P^{32}-deoxyribonucleoside triphosphates.

An oligonucleotide called LBS containing the YY1 consensus binding sequence was synthesized at the oligonuclotide facility of the Vollum Institute, Oregon Health Science University. A control double-stranded oligonucleotide (C2) that lacks an LBS binding site was kindly provided by K. Riggs, Columbia University. Sp1 and E2F oligonucleotides were purchased from Santa Cruz Biotechnology, Inc. All

oligonucleotide sequences are listed in Table 5.

Cotransfection and Transient-expression assays of c-myc expression

Hela cells were seeded at 3×10^5 cells per 35 mm^2 well in Dulbecco's modified Eagles' medium (DMEM) (GIBCO Laboratories, Inc), supplemented with 10% heat-inactivated fetal calf serum (Hyclone Labs, Inc.), penicillin (100 U/ml), and streptomycin (100 U/ml) (DMEM-10% IFBS-P/S), and grown at 37° C under 5% CO_2 atmosphere. The medium was changed at 17 hours after seeding. At 20 hours the cells were transfected with 8 ug of reporter plasmid (+66 c-myc promoter-CAT) and 2 ug of effector plasmids (containing HSV-1 IE genes) using the calcium phosphate technique (73). Plasmid pUC19 was used as a carrier DNA to bring the total amount of transfected DNA to 16 ug for each cotransfection. All samples were done in duplicate for each transfection. The transfected cells were shocked at four hours post transfection by removal of growth medium and addition 1 ml of 15 % (v/v) glycerol in DMEM. After 1 minute the glycerol solution was removed, and the cell were rinsed with PBS (phosphorus buffered saline), and allowed to continue growing in DMEM-10% IFBS-P/S medium. The medium was changed at 24 h post transfection and the cells are harvested at 44 hours post transfection. The cell lysates were used to measure the chloramphenicol acetyltransferase activity by the method of Nordeen et al. (99). The method measured the incorporating of (^{3}H)-Na-acetate (NEN Dupont, 3.3 uCi/mmol, 10 mCi/ml) into acetyl-chloramphenicol via a coupled reaction with acetyl coenzyme A synthetase. Acetylated (^{3}H) chloramphnicol was extracted by benzene and

its radioactivity was measured in a Beckman LS 6500 scintillation counter. Lysates were assayed for protein content by the Bradford method, and samples were normalized for protein content in each reaction.

Probe preparation for EMSA assay

The fragments of P_2 promoter region used as probes were generated by restriction digestion from -157/+515 c-myc-CAT plasmid (Fig. 6). The HindIII/EagI restricted fragment from wt-gD-392 CAT plasmid was used as a YY1- and Sp1-DNA binding complex control in electrophoretic mobility shift assay (EMSA). The FokI/HindIII restriction fragment from the pDHFR-210 CAT plasmid (-107 bp to -23 bp DHFR gene promoter region) and containing the E2F and Sp1 consensus binding sites was used as E2F-DNA binding complex control in EMSA assay.

All DNA fragments restricted from plasmids were isolated by the DEAE paper electroelution method (31). The Fragments were labeled by Klenow fill in α-P^{32}-deoxyribonucleoside triphosphates.

Nuclear extracts

Nuclear extracts were prepared by method of Dignam (29) from HeLa cell monolayers that were either mock infected or infected with HSV-1(KOS strain) at a multiplicity of infection of 10 for 8 hours. The protein concentration of the nuclear extracts was determined by Bradford protein assay (Bio-Rad Laboratory).

Mobility shift assays and Oligonucleotide competition assays

EMSA reaction mixtures at optimal Sp1/YY1 binding condition contained 20 mM n-(2-hydroxyethyl)piperazine-N'-(2-ethane sulfonic acid) HEPES (pH 7.6), 40 mM KCl, 1 mM $MgCl_2$, 50 mM NaCl, 0.15 mM $ZnCl_2$, 0.5 mM EDTA, 0.2 mM dithiothreitol, 5 % (v/v) glycerol, 1 ug of poly-dI-dC (Pharmacia Biotech), and 3 ug of nuclear extract in a total volume of 20 ul. The reaction mixture at optimal E2F binding condition contained a similar buffer but with addition of 2 ug of Salmon sperm DNA (Sigma) instead of 1 ug of poly-dI-dC, and no $MgCl_2$, but with addition of 10 mM EGTA. All probes used in the EMSAs had activity of 1.5×10^4 cpm. Following 20 min incubation at room temperature, 2 ul of 0.25% bromphenol blue were added and the samples were electrophoresed through polyacrylamide gels made with 4% acrylamide-0.13% bisacrylamide in a non-recirculating buffer with final concentration of 25 mM Tris, 0.1M glycine and 1mM EDTA, and running at 250 volts for 2.5 hours at 4^0 C. Gels were dried under vacuum and exposed to Fuji RX film with intensifying screens. For oligonucleotide competition assays, the above conditions were used but with 100- to 500-fold molar excess of unlabeled oligonucleotide competitor added before the addition of radiolabeled probe.

Orthrophenanthroline-Copper Ion DNA-Protein Complex Footprinting

Mobility shift assays were carried out as above but scaled up to 30-fold. The samples were electrophoresed at 4^0 C for 2.5 hours at 250 volts through polyacrylamide gels made with 4 % acrylamide-0.13% bisacrylamide in a non-recirculating buffer. After electrophoresis, the gels were immersed in 200 ml of 50 mM Tris-HCl, pH 8.0 buffer.

Then 20 ul of solution A (2 mM of 1,10-phenanthroline/ 0.45 mM of $CuSO_4$) and 20 ul of solution B (58mM of 3-mercaptopropionic acid) were added, and allowed to react with the gels for 12 minutes at room temperature. The reaction was quenched by the addition of 20 ml of 28 mM of 2,9-dimthyl-OP for 2 minutes. The gel were rinsed twice with distilled water twice and exposed to x-ray film overnight at room temperature. Gel segments containing DNA-protein complex and free probe were excised from the gel and placed next to a strip of DEAE paper, inserted into an 0.8 % of agarose slab gel. Following the addition of 2.5 ug of *Escherichia coli* tRNA as carried per strip, the DNA fragments were electrophoresed onto DEAE paper and isolated by method of Dretzen, et al. 1981. Dried DNA samples, containing 5000-10,000 cpm, were run on 8% of DNA sequencing gels along with the DNA probe that had been subjected to Maxam and Gilbert cleavage reactions.

Chapter 3

RESULTS

Section I: The role of HSV-1 immediate-early proteins in human c-myc gene transactivation

Since gene activation by HSV-1 generally involves three immediate early (IE) viral proteins, ICP0, ICP4, and ICP27, I chose to investigate the potential roles of these three proteins in the activation of the c-myc P_2 promoter. To precisely determine their roles, a series of cotransfection experiments was performed with a reporter plasmid and effector plasmids, containing IE genes that encode proteins ICP0, ICP4, and ICP27. The reporter plasmid, containing a wild type human c-myc promoter region (from +66 to +513 bp relative to the P_1 transcription start site), was fused to the choramphenicol acetyltransferase (CAT) gene (Fig. 14). The effector plasmids were added either singly or in different combinations. The resulting levels of CAT enzyme were determined by measuring the incorporation ^{3}H-Na-acetate into acetyl-chloramphenicol via a coupled reaction with acetyl coenzyme A synthetase. A summary of these experiments is presented in Figure 15, and a quantitation of the CAT assays is outlined in Table 4. The data is an average of three independent cotansfection experiments. In all experiments, CAT activity was measured in pmols of ^{3}H-acetylated chloramphenicol per mg protein, and the results are presented as the percentage of the CAT activity

relative to that seen with the target plasmid of +66 wt-c-myc CAT in the presence of effector plasmid ICP4. The CAT activity of +66wt-CAT reporter plasmid contrasfected with ICP4 was defined as 100% expression.

When the IE gene were cotransfected singly, ICP4 was seen to provide the greatest level of transactivation of c-myc-CAT (Fig. 15). Although ICP0 and ICP27 alone had little effect on the +66wt promoter, ICP0 provided a synergistic effect on ICP4-induced transactivation and produced a transactivation level of 137% relative to ICP4 alone. Furthermore, whenever a plasmid encoding ICP27 was added to the cotransfections in combination with plasmids encoding either ICP4 or ICP4+ICP0, a significant trans-repression occurred. The activity in the presence of ICP4+27 was only 45% that of ICP4 alone. When ICP27 was cotransfected with ICP4+0, the resulting CAT activity was more than 5-fold reduced from that of ICP4+0.

Section II: CT-I_2 and E2F are important cis-acting elements in c-myc promoter transactivation by HSV-1

Two cis-acting elements in the P_2 promoter region have been studied by two research groups (50, 53). One is the CT-I_2 element that locate at +117 bp relative to the P_1 transcription start site (Fig. 6). The other element is an E2F binding site locating at +100 bp in the c-myc promoter termed E2F-1 (Fig. 6). These two sites were shown to be essential elements for c-myc promoter transcription (50, 53). In addition, the E2F-1 site was shown to be required for the adenovirus E1A protein transactivation of c-myc gene expression (53). To determine the role of these elements in regulation of

c-myc gene expression by HSV-1, I used the c-myc +66 wild type promoter (c-myc +66wt) and the c-myc +66 mutant promoters that were mutated at either the CT-I_2 site (termed c-myc +66mt CT-I_2) or the E2F site (termed c-myc +66mt E2F). These promoter regions were fused to the choramphenicol acetyltransferase gene (CAT gene) as a reporter plasmid (Fig. 16). Each reporter plasmid was cotransfected into the HeLa cells with effector plasmids containing HSV-1 IE genes, which were added either singly or in different combinations. Results of this experiment are presented in Figure 17, and quantitation of the CAT assays is outlined in Table 4.

Both the CT-I_2 and E2F-1 site mutations resulted in a reduced level of transactivation by ICP4. Compared to the wild type promoter (c-myc +66wt -CAT), there was approximately a 30% decrease in CAT activity induced by ICP4 when the CT-I_2 site was mutated (plasmid +66mt CT-I_2-CAT) and about a 13% decrease when the E2F site was mutated (plasmid +66mt E2F-CAT). Moreover, the effects of these mutations were more pronounced whenever ICP0 was included in the cotrasfections. When ICP0 was added with ICP4, CAT activities observed with the CT-I_2 and E2F mutation decreased to 65% and 60% of wild type c-myc-CAT respectively. When ICP0 added with ICP4+ICP27, CAT activities of CT-I_2-CAT and E2F-CAT decreased relative to the wild type promoter about 2.5-fold and 2-fold respectively .

Section III: Electrophoretic mobility shift assays (EMSA)

The +66 c-myc promoter region used in this study covers 447 bp (from +66 bp to +513 bp relative to P_1 site). To detect proteins that bind to this region I restricted

this promoter region into three fragments termed probe I, probe II and probe III (Fig. 18). Computer analysis of the DNA sequence of this region indicated the following:

(1). Probe I region contains two potential E2F binding sites, E2F-(1) and E2F-(2) with inverted orientation, and two potential Sp1 binding sites, CT-I_2 and Sp1-x (Fig. 19-A). The E2F-(1) site (located at +100) has been shown to be an essential element in adenovirus induced c-myc gene transactivation and it binds specifically to transcription factor E2F (53). The CT-I_2 site, with an inverted orientation of a CT element at +117, is required for P_2 transcription and it specifically binds to the transcription factor Sp1 (50).

(2). Probe II region contains three potential Sp1 binding sites. Two of them (termed Sp1-x) contain a partial Sp1 consensus sequence (GGGCG), and the other is a CT element (CCCTCCC) that has been shown to bind the Sp1 transcription factor (50) (Fig. 19-B)

(3). Probe III region contains one potential Sp1 binding site (GGGCGG) and two sites that contain partial YY1 consensus binding sequences, referred to as YY1-(1) and YY1-(2). Both of these YY1 sites have one base pair mismatch with YY1 consensus binding sequence (CATNTT) (Fig. 19-C).

A. Cellular proteins form complexes with the c-myc P_2 promoter

To distinguish possible viral proteins from cellular transcription factors binding to the P_2 promoter region, I used either mockinfected (uninfected) or 8-hour herpes simplex virus type 1 (KOS strain) infected nuclear extracts from HeLa cells (described

in materials and methods). I then performed a series of eletrophoretic mobility shift assays (EMSA), using the c-myc probes (probes I, II, and III), asymmetrically labeled with ^{32}P at one end. These were incubated with one of the two nuclear extracts and complexes formed were separated by polyacrylamide gel electrophoresis and analyzed by autoradiography. As shown in Figure 20, the pattern of specific protein-DNA binding complexes observed with each of these probes was essentially the same when either noninfected (lanes 2, 5, and 8) or 8-hour HSV-1 infected nuclear extract (lanes 3, 6, and 9) were used. Since no virus-specific bands were observed, this suggested that the complexes formed on the +66 c-myc promoter region were mainly the result of binding of cellular factors instead of viral proteins.

B. Analysis of complexes formed within c-myc probe I (+66 to +212 bp)

Multiple protein-DNA complexes were observed with each probe. In order to identify cellular transcription factors involved in these bindings, unlabeled oligonucleotides: Sp1, E2F, YY1 containing the respective transcription factor binding site, E2F mutant containing a mutated E2F site, and C2 containing no known transcription factor binding site as a control, were used as competitors in EMSA reactions. All oligonucleotide sequences were listed in Table 5. In addition, a probe from the HSV-1 glycoprotein D (gD) promoter containing both Sp1 and YY1 binding sites was used as positive control in the oligonucleotide competition assays (data not shown).

