# IN VITRO STUDIES ON THE REGULATION OF HERPES SIMPLEX VIRUS TYPE 1 GENE TRANSCRIPTION

by

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# ABSTRACT

The herpes simplex virus type 1 (HSV-1) genes are temporally regulated during the lytic infection cycle, and have accordingly been classified into five major classes, namely α, αß, ß, ßτ and τ. This regulation of gene expression is known to occur both at the transcriptional and at the translational levels.

To investigate the molecular basis for the temporal regulation of HSV-1 gene expression observed in vivo, partially purified RNA Polymerase II was prepared from HEp-2 cells that were either mock-infected, infected with HSV-1 for 8h, infected with HSV-1 with cycloheximide present 0-7h and removed 7-8h post infection (cycloheximide reversed or "CR" cells), or infected with the immediate-early mutant tsLB2, for 8h at either the permissive or non-permissive temperatures. Whole cell extracts were also prepared from mouse L cells that either constitutively produce ICP4 (Z4 cells) or lack it (Lta cells). Using these systems, cloned fragments of HSV-1 DNA [each containing a promoter from a specific class of HSV-1 genes, namely immediate-early or α, delayed-early or ß and leaky-late or ßτ] were transcribed in vitro. The data obtained from these studies show the following: (i) viral proteins are not required for in vitro transcription from α and ß promoters, (ii) viral promoters in addition to ICP4 are required for optimal ßτ5 promoter transcription, (iii) viral proteins present in both 8h and in "CR" infected cells are

required for transcription in vitro from the βT5 promoter, and (iv) viral proteins present in the cells by 8h post infection negatively affect α and β gene transcription.

To investigate the factors that are involved in this differential transcription of the HSV-1 promoters, cellular extracts from uninfected and 8h infected HEp-2 cells were chromatographically fractionated on heparin-agarose, DEAE-Sepharose CL-6B and Sephacryl S-300 columns. The fractions isolated were then assayed by in vitro transcription assays for their ability to confer upon a partially purified preparation of Polymerase II from uninfected cells (Pol II-M) the features observed with a similar Pol II preparation from 8h HSV-1 infected cells (Pol II-H). By using these assays, an activity present in 8h infected cells was identified, that stimulates transcription from the βT5 promoter by Pol II-M. This fraction also inhibited transcription from certain α and β promoters by Pol II-M. Similar activities were not found in corresponding fractions from mock-infected cells.

To identify the viral proteins present, this active fraction was analysed by immunoblotting. Using monoclonal antibodies to specific HSV-1 proteins and antiserum to 7h infected cell polypeptides, the presence of the HSV-1 immediate-early proteins ICP0, ICP4 and the early proteins ICP6 and ICP8, were demonstrated in this fraction.

# INTRODUCTION

The herpes simplex viruses (HSV) are classified as members of the family Herpesviridae in that they possess a double stranded linear DNA in the form of a nucleoprotein core, a proteinaceous capsid containing 162 capsomeres, and an outer membrane or envelope derived from the virus-modified host nuclear membrane (Figure 1A)(191; refer to literature cited at end of thesis discussion). Based on their (a) relatively short reproductive cycle, (b) rapid spread in culture, (c) efficient destruction of infected cells, and (d) ability to establish latency primarily (but not exclusively) in ganglia, the herpes simplex viruses have been further classified into the subfamily Alphaherpesvirinae (261, 266). Two immunologically distinct genera of herpes simplex virus have been identified by immunological neutralisation, HSV-1 and HSV-2. Both types show cross reactivity antigenically and approximately 50% homology in their genomes. Therefore, although HSV-1 and HSV-2 differ in specific details (e.g., pattern of restriction enzyme cleavage sites in their DNA, host cell type specificity, mode of transmission and epidemiology of the viruses), the very close overall similarity seen suggests that many generalisations on the mechanism of gene regulation will cover both (317).

## LEGENDS TO FIGURES IN THE INTRODUCTION

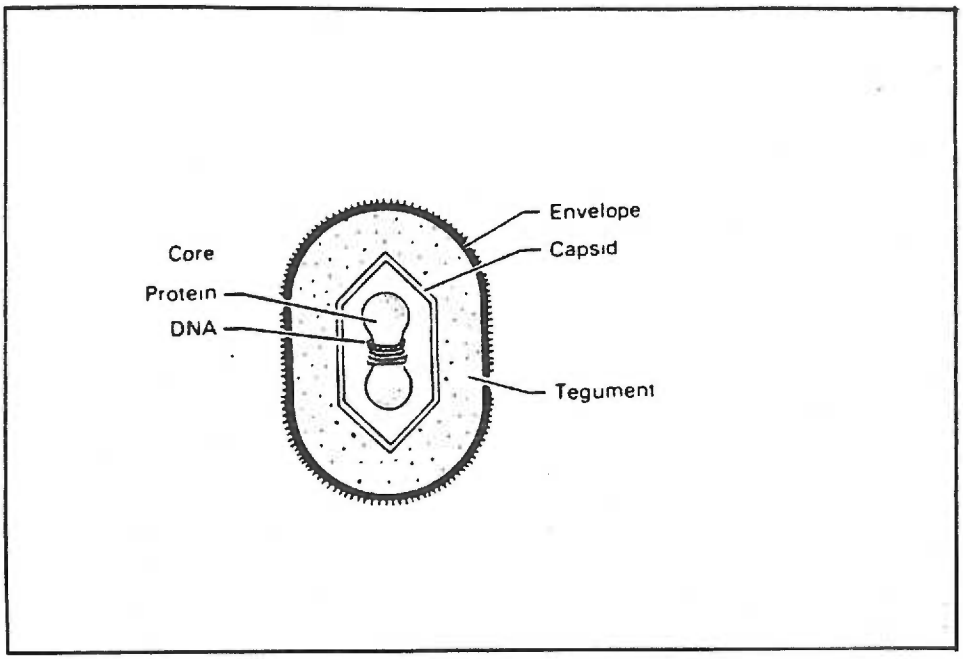
FIG. 1A.

Schematic representation of the herpes simplex virion. The herpes simplex virion is comprised of four structural elements : the viral genome arranged in a electron-opaque toroidal core, an icosadeltahedral proteinaceous capsid, an electron-dense amorphous tegument, and an outer envelope.

FIGURE 1B.

Diagrammatic representation of the herpes simplex virus type 1 genome in the prototypic arrangement. The horizontal lines represent the unique regions. The rectangular boxes denote the reiterated sequences. The two unique regions of the viral genome are each flanked by a pair of reiterated sequences. The length of the genome in map units is given at the top.

A


Envelope
Capsid
Core
Protein
DNA
Tegument

B

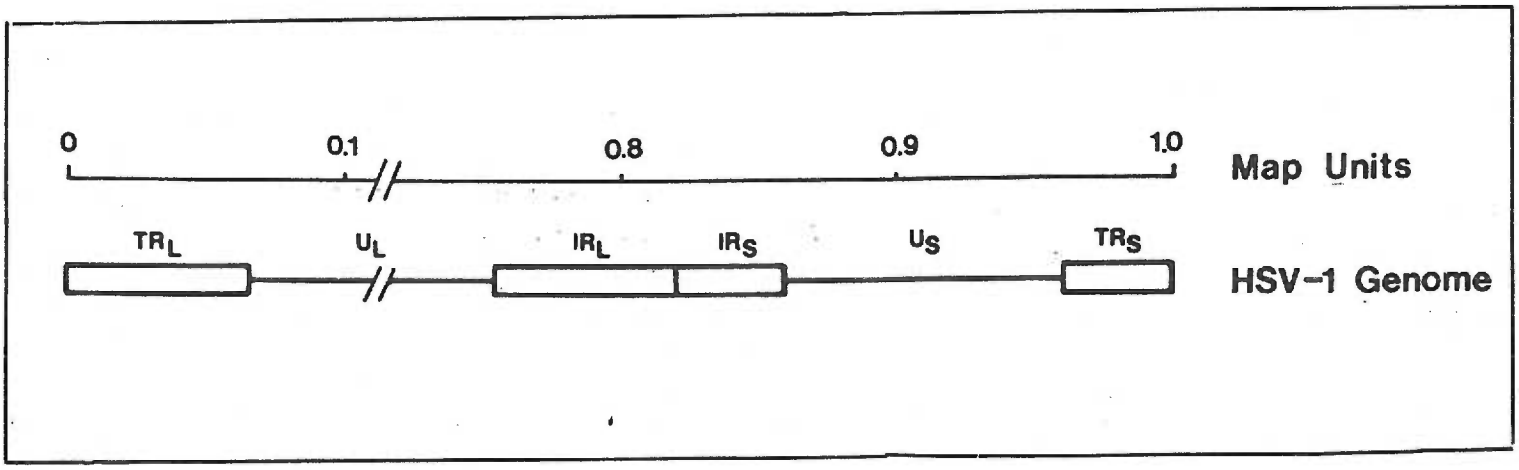

0
0.1
0.8
0.9
1.0
Map Units
TRL
UL
IRL
IRS
US
TRS
HSV-1 Genome

Fig. 1

**The HSV Virion.**

Morphologically, like the other herpesviruses, both HSV-1 and HSV-2 possess an outer membrane or envelope derived from the inner nuclear membrane of the cells which is modified by the insertion of viral glycoproteins (58, 124, 131, 223, 290, 294). These are interspersed in the membrane as spikes approximately 8 nm in length (332). Six HSV-1 glycoproteins (gA/B, gC, gD, gE, gG and gH) and their counterparts on HSV-2 have been identified immunologically (15, 78, 225, 226, 289, 290, 335, 337, Ackerman et al. Abstract no. 131 and Gompels et al. Abstract no. 135, 10th International Herpesvirus Workshop, Ann Arbor, MI). Underlying the envelope is an electron-dense, nondescript, amorphous region termed the tegument (263). This appears to be fibrous on negative staining (205, 206, 332), and variable in thickness depending on the location of the virion within the infected cell. [Virions accumulating in peripheral cytoplasmic vacuoles tend to have thicker teguments than virions accumulating in the perinuclear space (262)]. Several virally encoded proteins have been associated with the tegument (107, 314).

Inside the tegument is an icosadeltahedral capsid, approximately 100 nm in diameter. The 162 capsomere capsid is made up entirely of viral polypeptides (107, 292), of which at least five have been identified immunologically (45). These are VP5 (155-kd), VP19 (50-kd), VP22a (37-kd), VP24 (26-kd) and another 50 kd protein. Within the capsid is housed an electron-opaque toroidal core which contains the viral DNA

(103, 125). The precise arrangement of the DNA in the core is unknown, and the nature of the HSV-1 genome is further discussed below.

**The HSV-1 Genome.**

The HSV-1 DNA is in the form of a linear double-stranded DNA molecule of approximately 150-kbp (equivalent to a molecular weight of approximately $100 \times 10^6$ daltons) (19, 161). A unique feature of the herpesvirus genomes is their sequence arrangement, and HSV-1 is no exception. The HSV-1 DNA consists of two covalently linked components designated as L (long) and S (short), respectively comprising of 82% and 18% , of the total viral DNA (262) [Figure 1B]. Each of these components contains a segment of unique sequence DNA (designated as $U_L$ and $U_S$ and comprising 70% and 9.4% of the total DNA respectively) that is flanked by inverted repeats. The repeat regions flanking $U_L$ designated "ab" and "b'a'" each comprise 6% of the total DNA, while those flanking $U_S$ designated "c'a'" and "ca" each account for 4.3% of the viral DNA (316, 262). The repeats flanking the two unique regions differ in their size and average base compositions (316). Evidence for the presence of repeat sequences was derived in part from experiments where the genome was first heat denatured and then allowed to reanneal under dilute conditions (281). This resulted in two single-stranded loops of unequal size bridged by double-stranded DNA. The union of the two components (L and S) forms the joint region designated as

b'a'$_L$ a'$_S$ c', where $a_L$ and $a_S$ represent the "a" repeat sequences of the L and S components respectively (321). Since the "a" sequences at the junctions are identical (59, 166), and result from a sharing of the terminal nucleotides from each DNA component (203), the union segment is simply written as "b'a'c'". Thus, the configuration of the HSV-1 DNA in the prototype configuration may be theoretically represented as $a_Lb-U_L-b'a'c'-U_S-ca_S$ (262).

The directly repeated, terminally redundant 500-bp "a" sequence in HSV-1 (111, 316) has been shown by DNA sequencing, to consist of several short internal tandemly repeated sequences (59). These are a 20-bp direct repeat [DR1] followed consecutively by a 62-bp unique sequence [$U_b$], a 12-bp direct repeat [DR2] repeated 19-23 times, a 37-bp direct repeat sequence [DR4] repeated 2-3 times, a 58-bp unique sequence [$U_c$], and finally another copy of DR1. Simply, the "a" sequence can be represented as [DR1][$U_b$][DR2]$_{19-23}$[DR4]$_{2-3}$[$U_c$][DR1]. These "a" sequences have been shown to be the sequences which are required for cleavage and packaging of the viral DNA into the mature virion capsids during maturation (312, 315). The "a" sequences have also been shown to mediate virally trans-activated site-specific recombination during the inversion of the L and S components (38, 60, 201-204, 230, 286). Therefore, as a result of inversions and recombinations of the genome (63), HSV-1 DNA can exist as four isomers, differing solely in the orientation of the L and S components

(121). Figure 1B shows a simplified diagram of the HSV-1 genome in the prototype arrangements.

## An Overview of Gene Expression During the HSV-1 Lytic Cycle.

HSV-1, a nuclear replicating animal virus, has a rather wide host-range both in vivo and in vitro. It can infect almost any mammalian cell in culture and rapidly cause a cytopathic effect in its host cell after infection (262). A brief overview of the infectious cycle is as follows: after attachment and entry into the cell, the viral DNA within the new "de-enveloped" capsid is transported into the nucleus where viral DNA transcription and replication occur. After the genomic information has been completely expressed, assembly of the virion commences by packaging unit length of viral genome into empty procapsids which have been pre-assembled in the nucleus. This is followed by envelopment of the mature nucleocapsid by the inner nuclear membrane, transport of the now mature virion through the cytoplasm to the plasma membrane of the cell, and release of the as a mature virion. The entire process for HSV-1 requires approximately 18-24 hrs.

During the initial stages of infection, virion functions have been shown to be involved in the attachment, entry and transport of the nucleocapsid to the nucleus (262). Virus attachment is initiated by the interaction of anti-receptors on the viral envelope of the virus with specific receptors on the cell surface. Although polyclonal antibodies to the

FIGURE 2.

Sequence of events in the productive infection of host cells by herpes simplex virus. Infection is initiated by entry of the virion into the cell by a fusion process, following its attachment to the host membrane via cellular receptors. The endosome-protected virus is then transported to the nucleus, within which transcription of the viral genome occurs in a sequential coordinated fashion. Following replication of the DNA, the newly replicated viral genomes are packaged into pre-assembled capsids within the nucleus. The mature virion acquires infectivity by budding through the plasma membrane of the cell. (Modified from the sequence of events originally demonstrated by B. Roizman).

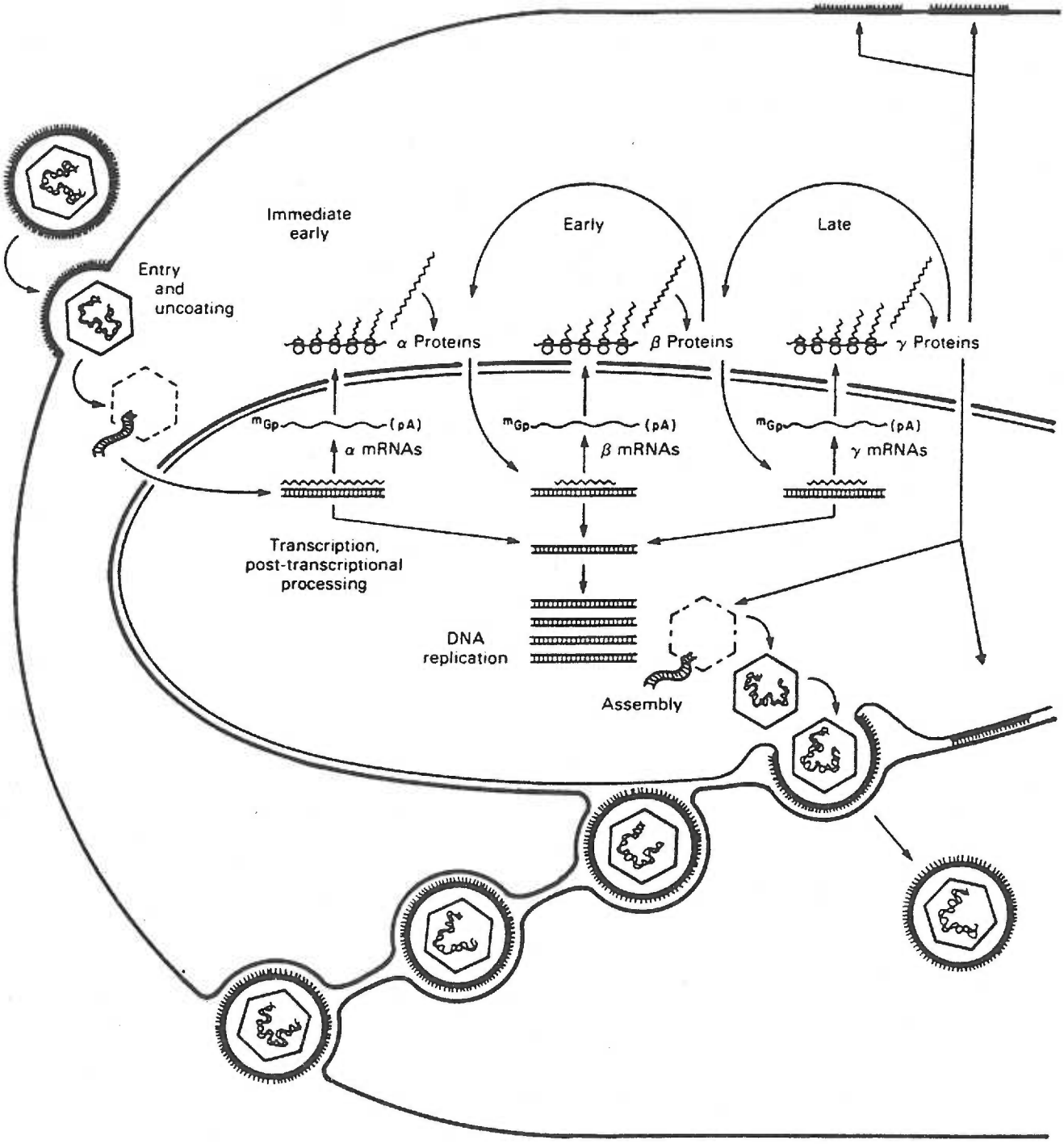

Immediate early
Early
Late
Entry and uncoating
α Proteins
β Proteins
γ Proteins
mGp (pA)
α mRNAs
β mRNAs
γ mRNAs
Transcription, post-transcriptional processing
DNA replication
Assembly

Fig. 2

glycoproteins, and especially monoclonal antibodies to gB, gC and gD, neutralise the virus (290), this does not conclusively prove that either one or more of these glycoproteins is the anti-receptor on the virion, since binding of the antibody to the virus may cause steric hindrance of adjacent proteins. The inability, however, to isolate deletion mutants in gD indicates an essential role for this protein in the infectious process. Attachment of the virus to the cell is presumed to lead to fusion of the virus envelope with the host membrane. This hypothesis is based on finding one of the viral glycoproteins (gE, the Fc receptor) in the host cell membrane following penetration of the virus (15). Virions with a temperature-sensitive mutation in the gB gene, have been shown to adsorb but not penetrate into the cell at the non-permissive temperature (274). This observation led to the identification of gB as the virion component possibly responsible for membrane fusion prior to entry (177, 185). Once the nucleocapsid is released into the cell, it is transported to the nuclear pores. Release of viral DNA into the nucleoplasm is shown to require a capsid function since viruses with temperature-sensitive mutations in a capsid protein [HSV-1(HFEM)tsB7] accumulate at nuclear pores at the non-permissive temperature, releasing the viral DNA into the nucleus only at the permissive temperature (14).