To analyze protein complexes formed in the +66 to +212 bp region (probe I

region) of the c-myc promoter, probes I from wild-type and mutant c-myc promoters were analyzed by EMSA. To optimize the protein-DNA binding complexes, I conducted EMSA reactions under conditions which optimal for either Sp1/YY1 or E2F binding (See Materials and Methods). In addition, double stranded oligonucleotides containing respective transcription factor binding sites were added as competitors in some of the reactions. Two different protein-DNA complex patterns were formed on the wild type probe I when conditions optimal for either Sp1/YY1 or for E2F were used (Fig. 21-A, lane 1 and 2). Even though complexes 1 and 5 appeared at both conditions, their binding affinities were apparently stronger at optimal Sp1 binding conditions than that at optimal E2F conditions. Complex 3 formed specifically at optimal Sp1 binding conditions (compare lane 1 with 2).

The pattern of protein-DNA complex formed at optimal E2F binding condition showed two unique bands, complexes 2 and 4 (lane 2), which were not observed at optimal Sp1 binding condition (lane 1). A 300-fold molar excess of an oligonucleotide containing an E2F binding site competed these two bands (lane 4), but the 300 fold excess of mutant E2F and C2 oligonuleotides failed to do so (lane 5 and 6 respectively). This suggested that complexes 2 and 4 were formed by a member of the E2F family of transcription factors.

When the wild type probe I was run in an EMSA under optimal Sp1 binding condition, band 3 was seen to be strongly competed by a 300-fold molar excess of an oligonucleotide containing an Sp1 binding site (lane 1 vs 3). When a c-myc probe I with mutated Sp1 site (CT-I_2 P-I) was used under Sp1 binding conditions, the bands 1

and 3 were reduced in their intensity, and band 5 was eliminated completely in comparison to the wild type probe I (lane 7 vs 1). Addition of an excess of Sp1 oligonucleotide essentially eliminated complex 3 (lane 9 vs lane 7). These results suggest that bands 3 and 5 are the result of binding of Sp1 or related proteins ("SpX"). To confirm Sp1 binding, and to determine if also YY1 protein bind to probe I region, I used unlabeled Sp1, YY1, E2F, E2Fmt, and C2 oligonucleotides as competitors in an EMSA reaction under the optimal Sp1/YY1 binding condition (Fig 21-B). Both E2F and YY1 oligonucleotides did not specifically affect any of complexes under this condition (lanes 3, 4, and 6). Addition of Sp1 oligonucleotide, however, inhibited the formations of the complexes 1 and 3 but not complex 5 (lane 5). This result indicated that complexes 1 and 3 may involve Sp1 or Sp1-related proteins (SpX). Taken together with the results shown in Fig. 21-A, it appear that complex 5 involves the CT-I_2 sequence but it is not likely formed by an Sp1 protein.

To further investigate the complexes formed on the wild type and mutant probe I under optimal E2F binding condition, I ran three probe I promoter fragments (wild type, CT-I_2 mutant, and E2F mutant probe I) in EMSAs with addition of specific oligonucleotids as competitors (Fig. 21-C). At the optimal E2F binding condition, the intensity of complex 1 was reduced compared that observed at the optimal Sp1 binding condition (Fig. 21-B). Moreover, the complex 3 did not appear at the optimal E2F binding conditions (Fig. 21-C, lane 1 and Fig. 21-B, lane 1). A 300-fold molar excess of Sp1 oligonucleotides reduced intensity of the complex 1, but had little effect on the other complexes (lane 2). Complexes 2 and 4 were competed by a 300-fold molar

excess of an oligonucleotide containing an E2F binding site (lane 3). Neither a mutant site (E2Fmt), an YY1 oligonucleotide containing YY1 binding site, nor a control oligonucleotide (C2) specifically affected any of the complexes except the complexes 6 and 7 (lane 6). When the CT-I_2 mutated probe I was used in EMSAs under these conditions (lane 7 through 11), the complex 1 was reduced in intensity, compared to that when wild type probe I was used (compare lane 7 to 1). Addition of the Sp1 oligonucleotides did not inhibit the formation of complex 1 and 5. This was probably due to an unfavorable binding condition for Sp1. The complexes 2 and 4 were strongly competed by the addition of an E2F oligonuluceotide but not by the E2F site mutated oligonucleotide (lanes 3, 9, and 14). When probe I with a mutated E2F site was used, complexes 2 and 4 were virtually eliminated as was expected (lanes through 12 to 16). In contrast to the wild type probe, complex 5 increased in intensity with the E2F-PI probe. A 300-fold molar excess of Sp1 oligonucleotes reduced intensity of the complex 1 but didn't affect the complex 5 (lane 13). From these results taken together, I conclude that the complexes 1 and 3 are formed by the binding of Sp1 or Sp1-related proteins (SpX); complex 5 may be the result of another protein binding to the CT-I_2 region. Complexes 2 and 4 appear to be the result of transcription factor E2F or proteins of E2F family binding to the E2F-1 site. Elimination of E2F binding on the E2F-1 site apparently enhanced the formation of complex 5 (lanes 12 to 16). Complex 6 was not reproducibly observed in our assays so we concluded this may be due to nonspecific protein binding. Complex 7 was also appeared to be due to an nonspecific binding because the excess of any kind of oligonucleotides competed this band even

though it consistently appeared in all experiments. In addition, increasing amount of poly-dI-dC DNA in EMSA reaction eliminated complex 7 (data not shown).

C. Analysis of complexes formed with c-myc probe II (+212 to +332 bp)

When probe II was used in EMSAs under Sp1 binding conditions, at least six different protein-DNA complexes were observed (Fig. 22, lane 2). Addition of Sp1 oligonucleotide inhibited formation of complexes 1, 2, and 4 (lanes 3 through 5). Increasing amounts of a YY1 site oligonucleotide showed only a weak competition with complexes 3 and 6 (lanes 6 to 8). No oligonucleotide that we tested was able to compete with complex 5. Moreover, addition of 5 ug of Poly-dI-dC in EMSA reaction had no effect on complex 5 (data not show). An interesting observation was that the complex marked with * increased as increasing amounts of the Sp1 oligonucleotide in EMSA reaction (Fig. 22, lanes 3-5). This particular complex was an unknown of origin. It was possible that large amount of Sp1 in nuclear extract excluded the unknown protein so that the complex only appeared when Sp1 protein was complexed with its oligonucleotide.

D. Analysis of complexes formed with c-myc probe III (+332 to +513 bp)

EMSA analysis using probe III showed that five major complexes were formed. Three of these (1, 2, and 3) were competed by the Sp1 site oligonucleotide (Fig. 23, lanes 3-5). Addition of a YY1 site oligonucleotide showed a small amount of inhibition on the formation of complex 4 but had little or no effect on the other complexes.

Increasing amount of poly-dI-dC did not affect the complex 4, it is most likely that this complex was due to binding of an unknown protein that interact with the YY1 binding site region. Complex 5 appeared to be the result of an nonspecific binding since it decreased as increasing amount of any kind of oligonucleotide (Fig. 23, lanes 5, 8, and 10), and also increasing amount of poly-(dI-dC)• poly-(dI-dC) DNA eliminated this complex.

Section IV. DNA binding specifity

The data from EMSAs and oligonucleotide competition experiments with three different probe I (wild type P-I, CT-I_2 P-I, and E2F P-I) indicated that complex 5 was due to Sp1 or Sp1-related proteins binding on the CT-I_2 site, and complexes 2 and 4 were due to binding of E2F-related proteins binding to the E2F-1 site. To locate binding sites of the other complexes formed on each individual probe, I used orthophenanthroline-copper ion (OP-Cu) DNA footprinting analysis. In this analysis, DNA probes selectively labeled with ^{32}P-deoxyribonuclesite triphosphate at one end were used in the EMSA reactions which were scaled up to 25-30-fold. DNA-protein complexes wee separated by electrophoresis through 4% of polyacrylamide gel at 4^{0}C. The gels were then immersed in the 1,10-phenanthroline-copper ion solution and allowed to digest for 10 minutes. The reactions were stopped by addition of 2,9-dimehyl-OP for 2 minutes. The digested gel were exposed to X-ray film. Bands corresponding to the free DNA probes and bound complexes were excised from OP-Cu digested EMSA gels. Eluded DNA were dried, dissolved in DNA sequencing

loading buffer, and loaded on an 8% sequencing gel along with the probes subjected to Max-Gilbert a chemical cleavage to establish a partial sequencing ladder (detail see materials and methods).

The following complexes were excised from EMSA footprinting gels and analyzed: a). Complexes 1 and 3 formed with Probe I (Fig. 21-B, lane 2); b). Complex 1+2 together, and Complex 4+5 together formed with Probe II (Fig. 22, lane 2); and c). Complex 1+ 2 together, Complex 3, and Complex 4 formed with Probe III (Fig. 23, lane 2). Even though the complexes 3 and 6 formed on probe II seemed to be specific for YY1, they were not excised from the gel due to the weak binding exhibited. Labeled DNAs isolated from EMSA gels digested with orthophenanthroline-copper ion reagent were analyzed on denaturing sequencing gels and subjucted to autoradiography. The resulting footprints obtained with Probe I, II, and III are presented in Figure 24, 25, and 26 respectively.

The region of Probe I protected by the complex 1 extended from +110 to +119 (GGAGGGAGGG) which covered CT-I_2 site (from +113 to 119) (GGGAGGG) (Fig. 24). Complex 3 protected the same region as the complex 1. These results were consistent with our results in oligonucleotide competition experiments, in which the complexes 1 and 3 were competed by the addition of anSp1 oligonucleotide. Thus Complexes 1 and 3 appear to be due to the binding of Sp1-related proteins to CT-I_2 site.

The region of the Probe II protected by Complex 1+2 extended from +229 to +235 that covered GGGCGAG sequence (Fig. 25, Complex (1+2)). This protected

region contained an entire potential Sp1 binding sequence (GGGCG), which had been mapped on the Figure 19-B as the first Sp1-x by computer analysis. Complexes 4+5 showed protections at two regions, A and B on the probe II (Fig. 25, complex (4+5)). The A region stretched from +229 to +235 which was exactly the same region being protected by complexes 1+2. I suggested that this protection was contributed by the complex 4 since addition of Sp1 oligonucleotide inhibited the formation of complexes 1, 2, and 4 together in oligonulceotide competition (Fig. 22, lane 3 to 5). The B region extended from +249 to +257 that covered GGGCGTCCT sequence, which also included a potential Sp1 binding sequence (GGGCG) that had been mapped on the Figure 19-B as the second Sp1-x. The B protection was most likely due to the contribution of complex 5. Complex 5, however, was not competed by Sp1 oligonucleotide. This indicated that a protein other than Sp1 and Sp1-related proteins (SpX) might be involved in the formation of this complex. These proteins might bind together on this entire B region with higher affinity than just SpX proteins alone bind to the GGGCG sequence. The region protected by Complexes 1+2 on the Probe III, extended from +376 to +382 (Fig 26, Panel A, complex (1+2)), which contained a entire Sp1 consensus binding sequence (GGGCGGG) that had been mapped on the Figure 19-C as Sp1-x. The region protected by Complex 3 was exactly the same region being protected by Complexes (1+2) extended from +376 to +382 (Fig 26, Panel A, Complex 3). These results were consistent with our results of oligonucleotide competition experiment, in which the Complexes 1, 2 and 3 were competed by the addition of Sp1 oligonucleotide. Thus we conclude that Complexes 1, 2, and 3 are due

to the binding of Sp1 and Sp1-related proteins to the GGGCGGG sequence from +376 to +382.

The region protected by Complex 4 on Probe III extended from +393 to +396 that covered TGGA sequence. The AGGA is an unknown transcription factor binding site. Taken together with result of that Complex 4 was not competed by any of oligonucleotide we tested in our oligonucleotide competition assay (Fig. 23), I presume that Complex 4 was due to the binding of an unknown cellular factor.

Chapter 4

Discussion

To investigate the specificity of the action of HSV-1 immediate early proteins ICP0, ICP4, and ICP27 on the transactivation of human c-myc gene, I performed a series of cotransfection experiments by using a reporter plasmid cotransfected with effector plasmids containing IE genes (ICP4, ICP0,and ICP27) either singly or in combination each other. When tested singly with a reporter plasmid containing a wild type promoter (+66wt c-myc-CAT), only ICP4 was able to activate the human +66 c-myc P_2 promoter. ICP0 and ICP27, when tested alone, gave a 2- and 6-fold repression of basal c-myc transcription, respectively (Fig. 15 and Table 4). ICP0, however, provided an added synergistic action (a 1.37-fold increase) on ICP4-mediated transactivation. ICP27, in contrast, inhibited the ICP4 and ICP0+4 mediated transactivation by 55% and 82% respectively (Table 4, Figure 15). Recent data suggests that ICP4 can modulate any promoter that contains a TATA homology. It was shown to form a tripartite complex with basic transcription factors, TBP and TFIIB, and it appears to operate through the general transcription machinery acting at the TATA box (26). ICP0 was shown to enhance expression of a spectrum of viral genes alone and it synergistically interacts with ICP4. ICP0 was also shown to physically interact with ICP4. The interaction of ICP0 with ICP4 could promote the ICP4's association

with its low-affinity DNA binding sites or with factors in the basal transcription machinery (146). ICP27 has been shown to function as a trans-repressor, and as a trans-activator, in presence of ICP4 and ICP0 depending on the target (124). ICP27 also showed the inhibition of nuclear localization of ICP0 (147). Therefore, several possible actions of ICP4, ICP0, and ICP27 could be postulated from our results. ICP4 clearly acted as a major transcription factor in HSV-1 induced human c-myc P_2 promoter transactivation. It is possible that ICP0 interacts specifically with ICP4 and promotes the association of ICP4 with the c-myc P_2 promoter or with other transcription factor. ICP27 could have a negative effect on the expression of ICP4 and ICP0 as well as P_2 promoter itself. If ICP27 acted specifically and directly as trans-repressor on P_2 promoter, the presence of ICP4 would down-regulation of ICP27 and lead a relief on this repression. On the other hand, if ICP27 acted specifically to repress the expression of ICP4 and ICP0, the consequent by lower level of ICP4 and ICP0 would result in lower induction of P_2 promoter activity. Furthermore, ability of that ICP27 inhibited the nuclear localization of ICP0 lowered synergistic effect of ICP0 on ICP4 transactivation. None of above possible effects of ICP27 could be excluded without any further investigation.

Two cis-acting elements, CT-I_2 located at +117 and E2F-1 located at +100 relative to P_1 transcription start site, were important for P_2 promoter transactivation. Mutations of CT-I_2 and E2F-1 sites resulted in a 87% and 54% decrease in basal transcription levels respectively (Table 4). ICP4 mediated transactivation, however, was only mildly suppressed by mutations in CT-I_2 and E2F-1 sites, showing a 30% and 13%

decrease in CAT activity respectively as compared to the wild type promoter with ICP4 alone. Most interestingly, transactivation mediated by ICP4+ICP0 decreased dramatically when these two promoter sites were mutated, resulting in about a 65% decrease when CT-I_2 was mutated and a 60% decrease when E2F-1 was mutated as compared to the wild type with ICP4+ICP0. These results indicated that CT-I_2 and E2F-1 sites were not indispensable for ICP4-mediated c-myc P_2 promoter transactivation, but they were more important for the ICP0 effects. ICP0 might influence transcription through these two sites either directly or indirectly by modulating Sp1 and E2F related proteins that bind to these two sites.

Taken together of the EMSA, oligonucleotide competition, and footprint analysis experiments, we demonstrated that the transactivation of human c-myc P_2 promoter by HSV-1 was not likely due to the binding of viral proteins on the P_2 promoter region. However, three eucaryotic transcription factors, Sp1, E2F, and YY1, have been shown to specifically interact with the P_2 promoter region. All three transcription factors are ubiquitously expressed in higher mammalian cells and are required for the constitutive and induced expression of a variety of genes (94, 71). They all had been shown to be modular in structure, each possessing distinct DNA binding and trans-activating domains. Sp1 was originally identified as a cellular transcription factor required for SV40 gene expression. It stimulates transcription by binding to GC-rich promoter elements embedded in variety of cellular and viral promoters. Recently, two structurally related transcription factors, Sp2, and Sp3, have been cloned, suggesting that Sp1 was a member of a multigene family, SpX. It has

been shown that the transactivation domains of the SpX proteins are modified with O-linked oligosachrides in vivo and phosphorylated by a protein kinase. It is as yet unclear whether these post-translational modifications play an important role in regulating SpX-mediated transcription. E2F was first identified as a cellular factor that interacted specifically with the E2 promoter of the adenovirus, so it was termed E2F. The activity of E2F had been shown to involve in many aspects of viral and cellular gene regulation. It has been demonstrated that E2F forms a complex with the retinoblastoma (Rb) protein and that leads to an inhibition of E2F transcriptional activity. YY1, a zinc finger-containing transcription factor, can either activate or repress transcription depending on the promoter context. A recent model suggests that YY1 influences transcription by its ability to recruit TFIIB and RNA Pol II on some DNA templates, or by its ability to interact with repressor proteins to suppress gene transcription (88).

No quantitative change was observed for any of these three transcription factors when the noninfected and HSV-1 infected HeLa nuclear extracts were analyzed in our EMSAs. This observation is different from adenovirus E1A-induced human c-myc transactivation. In this case, the immediate early protein, E1A, interactes with pRB causing the release the E2F from the pRB-E2F complex. The released E2F then activates c-myc transcription by binding to the E2F-1 site.

What are the mechanisms used by HSV-1 to transactivate c-myc gene expression? Several possibilities exist, although experiment evidence in support of any of one mechanism is lacking. Three viral proteins, ICP0, ICP4, and ICP27 are all

nuclear phosphoproteins, and phosphate has been shown to cycle on and off of each of them during the course of viral infection (1, 141, 147). Thus their potential role in phosphorylation could be involved in regulatory activities of the cellular transcription factors. Both Sp1 and YY1 have previously been shown to be modified by protein kinase. Perhaps these modifications result in a conformational change within the activation domain that allow to their interaction with the basal transcription machinery. Alternatively, the interaction of transcription factors by viral proteins might relieve the inhibition of transactivation so that transfection of viral regulatory gene, such as that for ICP4, can lead to superactivation. Viral proteins might also directly or indirectly function as coactivators, facilitating the interaction of Sp1 with the basal transcription machinery. Exactly how the three viral proteins (ICP0 ,4, and 27) modulate the cellular transcription components needs to be further investigated.

Table 2

Induction of Transfected Wild-type and Mutant c-myc Promoters by HSV-1 Superinfection

Reporter plasmid	+/- HSV-1 infection	CAT activity*	% of Induced +66 wt Activity
-101-myc-CAT	-	240	.77
-101-myc-CAT	+	34,189	110.2
+66wt-myc-CAT	-	246	.79
+66wt-myc-CAT	+	31,032	100
+66mt CT-I_2-myc-CAT	-	412	1.33
+66mt CT-I_2-myc-CAT	+	20,127	64.9
+66mt E2F-myc-CAT	-	408	1.31
+66mt E2F-myc-CAT	+	3,973	12.8

CAT activity=pmol OAc-CAM/mg protein/h. Data represents averages of 3 separate experiments, each with duplicate CAT assays.

From R.Millette unpublished results

Table 3

Induction of Transfected Wild-type and Mutant c-myc Promoters by HSV-1 Superinfection

Reporter plasmid	+/- HSV-1 infection	CAT activity*	% of Induced +66 wt Activity
+66wt-myc-CAT	-	104.5	5.6
+66wt-myc-CAT	+	1874.57	100
-157wt-myc-CAT	-	101.35	5.4
-157wt-myc-CAT	+	2054.9	109.6
-157mt CT-I_2-myc-CAT	-	122.34	6.5
-157mt CT-I_2-myc-CAT	+	1693.4	90.3
-157mt E2F-myc-CAT	-	67.8	3.5
-157mt E2F-myc-CAT	+	1006.2	53.7

CAT activity=pmol OAc-CAM/mg protein/h. Data represents averages of 3 separate experiments, each with duplicate CAT assays.

From H. Li, R. Millette unpublished results

Table 4

Quantity of CAT activity of cotransfection Assay

Reporter plasmid	Effector plasmid (ICPs)						
	none	4	0	27	4+0	4+27	4+0+27
+66 wt c-myc-CAT	11±2	100	6..6±1.8	2.2± 0.8	137±23	44±14	22±9
+66 mt CT-I_2-CAT	1.4±0.4	70±22	-	-	48±18	38±16	9±5
+66 mt E2F-CAT	5±0.9	87±30	-	-	55±25	46±6	12±5

CAT activity was measured in pmols ^{3}H-acetylated chloramphenicol/mg, and the results were presented as fold induction over the CAT activity seen with the target plasmid of +66 wt c-myc-CAT in the presence of effector plasmid ICP4. The CAT activity of +66 wt c-myc-CAT +ICP4 was defined as 100% expression. The data presented in Table 4 was average of 3 separate cotansfections.

Table 5

Oligonucleotides[1] used in EMSA

Sequence	Source of Sequence	Sequence
YY1[a]	LBF binding site of HSV-1 VP5-70bp[b]	CCAGGATCCA***GGGCCATCTT***GAATTTATCCTG G
C2[c]	uE3 binding site of murine IgH intronic enhancer	CCTTGCCATGACCTGCTTCCT
Sp1	Santa Cruz Biotech., Inc.	ATTCGATCG***GGGCGG***GGGCGAGC[d]
E2F	Santa Cruz Biotech., Inc	ATTTAAGT***TTCGCGC***CCTTTCTCAA[e]
E2F mt[f]	Santa Cruz Biotech., Inc	ATTTAAGT***TTCGATC***CCTTTCTCAA

[1] all oligonucleotides are double stranded

[a] originally be called LBS oligonucleotide that obtained from the Vollum Institute, Oregon Health Science University. Underlined base sequence is homology to YY1 binding site.

[b] bp are relative to cap site.

[c] a gift from K. Riggs and C. Calame, Columbia University.

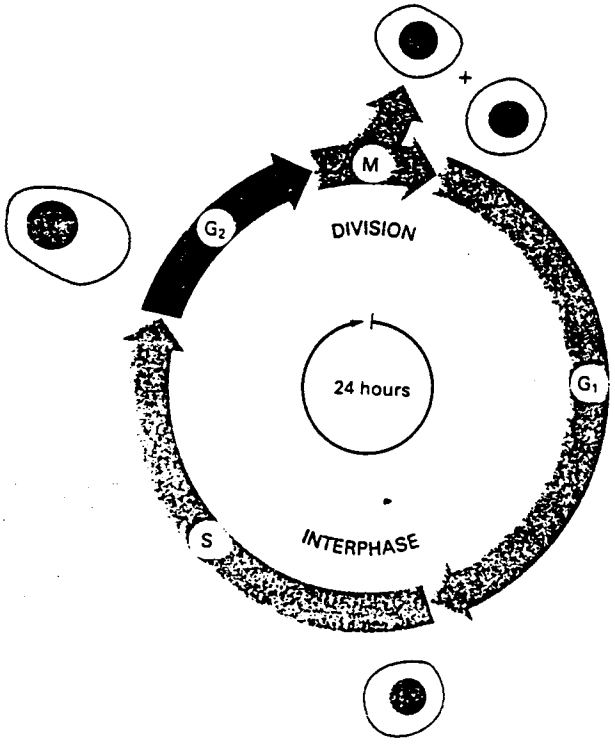
[d] underlined sequence is Sp1 binding site.

[e] underlined sequence is E2F binding site.

[f] E2F mutant oligonucleotide, bold two sites AT are substitution of CG

Figure 1

The Four Successive Phases of a Standard Eucaryotic Cell Cycle

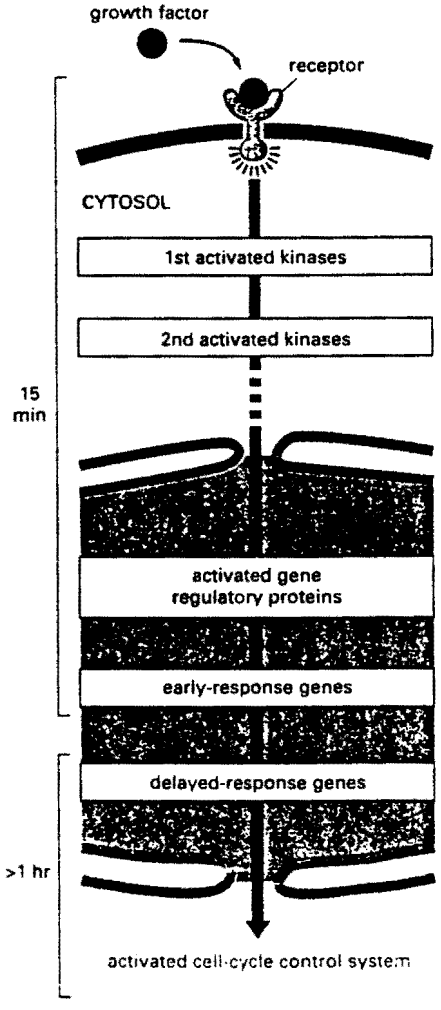


During interphase the cell grows continuously, during M phase it divides. DNA replication is confined to the part of interphase known as S phase. G_1 phase is the gap between M phase and S phase, G_2 is the gap between S phase and M phase.

From reference 3, page 865.

Figure 2

Typical Signaling Pathway for Stimulation of Cell Proliferation by a Growth Factor

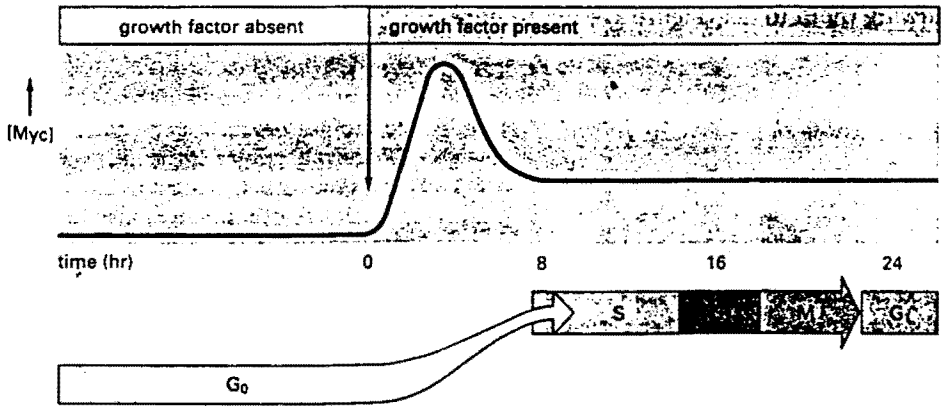


This simplified diagram shows some of the major steps. It omit many of the intermediate steps in the relay system.

From reference 3, page 901.