At all stages of infection, the HSV-1 genome is transcribed with cellular RNA polymerase II [Pol II] (3, 47). By using protein synthesis and DNA synthesis inhibitors, first

Honess and Roizman (131, 132) and subsequently other researchers, demonstrated different rates in accumulation of the viral polypeptides in infected cells during the virus growth cycle (119, 264, 291). Further, by using transcription inhibitors and amino acid analogues in studies on the kinetics of viral protein synthesis in infected cells, Honess and Roizman (134) demonstrated different requirements for either prior viral protein synthesis and/or RNA synthesis for the expression of the different classes of HSV-1 genes. Based on these described features, HSV-1 proteins were classified initially into three (131, 132), and later into five (224, 293) major classes: Immediate-early (α), Early (αß or $ß_1$), Delayed-early (ß or $ß_2$), Leaky-late (ßτ or $τ_1$), and True-late (τ or $τ_2$). Since the temporal expression of HSV-1 messenger RNA (mRNA) in general mirrors the cascade of protein synthesis (152, 168, 169, 304, 318, 319), the viral transcripts are also classified into α, αß, ß, ßτ and τ. Although well-accepted and widely used, this system of classification for the viral mRNAs does not correlate well with the presence of some classes of transcripts in the nucleus well before the mature protein appears in the cytoplasm.

In the first stage of gene expression a limited number of genes (the α genes) is transcribed in the absence of de novo protein synthesis (5, 144, 153, 168, 187). Thus, immediately after reversal of a protein synthesis block, by either cycloheximide or anisomycin, only α proteins are synthesised. The five α polypeptides ICP0, ICP4, ICP22, ICP27 and ICP47 [of

which at least 3 - ICP0, ICP4, and ICP27, are known to be regulatory proteins (120, 228, 232, 270)], reach peak levels by 2-4 hrs post infection (119, 132). Although the α mRNAs are efficiently transcribed by the pre-existing host cell transcriptional components, their transcription has been shown to be stimulated in trans by VP16, a structural protein (13, 14, 28, 56).

Hybridisation, intertypic recombination, marker rescue studies, and translation of hybrid arrested viral mRNAs, have been used to map the α genes within or near the L and S repeat regions (4-6, 40, 128, 153, 179, 186, 187, 208, 209, 244, 300, 326, 326, 328). Although α0 and α4 map within the repeat regions of the L and S components respectively, and are therefore present in 2 copies per genome, deletion studies have shown that only one copy of each gene is required for a productive infection (61, 230). The gene for α27 maps near the internal repeats of the L component, whereas those for α22 and α47 are contained partially within the internal repeats of the S segment and partially in $U_S$.

Following expression of the α proteins and prior to viral DNA synthesis, a more complex population of viral mRNAs (the ß mRNAs) is produced (304, 305, 319). The $ß_1$ genes (also known as the αß genes) e.g. ICP6, are expressed very soon after infection, and their mRNAs have often been mistaken for α mRNAs. The $ß_2$ genes (also known as the true ß genes) e.g. gB, gD, Tk, DNA polymerase, alkaline exonuclease, and other polypeptides mainly required for viral DNA replication, appear

slightly later and require functional α proteins for their expression (262). That prior synthesis of α proteins is required for ß transcription was shown by the lack of any ß gene transcription in the continued presence of a protein synthesis inhibitor, i.e. in the absence of α polypeptide synthesis (5, 144, 175). Based on studies with temperature-sensitive mutants of ICP4 where the normal transition from α to ß gene expression was observed only at the permissive temperature (325), the HSV-1 α protein ICP4 has been conclusively demonstrated to be required for stimulation of ß gene expression. The mechanism by which such a stimulation occurs, however, is as yet unresolved. The ß class of mRNAs reach a maximum peak of synthesis at 5-7 hrs post infection (132). By hybridisation, marker rescue and hybrid arrested translation studies, the ß genes have been shown to map throughout the entire viral genome (7, 32, 74, 129, 152, 240, 300).

Viral DNA replication has been the event used to differentiate between ß and later gene expression (97, 319). From studies using DNA synthesis inhibitors such as phosphonoacetic acid (PAA) or cytosine arabinoside (AraC) and DNA-negative temperature-sensitive mutants, it was concluded that late gene expression requires ß polypeptides and viral DNA replication (46, 132, 135, 146, 152, 165, 224, 322). Within the late class of HSV-1 genes two subclasses have been identified : (a) ßτ or leaky-late genes (e.g. ßτ5 capsid gene) which are expressed at low levels in the absence of viral DNA

replication, and (b) τ or true-late genes (e.g. gC or the glycoprotein C gene) which require viral DNA replication for optimal expression (293). Polypeptides of the ßτ class, in contrast to the ß polypeptides, are expressed at very low levels both before viral DNA synthesis and in the presence of DNA replication inhibitors (293). Both groups of late HSV-1 genes, encode a large group of proteins, mainly structural in function (129). Their genes are distributed throughout the viral genome, although some grouping of late genes does seem to occur in the vicinity of the major capsid protein (ICP5) gene (101, 165, 222).

Viral DNA synthesis follows soon after ß protein synthesis commences. Viral DNA synthesis is detected from 3-15 hrs post-infection, reaching a peak at about 6 hrs (132). HSV-1, (like the other herpesviruses), is unique among DNA animal viruses in that it specifies a large number of proteins (mainly ß proteins) that are involved in DNA synthesis (157). Examples are the major DNA binding protein [ICP8] (237), pyrimidine deoxykinase [ßTk or ICP36] (162, 164), alkaline exonuclease [ICP18] (207, 301), DNA polymerase α (235) and ribonucleotide reductase (43), of which ICP6 forms one of the two subunits (75, 137, 231). The ICP8 protein has been shown to bind to single-stranded DNA in a cooperative manner (268), and temperature-sensitive mutants in this gene fail to synthesise viral DNA at the non-permissive temperature (33, 46, 109, 330). The viral Tk has a wider substrate range than its cellular counterpart (163, 164), and although it is

essential for normal virus multiplication in experimental infections, it is dispensible in tissue culture (163, 262). The DNA polymerase has been shown to be the target of replication inhibitory drugs, and temperature-sensitive mutants in this gene have been shown to confer resistance to those same drugs (31, 46, 55, 165, 245). Besides ß proteins, viral replication involves the use of three origins of replication which are operationally defined as sequences which must be present in HSV-1 DNA for it to be amplified in permissive cells (202). Two of these sequences map within the "c" sequences of the S repeats and have been designated as $Ori_S$ (202, 298). The third replicative origin ($Ori_L$) maps in the L component of the viral genome at coordinates 0.410 - 0.412 (288).

The general mechanism by which replication of the viral DNA occurs is known, although some of the precise details are still lacking. At early times in infection circular and branched-linear forms of viral DNA have been found (100), whereas at later times primarily head-to-tail concatemeric forms have been observed (30). This, therefore suggests a rolling circle mechanism for HSV-1 DNA replication, although the typical theta structures associated with this method of DNA replication have not been detected (120, 142). The concatemers found late in infection are specifically cleaved at a direct repeat unit of 20 nucleotides (termed DR1) within the "a" sequences at the terminal repeats (202, 315). The unit lengths of viral DNA is packaged into empty capsids which

have been assembled in the nucleus (106, 107). Packaging of the DNA has been associated with specific binding of virion proteins to the "a" sequences (16). Two proteins required for packaging of the replicated DNA have been identified (25, 26, 57). The packaged DNA then forms the toroidal core structure observed in mature HSV-1 viruses by interaction with other viral DNA binding proteins. The DNA-containing nucleocapsids, along with constituents of the tegument, then bud through the inner nuclear membrane in regions which have been modified by the insertion of precursor forms of viral glycoproteins (123, 263). The nearly mature virions are then "secreted" in tubular shaped vesicles via the Golgi apparatus (similar to cellular soluble secretory proteins) during which maturation (i.e., glycosylation and final processing) of the envelope glycoproteins occurs (44, 136, 279). Egress of the mature HSV-1 from the cell has been suggested to occur by a mechanism similar to phagocytosis, but proceeding from the cell in an outward direction (82, 147). Figure 2 is a schematic representation of the major events in the lytic infectious cycle of HSV-1 in permissive cells as originally described by B. Roizman (257).

**Effect of the HSV-1 Lytic Cycle on Host Cells.**

During permissive infection, HSV-1 rapidly shuts off host macromolecular synthesis early in infection, leading eventually to structural and biochemical alterations that are ultimately fatal to the cell (262). Cellular DNA synthesis is

rapidly inhibited by the virus (264). Although stable RNA synthesis is inhibited by approximately 60% (213), the effect of HSV-1 on transcription of the host ribosomal genes appears to be varied. While transcription of the 4S and 28S ribosomal genes is inhibited to varying degrees, little inhibition in transcription of the 45S precursor is seen (318). Host protein synthesis, however, is uniformly inhibited (263). This is probably the result of a virus-mediated decrease in cellular mRNA stability (213) or an alteration in specificity of the polyribosomes for the different species of mRNAs present in an infected cell. An early event that has been described is the phosphorylation of two polypeptides in the 40S ribosomal subunit by a viral structural protein (94, 159). This causes a change in the proteins associated with polyribosomes after HSV-1 infection, as observed by Bartkoski (10), and possibly results in an alteration of ribosomal affinity for mRNAs. The structurally altered ribosomes may then prefer viral mRNAs over cellular mRNAs for translation. Support for this model was provided by the results of Stringer et al. (300) who have shown that as much as 90% of the polyribosome-associated mRNAs in infected cells are of viral origin. This inhibition in the translation of cellular mRNAs is accompanied by a cessation of maturation processes such as the glycosylation of host proteins (123, 294).

Data accumulated suggests a two-phased inhibition of host macromolecular synthetic processes (92). In the first stage a virion component has been demonstrated to be involved and de

novo protein synthesis is not required (94, 213-215). The isolation of vhs (virion host shut-off) temperature-sensitive mutants, which fail to shut-off host polypeptide synthesis in infected cells at the non-permissive temperature, provided strong evidence for the involvement of a virion component (248). The vhs mutants, however, also showed enhanced levels of α polypeptide synthesis suggesting an indiscriminate inhibition. Recently, the vhs mutation has been shown to increase the stability of the α mRNA (A. Oroskar and G. S. Read, Abstract no. 236, 11th International Herpesvirus workshop, Leeds, UK). Therefore, the protein associated with this gene may inhibit translation by decreasing the stability of cytoplasmic mRNA. The fact that this early shut-off occurs in physically or chemically enucleated cells (93), confirms the cytoplasmic site of action for the vhs protein. This stage of inhibition had been demonstrated by Read and Frenkel (248) to be due also to disaggregation of polyribosomes as suggested earlier by Nishioka and Silverstein (214). Thus, perhaps both mRNA stability and aggregation of polyribosomes are affected by the vhs protein and this brings about an inhibition of translation. The vhs mutation has been mapped by marker rescue studies to the Eco RI-A fragment (map units 0.49-0.63) of the HSV-1 genome (A. Oroskar and G. S. Read, Abstract no. 236, 11th. International Herpesvirus Workshop, Leeds, UK).

A second more complete and discriminating host shut-off is observed later, after de novo viral protein synthesis and

transcription (93, 132, 133, 213, 215, 283). Although this shut-off coincides with ß protein synthesis, experimental results do not eliminate the possible involvement of τ gene products (262).

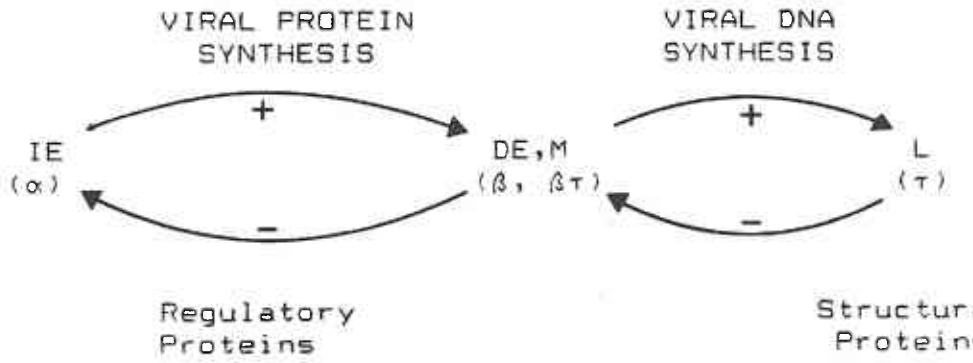
**Gene Regulation Studies in HSV-1.**

The preceding description of the HSV-1 infectious cycle has indicated a complex coordinated regulation of HSV-1 genes, which are thus expressed in a specific sequential manner. The viral genes are transcribed by the cellular RNA polymerase II [Pol II] (3, 47), and therefore, share many features with eukaryotic genes (9, 11, 284, 285, 300). Central to the understanding of HSV-1 gene regulation, therefore, is comprehension of the mechanism by which the infected cell differentiates between host, immediate-early, early and late genes. In 1974 Honess and Roizman (132) by the use of transcription and translation inhibitors demonstrated regulatory process which turn on and off synthesis of the sequentially synthesised polypeptides. By the use of amino acid analogues, they later demonstrated that the 'on' and 'off' processes were due to viral proteins of one temporal group regulating those of another group at the transcriptional or post-transcriptional level. By infecting and maintaining HEp-2 cells in media containing amino acid analogues [e.g., canavanine instead of arginine, and azetidine-2-carboxylic acid instead of proline and hydroxyproline], they observed α protein synthesis but no transition to later gene expression.

FIGURE 3.

Schematic representation of the cascade regulation of the herpes simplex virus type 1 genome (as originally proposed by Honess and Roizman, 1974). The Immediate-early (or the α) genes are shown to positively regulate transcription from the Delayed-early (or ß) genes, the products of which are shown to activate Late gene transcription. In addition, the proteins of each class of gene is shown to repress transcription from the preceding class of genes. The positive or negative effects of the viral proteins is represented by a + or −, respectively.

## CASCADE REGULATION
## HERPES SIMPLEX VIRUS TYPE 1




α = ICP0, ICP4
β = gB, TK
βτ = ICP5
τ = gD, VP5

ICP = Infected Cell Polypeptide

**Fig.3**

However, these analogues added to cells already synthesising normal α proteins allowed the synthesis of later gene products. This, therefore, showed a requirement for normal functional α proteins in later gene expression. On the other hand, addition of the analogues after the onset of ß and τ mRNA synthesis led to sustained synthesis of all groups of HSV-1 polypeptides. This indicated the requirement for later polypeptides in the repression of earlier gene expression. Figure 3 diagrammatically represents a simplified version of the cascade regulation of gene expression in HSV-1 as suggested by Honess and Roizman (132, 133).

I. Positive Regulation of HSV-1 Gene Expression.

1. Immediate-early gene regulation.

(A) Transcription of immediate-early genes in absence of viral proteins.

Transcription of immediate-early (or α) genes has been demonstrated to be independent of de novo protein synthesis. Thus the α genes were shown to be transcribed in the host cell in the presence of protein synthesis inhibitors such as cycloheximide, anisomycin or puramycin (5, 132, 144, 153, 168, 180). Their mRNAs are translated immediately after reversal of the translation block, even in the presence of the transcription inhibitor actinomycin D (132, 133). This demonstrated that the transcription of α genes is indifferent to subsequent viral transcription and translation. Accordingly, in vivo transfection studies have shown efficient expression from α promoters upon their introduction into

uninfected host cells (112). Recombinant constructs carrying the coding sequences of either a ß gene (Tk) a or a eukaryotic gene (chicken ovalbumin) fused to the transcriptional control sequences of the α4 gene in the correct orientation [α4/Tk or α4/ovalb chimaeric genes] were made (127, 233, 234). These chimaeric genes were then recombined into viral DNA and assayed for expression (233). Alternatively, the chimaeric constructs were transfected into cells and assayed either in transient assays, or as part of the cellular genome in stably transformed cells (127, 234). It was found that the chimaeric genes [α4/Tk or α4/ovalb] in all situations were transcribed in the presence of a protein synthesis block and in the absence of any other viral protein synthesised de novo. The chimaeric genes carrying α4 promoters were thus transcribed as expected of bona fide α genes.

(B) Enhancement of immediate-early gene transcription by a virion factor

Transcription of the Tk gene bearing α4 regulatory sequences [α4/Tk] was shown to be enhanced by superinfection with ultraviolet-inactivated $Tk^-$ virus under conditions that inhibited de novo protein synthesis (233). Further, a virus bearing a temperature-sensitive mutation in a γ function required for uncoating [HSV-1 (HFEM)tsB7] was unable to stimulate expression from α promoters in its own genomic DNA at the non-permissive temperature (13, 14). [This mutant virus was unable to release its genome from the capsid at the non-permissive temperature.] However, this virus was able to

fully activate a resident α4/Tk chimaeric gene already integrated into the recipient cell genome (13, 14, 127). This suggested that the α4 promoter in the α4/Tk gene was stimulated in trans by a τ function, possibly a virion tegument protein that was released into the nucleus before the disruption of the viral capsid. Since stimulation of α gene expression is a nuclear event, it requires co-transport of the virion component responsible for α gene induction along with viral DNA into the host nucleus. Subsequent studies involving cotransfection of cloned restriction fragments (representing the entire HSV-1 genome) along with the α4/Tk chimaeric genes in transient expression assays revealed a trans-activating function located in the Eco RI-I fragment of the HSV-1 genome (28). Subcloning, insertional mutagenesis, hybrid-arrest translation, and immunoprecipitation studies led to the identification of a 65 kilodalton virion tegument phosphoprotein (Vmw65 or VP16) as the α trans-activator (56, 182, 183).

(C) Transcriptional control regions of immediate-early genes

Studies on the transcriptional control sequences of the α4, α0, α22/α47 and α27 genes have revealed the existence of two "physically separable and movable regions", the promoter region and the regulatory region (170, 181). The promoter region (located within a 110-bp region upstream from the transcription start site) allows basal level expression of genes from the α promoters, but is not induced in trans by VP16. The regulatory region, that is responsive to

superinfection and trans-induction by VP16, is located upstream from -110-bp relative to the transcription start site. Deletion analyses of this region led to the identification of AT-rich DNA elements that contain one or more copies of the consensus sequence TAATGARATTC (R=Purine) (48). This TAATGARATTC sequence when placed upstream to the ßTk promoter (at -80-bp relative to the cap site of the Tk transcript) or other heterologous promoters conveys to these promoters the α characteristic of trans-inducibility by VP16 (127, 181-183). The TAATGARATTC sequences are embedded in GC-rich sequences, which are also found within the promoter region. These GC-rich sequences have been shown to contain binding sites for Sp1, a general factor required for transcription from most eukaryotic genes (108, 149, 150). The interaction of the Sp1 factor with its predicted binding sites in the α control region may provide much of the basal strength of the α promoters. The isolated VP16 protein is not known to have any DNA binding properties. Therefore, the stimulation of α genes by VP16 at the TAATGARATTC sequences, may be by indirect interaction of the protein at these sequences via Sp1 or other cellular protein(s) (120).

2. Delayed-early gene regulation.

The pioneering studies of Honess and Roizman (132) demonstrated a lack in accumulation of functional mRNA for ß polypeptides in cells which were treated with and maintained in cycloheximide-containing medium throughout infection. This indicated a role for one or more of the HSV-1 α proteins in

either the production or the maintenance of ß mRNA. Subsequently, Honess and Roizman (133) using amino acid analogues, demonstrated the necessity for functional α polypeptides in the production of ß mRNAs. Later experiments by Leiden et al. and Leung et al. (175) showed that activation of the ßTk gene (integrated into the cellular genome) by infecting HSV-1 occurred only under conditions where viral protein synthesis was permitted. This confirmed the original observation that ß gene expression is dependent on prior viral protein synthesis. Experiments with other viral ß genes (273) and hybrid genes consisting of Tk upstream sequences fused to the coding sequences of either the ß-interferon, the ovalbumin, or the bacterial chloramphenicol acetyl transferase gene [CAT] (127, 234, 252), also demonstrated that prior α gene expression is required for transcription from these ß promoters.

A. Proteins required for ß gene transcription

(i) The Immediate-early protein ICP4

The first α polypeptide to be recognised as important in HSV-1 gene regulation was ICP4. By using temperature-sensitive mutants of the α4 gene several groups demonstrated that the protein produced by this mutant gene at the non-permissive temperature failed to allow synthesis of early and late RNAs and proteins (27, 72, 239, 324). At the non-permissive temperature, the temperature-sensitive mutants of ICP4 were found to permit synthesis of all the α gene products but proved to be incapable of transporting the ICP4 protein

synthesised to the nucleus. (27). A need for the continued presence for the functional form of the ICP4 protein for the expression of the later genes was therefore suggested by Watson and Clements (325). Using a temperature-sensitive mutant of ICP4, they showed that normal ß and τ gene expression occurred at the permissive temperature during late times in infection. By shifting from the permissive to the non-permissive temperature, even at late times in infection, expression could be reverted to only α mRNA synthesis.

Cotransfections experiments have demonstrated that the protein produced by a plasmid-borne α4 gene enhanced transcription of chimaeric genes under the control of ß promoters plasmid (218-220). Similar experiments further demonstrated that plasmids containing the temperature-sensitive mutant form of the α4 gene did not transactivate cotransfected ß promoters at the non-permissive temperature (64). These experiments provided conclusive proof for the involvement of ICP4 in the trans-activation of ß genes. Using a cell line that constitutively expresses an integrated α4 gene, Persson et al (228) have provided more direct evidence for trans-activation of HSV-1 genes by ICP4. By superinfecting this ICP4 producing cell line with wild type HSV-1(KOS) they demonstrated transcription from some ß promoters (namely ICP6, ICP8, gB, gD and gE) under conditions that precluded de novo protein synthesis (i.e. in the presence of protein synthesis inhibitors).

Reports that the immediate-early E1A function of adenovirus can be replaced by the ICP4 protein (310), and that ICP4 can induce synthesis of cellular stress proteins (217) have conclusively demonstrated that ICP4 is a regulatory protein. Activation of the eukaryotic ß-globin gene by cotransfection with the α4 gene indicated that a wider range of genes can be induced by ICP4 (86). By mapping temperature-sensitive and deletion mutants in the α4 gene, it has been shown recently by DeLuca et al. (65) that only 700-bp within the 3' region of the α4 gene (corresponding to the N-terminal 10-20% of the ICP4 protein) is required for trans-activation of ß genes.

The mechanism of ICP4 action on the various genes is as yet unknown. Analysis of the sequences required for HSV-1 mediated stimulation of the ßTk gene transcription has localised the ICP4 target signals within the Sp1 and CTF ('CCAAT box' transcription factor) binding sites (80). However, no consensus sequences for ICP4 induction have been identified among the ß gene promoters studied (80, 84, 85, 150). ICP4 protein has been shown to bind to HSV-1 DNA, albeit in the presence of cellular proteins (96). Cumulatively, the data suggests a role for ICP4 in the activation of transcription via a modification and/or activation of cellular regulatory factors in a fashion similar to that suggested for the adenovirus E1A activation of genes (210).

(ii) Immediate-early protein ICP0

A second α gene product, ICP0 or Vmw110, has also been implicated in the positive regulation of the later classes of HSV-1 genes. However, debate as to the actual necessity and extent of ICP0-mediated regulation exists. The first evidence for the role of ICP0 in the trans-activation of HSV-1 ß genes was presented by O'Hare and Hayward (219, 220). By cotransfection of plasmids containing the α0 gene with plasmids carrying the bacterial chloramphenicol acetyl transferase (CAT) gene placed under the control of various ß promoters, they showed that ICP0 could enhance CAT gene expression in the absence of other HSV-1 proteins. This trans-activation of transcription from ß promoters by both ICP4 and ICP0 has also been demonstrated by Quinlan and Knipe (246) and Gelman and Silverstein (105), using the ICP8 and Tk promoters respectively. However, recent studies by Sacks et al. (Abstract no. 3. 11th International Herpesvirus Workshop, Leeds, UK) have shown that deletions in the α0 gene (resulting in a non-functional truncated ICP0 polypeptide) do not appreciably block viral polypeptide synthesis, viral DNA replication, or DNA encapsidation during productive infection. A more detailed analysis of different in-frame deletions and insertions within the α0 gene further demonstrated that ICP0 alone is not sufficient for trans-activation of later HSV-1 gene classes, but that sequences in its second exon are required for trans-activation in cooperation with ICP4 (R. D. Everett, Abstract no. 14, 11th International Herpesvirus Workshop, Leeds, UK). Thus, the debate whether ICP0

independently trans-activates ß genes or functions to 'fine tune' the action of ICP4, is as yet unresolved.

B. Transcription of delayed-early genes in absence of viral proteins.

(i) In vivo studies

Despite the afore-mentioned requirement for functional α protein in ß gene expression during infection of host cells, ß genes have been demonstrated to be efficiently expressed when present in uninfected cells (317). By inserting synthetically synthesised nucleotide linker molecules at desired locations in the transcriptional control regions of the ßTk gene, McKnight et al. (194-197) constructed clusters of point mutations (linker scanning or LS mutations) without altering distances between the different transcriptional regulatory sequences. These LS mutant DNAs were micro-injected into uninfected Xenopus laevis oocytes which were then assayed for HSV-1 Tk transcription. From their data, three distinct regions within a 105-bp region were found to be required in cis for optimal transcription from the ßTk promoter. The Proximal signal consists of the Goldberg-Hogness box (or TATA box) and areas immediately surrounding the transcription start site (195, 197). Two Distal signals (DSI and DSII) extending from -46bp to -105bp (relative to the transcription start site) were also identified. Further work in a similar system characterised a hexanucleotide GC-rich sequence [GGGCGG] within each DS box (198). It was also shown that the GC sequences per se (and not complementarity between DSI and

DSII) were important for efficient transcription from the Tk promoter. Recently, McKnight et al. (198) have further shown that, despite the apparent similarities in their sequences, DSI and DSII are not equivalent in function. In fact, mutations introduced within DSI alone have little effect on the transcription pattern from the Tk promoter. Similar, but not identical, GC-rich sequences that are important for the regulated expression of the gD gene have been identified (84,85). However, it should be mentioned that the level of ßTk transcription in uninfected cells can be enhanced by ICP4 (173).

(ii) In vitro studies.

Results from in vitro transcription experiments have demonstrated transcription from ß promoters by systems lacking viral proteins. Using uninfected whole cell extracts (WCE) prepared by the method of Manley et al. (184) from HeLa cells, Frink et al. (102) and Draper et al. (74) have shown accurate recognition and initiation from several ß promoters. Supporting results were obtained by Read and Summers (249) who demonstrated correct transcription from the ßTk gene using a similar uninfected WCE system. Recently, Pizer et al. (229), using uninfected HeLa cell nuclear extracts prepared by the method of Dignam (71), reported accurate transcription from the ßgD promoter. These results indicate that accurate transcription from ß promoters by uninfected HeLa cell polymerase II can be acheived.

The picture which emerges from the transfection and in vitro transcription studies is that functional viral proteins are required for ß gene expression, only when the ß exists as a part of the viral genome. In cells biochemically transformed with the Tk gene, however, expression from the ßTk promoter can be up-regulated by a wild-type superinfecting virus (173).

3. Late gene regulation.

As mentioned in the overview of the HSV-1 infectious cycle, late gene expression occurs following the onset of viral replication (132, 133). However, the actual requirement for viral DNA synthesis in late gene expression was initially in question. Using hydroxyurea and mitomycin C to inhibit DNA replication in HeLa cells, one group of workers did not observe expression of the late genes in the absence of viral DNA synthesis (236, 300, 304, 305, 319, 322). Using the same replication inhibitors in HEp-2 cells, Honess and Roizman (132), however, found reduced but significant levels of late protein synthesis in the absence of viral DNA replication. Since the effect of the various drugs on DNA replication was later demonstrated to vary with cell type and the stage in life-cycle (317), several groups undertook the careful re-examination of the role of viral DNA replication in HSV-1 late gene expression. Using quantitative hybridisation and Southern blots to identify the size-fractionated viral late mRNAs, it was concluded that there are two classes of late genes : (a) ßγ or $\gamma_1$ genes which are transcribed at low levels

prior to viral DNA, and (b) $\tau$ or $\tau_m$ genes which stringently require viral DNA replication for detectable levels of transcription (46, 128, 129, 152, 224, 293). Some groups, however, have demonstrated extremely low level of $\tau$ gene expression from unreplicated viral genomes (224).

Proteins required for Late Gene Transcription

(i) The Immediate-early protein ICP4

As described earlier in this review, Watson and Clements (325) had demonstrated that a functional ICP4 protein is required for late gene expression. However, data from Persson et al. (228) demonstrated that ICP4 alone did not suffice to trans-activate late gene transcription. By superinfecting a cell line transformed with and constitutively expressing ICP4 in the presence of cycloheximide, Persson et al. (228) demonstrated a lack of transcription from the gC promoter despite the presence of functional ICP4 protein in the cell.

(ii) The Immediate-early protein ICP27

In recent studies, a second $\alpha$ protein has been postulated in the control of $\beta\tau$ and $\tau$ gene expression. Recent isolation of temperature-sensitive mutations in the $\alpha$27 gene has shown that cells infected at the non-permissive temperature with these mutants show a reduction in the level of $\beta\tau$ and $\tau$ polypeptides, even in the presence of significant $\beta$ protein synthesis and DNA replication (270). Although this shows a positive regulation of $\beta\tau$ and $\tau$ gene expression by ICP27, these results somewhat contradict the very basis for late gene classification -- the requirement for $\beta$ protein synthesis and

DNA replication. Experiments with transient expression systems have demonstrated that a functional ICP27 does not stimulate transcription from ß promoters (64, 85, 219). These results indirectly support the idea of late gene activation by ICP27, circumventing the requirement for prior ß gene expression. However, these results do not rule out the possible involvement of ß protein(s) (not required for replication) in late gene expression. These ß proteins may require functional ICP27 for their synthesis, and thus not be produced at the non-permissive temperature in the temperature-sensitive ICP27 infected cells. Alternatively, these ß proteins may be turned on by one or more α proteins (other than ICP27) but require a functional ICP27 to activate late gene expression. Ruyechan et al. (269) have previously reported a locus, Cr, near the right hand terminal of $U_L$ as important for the expression of the γgC gene. Since the α27 gene has subsequently been mapped in this region (183), this provides further support for the involvement of α27 in the expression of late genes.

(iii) The Delayed-early protein ICP8

Using a temperature-sensitive mutant of the major DNA binding protein (ß gene ICP8), Conley et al. (46) and Godowski and Knipe (109) showed a reduction in the expression of late genes at the non-permissive temperature. Since ICP8 is a part of the viral replication machinery (33), whether ICP8 acts directly on late gene transcription or via the requirement for replication is open to question.

B. Transcriptional Control Regions of Late Genes.

Studies with βτ and τ promoters have revealed differences in the transcriptional control regions. These differences may provide part of the basis for their temporal distinction. By fusing varying lengths of the βτ5 transcription control region to the bacterial CAT gene, Costa et al. (51) have shown that sequences from -125-bp to the transcription start site are required for trans-activation by superinfecting virus in transient assay systems. The τgC gene control regions delineated (130), however, deviate from the general requirement for far upstream sequences in the trans-activation by viral proteins. Homa et al (130) created a series of deletions in the 5' transcribed non-coding and untranscribed upstream regions of the τgC gene, and assayed their effects on transcription from the gC promoter in the course of infection. By this method, they observed a requirement for sequences -35 to +124, relative to the transcription start site, for regulated expression of the gC gene. Fusion of this 158-bp region to the transcribed portions of the HSV-1 βTk gene, or the bacterial CAT gene, resulted in their trans-activation by α0 and α4 gene products. The presence of this 158-bp region upstream to the Tk control regions further caused expression of the Tk gene to be inhibited by Phosphonoacetic acid (PAA) - a feature associated with τ genes. Sequence analysis within this 158-bp region of the τgC promoter showed the presence of a TATA box and the leader sequence (present at the N-terminal end of all secretory proteins). Thus, it appears that unlike

α and ß genes, the TATA box and all sequences downstream to it are all that is required for the regulated expression of the γgC gene.

C. Regulation of late genes can be similar to that of delayed-early genes.

(i) In vivo studies

This perfect scenario for late gene expression is marred by some conflicting results obtained with other in vivo studies. Contrary to the hypothesis for requirement of viral products in late gene expression, Sandri-Goldin et al (273) have shown expression of γ genes, in the Eco RI-F region of the genome, when these genes are present as integrated copies in the uninfected cell genome. Similarly, it has been shown that the entire γgC gene or chimaeric constructs bearing the ß Tk gene under the control of a γ promoter are regulated as ß genes when integrated into the cellular DNA (193, 282). Lack of viral DNA replication (due to the presence of Phosphonoacetic acid or absence of a viral replication origin on the plasmids) did not inhibit viral induction of γ-Tk chimaeric or γgC genes. Infection of these cells, carrying integrated γ-TK or γgC genes, with HSV-1 carrying temperature-sensitive mutations in the α4 gene [HSV-1(ts502 305)] did not induce transcription from the γ promoters at the non-permissive temperature. However, viruses with temperature-sensitive mutations in the ß8 gene [HSV-1(tsHA1 305) did induce the γ promoters, even at the non-permissive temperature. The γ-Tk construct showed activation in trans

when cotransfected separately with plasmids containing the α0 gene, or the complete or truncated forms of the α4 gene (lacking 40 % of the coding sequence at the 3' end) (193). These results indicate that τ promoters outside the environment of the viral genome are trans-activated by ICP4 like ß promoters, and are unaffected by absence of viral replication. However, a similar τ-Tk construct, when integrated into the viral genome, was regulated like a true τ gene. Thus, its expression was found to be inhibited on the addition of the replication inhibitory drug PAA (193). Transfection of chimaeric Tk genes bearing the ßτ5 promoter also could not be distinguished from bona fide ß promoters in uninfected cells (68). Thus, it appears, that the genome environment in which the late gene is located (viral or cellular) plays an important role in the regulation of a τ gene.

(ii) In vitro studies

As noted with late gene expression in vivo, considerable variation in the recognition of late promoters by in vitro transcription systems has been observed. Read and Summers (249) using uninfected HeLa WCEs showed accurate and efficient initiation from ßτ5 and τ promoters in the Tk gene region. This observation was further extended by Chisholm and Summers (34), who used a similar system to show efficient transcription from the ßτ5 promoter. However, Frink et al. (102), also using an uninfected HeLa WCE system, were unable to show transcription in vitro from other ßτ and τ genes.

Recently, using an uninfected nuclear extract in transcriptional studies, Pizer et al. (229) have reported a low level of transcription from the ßτ5, τgC and the τUS7 promoters. This low level of transcription, however, was greatly enhanced upon the addition of a partially purified infected cell fraction enriched for the ICP4 protein.

The above in vivo and in vitro studies seem to indicate, therefore, a differential response of late promoters to viral polypeptides depending on whether the genes are in the normal viral genomic environment or not. Further, the demonstration by Wright et al (334) of τ promoter expression in uninfected murine erythroleukaemic cells undergoing chemically induced differentiation, indicates that the response of late promoters to viral polypeptides may be dependent on the cell stage as well.

II. Negative Regulation of HSV-1 Genes.

During the early years of HSV-1 gene expression research, Honess and Roizman (132) demonstrated that by inhibiting either ß protein synthesis (by using a cycloheximide block) or ß mRNA synthesis (by using an actinomycin D block following a cycloheximide block), the α polypeptides were made in excess and over a prolonged period of time. Moreover, it was shown that when ß polypeptides were synthesised a rapid repression of α polypeptide synthesis occurred. Similar experimental regimens, but at later times in infection, had shown that the decline in the rate of ß polypeptide synthesis was due to the production of the next class of proteins, the late proteins

since lack of late gene expression retarded the shut-off of early gene expression (133). When cells were infected in the presence of the amino acid analogue canavanine, Harris-Hamilton and Bachenheimer (119) found that IE proteins accumulated at the same levels as in untreated control cells, even though protein synthesis was restricted to mainly the IE class. This indicated that α genes are under negative control in both untreated and canavanine treated cells, probably by arginine-poor proteins which are, therefore, synthesised and can function normally despite the presence of canavanine in the medium.

A. Proteins Involved in Negative Regulation of Transcription

(i) The Delayed-early protein ICP8

Despite some promising findings on negative control in HSV-1 infected cells, at present only one ß protein has been shown to be involved in the shut-off of α synthesis. When cells were infected with a temperature-sensitive mutant in the ICP8 gene at the non-permissive temperature, Godowski and Knipe (109) observed that α (and some ß, ßτ and τ) polypeptides were over-produced. This suggested a role for ICP8 in the negative regulation of viral genes. This hypothesis has been strengthened by results from transfection assays in which the ICP8 gene, when present in a 2-3 fold excess, was shown to inhibit α4 gene transcription (246). Both of these observations have been further supported by nuclear run-off assays of Godowski and Knipe(40). By assaying transcription initiated at different times after infection

with HSV-1(KOS)1.1ts18 (carrying a temperature-sensitive ICP8 gene), they observed a failure to shut off α gene transcription at the non-permissive temperature.