Figure 3

The Response of c-Myc to a Growth Factor



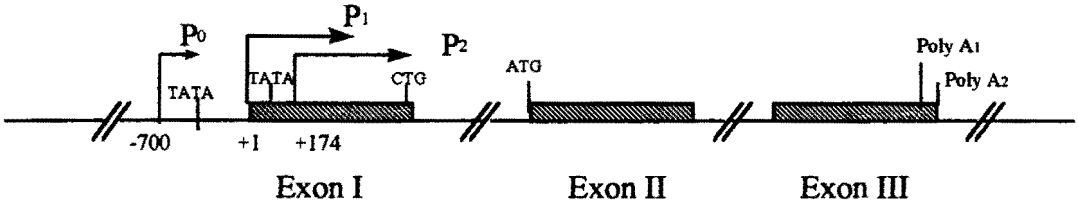
Cell-Division Controls in Multicellular Animals

c-Myc is the product of the early-response gene myc. The graph shows the changes in the concentration of Myc protein following a sudden increase in growth factor concentration to a new steady value, which causes the cell to exit G0 and to proliferate. The changes in Myc concentration reflect changes in myc gene transcription, stimulated by exposure of the cell to the growth factor. Myc protein itself inhibits myc transcription, and this negative feedback is to explain why the level of Myc declines from its initial peak to a lower steady value.

From reference 3, page 901.

Figure 4

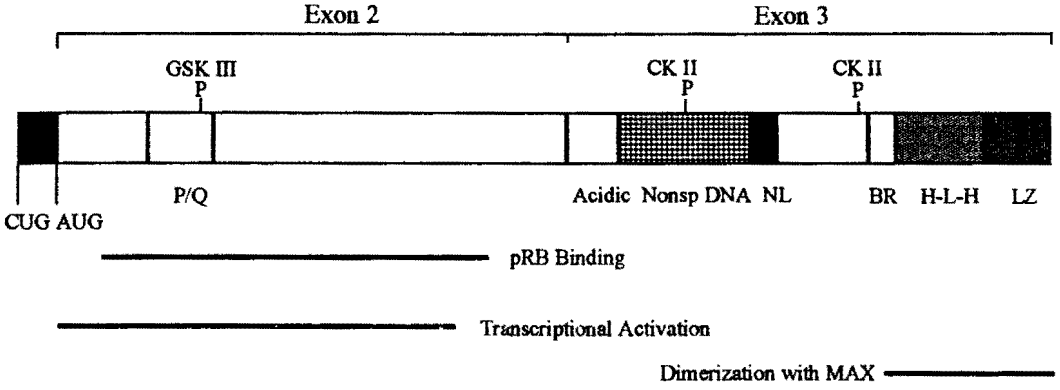
Topography of Human c-myc Gene



Abbreviation used:
P_0, P_1, P_2: three c-myc promoters. CTG, AUG: major translation start codons. Poly A_1, Poly A_2: polyadenylation site.

Figure 5

Summary of c-Myc Polypeptide and Some of Its Functional Domains



Abbreviations used:
CUG, initiation site within exon 1. AUG, initiation site defining beginning of exon 2. P/Q, proline/glutamin rich region. Nonsp DNA, nonspecific DNA-binding region. NL, nuclear localization signal. BR, basic region. H-L-H, helix-loop-helix protein dimerization domain. LZ, leucine zipper. GSKIII, phosphorylation by glycogen synthase kinase. CK II, phosphorylation by casein kinase II.

From reference 82, page 812.

Figure 6

Mapping Proximal Control Elements of P_1 and P_2 Promoter

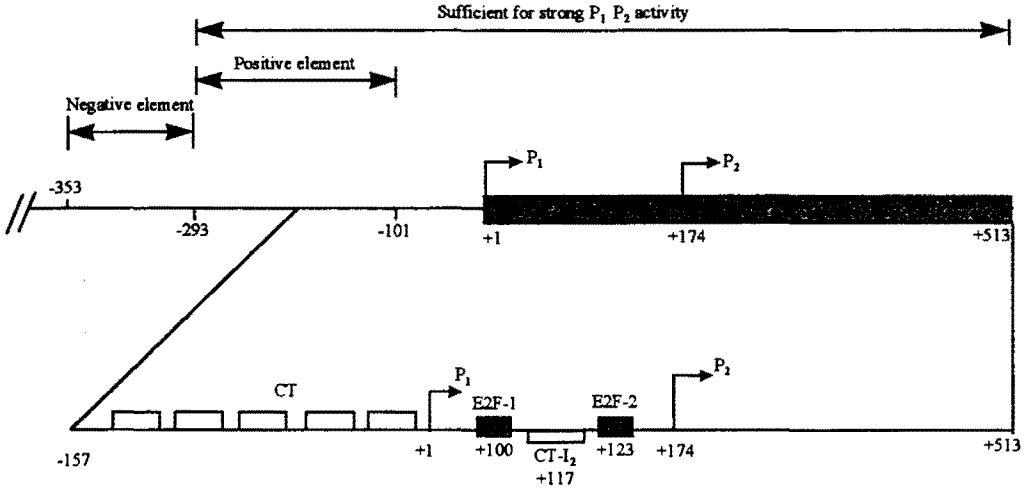
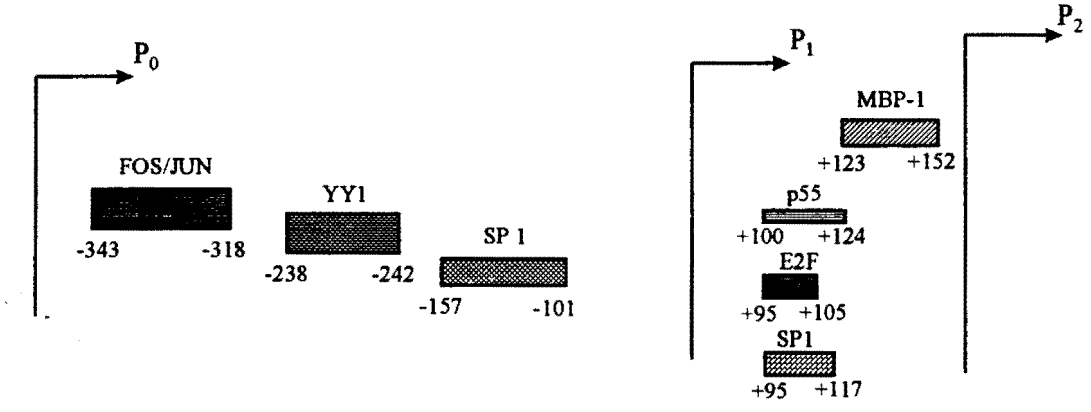


Figure 7

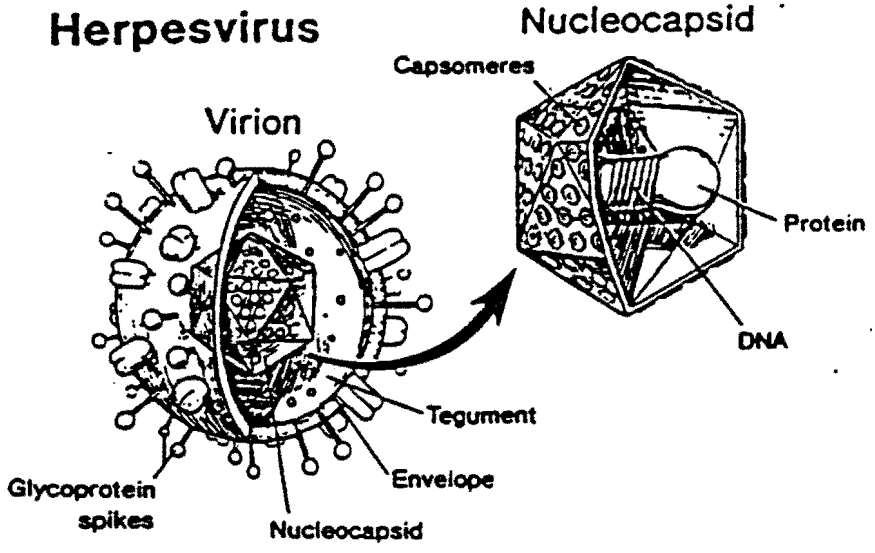
Summary of Some Human c-myc Transcriptional Factor Binding Sites



From reference 82, page 832.

Figure 8

Herpes Simplex Virion Structure

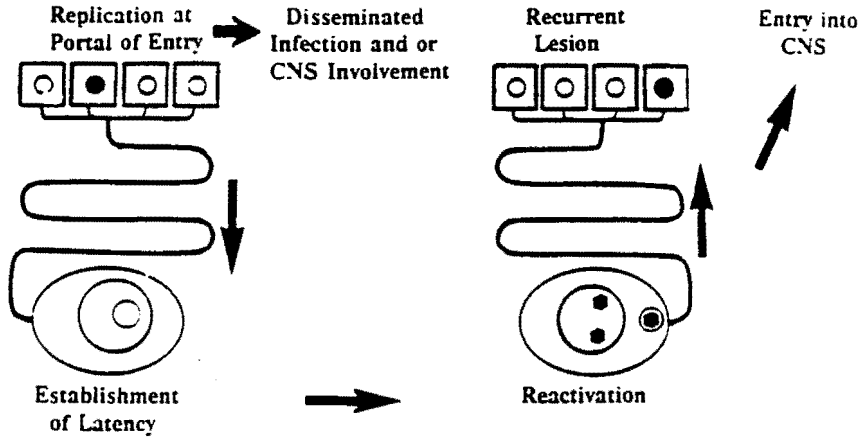


The protein of the herpesvirus is surrounded by the DNA, much like thread on a spool. A protein structure called the capsid is in the shape of an icosahedron and surrounds this DNA genome core. This combined structure is called nucleocapsid. An additional phospholipoprotein envelope surrounds the nucleocapsid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

From reference 92, page 294.

Figure 9

Natural Course of HSV Infection in Vivo



Virus first replicates in epithelial cells(squares) at the portal of entry, then moves through neurites (curved lines) to establish latent infections in neurons (ovals).

From reference 116, page 18.

Figure 10

Viral Genome Structure of Herpes Simplex Virus Type 1

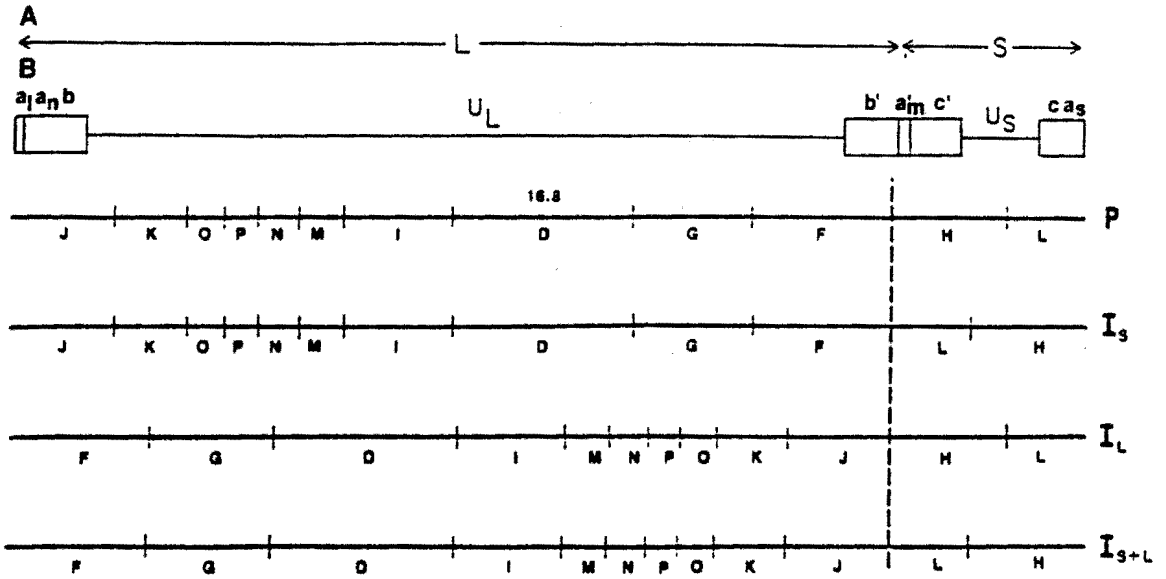


FIG. 2. Schematic representation of the arrangement of DNA sequences in the HSV genome. **A:** The domains of the L and S components are denoted by the *arrows*. The second line shows the unique sequences (*thin lines*) flanked by the inverted repeats (*boxes*). The letters above the second line designate the terminal *a* sequence of the L component (a_L), 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 reference 39, page 1049.

Figure 11

Replication of Herpes Simplex Virus in Susceptible Cell

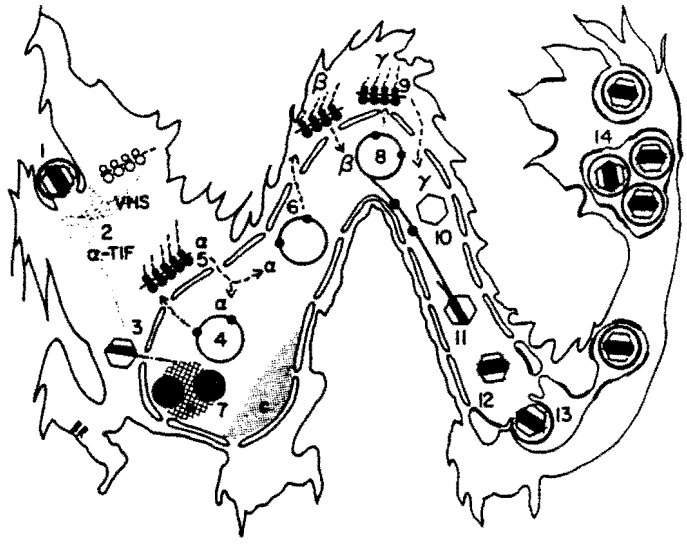


FIG. 3. Schematic representation of the replication of HSV in susceptible cells. **1:** The virus initiates infection by the fusion of the viral envelope with the plasma membrane following attachment to the cell surface. **2:** Fusion of the membranes releases two proteins from the virion. VHS shuts off protein synthesis (broken RNA in open polyribosomes). α-TIF is transported to the nucleus. **3:** The capsid is transported to the nuclear pore where viral DNA is released into the nucleus and immediately circularizes. **4:** The transcription of α genes by cellular enzymes is induced by α-TIF. **5:** The 5 αmRNAs are transported into the cytoplasm and translated (filled polyribosome); the proteins are transported into the nucleus. **6:** A new round of transcription results in the synthesis of ß proteins. **7:** At this stage in the infection, the chromatin (*c*) is degraded and displaced toward the nuclear membrane, whereas the nucleoli (*round hatched structures*) become disaggregated. **8:** Viral DNA is replicated by a rolling circle mechanism, which yields head-to-tail concatemers of unit-length viral DNA. **9:** A new round of transcription/translation yields the γ proteins, consisting primarily of structural proteins of the virus. **10:** The capsid proteins form empty capsids. **11:** Unit-length viral DNA is cleaved from concatemers and packaged into the preformed capsids. **12:** Capsids containing viral DNA acquire a new protein. **13:** Viral glycoproteins and tegument proteins accumulate and form patches in cellular membranes. The capsids containing DNA and the additional protein attach to the underside of the membrane patches containing viral proteins and are enveloped. **14:** The enveloped proteins accumulate in the endoplasmic reticulum and are transported into the extracellular space.