(ii) The Immediate-early protein ICP4

By analysis of temperature-sensitive mutants in the α gene, it has been shown by a number of workers that the non-functional ICP4 protein produced at the non-permissive temperature results in the overproduction of α transcription and translation products (66, 72, 238, 239, 324, 325). Transfection experiments with deletions of the α4 gene (which synthesised non-functional forms of the ICP4 protein) also showed lack of repression of α gene transcription (193). The finding that the action of the temperature-sensitive form of ICP4 was dominant over that of the wild type ICP4 protein, indicated a trans rather than a cis mechanism of ICP4 function (238, 239). A more definitive proof for trans repression of α genes by the ICP4 product was demonstrated in transient expression systems, where plasmid-borne α4 gene efficiently repressed expression from α promoters on cotransfected plasmids (219,220).

(iii) The Immediate-early protein ICP27

The ICP27 protein has also been found to play an essential role in the regulation of HSV-1 gene expression, as mentioned earlier. Experiments with temperature-sensitive mutants in the α27 gene have shown that ICP4 and ICP27 proteins are over-produced at the non-permissive temperature (270). Therefore, in addition to its positive role in

activation of late gene expression, ICP27 has now been demonstrated to be involved in repression of the α0 and α4 gene expression. Since the ICP4 protein (present in the α27 temperature-sensitive mutant presumably in a functional form) did not mediate repression, it appears that while a functional ICP4 protein is required for the negative regulation of HSV-1 genes, ICP4 alone is not sufficient to account for the negative effects associated with it.

B. Negative Regulation at the Level of Transcription Control

Although much research has been aimed at the shut-off of α genes at the transcriptional level, possible modulation of α gene activity at the translational level cannot be ignored (152, 153, 284, 285). At later times in infection Harris-Hamilton and Bachenheimer (119) found higher amounts of α0 and α4 mRNA present, despite the insignificant levels of these proteins seen in infected cells at that time. The existence of mRNA which are not translated indicate that the shut-off of α gene translation is due to the inability of the α mRNAs to compete efficiently with the β and τ mRNAs for the host translation system. The reasons for this could be differences in methylation in the different classes of mRNAs (12), or differences in functional stability between the α mRNAs and the later classes of mRNAs (248).

From the preceding account of gene regulation in HSV-1, it appears that positive control of expression is mediated mainly at the transcriptional level, whereas negative

regulation of the HSV-1 genes could be due to both transcriptional and translational modulation.

**Gene Regulation of the other Herpesviruses at the Transcriptional level.**

The temporal mode of gene expression described for HSV-1, is a feature shared by all members of the Herpesviridae (262). Since most of of the research on herpesvirus gene expression has been concentrated on HSV-1, similar information on the other herpesviruses is still quite incomplete. Where available, however, studies indicate that as in HSV-1 the viral proteins first synthesised are important for the expression of the succeeding class(es) of genes. In this review, such studies on the Pseudorabies virus, Epstein-Barr virus and the Human Cytomegalovirus are described.

Pseudorabies Virus.

Pseudorabies virus (PRV), a virus of swine, is formally known as Suid herpesvirus 1 (191). Like HSV it is a member of the Alphaherpesvirinae (262), and therefore shares many of its characteristics. The PRV genome is also transcribed by cellular RNA Polymerase II and shows different transcription patterns at different stages of infection (21, 87, 91). Three temporal classes of PRV genes have been described - immediate-early (IE), early (E) and late (L) (87, 117) - based on their kinetics of expression and abundance at various stages of infection.

The IE gene is transcribed from within the internal repeats of the genome to give a single 6-kb mRNA (90, 91, 138, 247). The RNA accumulates in the presence of cycloheximide, and on removal of the drug the mRNA is immediately translated into a 180-kd multifunctional protein called IE (21, 22, 90, 247). Temperature-sensitive mutants defective in this IE function show, at the non-permissive temperature, an over-production of IE protein and a lack of transition to E and L gene expression (138). This implies that the 180-kd IE protein is continuously required for the orderly transcription pattern of PRV, i.e., self-repression of IE transcription, and stimulation of later gene expression. Co-transfection studies have shown that plasmids bearing the gene for the 180-kd protein are capable of stimulating expression of recombinant genes under the control of promoters from HSV-2 βTk (221), human and rabbit β-globin, α2(1) collagen, SV40 Late (3) genes. The mechanism by which the IE protein regulates transcription is, however, unknown. Recently in vitro transcriptions with extracts prepared at the permissive and non-permissive temperatures from cells infected with PRV carrying a temperature-sensitive mutation in the IE gene (tsG), showed that the stimulation of viral late gene transcription occurred only in the extracts prepared at permissive temperature. This demonstrates that activation of late gene transcription in vitro was due to the 180-kd protein (1).

The E genes are transcribed from various parts of the PRV genome (91) at early times in infection, and accumulate up to 3-4 hrs post-infection (21). The E genes, activated presumably by the 180-kd protein, are transcribed before initiation of viral DNA synthesis, as shown by E mRNA synthesis in DNA$^-$ temperature-sensitive mutants at the non-permissive temperature (21). A few of the E proteins have been shown to be required for the subsequent DNA replication e.g., the 136-kd major DNA binding protein, two smaller acid-soluble DNA binding proteins of 10-kd and 15-kd respectively, thymidine kinase, DNA Polymerase and a DNase (23, 114, 116, 118, 216). Viral DNA replication, though not required for E initiating transcription, is required for repression of E gene transcription. This is shown by the failure of DNA$^-$ temperature-sensitive mutants to shut off E gene transcription at the non-permissive temperature (21).

The L genes are transcribed from sequences on the genome not transcribed at immediately-early and early times during infection (21, 87). Late transcription starts by 2.5 hrs post-infection (after viral DNA synthesis has started) and the mRNAs accumulate abundantly thereafter upto the end of the infectious cycle (21). The transition from E to L gene expression in PRV is believed to require DNA synthesis (155, 156). However, different DNA$^-$ temperature-sensitive mutants showing different degrees of E to L transition have been isolated (21). This, therefore, implies that other proteins,

whose presence maybe dependent on a replicating virus DNA, are involved in L gene activation.

Human Cytomegalovirus

The Human Cytomegalovirus (HCMV) is a member of Betaherpesvirinae and shows characteristic cytomegalia (enlarged cells) upon infection (262). It is the largest member among the human herpesviruses, and with a 240-kbp genome is 50 % larger than the HSV-1 genome (120). The genome organisation of HCMV is similar to that of HSV, in consisting of $U_S$ and $U_L$ components bounded by homologous repetitive sequences (262).

Despite its isolation early in the herpesvirus research era, very little information on HCMV gene expression during lytic infection is currently available (120). Following adsorption of the virus to the cell (presumably via cell-surface receptors and viral glycoproteins), the virus uncoats within the cytoplasm. The nucleocapsid is then conveyed rapidly to the nucleus, where a temporally controlled expression of its genome occurs (67, 296, 323). HCMV is thought to be more dependent on cellular functions than HSV for its gene regulatory processes (262).

The first phase of genome expression is called the immediate-early (IE) period. Transcription of the IE genes occurs from a single unidirectional promoter complex (2, 297), even in the absence of protein synthesis, prior to DNA replication (120, 145). Upon reversal of a translation block initiated at the start of infection, IE proteins of 75-kd, 72-

kd and 68-kd are produced (145, 253). The products of mRNAs IE-1 (the 72-kd protein) and IE-2 (the 68-kd protein) are the most abundant species detected at immediate-early times in the normal infection cycle and after reversal of a translation block (253). These have also been detected by immunofluorescence in the nucleus, after transfection of DNA containing the IE genes (120). Cotransfection of plasmids carrying both the EI-1 and EI-2 genes with recombinant plasmids containing the bacterial CAT gene under the control of HCMV or heterologous viral promoters, showed trans-activation from these promoters by the HCMV IE proteins in transient assay systems (120). This suggests the importance of IE proteins in the gene expression of later genes in yet another human herpesvirus.

Epstein-Barr Virus.

The Epstein-Barr virus (EBV) is classified as a member of the Gammaherpesvirinae. Having a very restricted host range, it is able to replicate only in B- and T-lymphocytes (262). Even in a permissive host, infectious progeny are sometimes not produced due to an arrest at a pre-lytic stage in the viral replication cycle (120).

The EBV lytic cycle shares many features with the HSV-1 lytic cycle (83). EBV enters by fusion of its envelope with the cell membrane (73, 167). A set of genes (the early genes) are transcribed prior to DNA synthesis. A second set of genes (the late genes), however, require prior viral DNA synthesis for their expression. The late viral mRNAs are translated

into structural proteins in the cytoplasm, and are transported back to the nucleus where the nucleocapsid is assembled. After assembly, the capsid acquires an envelope from the outer membrane of the nucleus.

As obvious from the preceding paragraph, the EBV genes are operationally divided into early (E) and late (L) based on whether or not they require DNA synthesis for their expression (120). E genes are defined as those which are transcribed in the presence of a protein synthesis inhibitor, translated immediately after removal of that inhibitor and expressed prior to DNA replication (37, 271). L genes are classified as those which require prior transcription, translation and viral DNA synthesis for their expression (302). Neither class of genes show any clustering on the EBV genome based on class or function (120).

The pre-replicative E genes code for early antigen (EA) proteins, which are further subclassed as R (restricted) or D (diffuse) based on their distribution in the infected cell (36, 120). Being produced soon after infection and prior to L gene expression or replication, the EAs are presumed to have regulatory or enzymatic activities. One EA shown experimentally to be a strong trans-activator of viral genes is the 54-kd phosphoprotein MS-EA(D) (37, Kenney et al 1986 Abstract no. 139, Leiberman et al 1986 Abstract no. 151, 11th International Herpesvirus Workshop, Leeds, UK). This protein is coded for by a 1.9-kb mRNA transcribed from an open reading frame on the left side of the Bam HI-M fragment (Siebl et al

1986 Abstract no. 152, 11th International Herpesvirus Workshop, Leeds, UK). Cotransfection experiments with plasmids carrying the MS-EA(D) gene and recombinant constructs containing the bacterial CAT gene under the control of promoters from either EBV, SV 40, Adenovirus or HSV-1 showed promiscuous trans-activation of all these promoters by the MS-EA(D) protein (120). This trans-activating gene has also been identified in the P3HR-1(EBV) cell line which spontaneously produces infectious EBV due to the presence of a rearranged form of EBV DNA (35). Therefore, the MS-EA(D) protein may play an important regulatory role in the expression of lytic cycle EBV genes.

A second region important in the activation of EBV lytic cycle genes has also been suggested. This region has been identified in non-rearranged Bam HI-Z fragments (found in the virus) and in the heterogeneous or rearranged Bam HI-WZ, or 'het' DNA fragment (found in P3RH-1 cells) (52, 309). Cloned DNA from the non-rearranged Bam HI-Z fragment or rearranged het DNA has been shown to encode a polypeptide which, in transfection assays, was shown to trans-activate synthesis of several EBV E polypeptides (52, 309). Recent experiments have shown that cotransfection of the open reading frame BZLF1 (contained within the Bam HI-Z region) with other EBV genes, activated expression of the latter (E. Manet et al. Abstract No. 154, 11th International Herpesvirus Workshop, Leeds, UK). The pattern of transcription activated by the BZLF1 open reading frame was similar to that observed in the absence of

DNA replication. Thus, E genes were shown to be activated by the polypeptide encoded by the BZLF1 open reading frame. Therefore, like the MS-EA(D) protein, the protein coded for by the BZLF1 open reading frame appears to be a trans-activator of EBV E gene expression.

## Summary of Herpesvirus Gene Regulation

As described in the preceding review, gene expression in herpesviruses is temporally coordinated into specific phases of expression. Generally, these may be classified into three phases: immediate-early (IE), early (E) and late (L). The herpesvirus genes expressed during these phases are therefore named as IE, E and L.

The IE genes are the set of genes transcribed soon after infection by the existing cellular polymerase II, and in the absence of de novo protein synthesis. The IE mRNAs are, therefore, the only ones translated immediately after removal of a translation inhibitor added at the start of infection. Although only a small percentage of the viral genomes comprise the IE genes, their gene products are important for subsequent viral gene expression. In fact, in the absence of fully functional IE gene products, gene expression of the later classes of genes have been shown not to occur.

The E genes are those which are expressed next in the course of infection. Like the IE proteins, the E proteins too may be important in the activation of the subsequent class of genes. However, they are also required for the repression of the IE gene transcription. The E gene products also include proteins and enzymes required for viral DNA synthesis, an event which closely follows E gene expression.

DNA replication appears to be the event which sets apart the L genes from the rest. Prior to DNA synthesis the L genes are expressed at exceedingly low levels. After replication,

however, the L gene expression level is visibly boosted. The products of the L genes are mainly structural in function. However, the L proteins (like the E proteins) may be involved in the repression of the preceding classes of genes. Furthermore, as research in HSV-1 has shown, the L proteins are also required for the enhancement of the IE gene expression in the next round of infection.

As shown by the numerous transfection assays, stimulation of gene expression in the herpesviruses appears to be in *trans* as opposed to the *cis* stimulation of transcription by enhancers. The absence of homology among the promoters affected by these regulatory proteins and the lack in physical similarity among the proteins themselves, suggests that their action is mediated by cellular protein(s) -- probable mechanisms of which will be discussed in Part IV.

**Objectives of this Dissertation**

The objectives of this dissertation were mainly twofold.

1. (a) To study transcriptional regulation of the herpes simplex virus genes in vitro, and
   (b) To demonstrate in vitro promoter selectivity of the HSV-1 genes by the cellular transcription system similar to that observed during the temporal expression of HSV-1 genes in vivo
2. To identify the factors that may be responsible for that promoter selectivity.

The approaches used to demonstrate promoter selectivity of HSV-1 genes in vitro were the following :

A. Preparation of cell-free transcription systems from

(a) Human (HEp-2) or mouse (L) cells that were uninfected or untransformed (so as to contain no viral proteins) to observe the effect of a virus-unmodified system on HSV-1 gene expression;

(b) HEp-2 cells infected at the non-permissive temperature with temperature-sensitive mutants of ICP4 gene (so as to contain functional forms of all the IE proteins, except ICP4) to observe the effects of an ICP4 deficient system on HSV-1 gene expression;

(c) HEp-2 cells infected at the permissive temperature with the ICP4 temperature-sensitive mutant, or with the wild type HSV-1(F) under normal conditions for 8 hrs (so as to contain all viral proteins present in infected cells at 8h post-infection) to observe the effect of 8h infected cell proteins on HSV-1 gene expression;

(d) HEp-2 cells infected with the wild type HSV-1(F) virus in the presence of a protein synthesis inhibitor that is later removed 1 hr prior to harvesting [so as to contain mainly the IE proteins which had been shown in vivo to be the class of proteins synthesised immediately on reversal of a translation block, ()] to study the effect of a

transcription system containing mainly the IE proteins on HSV-1 gene transcription; and

(e) Mouse L cells (lacking the native thymidine kinase activity) stably co-transformed with the HSV-1 ICP4 and Tk genes (so as to constitutively express the ICP4 protein at low levels) to observe the effect of ICP4 alone on HSV-1 transcription, in the absence of the other hypothesised HSV-1 regulatory proteins.

B. The utilisation of these cell free preparations individually in in vitro transcription run-off assays, to observe their preference (or selectivity) in transcribing cloned linearised templates containing specific classes of HSV-1 promoters (namely, α, ß and ßτ).

The approach used to identify the factors that may be involved in the observed promoter selectivity by cellular Polymerase II from mock infected and HSV-1 infected cells on HSV-1 genes were as follows :

1. Chromatographic isolation of protein factors present in whole cell extracts from mock infected and 8 h HSV-1 infected HEp-2 cells on Heparin-agarose, DEAE Sepharose-CL6B and Sephacryl S-300 columns;
2. Addition of the individual protein fractions to RNA Polymerase II from uninfected HEp-2 cells (Pol II-M) in

in vitro transcription assays with linearised templates containing different temporal classes of HSV-1 promoters.

3. Immunological identification by monoclonal antibodies and antisera of the HSV-1 proteins present in the fractions that induce Pol II-M to manifest transcriptional properties associated with 8 h HSV-1 infected cells.

Finally, possible models for the mechanisms of gene expression in DNA viruses are discussed (Part IV) in view of the data available currently from HSV and other DNA virus systems.

# In Vitro Transcription of Herpes Simplex Virus Genes: Demonstration of Promoter Selectivity in Vitro

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# ABSTRACT

To investigate the molecular basis for the temporal regulation of HSV-1 gene expression observed in vivo, we have prepared partially purified RNA polymerase II from HEp-2 cells that were either mock-infected, infected with HSV-1 for 8 h, infected with HSV-1 with cycloheximide present 0-7 h and removed 7-8 h post-infection (cycloheximide reversed or "CR" cells), or infected with the immediate-early mutant tsLB2, for 8 h at either the permissive or non-permissive temperature. We also prepared whole cell extracts from L cells that either constitutively produce ICP4 (Z4 cells) or lack it (Lta). Using these systems, we have transcribed in vitro cloned fragments of HSV-1 DNA, each containing a promoter from a specific class of HSV-1 genes, namely immediate early (IE or α), delayed early (DE or ß), and leaky-late (LL or ßτ). The data obtained from these studies show the following: (i) viral proteins are not required for in vitro transcription from α and ß promoters, (ii) viral proteins in addition to ICP4 are required for optimal ßτ promoter transcription, and (iii) viral proteins present in both 8h and in "CR" infected cells are required for transcription in vitro from the ßτ5 promoter, and, (iv) viral proteins present in the cells by 8 hours post-infection negatively effect α and ß gene transcription.

# INTRODUCTION

The classification of herpes simplex virus type 1 (HSV-1) genes into immediate early (IE, α), early (DE, ß), leaky-late (LL, ßτ) and late (τ) is based on the "cascade" regulation of gene expression observed in infected cell cultures (23,24,25,27). Although the HSV-1 genes are transcribed by the host RNA polymerase II throughout the infective cycle (11), viral gene products are required as mediators in the transcriptional control of HSV-1 genes. It has been shown that upon infection, even in the absence of de novo protein synthesis (1,31,), a virion protein promotes expression of the α genes (2,6,9,32,44). The products of two or more of the α genes are believed to be required for the sequential expression of the later classes of genes (8,27,29,30). While deletion mutant (14) and temperature-sensitive mutant (13,16,44,45) studies have clearly shown the necessity of ICP4 in the positive regulation of ß, ßτ and τ genes, transfection studies have demonstrated the importance of both ICP4 and ICP0 for maximal expression from ß promotors (18,20,35,40,41a). However, a third α gene product, ICP27, along with ICP4 has been implicated in the positive control of later genes (48), and in the negative regulation of α genes (41b,48). In addition, negative regulation of α gene expression ( and therefore, perhaps indirectly, of the later genes ) has been demonstrated to be due to some ß gene products, e.g., ICP8 (21,22).