From reference 39, page 1051.

Figure 12

Regulation of HSV-1 Gene Expression

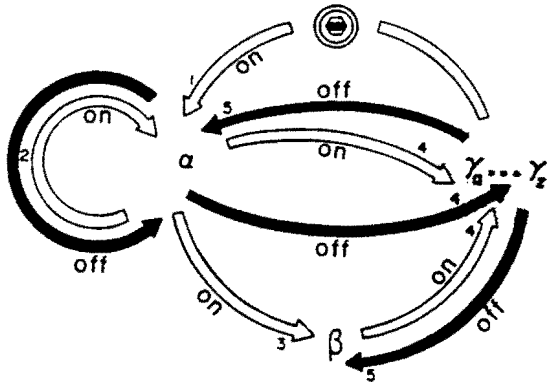
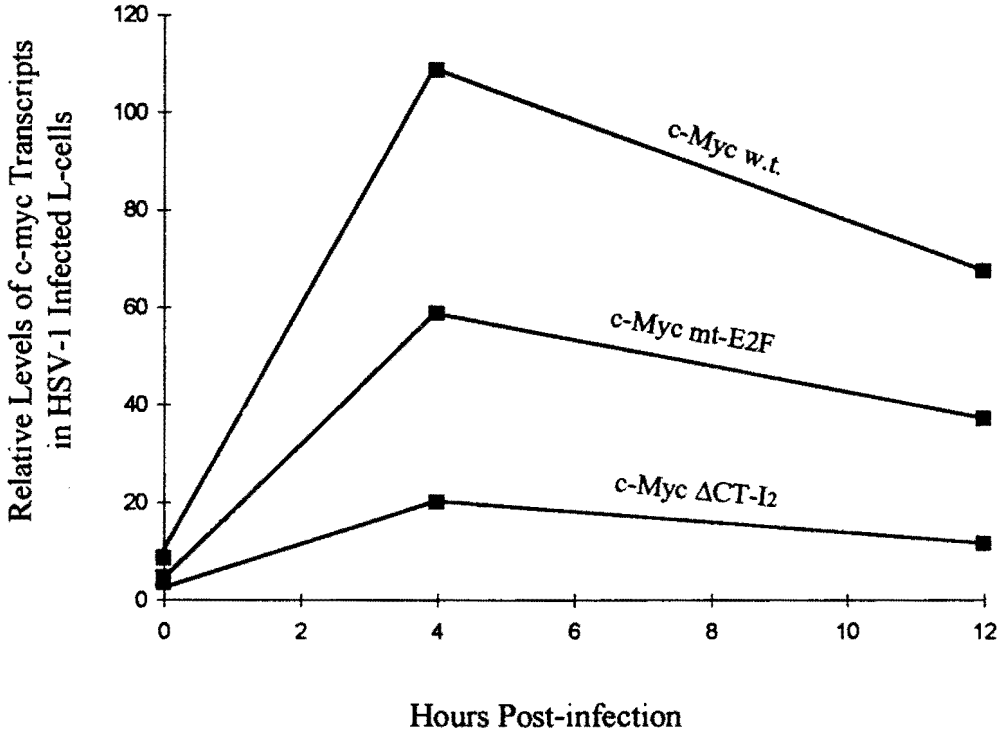


FIG. 5. Schematic representation of the regulation of HSV-1 gene expression. *Open* and *filled arrows* represent events in the reproductive cycle which turn gene expression "on" and "off," respectively. **1:** Turning on of α gene transcription by α-TIF, a γ protein packaged in the virion. **2:** Autoregulation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

From reference 39, page 1053.

Figure 13

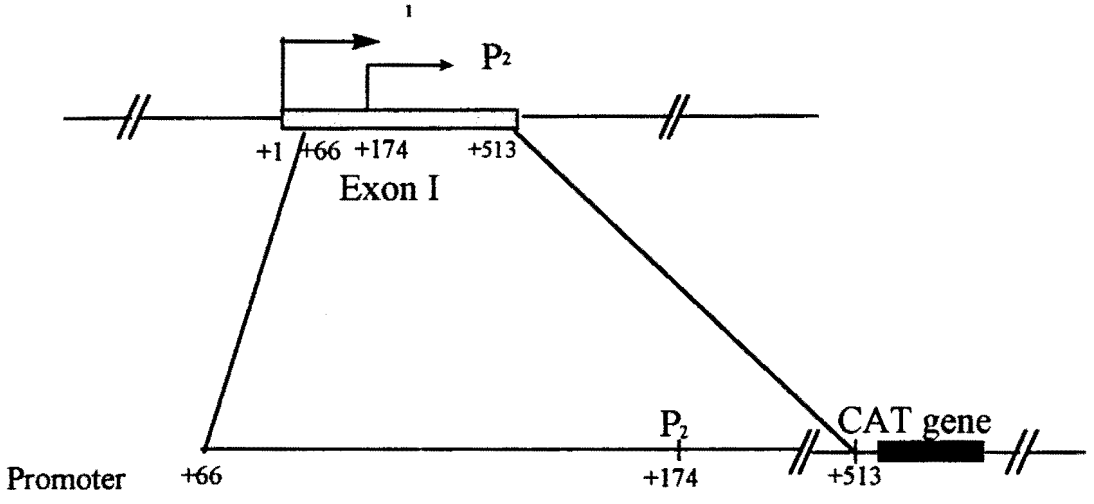
Induction of Wild-type and Mutant c-myc Promoter Following HSV-1 Infection



From R. Millette unpublished result

Figure 14

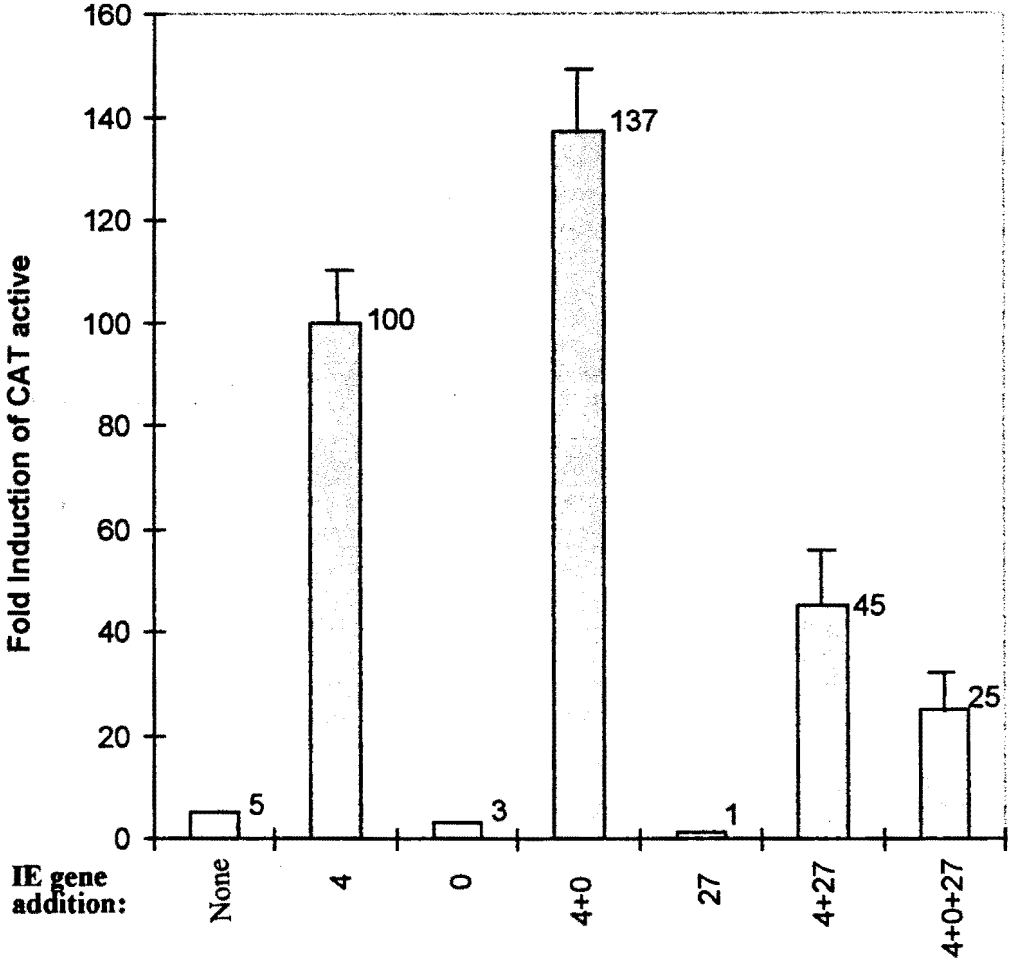
Construct of c-myc Pomotor and CAT Region in c-myc Wild Type +66wt-CAT Plasmid



The c-myc wild type promoter region from +66 to +513 bp (relative to P_1 transcription start site, and including the P_2 start site), was fused to upstream of the chloramphenicol acetyltransferase (CAT) gene in pBR322-CAT plasmid.

Figure 15

Results of Transient Expression Assays Using Wild Type+66 c-myc-CAT as Reporter Plasmid Cotransfected with HSV-1 Genes



Effects of exogenous of ICP4, ICP0, and ICP27 on +66 wild type c-myc CAT promoter. The CAT activity is normalized on 100% of c-myc +66wt transactivation by ICP4.

Figure 16

Construct of Promoter and CAT Region in c-myc +66 Wild Type-CAT, +66 Mutant CT-I_2-CAT and E2F-CAT plasmids