In contrast to the in vivo studies, in vitro studies thus far have proved less successful in elucidating the role of viral proteins in the temporal regulation of HSV-1 genes. Some studies with uninfected cell extracts have demonstrated efficient transcription from ß (19,28,47) and ßτ (7) promoters in vitro, whereas, other studies with similar systems have failed to show transcription in vitro from other ßτ promoters (19). A significant advance in this area is the recent report by Pizer et al. (43) which shows that the addition of partially purified ICP4 to an uninfected cell transcription system confers upon it the ability to efficiently transcribe from ß and ßτ promoters.

To better understand the role of both host and viral proteins in the regulation of HSV-1 genes, we have used an in vitro system that displays many features of HSV-1 transcriptional regulation that have been observed in vivo. We have previously reported (17) that the α47 promoter was transcribed by a partially purified RNA polymerase II preparation from mock-infected HEp-2 cells, but not appreciably by a similar system from 8h HSV-1 infected system. We have now extended these studies by using cloned viral DNA fragments, each containing a different temporal class of HSV-1 promoter, as template for in vitro transcription by partially purified polymerase II (3) prepared from cells that were either uninfected or HSV-1 infected under conditions, so as to contain either (i) no viral polypeptides, (ii) all α polypeptides, (iii) mainly α polypeptides, or, (iv) the full complement of viral polypeptides present at 8 h post

infection. In addition, we have used whole cell extracts from mouse L cells that either constitutively express the α polypeptide, ICP4, (Z4 cells), or lack it (Lta cells).

The results presented here provide evidence for transcriptional regulation of α, ß and ßτ genes in vitro, and direct support for several features of the in vivo model of cascade regulation, i.e., while transcription of the α genes do not necessitate the presence of viral proteins, transcriptions from the later genes do require the presence of viral proteins made de novo. This system may further provide a basis for analysis of cellular and viral transcriptional components involved in this regulation.

## MATERIALS AND METHODS

**Viruses and cells for polymerase II preparation:** HEp-2 cells and HSV-1(F1) were propagated and harvested in 850 $cm^2$ plastic roller bottles as previously described (39). For RNA polymerase preparations, cells at 80-85% confluency in 850 $cm^2$ roller bottles were either mock-infected or infected with virus at 20 pfu/cell for 8 hrs. To prepare cells that contained viral polypeptides of mainly the α class, cells were infected with HSV-1 in the presence of cycloheximide (75 ug/ml). At 7 h post infection the drug was removed and at 8 h the cells were harvested (cycloheximide reversed or "CR" cells). To assess the role of the immediate-early proteins ICP0,-22,-27 and -47 in transcription, in the absence of a functional ICP4, cells were infected at either the permissive temperature (32°C) or at the non-permissive temperature (39°C) with the ICP4 mutant of HSV-1, tsLB2, [obtained from I. Halliburton, Univ. of Leeds, UK]. Partially purified RNA polymerase II (Pol II) preparations were carried out as described (3). Table 1 summarizes the cells used and the Pol II preparations derived from them.

**Cells for whole cell extract preparation:** Mouse L cells lacking thymidine kinase (Lta) and a derived cell line (Z4) stably co-transformed with the HSV-1 ICP4 gene and thymidine kinase (Tk) gene (42), were obtained from R. Persson and J. Smiley, Hamilton, Ontario, and were propagated to 80-85% confluency in Dulbecco Modified Eagles medium supplemented with 5% fetal bovine serum (DME + 5% FBS) in 850 $cm^2$ roller

bottles. HAT medium (DME + 5% FBS containing 0.1 mM hypoxanthine, 4 µM aminopterin and 16 µM thymidine) was used to select $Tk^+$ cells. Cells were harvested and washed twice in phosphate buffered saline. Cells were resuspended in 1 volume (w/v) of TGMED (50 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM Phenyl Methyl Sulphonic Fluoride and 25% v/v glycerol) and frozen at -70·C until use. Whole cell extracts (WCE) were prepared from both Lta and Z4 cells as described (34).

**DNA:** Recombinant plasmids containing HSV-1 DNA inserts having α, ß and ßγ promoters were purified by the alkaline lysis method (4) followed by banding in CsCl gradients in the presence of Ethidium bromide. Plasmids were digested with appropriate restriction enzymes using the three buffer system of Maniatis et al (33). Fragment inserts were separated by electrophoresis in agarose gels and isolated by absorption to DE-81 paper as described (17). Plasmids and HSV-1 inserts used are listed in Table 2.

**In vitro transcriptions with Pol II:** Transcription assays were performed as described (17). In the more recent experiments this procedure was modified using the "spun column" technique to isolate RNA (33). Briefly, reaction mixtures in 25 µl total volume contained DNA at 12-24 µg/ml, 50 mM Tris-HCl (pH 8), 0.16-0.18 M KCl, 3-7 mM $MgCl_2$, 0.6mM each of ATP, GTP, and UTP, 50uM $\alpha$-$^{32}$P-CTP at 7-20 µCi/nmol (New England Nuclear Corp., Boston, MA), and 5-10 µl of Pol II.

Transcriptions were performed at 37°C for 60 minutes and stopped by addition of 0.3 ml of stop buffer (0.3 M NaOAc, 0.5% sodium dodecyl sulfate, 2 mM EDTA) and 10 ug E. coli tRNA. The mixtures were extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). The aqueous phase was centrifuged for 4 mins at 2000xg through a 0.7 ml Sephadex G-50 column equilibrated with stop buffer (33). RNA was precipitated from the eluate with 2 volumes of cold absolute ethanol, washed with 80% cold ethanol, and dried in vacuo. Transcripts were glyoxalated and analyzed by electrophoresis in 1.4% to 2% agarose gels containing 5% glycerol (38). To analyse small transcripts, the RNAs were dissolved in 100% formamide containing 0.02% w/v of both bromphenol blue and xylene cyanol, heated at 65C for 3 minutes, quickly chilled and analyzed by electrophoresis through 5% polyacrylamide gels containing 8M urea (36).

**In vitro transcription with whole cell extract (WCE):** Transcription was carried out essentially as described by Manley et al (34), but using reaction mixtures containing 50% (v/v) WCE, linear HSV-1 DNA templates at 0.04 µg/µl, 120 mM KCl and ribonucleotides as given above for Pol II transcriptions. Incubation was at 30°C for 90 minutes.

**Western Blots of WCE with anti-ICP4 monoclonal antibodies:** Approximately 300 µg of WCE protein was electrophoresed in a 8.5% denaturing polyacrylamide gel as described (39). Proteins were then electrophoretically transferred onto nitrocellulose paper (BA-85, 0.45 µm pore size) in Transfer

Buffer, pH 8.3 ( 25mM Tris base, 192 mM glycine and 20% v/v methanol) at 200mA for 7 hrs at 4°C. The proteins on the NC were then blocked in BLOTTO (26) for 24 hrs at room temperature. The blocked proteins were then reacted with a 1:1000 dilution of the monoclonal antibody A005, (kindly provided by Bill McClements of Merck Sharp & Dohme Labs) for 17 hrs at 4°C, washed 10 times with BLOTTO at room temperature with shaking, reacted with $^{125}$I-labelled Protein A at 5x10$^{6}$ cpm/ml BLOTTO (ICN Radiochemicals, Irvine, CA), washed 10 times as before, air dried, and exposed to XAR X-ray films without intensifying screens.

## RESULTS

**In vitro transcription from an immediate-early promoter:** We previously reported that a partially purified RNA polymerase II preparation from mock-infected, but not from HSV-1 infected cells, preferentially transcribed RNA from an immediate early (IE) promoter in vitro (17). To extend these observations and previous ones on in vitro promoter selectivity (3,17), we used the 2.9-kb EcoRI/SalI fragment containing the IE mRNA-1 (ICP0) promoter as template for in vitro transcription by various RNA polymerase preparations from uninfected and infected cells. As we have shown previously for the IE RNA-5 promoter (17), an RNA polymerase preparation from mock-infected cells (Pol II-M) but not from 8 h infected cells (Pol II-H) produced appreciable amounts of the specific IE-1 run-off product (1.4-kb) (Fig.1A. lanes M and H). Pol II-CR, prepared from cycloheximide reversed infected cells (i.e. cells infected and maintained in the presence of cycloheximide from 0-7 h and then incubated without the drug from 7-8 h) quite efficiently produced the same 1.4-kb runoff product (Fig.1. lane CR). Pol II-ts32, prepared from IE mutant tsLB2 infected cells maintained at permissive temperature, showed a transcription pattern similar to that of Pol II-H (Fig.1. lane 32°), whereas Pol II-ts39 from cells infected with tsLB2 and maintained at non-permissive temperature gave a transcription pattern faintly resembling that of Pol II M (Fig.1. lane 39°). The lower amount of product in this case reflects a general lower activity of the tsLB2-39° polymerase preparation. The 2.9-kb

product is the result of the end-to-end transcription that is usually observed with these transcription systems. To verify that the 1.4-kb transcript originated from the IE RNA promoter, we used a 1.8-kb EcoRV/Sst II truncated ICP0 template (Fig. 1B.). The expected 1.1-kb product was produced in significant amounts by Pol II-M, but only in low amounts by Pol II-H. Similar results were obtained using the BamHI-Z fragment containing the IE RNA-5 (α47) promoter. In this case we observed significant amounts of the specific 1.28-kb runoff product with PolII-M, -CR, and -ts39, whereas PolI-H and -ts32 yielded mainly end-to-end products (data not shown).

**In vitro transcription from a delayed-early promoter:** To test for promoter selectivity on a delayed-early (DE,ß) template, we transcribed the 2.7-kb SalI/KpnI fragment (map units 0.361-0.378) containing the major glycoprotein B (gB) promoter (Fig.2). From sequence analyses of the gB gene (5) and from our in vivo and in vitro mapping data of the 5' ends of mRNAs produced from this region (Cleveland and Millette, manuscript submitted), we expected initiation from the major gB start site to yield a 1.28-kb runoff product. Using the 2.7-kb gB template, we found that the expected 1.28-kb RNA was produced mainly by Pol II-M and Pol II-CR (lanes M and CR) and to a lesser extent by Pol II-ts39 (lane 39°). On the other hand, Pol II-H and Pol II-ts32 produced mainly a 0.78-kb run-off product and negligible amounts of the 1.28-kb RNA (lanes H and 32°). By transcribing various truncated templates (data not shown, J. Cleveland Ph.D Thesis), we have shown that the smaller product originates 500 bp downstream from the major gB

start site, and by S1 nuclease analyses we have further shown that this in vitro product coincides with the mapping of a minor in vivo RNA (Cleveland and Millette, manuscript submitted). The mapping of products from the SalI/KpnI template were verified by transcribing a truncated template, the 1.1-kb SstII fragment . With this template, a 0.6-kb transcript which corresponds to the 1.28-kb mRNA observed in Fig 3 was produced by Pol II-M but not by Pol II-H (data not shown).

**In vitro transcription from a Leaky-late promoter:** The 5' end of the βτ, ICP5 transcript has been mapped within the SalI fragment (0.261-0.271 m.u.) by Costa et al (10). When this DNA fragment was used as template for transcription, the 1.0-kb run-off transcript predicted from mapping (10), and sequence data (12) was obtained with Pol II-CR, Pol II-H, Pol II-ts32, and Pol II-ts39 (Fig.3, lanes CR,H, 32°, and 39°), whereas mainly the end to end product was obtained with Pol II-M (lane M). The products of the ICP5 promoter were verified by using a 1.26-kb HpaI/HindIII truncated template. This yielded the approximate expected run-off product of 0.8-kb with Pol II-H, but not with Pol II M (data not shown).

**In vitro transcriptions with WCE from Lta and Z4 cells:** To analyse the transcriptional effect of ICP4 in the absence of other viral polypeptides except tk, we prepared whole cell extracts (WCE) from Lta cells (tk- mouse L cells) and their derivative cell line Z4 (containing and expressing the HSV-1 ICP4 and Tk genes, 42). For these experiments, we chose the

WCE over the Pol II system to ensure against potential loss of ICP4 during the purification process. The presence of ICP4 in the Z4 but not Lta WCE was verified by Western blot analysis (Fig. 4) using monoclonal anti ICP4 (generously provided by William McClements, Merck Sharp and Dohme). The transcription results show that the 1.4-kb RNA corresponding to the ICP0 specific product (Fig.5) were made by both the Z4 and Lta extracts, though a slight reduction in the amount of product made by the Z4 WCE was observed. Similar results were obtained with the α47 promoter using the 1.9-kb BamHI-Z template (data not shown). With the ß (gB) template, small amounts of the 1.28-kb product from the major gB promoter using either extract were observed. Both extracts however, produced substantial amounts of the 0.78-kb transcript, that initiates downstream from the gB promoter. With the ßτ (ICP5) template, neither whole cell extract produced visible amounts of the 1.0-kb ICP5 runoff product from the 1.7-kb Sal I fragment (Fig 5).

# DISCUSSION

By using in vitro transcription systems comprised of either whole cell extracts (WCEs) or partially purified RNA Polymerase II (Pol II), we have demonstrated promoter selectivity, that parallels in many respects the gene regulation that is observed during in vivo cascade regulation of HSV-1 genes. The main conclusions that can be drawn from this study are the following: (i) in vitro transcription from the α and ß promoters (α0, α47, ßgB and ß1.2-kb RNA in the Hind III-K region) requires no viral proteins, but only the pre-existing host cell transcriptional components; (ii) proteins present in cycloheximide reversed HSV-1 infected cells do not manifest negative regulation of transcription from the α and ß promoters studied here; (iii) transcription from the ßτ5 promoter requires proteins, that are presumably viral, present in both cycloheximide reversed and untreated 8 h HSV-1 infected cell Pol-II preparations; and (iv) proteins present in 8 h infected cells, exhibit a negative effect on α and ß transcription in our system. Transcription studies with whole cell extracts (WCEs) from Z4 cells which are transformed with, and constitutively express the ICP4 gene product (42), show that the presence of that viral protein, in the absence of other known regulatory proteins, is not sufficient to stimulate detectable levels of transcription from the ßτ5 promoter. [Although Z4 cells contain the complete protein coding sequences for the HSV-1 IE protein ICP47 and the E

protein Tk, Persson et. (1985) have shown in control experiments that these proteins per se do not play a role in regulation of HSV-1 genes (42)] Therefore, Z4 cells are established as cells expressing viral proteins, of which the sole known HSV-1 regulatory protein present, is ICP4.] Therefore, the picture that emerges from the results presented in this paper suggests that host proteins alone can suffice for expression from the α and ß promoters tested here, whereas, additional proteins present in infected cells are required for the activation of the ßγ5 promoter.

Results of our in vitro transcription with the α0 and α47 promoters provided further support for the current theories on α gene expression based on results observed in vivo. Although a virion protein, VP16, has been demonstrated to induce high levels of α gene transcription (2,6,9,32), those same studies had also shown that for basal levels of α transcription, the virion protein is not absolutely essential (40). This point is also supported by transfection studies with the α4 promoter (40, 41), where the promoter is efficiently expressed when transfected into uninfected cells.

From the work of Honess and Roizman (1974) and similar subsequent studies, ß genes during the normal infectious cycle of HSV-1 have been shown to require immediate-early polypeptides (8,25,27,30,31). This point has also been supported by recent transfection studies, which have indicated the involvement of either or both ICP0 and ICP4 in ß transcription (16,18,20,41a,42,45,46,49). Our results showing

transcription from the ßgB and the ß1.2-kb RNA promoter by an uninfected cell system, therefore, appear to be in contradiction. However,the remarkable work on the analysis of control regions required for efficient transcription of the ßTk gene done by McKnight et al. (37), is an example of efficient ß gene transcription in uninfected cells. Expression from ß promoters in uninfected cell systems has also been demonstrated in transfection studies on promoters for the HSV-1 ßTk and HSV-2 ß38K protein genes (40,41a). Furthermore, transcription in vitro from the ßTk and ßgD gene promoters in uninfected cell free systems has been demonstrated (19,28,43,47). These studies (19,28,37,40,41,43,47) therefore suggest that for the expression of some ß genes viral proteins are not an absolute necessity. However as demonstrated by O'Hare and Hayward (40), elevated expressions from ß promoters require viral proteins.

Studies on the transcription of ßτ genes have provided rather diverse results. Early results with uninfected WCE showing transcription from ßτ and τ promoters located within or near the tk region (47), and later observations on transcription from the ßτ5 promoter by a similar system (7) indicate that ßτ transcription is indifferent to the presence of viral proteins. Transcription of ßτ5 has also been shown in vitro with nuclear extracts from uninfected cells (43). These results are in direct contrast to results we have presented here, showing that detectable transcription from the

ßγ5 promoter required the presence of proteins present in the Pol II preparations from 8 h HSV-1 infected HEp-2 cells, untreated or cycloheximide reversed. Our findings however, are in agreement with those of Frink et al (19), which also show that ßγ promoters are not appreciably transcribed by an uninfected whole cell extract.

Transfection studies by Dennis and Smiley (1984) which showed that the ßγ5 promoter could be transactivated only in the presence of a superinfecting virus further support our results with the ßγ5 promoter. Therefore, the positive transcriptional results observed on the ßγ5 promoter by uninfected WCE systems may be instances of the well-known lack of promoter selectivity of such a system.

Studies by Persson et al. (1985) have demonstrated that cells transformed with and constitutively expressing the ICP4 gene (Z4 cells), when infected with HSV-1 under conditions that precluded viral protein synthesis, were able to allow only marginal levels of transcription from the ßγ5 promoter of the virus. This demonstration that despite the presence of the ICP4 protein, in the absence of the other proposed viral regulatory proteins (e.g., ICP0, ICP27 and ICP8), the cell transcription system is unable to detectably activate the ßγ5 promoter. Our results showing lack of ßγ5 transcription by Z4 WCEs (which contain ICP4 as the sole viral regulatory protein, Figure 4) therefore, is in agreement with the observations of Persson et al. (1985). however, results from experiments by Pizer et al. (1986) showing an elevated expression from this

promoter on the addition of a partially purified ICP4 extract to an uninfected in vitro system, are in contrast to the results presented here. However, since the ICP4 enriched extract used (43) in those experiments is partially purified, and may therefore contain other viral proteins, the results of Pizer et al. (1986) do not conclusively demonstrate that the viral protein ICP4 alone is sufficient to enhance the level of transcription from the ßτ5 promoter.

In conclusion, from the results obtained in our present study, an in vitro representation of HSV-1 promoter selectivity approximating the promoter selectivity shown thus far in in vivo test systems has been presented. In view of this, we present our in vitro partially purified RNA Polymerase II system as a valid system for the study of expression of virus and cellular genes in vitro.

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FIG. 1. In vitro transcription from the Immediate-early RNA-1 (ICP0) promoter. (A) The 2.9-kbp EcoRI-SalI template containing the IE-1 (ICP0) promoter was transcribed in vitro with Pol II-M (lane M), Pol II-H (lane H), Pol II-CR (lane CR), Pol II-ts32. (lane 32) and Pol II-ts39. (lane 39). RNA products were extracted, glyoxylated as described, and assayed by electrophoresis through 1.4% agarose gels. (B) Transcription of a truncated template, the 1.8-kbp SstI-EcoRV fragment to confirm the origin of this product shown are transcription products from Pol II-M (lane M), and Pol II-H (lane H). . 5' 32P-labelled HindIII-restricted lambda DNA used as markers are shown in lanes . (C) Maps of the templates used and the in vitro runoff products obtained.

FIG. 2. In vitro transcription from a Delayed-early (gB) gene. (A) The 2.7-kbp SalI-KpnI DNA fragment (coordinates 0.361-0.378) was transcribed with Pol II-M (lane M), Pol II-H (lane H), Pol II-CR (lane CR), Pol II-ts32. (lane 32) and Pol II-ts39 lane 39). The transcription products were assayed as described. (B) Mapping of the gB gene specific 1.28 and 0.78-kbp transcripts according to Cleveland and Millette, manuscript submitted.

FIG. 3. In vitro transcription from a Leaky-late (ßγ5) promoter. (A) The isolated 1.7-kbp SalI DNA fragment containing the ßγ5 promoter was transcribed in vitro as described with Pol II-M (lane M), POl II-H (lane H), Pol II-CR (lane CR), Pol II-ts32. (lane 32) and Pol II-ts39. (lane 39). RNA products were analysed as described. The ßγ5 specific transcript migrates at 1.0-kbp. Lane contains the 5' 32P-labelled HindIII-restricted lambda DNA markers. (B) Mapping of the ßγ5 template and the runoff obtained in vitro.

FIG. 4. Transcription by whole cell extracts from Z4 and Lta cells. Whole cell extracts (WCE) from mouse Lta ($tk^-$) cells and derived cell line, Z4, ($tk^+$, $ICP4^+$) were used in in vitro transcriptions with DNA fragments containing α0 (lanes 1 and 2), gB (lanes 3 and 4), and βγ5 (lanes 5 and 6) promoters. Transcription and analysis of products was as described in the text. Lanes contain the 5' 32P-labelled HindIII-restricted lambda DNA markers.

FIG. 5. Western Blot analysis of Lta and Z4 WCE using monoclonal anti-ICP4 antibody. Approximately 300 μg of WCEs from Lta (lane Lta) and Z4 (lane Z4) cells were used in Immunoblots with the monoclonal anti-ICP4 antibody, Aα005 at a 1:1000 dilution, as described in the Materials and Methods. Lanes MI and Inf show the 35S-labelled protein extracts from uninfected and HSV-1 infected HEp-2 cells, respectively, used as markers.

**TABLE I**

| CELLS USED FOR POL II PREPARATION | POL II DESIGNATION |
|---|---|
| 1. Mock infected HEp-2 | Pol II-M |
| 2. HEp-2 infected with HSV-1(F1) at 20 pfu/cell, and harvested 8 hpi. | Pol II-H |
| 3. HEp-2 cells infected with HSV-1(F1) treated with cycloheximide at 50 ug/ml from 0--> 7 hours, and harvested at 8 hpi. | Pol II-CR |
| 4. HEp-2 cells infected with HSV-1(tsLB2), maintained at 32·C, and harvested at 8 hpi. | Pol II-ts32 |
| 5. HEp-2 cells infected with HSV-1(tsLB2), maintained at 39·C, and harvested at 8 hpi. | Pol II-ts39 |

TABLE 2: Temporal classes of HSV-1 Promotors used as templates in in vitro transcription

| Plasmids | HSV-1 Promotor | Transcription templates |
|---|---|---|
| 1. pKSBBO | IE mRNA-1 | 2.9 kb EcoRI/SalI<br>1.8 kb SstII/EcoRV |
| 2. pGTSa2 | DE gB | 2.7 kb SalI/KpnI<br>1.1 kb SstII/SstII |
| 3. pIM 10 | M VP5 | 1.7 kb SalI/SalI<br>1.3 kb HpaI/HindIII |

# Transcription of IE-1 (ICP0) RNA

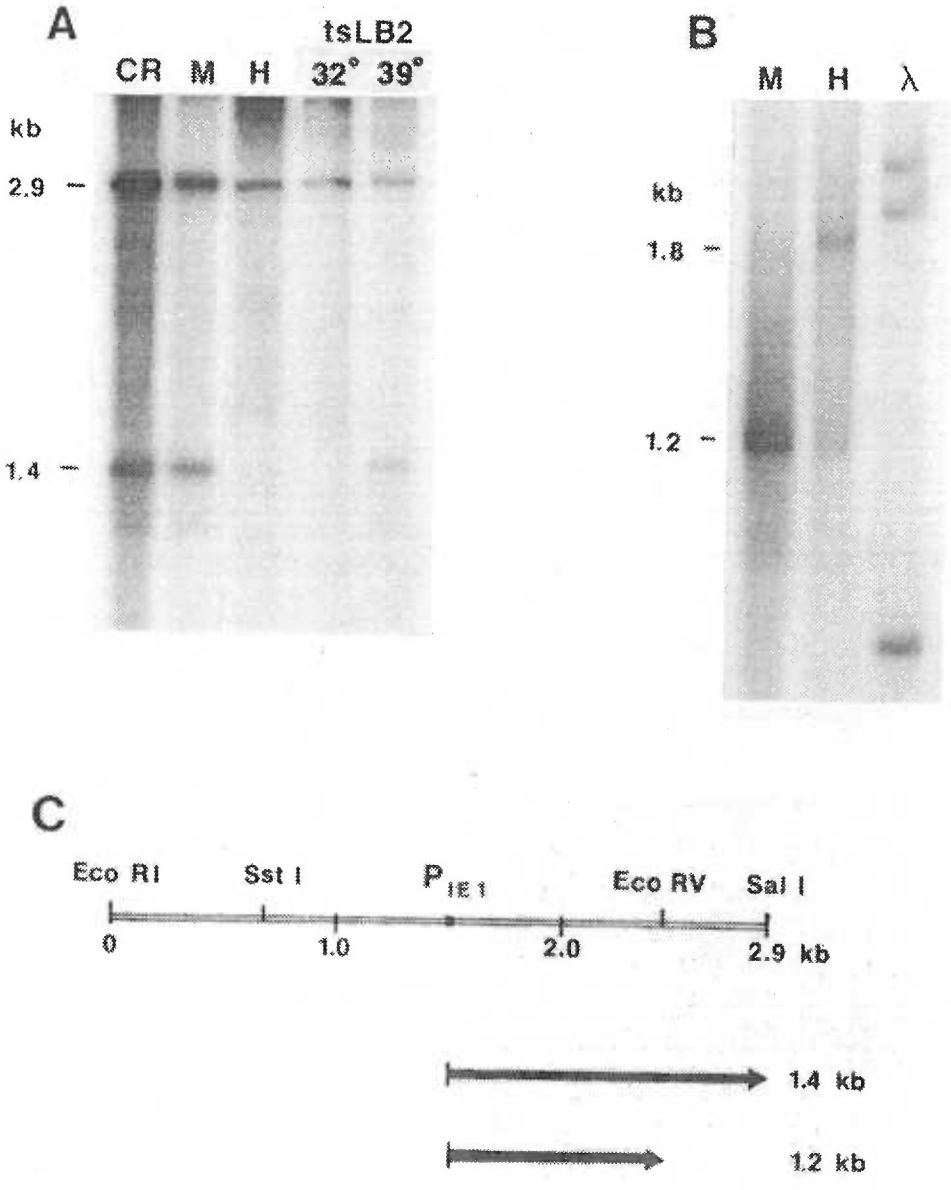



Fig.1

# Transcription of gB RNAs

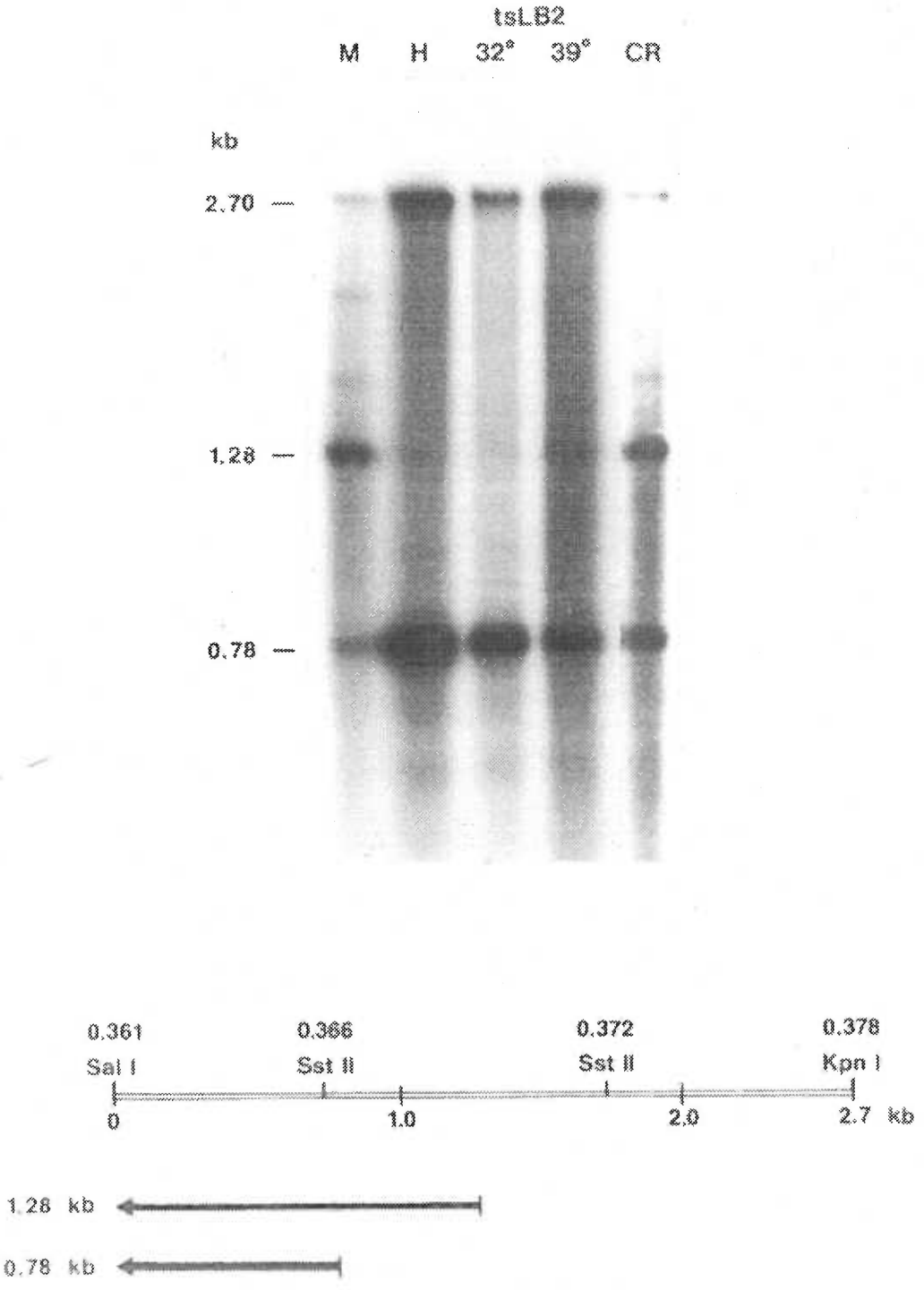



Fig.2A

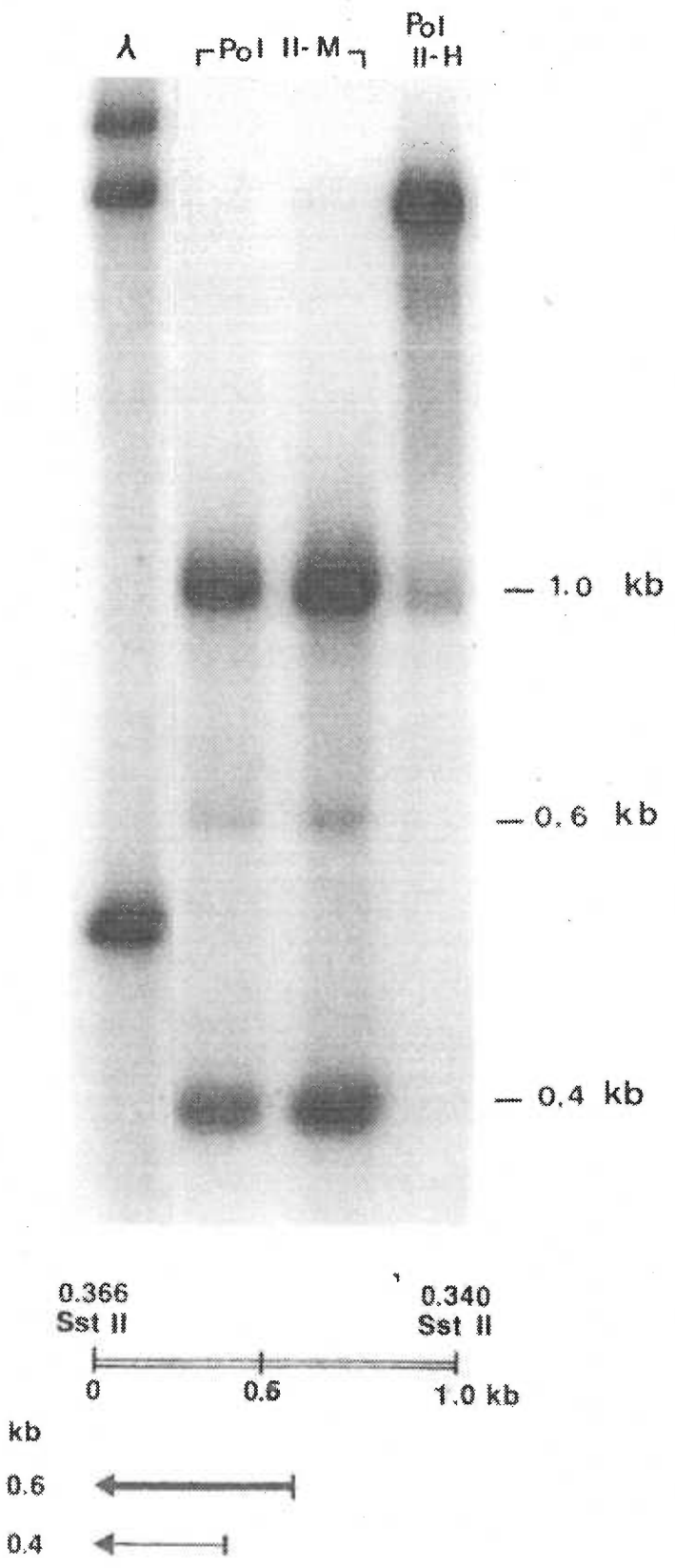
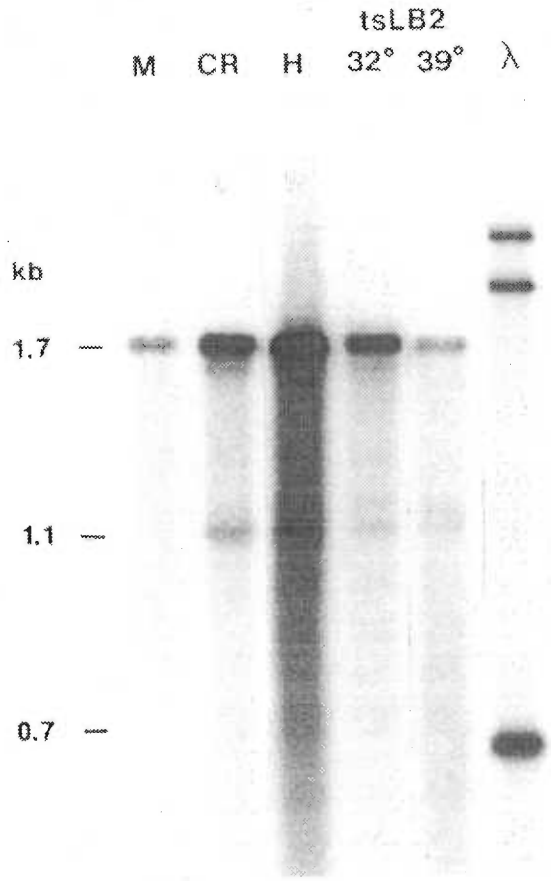

gB Truncation
λ
Pol II-M
Pol II-H
1.0 kb
0.6 kb
0.4 kb
0.366 Sst II
0.340 Sst II
0
0.6
1.0 kb
kb
0.6
0.4