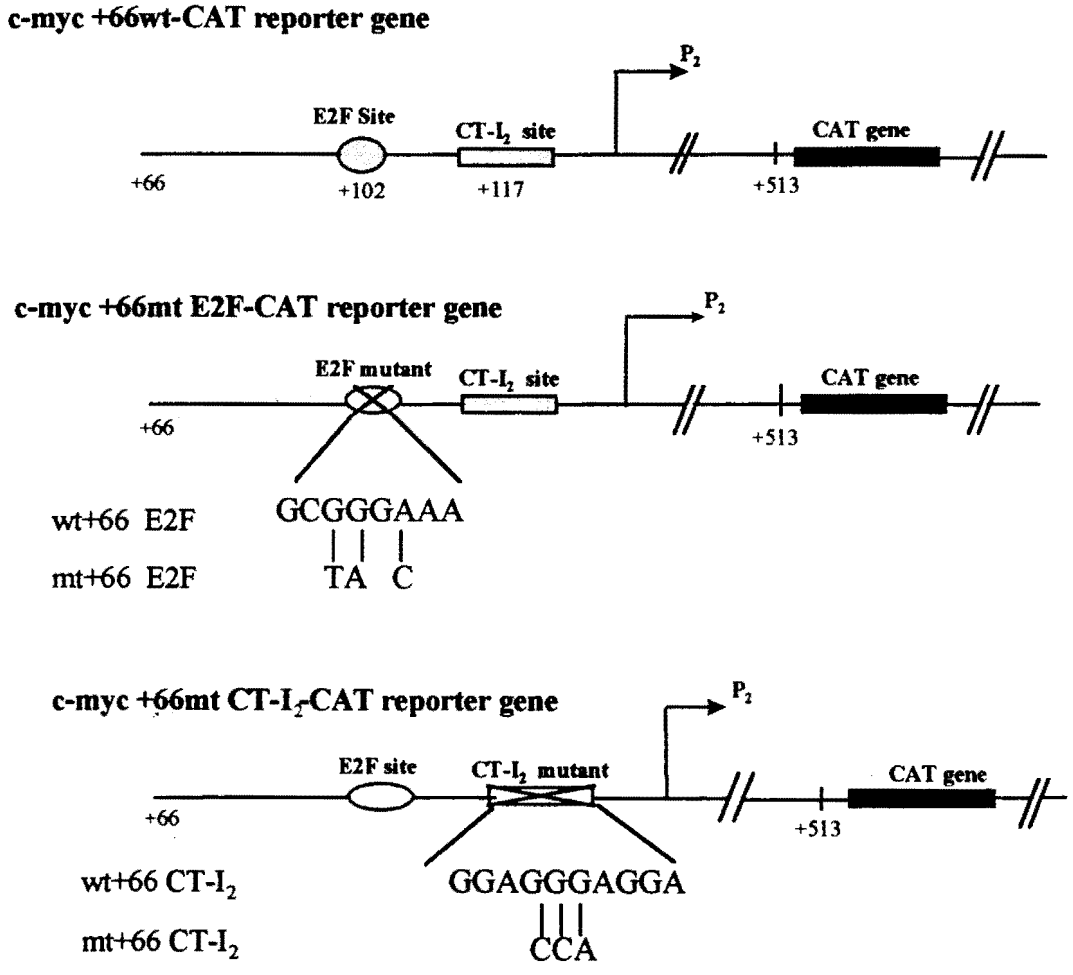
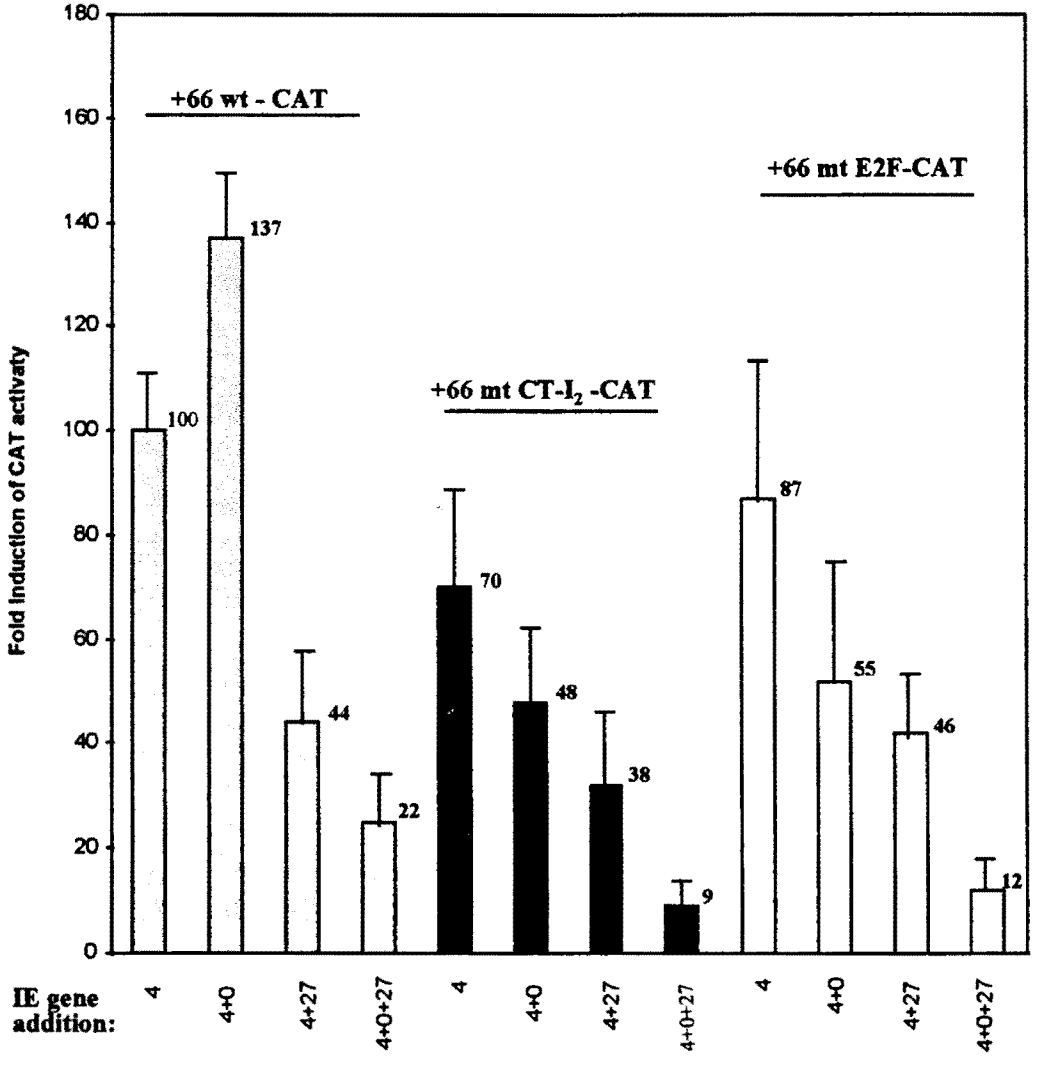


Diagram of the +66 c-myc promoter constructs of three reporter plasmids used in transient expression assays. The CT-I_2 and E2F sites are indicated by shielded box and oval respectively. The crossed box and oval represent mutated site for CT-I_2 and E2F respectively. The three specific mutated nucleotides in these two sites are indicated below the sequence of each site.

Figure 17

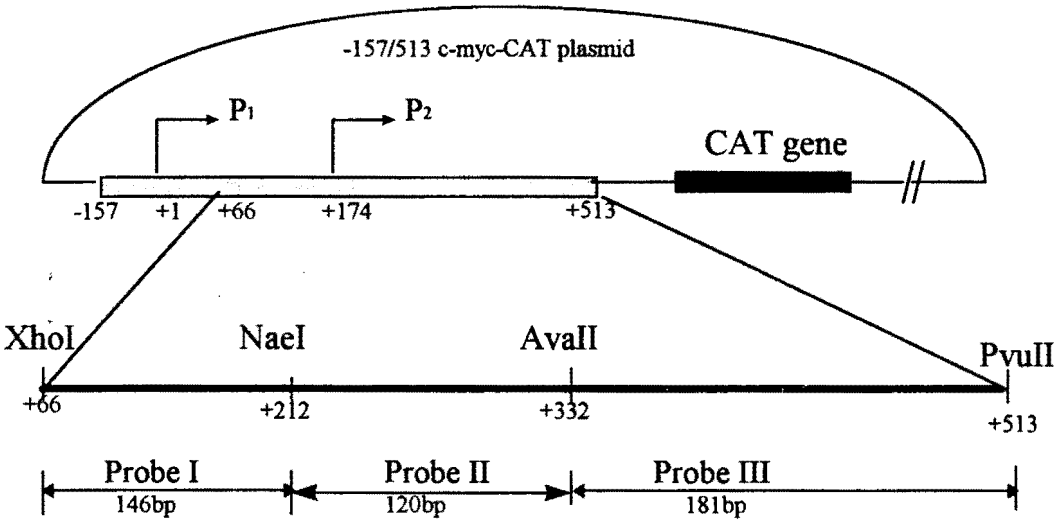
Results of Transient Expression Assays Using wild type+66wt c-myc-CAT or mutant +66mt-CT-I_2-CAT and +66mt E2F-CAT as Reporter Plasmids Cotransfected with HSV-1 Genes



Mutation on transcription factor binding sites (CT-I2, E2F) exhibited different degree effect on +66 c-myc promoter transactivation by HSV-1. The CAT activity was normalized on 100% of c-myc +66wt transactivation by ICP4.

Figure 18

Map of Probes Used in EMSA



+66 wild type P_2 promoter region (from +66 to +513) was excised from -157 wild type c-myc promoter CAT plasmid (-157/513 c-myc-CAT) by restriction digestion of XhoI and PvuII. This P_2 promoter region was further digested by restriction enzyme NaeI and AvaII to generate three probes. Each probe, then, was asymmetrically labeled on either lower or upper strand by a Klenow fill-in reaction. The labeled probes were used in EMSA at 1.5×10^4 cpm per reaction.

Figure 19

Map of Potential Cellular Transcription Factor Binding Sites on Human +66 c-myc P_2 Promoter Region

A: c-myc probe I (XhoI-NaeI), 144 bp (from +66 to +210)

tcgaga agggcagggc ttctcagagg cTTgGCGgga aaaagaacgg aGGGAGGGaT CGCGC tgagt ataaaagccg gttttcgggg
E2F-(1) CT-I_2 E2F-(2)
ctttatctaa ctcgctgtag taattccagc gagaggcaga gggagcgagc GGGCGgcc
Sp1-x

B: c-myc probe II (NaeI-AvaII), 120 bp (from +211 to +331)

gg ctagggtgga agagccGGGC Gagcagagct gcgctgcGGGC Gtcctggga agggagatcc ggagcgaata gggggcttcg cctctggccc
Sp1-x Sp1-x
agCCCTCCCg ctgatccccc agccagcg
CT element

C: c-myc probe III (AvaII-PvuII), 181 bp (from +332 to +513)

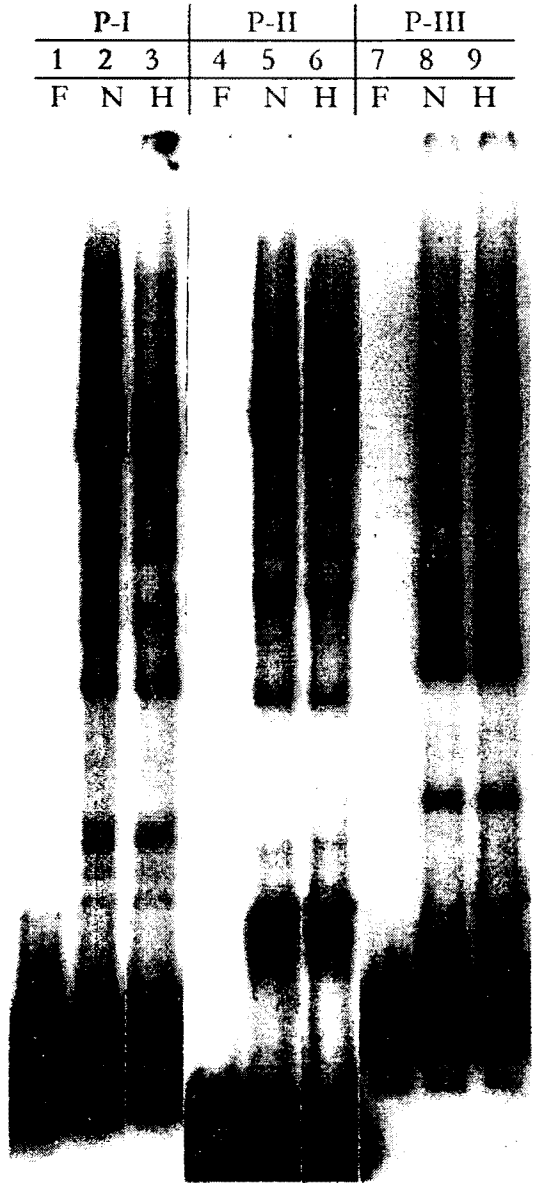
gt ccgcaaccct tgccgcatcc acgaaacttt gcccatagca gcGGGCGggC AcTTTgcact ggaacttaca acacccgagc aaggacgcga
Sp1-x YY1-1

ctctcccgac gcggggaggc tattctgccC ATTTggggac acttccccgc cgctgccagg acccgcttct ctgaaaggct ctccttgcag
YY1-2

* Underlined sequence indicate potential transcription factor recognition sites. Capital letters are those that match consensus binding sequence of transcription factor. Base pairs are relative to P_1 transcription site.

Figure 20

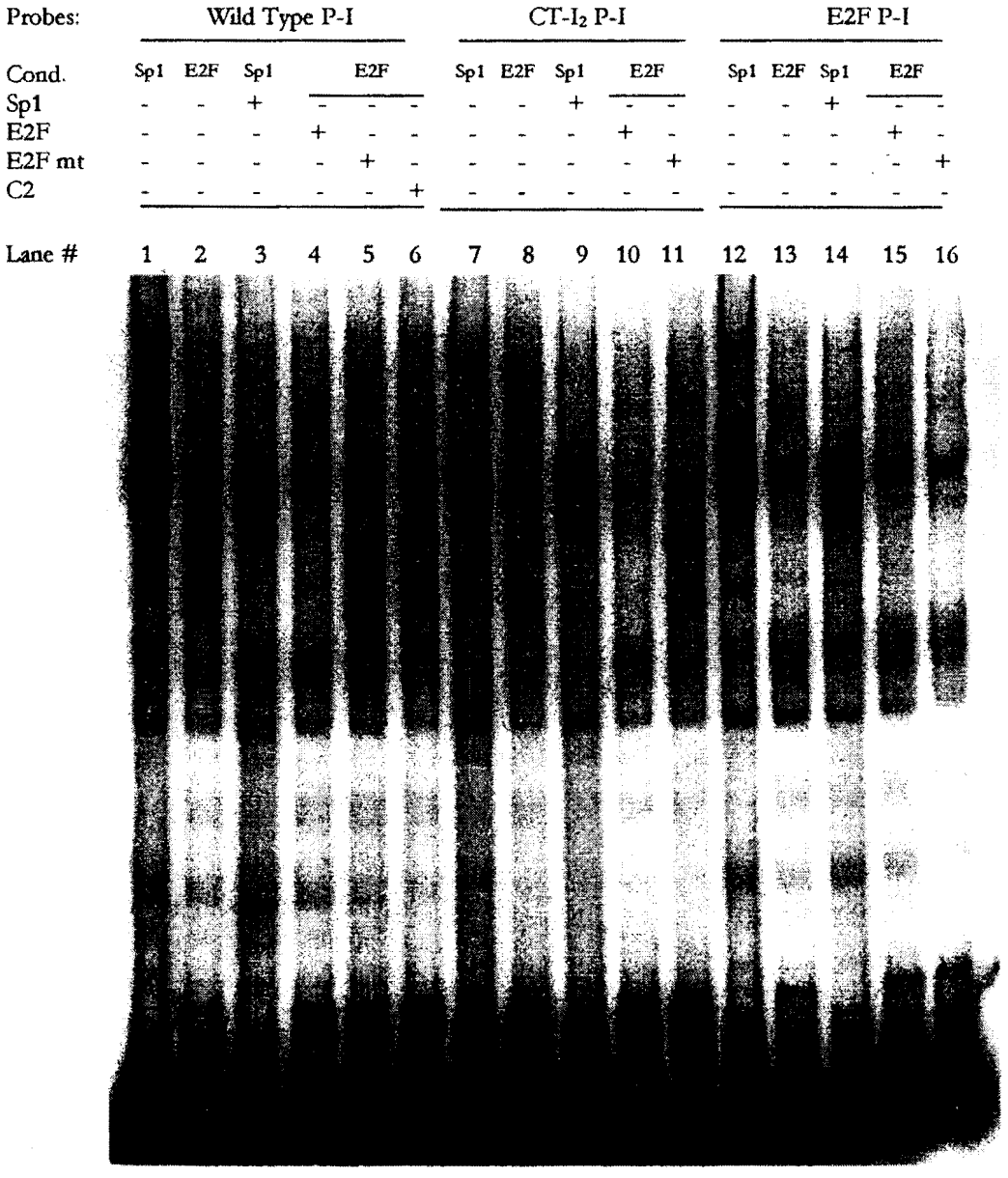
Comparison of Protein-DNA Complex Patterns Between Noninfected and HSV-1 Infected Hela Nuclear Extracts Formed on the Three Probes