Fig.2B


tsLB2
M
CR
H
32°
39°
λ
kb
1.7
1.1
0.7

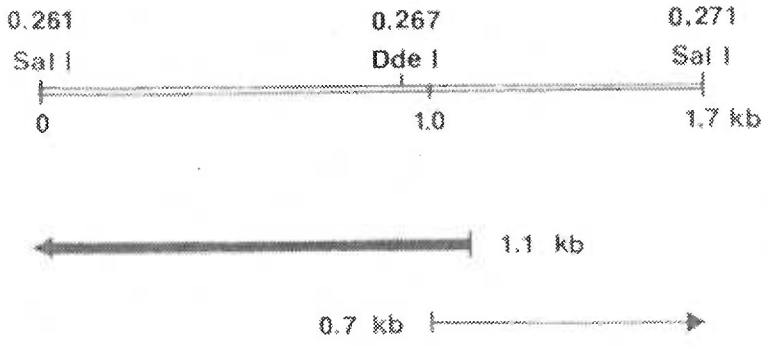

0.261
Sal I
0.267
Dde I
0.271
Sal I
0
1.0
1.7 kb
1.1 kb
0.7 kb

Fig.3

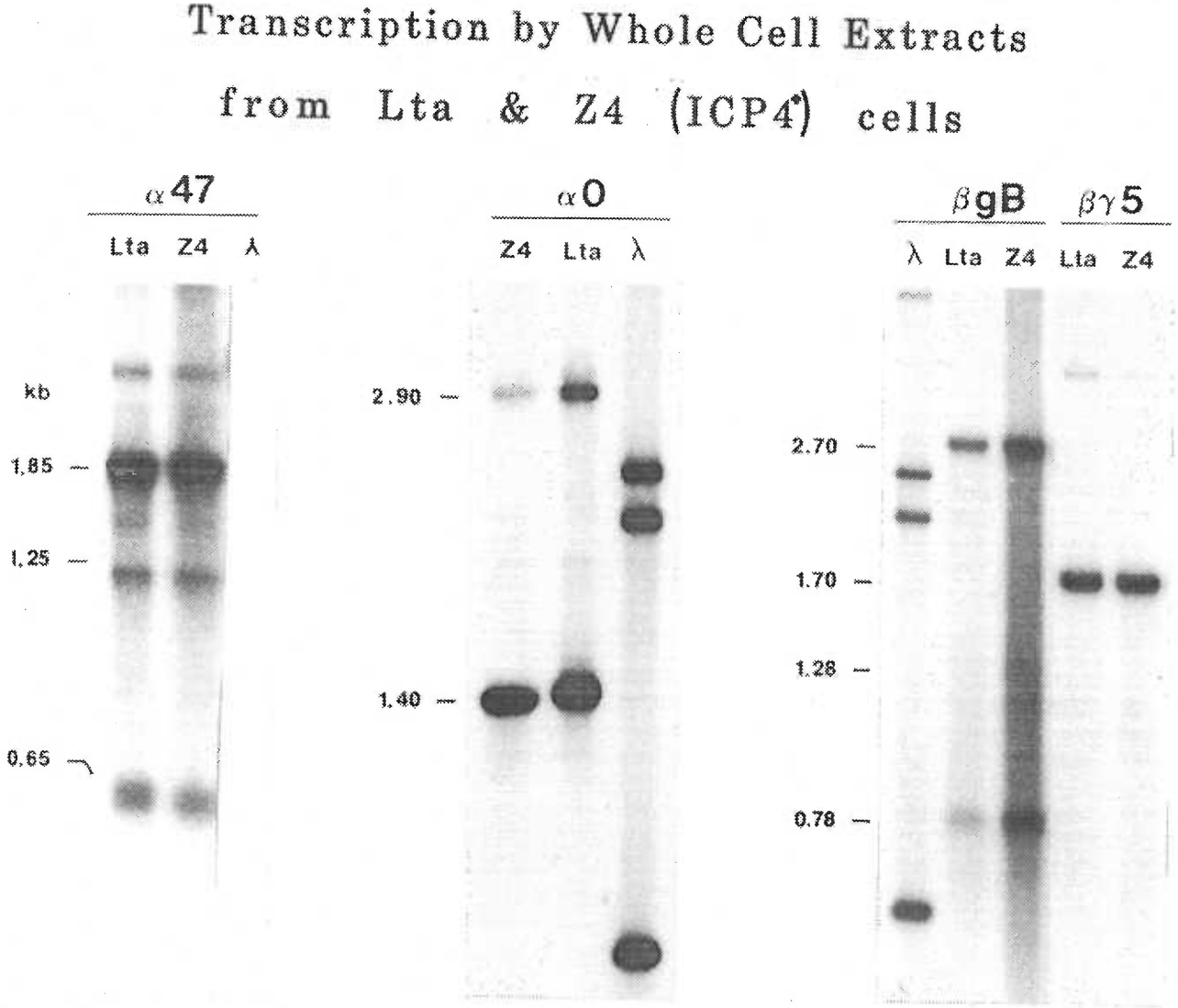

Transcription by Whole Cell Extracts
from Lta & Z4 (ICP4⁻) cells
α47
Lta
Z4
λ
kb
1.85
1.25
0.65
α0
Z4
Lta
λ
2.90
1.40
βgB
βγ5
λ
Lta
Z4
Lta
Z4
2.70
1.70
1.28
0.78

Fig.4

Western Blot

Anti-ICP4

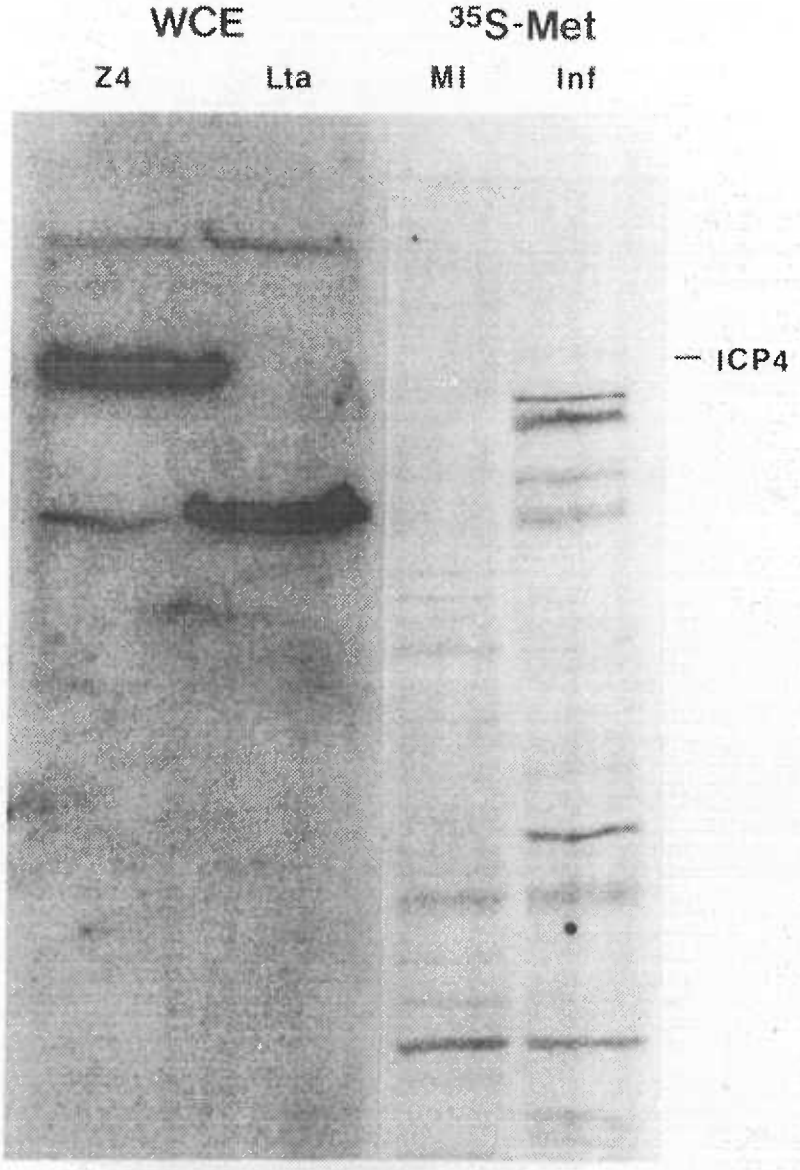



Fig.5

# FACTORS INVOLVED IN THE IN VITRO TRANSCRIPTIONAL CONTROL OF HERPES SIMPLEX VIRUS – 1 GENES

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## ABSTRACT

The herpes simplex virus type 1 (HSV-1) genes have been shown to be temporally regulated during the lytic infection cycle (Honess, R. W. and B. Roizman, 1974, J. Virol. 14: 8-19), and have accordingly been classified into five major classes, namely α, αβ, β, βτ and τ. This regulation of gene expression has been shown to occur both at the transcriptional (Honess, R. W. and B. Roizman, 1974, J. Virol. 14: 8-19) and at the translational (Kozak, M. and B. Roizman, 1974, Proc. Natl. Acad. Sci. USA. 71: 4322-4326) levels. Previously, we have shown that transcription of some α and β promoters occurs preferentiallly by partially purifed RNA Polymerase II prepared from uninfected HEp-2 cells (Pol II-M), whereas, partially purified RNA Polymerase II prepared from 8 h HSV-1 infected HEp-2 cells (Pol II-H) preferentially transcribes from the βτ5 promoter (J. Lahiri, K. E. Sampson and R. L. Millette, Manuscript submitted). To investigate the factors that are involved in this differential transcription of the HSV-1 promoters, cellular extracts from uninfected and 8 h HSV-1 infected HEp-2 cells were chromatographically fractionated on heparin-agarose, DEAE-Sepharose CL-6B and Sephacryl S-300 columns. The fractions isolated were then assayed by in vitro transcription assays for their ability to confer upon Pol II-M the features observed with Pol II-H transcription. By using these assays, we have identified an activity present in 8 h infected HEp-2 cells, that stimulates transcription from the βτ5 promoter by RNA polymerase II from

uninfected cells (Pol II-M). This fraction also inhibited transcription from certain α and β promoters by Pol II-M. Similar activities were not found in corresponding fractions from uninfected cells. To analyse the viral proteins in this fraction, we demonstrated by immunoblotting the presence of HSV-1 Immediate-early proteins ICP0, ICP4 and possibly the Early proteins ICP6 and ICP8 in this fraction.

## INTRODUCTION

During the lytic infection cycle, the herpes simplex virus type 1 (HSV-1) genes had originally been demonstrated by Honess and Roizman (1974, 1975) to be sequentially expressed. By using drugs that inhibited transcription and translation at different stages in the lytic infection cycle, Honess and Roizman, and other groups (1,17,20,21,27,37) have shown the HSV-1 genes to be expressed in 3 major phases, which they classified as α, ß and τ genes (19,24). Subsequent finer inspection of the expression kinetics of these genes, however, showed further differences within them (18,36). Two groups of ß genes were identified, the Early (αß or $ß_1$) and the Delayed-early (true ß or $ß_2$) genes, depending on how soon after infection they were expressed. The τ genes were also subdivided into Leaky-late (ßτ or $τ_1$) genes and the True-late (true τ or $τ_2$) genes, based on how stringently they required viral DNA synthesis for their expression. Accordingly, the temporally different classes of HSV-1 genes, are now classified as α, αß, ß, ßτ and τ (38).

The mechanism of regulation of gene expression in HSV-1, has been the subject of extensive research over the past few years. In vivo studies have shown that for the expression of ßτ genes, HSV-1 proteins are required. The ßτ promoter most studied is that of the ßτ5 gene, which codes for the major capsid protein, ICP5, in the virus (5). By using a chimaeric gene containing the ßτ5 promoter fused to the coding sequences of the ßTk gene, Dennis and Smiley (1984) showed

that the transfected ßτ5 promoter was activated in *trans* only when infected with a $Tk^-$ but otherwise fully competent HSV-1 virus. By infecting mouse cells transformed with and expressing the α4 gene, with wild type HSV-1 in the presence of cycloheximide, Persson et al (1985) observed marginal transcription from the ßτ5 gene. These studies therefore, demonstrate a necessity for other α proteins besides ICP4, for *trans*activation of the ßτ5 gene.

Previous in vitro transcription studies from this laboratory on the ßτ5 promoter, have shown that proteins present in 8 h HSV-1 infected cells (either untreated or following reversal of a cycloheximide block) are essential for detectable in vitro transcription from the ßτ5 promoter (manuscript submitted). We have also demonstrated a lack of transcription from the ßτ5 promoter using in vitro systems that either lack (a) viral proteins, (b) a functional ICP4 protein, or, (c) all the known regulatory proteins except ICP4. These results provide further support for the observation on ßτ5 gene activation reported by Dennis and Smiley (1984) and Persson et al (1985).

In their early studies, Honess and Roizman (1974, 1975) had reported the involvement of viral proteins in the turn-off of expression of the preceding class of genes. However to date, only one ß protein, (ICP8) has been demonstrated to be important in the inhibition of α gene expression. Using viruses carrying a temperature sensitive mutation in the ß8 gene, Godowski and Knipe (1983) have shown over-production of

α polypeptides at the non-permissive temperature. Transfection assays have also shown inhibition of α4 gene transcription in trans by the ß8 gene product (34). These observations have been recently confirmed by nuclear run-off assays (16) which showed a failure in the shut-off of α gene transcription at the non-permissive temperature on infection with HSV-1(KOS)1.1ts18, which carries a temperature sensitive mutation in the ß8 gene.

Several groups have independently shown the involvement of the α proteins in negative regulation of α gene expression (4,6,13,17,20,24,31,32). By using various temperature sensitive mutants of the α4 gene in temperature shift experiments, it was demonstrated that the functional ICP4 protein (made at the permissive temperature) was required both for the activation of later genes and for the inhibition of α genes. A trans-mechanism for ICP4 regulation was further suggested, since the action of the temperature sensitive form of the ICP4 protein was dominant over that of the wild type protein (31).

Recent experiments with α27 temperature sensitive mutant viruses has demonstrated that at the non-permissive temperature, ICP4 and ICP27 proteins are overproduced (35). This result therefore indicates that ICP27 may also be involved in the negative regulation of α genes.

To address the question as to why the different classes of HSV-1 promoters are differentially transcribed by cellular RNA Polymerase II at different times after infection, we have

chromatographically isolated fractions from both uninfected and 8 h HSV-1 infected HEp-2 cells over heparin-agarose, DEAE-Sepharose CL-6B and Sephacryl S-300 columns. These fractions have been added to in vitro systems containing partially purified RNA Polymerase II from uninfected HEp-2 cells (Pol II-M) and templates containing promoters from HSV-1 α0, α47, ßgB, a 1.2-kb ß RNA (2), or ßγ5 genes. By in vitro transcriptional stimulation assays, we have identified a protein fraction from 8 h HSV-1 infected HEp-2 cells that can stimulate transcription from the ßγ5 promoter. This fraction also inhibits transcription from the α and ß promoters used in this study. To determine what viral proteins are present in this active fraction, we have identified by immunoblotting procedures using monoclonal antibodies, the HSV-1 α proteins ICP0 and ICP4 in this fraction. By using antiserum to 7 h HSV-1 infected cell polypeptides, we have also identified a protein that comigrates with the HSV-1 ICP8 in this fraction.

## MATERIALS AND METHODS

**Cells and Viruses for Partially Purified polymerase II Preparation :** HEp-2 cells and HSV-1 (F1) viruses were propagated and harvested as previously described (). Preparation of partially purified Polymerase II from mock-infected and 8 h HSV-1 infected HEp-2 cells was by the method of Beck and Millette (3).

**Fractionation of HEp-2 Cell Extracts :** Whole cell extracts (WCE) were made from mock-infected and 8 h HSV-1 infected HEp-2 cells by a modified Beck and Millette method (3). Briefly, approximately 10 gms of cells (equal to approximately $7 \times 10^9$ cells) from 20 Roller bottles was thawed and diluted to approximately $2 \times 10^8$ cells/ml in TGMED (50 mM Tris-HCl pH 7.9, 25 % v/v glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM Dithiothreitol) containing 0.1 mM PMSF (phenyl methyl sulphonyl fluoride) and 0.5 trypsin inhibitory units/ml of Aprotinin (Sigma). The diluted cells were then suspended in 0.3 M Ammonium sulphate, and sonicated by a Heat Systems Ultrasonic W-375 sonifier on a 70 % duty cycle at setting 4 for six 10 second pulses at intervals of 30 seconds. The sonicate was then centrifuged in a Spinco SW 41 rotor for 1 hr at a speed of 200,000 x g. The resulting high speed supernatent was then dialysed on ice for 5 hrs against 2 changes of TM (50 mM Tris-HCl pH 7.9, 1 mM EDTA, 1mM Dithiothreitol, 12.5 mM $MgCl_2$, 20 % v/v glycerol) containing 0.1 M KCl. The WCEs were then subjected to fractionation steps primarily as described by Jones et al (1985). Briefly,

the WCEs were individually first applied to Heparin-Agarose (Biorad) columns equilibrated in TM + 0.1 M KCl. The peak fractions (as observed on 280nm wavelength) of the 0.4 M KCl eluate was dialysed for 60 mins against TM + 0.03 M KCl, to a final concentration of 0.1 M KCl. The dialysed Heparin-agarose 0.4 M eluate (HA-0.4) was then applied to a DEAE Sepharose CL-6B (Pharmacia) column equilibrated in TM + 0.1 M KCl. The peak flow-through fractions (identified at 280nm wavelength) obtained at 0.1 M KCl were then adjusted to 65 % saturation by adding Ammoniun sulphate, redissolved in TM + 0.1 M KCl, and desalted on a 0.1 m KCl equilibrated Sephacryl S-300 (Pharmacia) column. The peak fractions obtained from both mock-infected and HSV-1 infected cells were designated as M.1 and H.1, respectively, and stored at -70°C till further used.

The peak fractions eluted at 0.225 M KCL from the CL-6B columns, were dialysed for 30 mins against TM + 0.03 M KCl. The fractions M.2 and H.2 from mock-infected and HSV-1 infected cells respectively, were quick frozen and stored at -70°C until further used.

The peak fractions eluted at 0.5 M KCl from the CL-6B column, were dialysed for 30 mins against TM + 0.05 M KCl containing 0.1 % NP-40. The dialysed 0.5 M eluates from mock-infected cells were named M.5, while their counterparts from HSV-1 infected cells were called H.5. These fractions, like the other fractions, were stored at -70°C until used for transcriptional or immunoblotting studies.

Figure 1 shows a schematic representation of the preparation and fractionation of whole cell extracts from uninfected or HSV-1 infected HEp-2 cells.

**DNA :** Recombinant plasmids containing cloned HSV-1 genes and gel-purified transcription templates cleaved from the plasmids, were isolated as previously described (11). The templates used for in vitro transcription [αO, α47, ßgB, 1.2-kb ßmRNA from Hind III-K fragment, and ßγ5] have been described elsewhere (manuscript submitted).

**In Vitro Transcription :** In vitro transcriptions were carried out essentially as described (manuscript submitted). Conditions were adjusted such that the addition of 8 µl of protein fractions/25 µl reaction volume did not alter the previously described reaction conditions. In control transcription with Polymerase II from uninfected (Pol II-M) or 8 h HSV-1 infected (Pol II-H) cells, where no fractions were added, 8 µl of the TM + 0.1 M KCl buffer was added instead. In transcriptions with the H.2 fraction described above, 5 µl of the same buffer was added to the 3 µl of fraction in the system. Since the H.2 fraction was found to contain HSV-1 encoded DNA exonuclease, 1 mM $CaCl_2$ and 0.5 mM $ZnCl_2$ were added to inhibit degradative action of the DNase enzyme on the transcription templates. In all transcription reactions, 5 µl of either Pol II-M or Pol II-H was added, as the experiment demanded.

**Immunoblotting of proteins :** Immunoblotting of the fractions was done by the method of Johnson et al (22) and has

been described in more detail previously (manuscript submitted). Antibodies used were (a) the monoclonal antibody to ICP0 protein (H1083) obtained from L. Pereira, California Department of Health Services; (b) the monoclonal antibody to ICP4 protein (Aα005) obtained from W. McClements, Merck Sharp and Dohme Laboratories; and (c) hyperimmune rabbit anti-HSV-1 serum (F69) obtained from P. Schaffer, Dana-Farber Cancer Institute. Dilutions of the antibodies used were as follows :- 1:1000 for Aα005, 1:250 for H1083, and 1:50 for F69. Immunoblotting with H1083 (the monoclonal antibody to ICP0) was done using a second labelled antibody ($^{125}$I-labelled sheep anti-mouse Immunoglobulins from Amersham Corporation, Arlington Heights, Il) in place of the $^{125}$-labelled Protein A (ICN Radiochemicals, Irvine, CA).

# RESULTS

**Turn-off of Transcription from the α Promoters :** In previous in vitro transcription studies we have shown that a partially purified RNA Polymerase II from mock-infected HEp-2 cells (Pol II-M), efficiently transcribed RNA from two α promoters - those for α0 and α47 genes, respectively ( , manuscript submitted). A similar preparation from 8 h HSV-1 infected HEp-2 cells (Pol II-H), on the other hand, showed little transcription from these α promoters.