Each of the end-labeled DNA probes was incubated with either noninfected or HSV-1 (KOS) infected crude HeLa nuclear extract in gel mobility shift assays. The F, N, and H represented free probe, noninfected, and HSV-1 infected nuclear extract used respectively

Figure 21-A

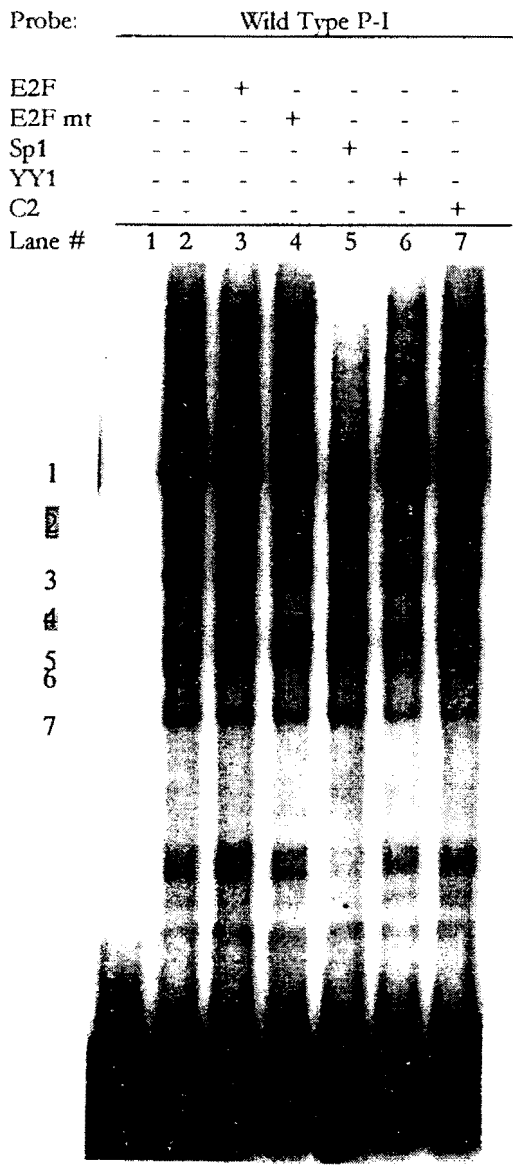
Comparison of Protein-DNA Complex Patterns Formed on Wild-type Probe I with Those Formed on CT-I_2 and E2F Mutated Probe I as well as Their Oligonucleotide Competitions at Optimal Sp1 and E2F Binding Conditions



The selected optimal binding conditions for Sp1/YY1 and E2F in the EMSA and oligonucleotide competition were indicated as Sp1 and E2F respectively under the probe. Oligonucleotides indicated above the lanes were added at the amount of 300-fold molar excess to the reacrion. The numbers on the left side of the picture represented the order of complexes formed on the Probe I..

Figure 21-B

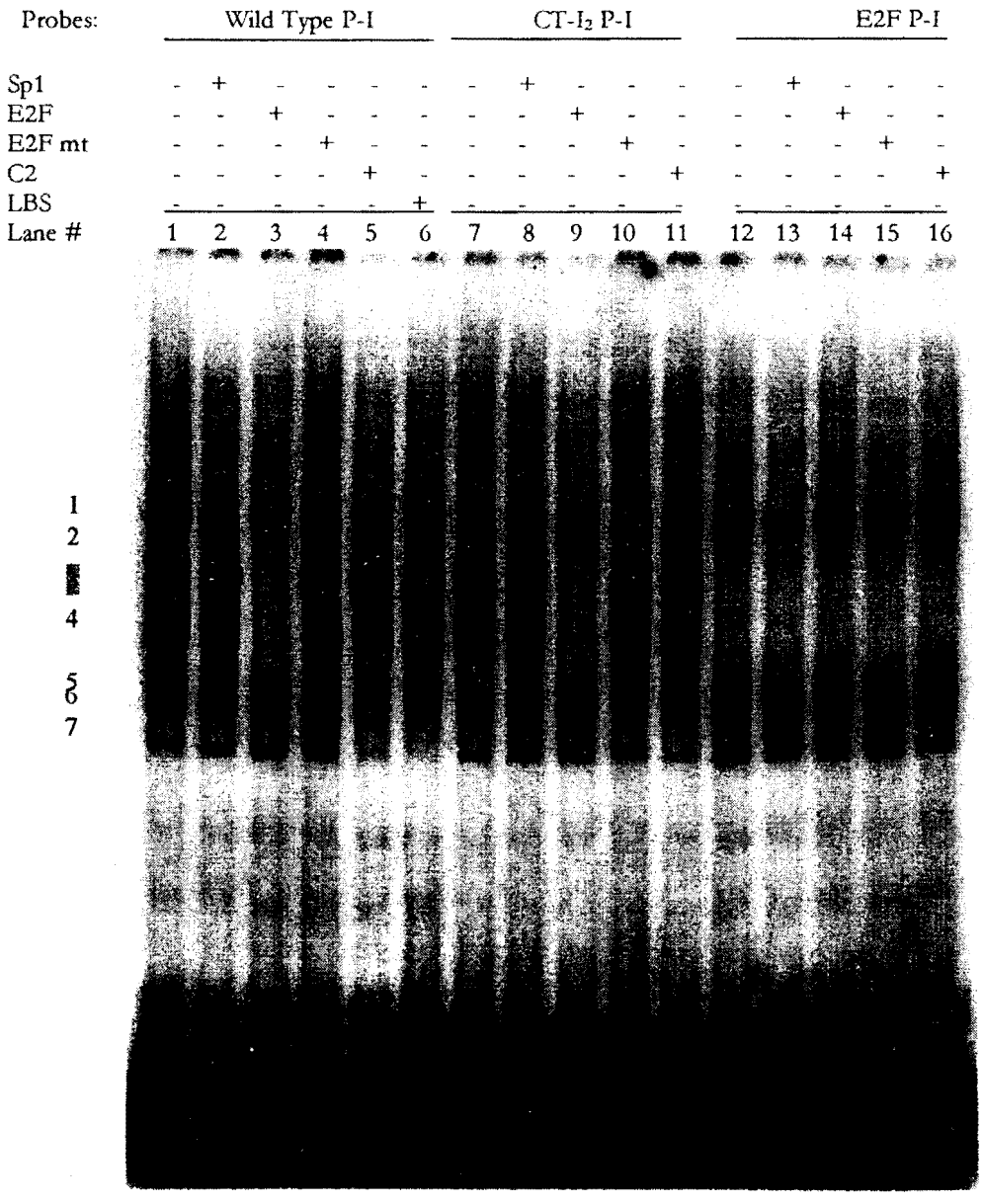
Sp1, YY1, and E2F Oligonucleotide Competitions for Interaction of Cellular Proteins with Wild Type c-myc Probe I at Optimal Sp1 Binding Condition



Oligonucleotides indicated above the lanes were added at the amount of 300-fold molar excess to the reacrion. Complexes 1 and 3 on the wild type probe I were specifically competed by Sp1 oligonucleotide but not YY1 at optimal Sp1/YY1 binding condition. The numbers on the left side of the picture represented the order of complexes formed on the Probe I.. The shaded numbers were complexes that were not able to observed at this binding condition.

Figure 21-C

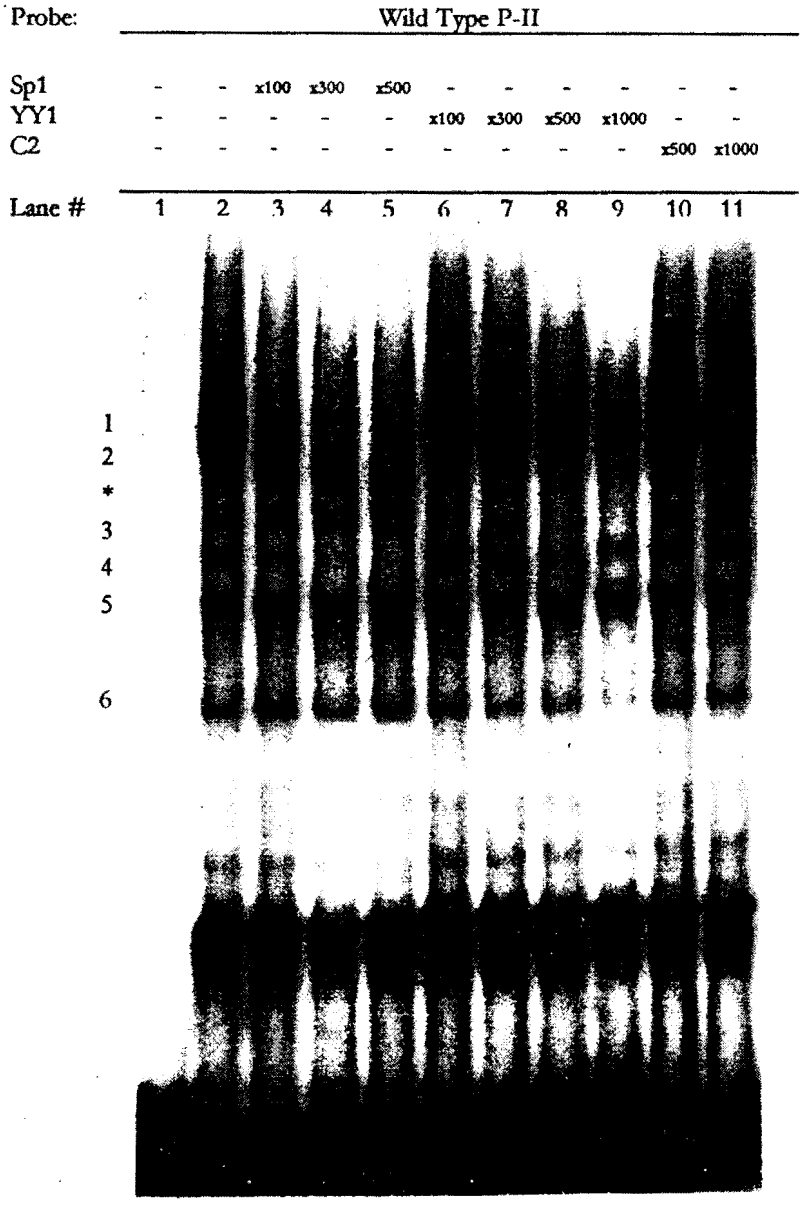
Comparison of Protein-DNA Complexes Formed on Wild-type Probe I with Those Formed on CT-I_2 and E2F Mutated Probe I: Oligonucleotide Competition at Optimal E2F Binding Condition



The wild type probe I, CT-I_2 site mutated probe I, or E2F site mutated probe I was incubated with HSV-1 infected nuclear extract at optimal E2F binding conditions. Oligonucleotides indicated above the lane were added at 300-fold molar excess to the reaction mixtures.

Figure 22

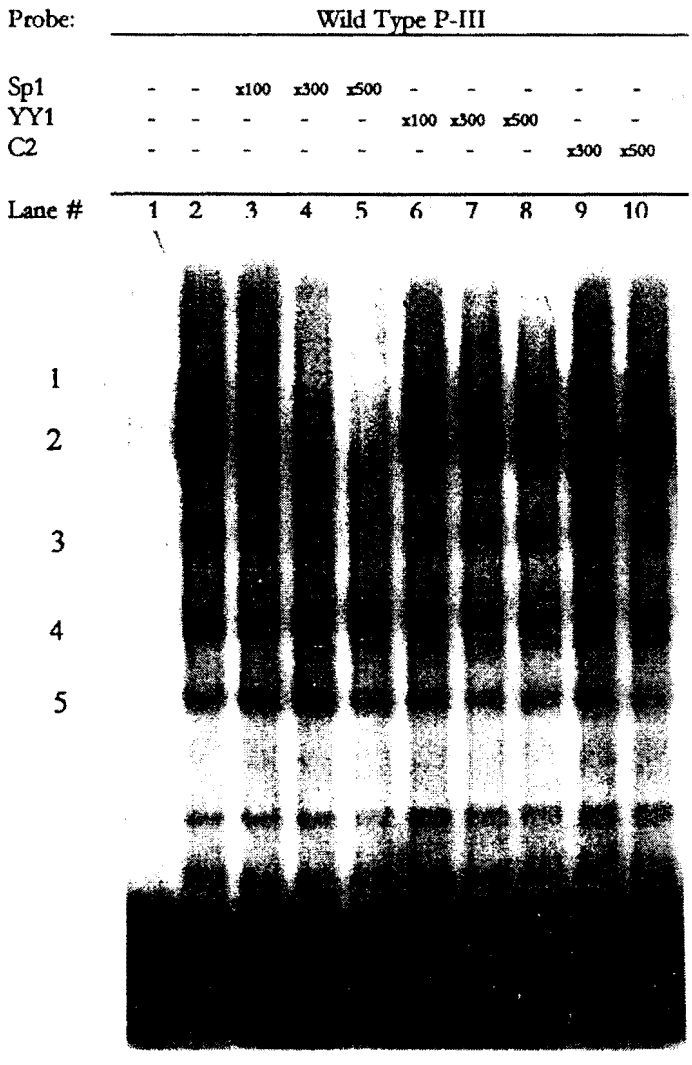
Oligonucleotide Competitions for Interaction of Cellular Proteins with c-myc Probe II at Optimal Sp1 Binding Condition



The oligonucleotide competition assays were carried out with an end-labeled DNA probe II (120 bp), the noninfected Hela nuclear extract (3ug), and the unlabeled oligonucleoteds in the amounts indicated above each lane. Locations of major complexes were indicated by numbers.

Figure 23

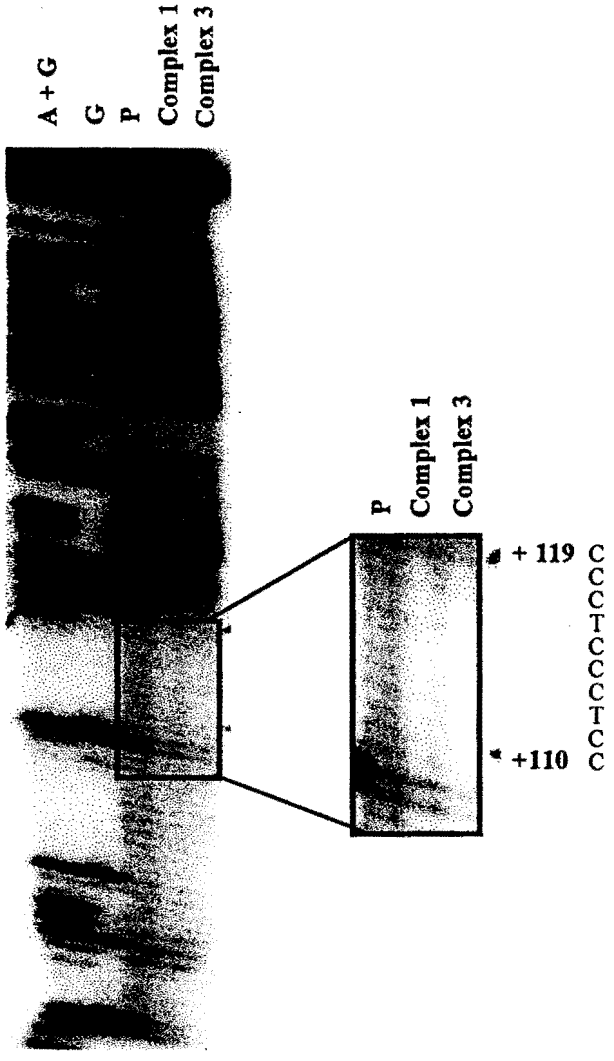
YY1 and Sp1 Oligonucleotides Competition for Interaction of Cellular Proteins with Wild Type c-myc Probe III at Optimal Sp1 Binding Condition



The oligo competition assays were carried out with an end-labeled DNA probe III (120 bp), the noninfected Hela nuclear extract (3ug), and the unlabeled oligonucleoteds in the amounts indicated above each lane. Locations of major complexes were indicated by numbers.

Figure 24

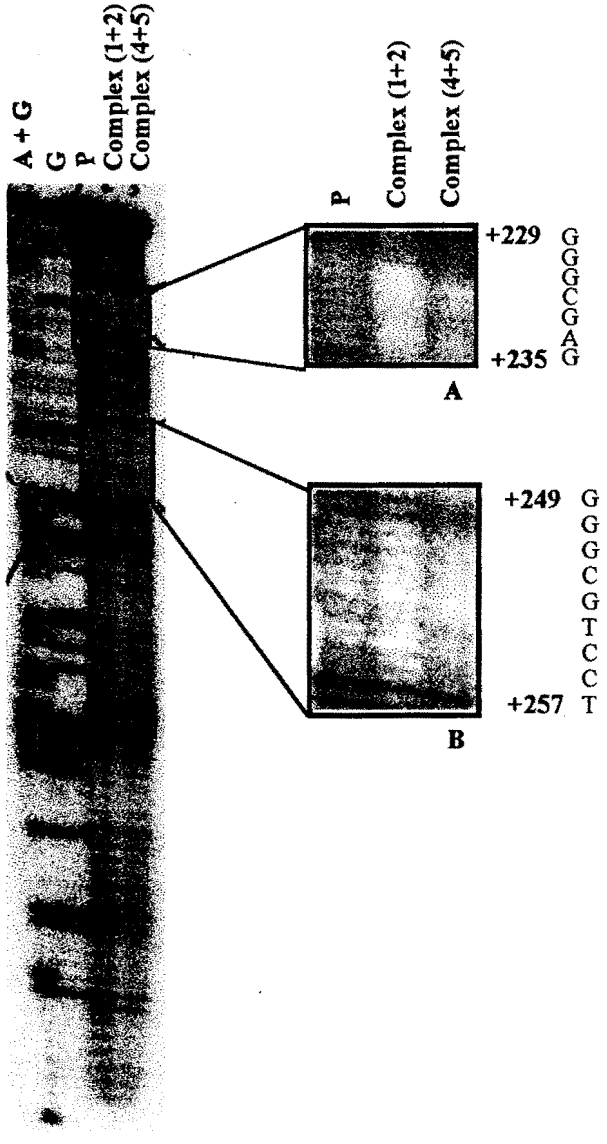
Orthophenanthroline-Copper ion (OP-Cu) DNA Footprinting Analysis of Major Complexes on the Probe I



A footprinting reaction was carried out within the acrylamide gel matrix (See materials and methods) . Free DNA probe I, complex 1 and 3 (Fig. 21-A) were excised respectively from OP-Cu digested EMSA gel. Eluded DNA were then dried and loaded on a 8% of sequence gel along with the Max-Gilbert cleavage of probe I. Lanes A+G and G: DNA fragments produced by partial cleavage of the end labeled probe I at adenines plus guanines and at guanines respectively. P: unbound DNA probe I. Complex 1 and complex 3: complex 1 and 3 formed on the P-I. The observed footprints were blown up to the right.

Figure 25

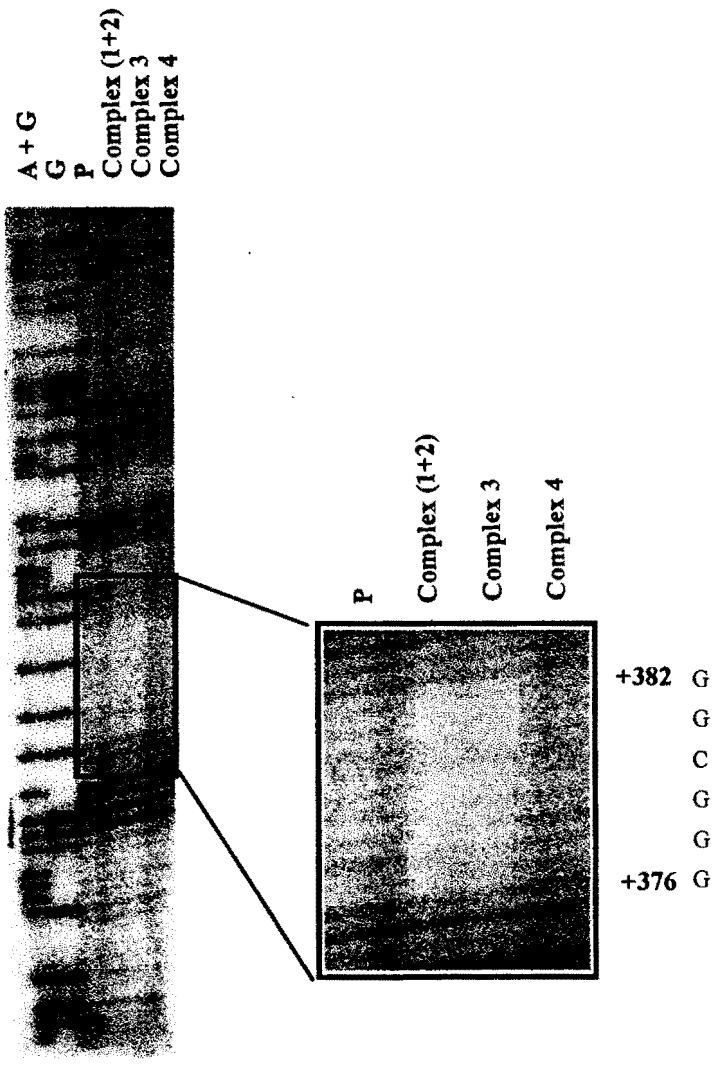
Orthophenanthroline-Copper ion (OP-Cu) DNA Footprinting Analysis of Major Complexes on the Probe II



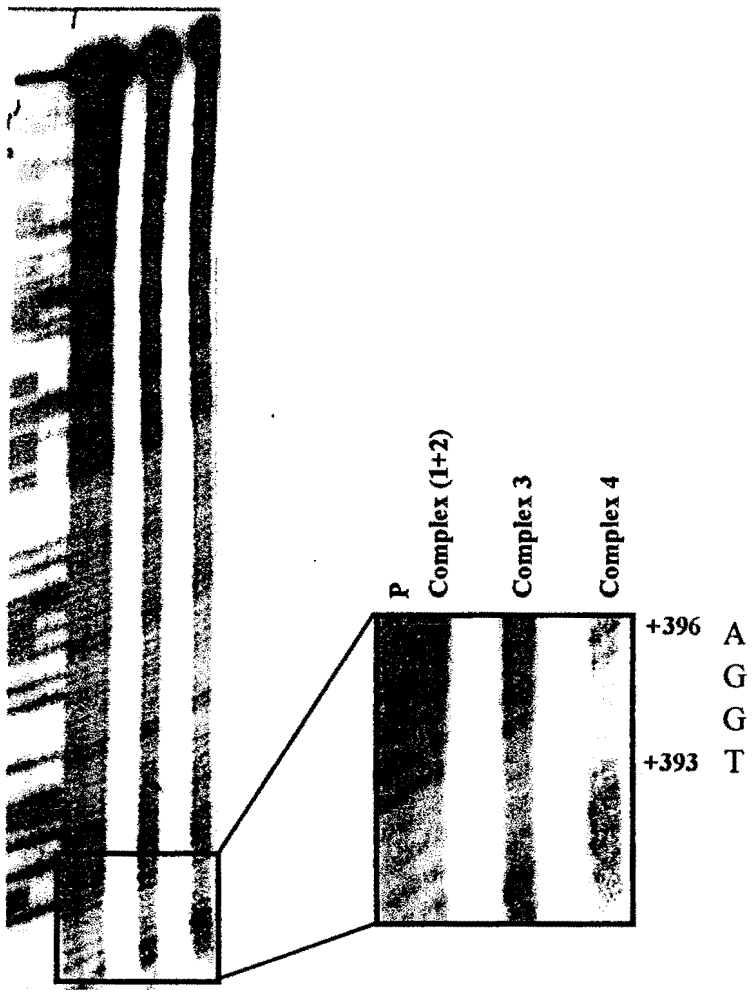
Free DNA probe II, complex 1+2, and complex 4+5 together (Fig. 22,) were excised from OP-Cu digested EMSA gel respectively. Eluded DNA were then dried and loaded on a 8% of sequence gel along with the Max-Gilbert cleavage of probe II. Lanes A+G and G: DNA fragments produced by partial cleavage of the end labeled probe II at adenines plus guanines and at guanines respectively. P: unbound DNA probe II. Complexes (1+2): complex 1 and complex 2 together excised from OP-Cu digested EMSA gel. Complexes (4+5): complex 4 and complex 5 together excised from OP-Cu digested EMSA gel. The observed footprints were blown up to the right.

Figure 26

Orthophenanthroline-Copper ion (OP-Cu) DNA Footprinting Analysis of Major Complexes on the Probe III



Panel A



Panel B

Free DNA probeIII, complex 1+2 together, complex 3, and complex 4 (Fig. 23) were excised from OP-Cu digested EMSA gel respectively. Panel A was footprinting samples run for 1.5 hours. Panel B is footprinting samples run for 3 hours. A+G and G: DNA fragments produced by partial cleavage of the end labeled probe III at adenines plus guanines and at guanines respectively. P: unbound DNA probeIII. Complex (1+ 2), complex 3, and complex 4: complex 1+2 together, complex 3, and complex 4 excised from OP-Cu digested EMSA gel respectively. The observed footprints were blown up to the right.

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Table 1

Stimulation of c-Myc/CAT Expression by HS V-1 Superinfection [a]

Plasmid[b] Transfected	HSV-1 Superinfection	pmol ^{3}H- Oac incorp.[c] per mg protein
c-myc-101-CAT	-	375
c-myc-101-CAT	+	28,800
c-myc-101-CAT	+ (UV-irrad.)	1,740
c-myc -353-CAT	-	227
c-myc -353-CAT	+	23,500
c-myc -353-CAT	+ (UV-irrad.)	987

[a] 6×10^5 Hela cells were transfected with 8 ug of the indicated plasmids plus 8 ug pUC18 by the Ca-phosphate technique. 22 hour later the cells were infected with HSV-1 at moi=3, and 24 hour after infection the cells were harvested of CAT assay.

[b] c-myc-101: 101 bp of human c-myc promoter (relative to the P1 start site) coupled to the CAT gene; c-myc-353: 353 bp of c-myc promoter coupled to the CAT gene (both gifts of N.Hay).

[c] Incorporation of 3H-Oac into mono-and di-acetylated chloramphnicol, measured by the method of Nordeen, et al. Average of 3 transfection experiments is presented.

From R. Millette unpublished results