To investigate the involvement of HSV-1 proteins in the inhibition of α gene transcription, we chromatographically fractionated whole cell extracts from mock infected and 8 h HSV-1 infected HEp-2 cells on Heparin-agarose, DEAE Sepharose CL-6B and Sephacryl S-300 columns, Figure. 1.). The fractions isolated were then tested for their stimulation or inhibition of transcription from cloned viral promoters by Pol II-M. The fractions eluted at 0.1M and 0.4M KCl from Heparin-agarose columns, and at 0.1M, 0.225M and 0.5M KCl from the CL-6B columns, were designated as either MI-HA-0.1, MI-HA-0.4, M.1, M.2 and M.5 (for fractions derived from mock-infected cells), or, 8h-HA-0.1, 8h-HA-0.4, H.1, H.2 and H.5 (for fractions obtained from HSV-1 infected cells). Due to excessive DNase activity, however, the HA-0.4 fraction from infected cells, and therefore its control fraction (MI-HA-0.4) were not assayed. The HA-0.1 fractions from both mock-infected and HSV-1 infected cells were assayed for transcriptional

activity, but as predicted by Dynan and Tjian (1983), this fraction did not appear to possess any relevant transcriptional activity.

First, we tested the effects of the various DEAE-Sepharose CL-6B fractions (Figure 1) from both uninfected and 8 h HSV-1 infected cells individually, on the transcription from two HSV-1 α promoters by Pol II-M.

Using the 1.9-kb Bam HI-Z fragment of HSV-1 DNA, which contains the α47 promoter, the expected 1.25-kb α47 specific product was transcribed well in the presence of fractions M.1, M.5 and H.1 (Figure 2). The α47 specific product was transcribed at slightly reduced levels on the addition of fraction H.2, and, negligibly transcribed in the presence of fraction H.5, giving a transcriptional pattern similar to that observed with Pol II-H. The addition of fraction M.2 to Pol II-M, however, caused a dramatic increase in α47 transcription by Pol II-M.

To verify these observations on another α promoter, similar in vitro transcription assays were done by adding the CL-6B fractions to Pol II-M, using the 2.9-kb Eco RI/Sal I fragment, containing the α0 promoter, as template. As expected, the 1.4-kb α0 specific product was transcribed in the presence of fractions M.1, M.5 or H.1 (Figure 3). The same α0 specific product was transcribed at slightly reduced levels in the presence of fraction H.2, and, hardly transcribed in the presence of fraction H.5 or with Pol II-H. As with the α47 template, a marked stimulatory effect on α0

transcription by Pol II-M was observed on the addition of fraction M.2.

The results obtained above demonstrate that the CL-6B H.5 fraction from 8 h HSV-1 infected HEp-2 cells contains proteins which can inhibit transcription from the α promoters, whereas, fraction M.2 from mock infected cells contains proteins which can stimulate transcription from these promoters.

**Turn-off of Transcription from ß Promoter :** Our previous results using templates containing the ßgB promoter, showed that this promoter is transcribed efficiently by Pol II-M. Pol II-H however, preferentially transcribed RNA from a minor downstream promoter located approximately 500-bp from the major gB cap site (manuscript submitted). To see if this lack of efficient transcription from a ß promoter by Pol II-H could be due to the presence of HSV-1 infected cell proteins, the CL-6B fractions (Figure 1) were added as before to Pol II-M in in vitro transcription assays, using the 2.7-kb Sal I/Kpn I fragment as template. Transcription from the major gB promoter was indicated by the production of a 1.28-kb run-off product.

As observed with the α promoters, the results indicate that addition of fractions M.1 or M.5 had little effect on gB transcription by Pol II-M. However, addition of fractions H.1 or H.2 slightly reduced efficiency of gB transcription by Pol II-M, whereas, the presence of fraction H.5 resulted in a reduction in gB transcription, as compared to its transcription by Pol II-M alone. As noted with the α

promoters, addition of fraction M.2 to Pol II-M enhanced transcription of the ßgB promoter by Pol II-M. Similar results have been obtained with another ß template,- the 1.2-kb ß RNA in the Hind III-K region of the HSV-1 genome (data not shown).

The start site of the 0.78-kb RNA produced preferentially by Pol II-H from this 2.7-kbp Sal I/Kpn I template, has been mapped to approximately 500-bp downstream from the ßgB cap site (J. L. Cleveland, Ph.D Thesis, Wayne State University, Detroit, MI). In keeping with the differences observed in transcription from the gB and 0.78-kb RNA promoters by Pol II (mentioned in an earlier paragraph), the addition of the various CL-6B fractions to Pol II-M provided results from this promoter that were distinct from those observed with the gB promoter. Addition of fractions H.1 or M.1 did not alter the effect of transcription by Pol II-M on the 0.78-kb RNA. The addition of fraction M.5, however, reduced the ability of Pol II-M to transcribe from this promoter. Enhanced transcription from the 0.78-kb RNA promoter by Pol II-M was observed on the addition of fraction M.2. This stimulatory effect was further enhanced on the addition of either fraction H.2 or fraction H.5 to Pol II-M.

Therefore, from the results shown thus far, we have identified a fraction, H.5, from 8 h HSV-1 infected HEp-2 cells that exerts a marked inhibitory effect on α and ß transcription, and fraction M.2 from mock infected cells that stimulates transcription from these promoters.

**Turn-on of Transcription from the ßγ5 Promoter :** In contrast to our in vitro transcription results observed with the α and ß promoters, we showed previously that Pol II-H but not Pol II-M preferentially transcribes from the ßγ5 promoter (manuscript submitted). To investigate the role of HSV-1 proteins in ßγ5 transcription, the CL-6B fractions from uninfected and 8 h HSV-1 infected HEp-2 cells were added, as before, to Pol II-M and used to transcribe in vitro the 1.7-kb Sal I fragment containing the ßγ5 promoter. As indicated by the production of the 1.0-kb and 1.1-kb RNAs [expected from mapping data by Frink et al (1983)], transcription from the ßγ5 promoter was obtained with Pol II-H, but not with Pol II-M. As was the case with Pol II-M alone, no detectable stimulation of transcription from the ßγ5 promoter was observed by the addition of fractions M.1, M.2, M.5 or H.1 to Pol II-M. However, the addition of fractions H.2 or H.5 (separately or together) produced a transcriptional pattern similar to that observed with Pol II-H alone.

These results, therefore, have identified two DEAE Sepharose CL-6B column fractions from 8 h HSV-1 infected HEp-2 cells (fractions H.2 and H.5), which can individually confer upon Pol II-M the ability to transcribe from the ßγ5 promoter. That this is due to the affects of virus-coded functions on transcription, either directly or indirectly via cellular proteins, is shown by the absence of stimulation of Pol II-M by the corresponding protein fractions from mock-infected cells, namely, fractions M.2 and M.5, respectively.

**Western Blots with HSV-1 Antiserum:** Since CL-6B fractions H.2 and H.5 from 8h HSV-1 infected HEp-2 cells had been found to affect transcriptional regulation in vitro, it was important to identify the HSV-1 proteins present in these fractions. Using HSV-1 antiserum (provided by P. Schaffer, Univ. of Chicago), Western Blots were performed with the HA-0.4, and CL-6B fractions from uninfected and 8 h HSV-1 infected HEp-2 cells. Results shown in Figure 6, indicate that proteins which comigrate with ICP 4, 6 and 8 are present in the HA-0.4 fraction, and in the CL-6B fractions H.2 and H.5. Proteins comigrating with ICP6 and ICP8 are also seen in Pol II-H. The antiserum was largely unreactive with the corresponding fractions and Pol II from uninfected cells.

**Western Blots with Monoclonal Antibody, Aα005, to ICP4 :** To confirm that ICP4 is present in the 8h HSV-1 infected fractions, the same fractions were electrophoretically separated on 8.5 % Polyacrylamide gels under denaturing conditions, and analysed in immunoblots using Aα005, a monoclonal antibody to the ICP4 protein (obtained from W. McClements, Merck, Sharp and Dohme Labs). As shown in Figure.7, ICP4 is present in abundant quantities in the whole cell extracts and in fractions HA-0.4 and H.2. It was also detectable in Pol II-H, and in very low levels in fraction H.5. As expected, it was not detected in the protein fractions and in the partially purified Pol II preparation from uninfected HEp-2 cells.

**Western Blots with H1083, Monoclonal antibodies to ICP0 :** To confirm the presence of ICP0 in the transcriptionally active fraction from 8 h HSV-1 infected cells, the CL-6B fraction H.2 was reacted with H1083, a monoclonal antibody to the ICP0 protein (obtained from L. Pereira, California Department of Health). Using the monoclonal antibody, H1083, at a dilution of 1:250 a protein that comigrated with the ICP0 protein in HSV-1 infected cellular extracts, was identified (Figure. 8). That the protein identified was indeed a virally coded protein, was further shown by its absence from the corresponding fraction from mock-infected cells, M.2. Since from the previous immunoblots, fractions H.2 and H.5 have been shown to possess identical protein profiles qualitatively, we presume this protein identified by H1083 to also be present in H.5.

## DISCUSSION

Previously,we have demonstrated in vitro promoter selectivity on HSV-1 genes by a partially purified RNA Polymerase II preparation from mock infected and HSV-1 infected HEp-2 cells (manuscript submitted). In this publication, we have shown this effect to be mediated by proteins present in extracts from 8h infected HEp-2 cells. We have chromatographically fractionated whole cell extracts from mock-infected and 8h HSV-1 infected cells over heparin-agarose, DEAE Sepharose CL6B and Sephacryl S-300 columns. To assay for their transcriptional characteristics, these fractions were individually added to a partially purified RNA polymerase II preparation from uninfected cells (Pol II-M). Results from such transcription assays have identified a protein fraction from 8h infected whole cell extracts, which inhibited transcription from the α and ß promoters by Pol II-M. This fraction was also found to stimulate transcription from the ßτ5 promoter by Pol II-M. From these results, it can be concluded that both transcriptional effects observed in this system - activation of ßτ5, and the repression of α and ß transcription - are due to polypeptides present mainly in the infected cell fractions. Whether these diametrically opposing activities observed (the repression of α and ß transcription, and the stimulation of ßτ5 transcription) are due to a single activity or due to seperate activities which coexist in one

fraction, can be resolved only by further chromatographic fractionation of these fractions.

We initially fractionated whole cell extracts from mock-infected and 8h infected cells by adsorption on heparin-agarose columns (). The fractions eluted from heparin-agarose columns at 0.1M KCl (HA-0.1) were assayed in in vitro transcription with the 2.9-kbp template containing the αO promoter. As originally shown by Dynan and Tjian (1983), this fraction from both mock infected and HSV-1 infected cells, was found to have negligible transcriptional activity (Figure. 2 lane M.1). In subsequent studies, therefore, this fraction was not assayed. The 0.4M Heparin-agarose column eluate from HSV-1 infected cells, was found to contain excessive DNase activity, and could not, therefore, be used successfully in transcription assays. However, all its components were fractionated on the CL-6B column, and individually assayed for inhibitory or stimulatory activities on Pol II-M transcription of the viral promoters. The DNase activity associated with the 8H-HA-0.4 fraction, was found to elute at 0.225M KCl from the CL-6B column. This was concluded from results showing the degradation of template (data not shown). In transcription, this exonuclease activity was inhibited by the addition of 0.5 mM $ZnCl_2$ and 1 mM $CaCl_2$.

Our previous in vitro transcription studies (manuscript submitted) showed lack of transcription from α and ß class promoters by a partially purified preparation of Pol II from 8 h HSV-1 infected HEp-2 cells (Pol II-H). The lack of

transcription observed could have been due to either (a) inactivity of the infected cell transcription system in such experiments; (b) a non-specific inhibition of transcription by proteins comprising the infected cell transcription system; or, (c) presence of specific repressors of α and ß transcription. The possibilities of an inactive infected cell transcription system or of non-specific inhibition are proved to be incorrect by its ability to correctly transcribe from the ßτ5 promoter (Figure. 5 lane Pol II-H). To test the possibility that lack of transcription from the α and ß promoters by Pol II-H was due to a specific inhibition of transcription from these promoters by Pol II-H, infected cell fractions obtained from the DEAE Sepharose-CL-6B column was added to Pol II-M in transcription assays. As the results demonstrated, transcription from the α0, α47. ßgB, and the 1.2-kb ß RNA by cellular Pol II was inhibited upon the addition of specific fractions (namely fractions H.2 and H.5) to Pol II-M (Figures. 2, 3 and 4). These infected cell fractions, however, activated transcription from the ßτ5 promoter by Pol II-M (Figure 5). This therefore, indicates a specific inhibition of α and ß transcription by the cellular transcription system upon the addition of particular HSV-1 proteins which are present mainly in fractions H.2 and H.5.

In contrast to the preceding section, a stimulatory effect on α and ß transcription by Pol II-M was observed in the presence of the mock-infected cell fraction M.2 (Figures. 2, 3 and 4 lanes M.2). This fraction also stimulated

transcription from the 0.78-kb RNA (Figure. 4 lanes Pol II-M and M.2). However, no transcription from the βγ5 promoter was observed on the addition of fraction M.2 to Pol II-M (Figure 5. lane M.2). Therefore, these results indicate an enhancement in transcription by Pol II-M in the presence of fraction M.2 only on promoters that are even marginally transcribed by Pol II-M alone. This enhanced transcription is perhaps due to the presence of stimulatory factors in fraction M.2 that are absent in our partially purified preparation of Pol II-M.

Using in vitro run-off transcription assays, we have previously shown that detectable transcription from the βγ5 promoter and the 0.78-kb RNA promoter (on the gB template) occurred only with partially purified RNA Pol II prepared from 8 h HSV-1 infected HEp-2 cells [either untreated (Pol II-H) or cycloheximide reversed (Pol II-CR)]. A similar Pol II preparation from uninfected HEp-2 cells (Pol II-M), on the other hand, had displayed negligible transcription from these promoters (manuscript submitted). In this publication, we have demonstrated that Pol II-M is able to transcribe from the 0.78-kb RNA and βγ5 promoters when it is supplemented with proteins present in either fraction H.2 or fraction H.5 eluted from CL-6B columns at 0.225M and 0.5M KCl, respectively, (Figure. 4. lanes H.2, H.5, H.2+H.5 ). The observed stimulation of transcription from these promoters by protein fractions from infected cells, could be due to either (i) virally encoded trans activating factor(s), or (ii) a virally

induced increase in amount of, or activation of, cellular transcription factors. Further investigation will be needed to distinguish between the two possibilities. However, data from band-competition studies with ßτ5 promoter using whole cell extracts and the CL-6B 0.2M fractions from mock-infected and infected cells, seem to support the latter possibility. In these studies, a single ßτ5-specific band of similar mobility was demonstrated with both uninfected and infected cell proteins (D. Tomar, J. Lahiri, K. S. Sampson and R. L. Millette, manuscript in preparation). This therefore suggests the formation of a ßτ5-specific transcription complex by similar factors present in both infected and uninfected cells. Activation of transcription from the ßτ5 promoter in infected cell may perhaps be due to a virally induced increase in the amount of the factor(s) present to concentrations required for transcription. Alternatively, the factors in the transcription complex may be virally modified to the more activated form required for efficient transcription to occur.

By immunoblotting of the transcriptionally active fraction H.2, we have identified the HSV-1 immediate-early proteins ICP 0 and 4, and a protein comigrating with ICP8 (Figures. 6, 7, and 8). Since immunoblotting of H.2 and H.5 with antiserum to HSV-1 infected cell proteins show an identical protein profile qualitatively (Figure. 6), it can be further concluded that repression and activation of the HSV-1 genes by cellular Pol II are due (directly or indirectly) to

HSV-1 proteins that can be eluted at either 225 mM or 500 mM KCl from DEAE-Sepharose CL-6B columns.

Numerous temperature-sensitive, deletion mutants (4,9,31-35,39,40) and transfection assays (12,14,28,29,34) have shown the importance of the HSV-1 IE polypeptide ICP4 in the positive regulation of the later classes of HSV-1 genes. Similar studies have also shown the involvement of ICP4 in negative regulation of the immediate-early genes. (29). Persson et al (1985) have assayed transcription of the different classes of HSV-1 genes in cells transformed with, and constitutively expressing the α4 gene product (Z4 cells), in the presence of, and following reversal of a cycloheximide block. Their results indicated the inability of ICP4 alone to effectively either repress α transcription or stimulate βτ5 transcription during infection. Our earlier and present results have shown that βτ5 transcription is stimulated by cellular transcription systems that contain ICP4 and other known viral regulatory proteins (Pol II-H, Fractions H.2 and H.5, Figure. 6), but not by those containing ICP4 as the sole regulatory protein [Z4 whole cell extracts, (manuscript submitted)]. These results, therefore, are in agreement with ICP4 being one of the proteins (but not the sole protein), required for the efficient turn-on of βτ5 expression.

By immunoblotting with monoclonal antibodies to the HSV-1 IE protein ICP0, we have demonstrated a lack of ICP0 in our preparation of Pol II-H which preferentially transcribes later promoters. By similar immunological analysis, ICP0 was

however demonstrated in the transcriptionally active fraction from 8 h HSV-1 infected cells. The importance of ICP0 in HSV-1 gene regulation was originally shown in cotransfection assays by O'Hare and Hayward (1984). By cotransfection experiments, they had demonstrated the stimulation in trans of plasmid borne promoters for the HSV-1 ßTk and HSV-2 ß38K protein, by a fully expressive plasmid borne α0 gene. Recently, however, the extent of ICP0 involvement in HSV-1 gene regulation has been questioned (7). Recent experiments with complete and partial deletions (Sacks et al Abstract No. 13 11th. International Herpesvirus Workshop, Leeds, UK) or frame-shift mutations (R. Everett, Abstract No. 14, 11th International Herpesvirus Workshop, Leeds, UK) in the α0 gene have shown that the absence of a fully functional ICP0 did not cause any blocks in the normal lytic cycle of HSV-1. These studies indicate that ICP0 when present, may simply augment the function of the other proteins. The transcription results in this publication showing activation of transcription from the ßγ5 promoter possible by a system which appears to lack ICP0, support the idea that the HSV-1 IE protein ICP0 may not be essential in HSV-1 gene regulation.

As mentioned in the Introduction in vivo studies () have shown the importance of ICP8 in negative and positive regulation of HSV-1 genes. The possibility that ICP8 is present in our transcriptionally active fractions described in this publication, was indicated in immunoblotting experiments with antiserum to HSV-1 infected cell proteins (Figure. 6).

Definitive proof of its presence in the transcriptionally critical fractions, however, must await its detection by monoclonal antibody to the purified ICP8 protein.

To conclude, we have used a partially purified in vitro transcription to analyse protein fractions that are transcriptionally active in HSV-1 gene expression. By a preliminary biochemical dissection of this system, we have identified protein fractions from 8h HSV-1 infected cells that is capable of efficiently activating transcription from the ßτ5 promoter. These fractions have also been able to inhibit transcription from α and ß promoters. Preliminary immunological assays have identified 3 of the presently known HSV-1 regulatory proteins (ICP0, 4 and 8) along with ICP6 in the transcriptionally active fractions. Continuing studies with this system may therefore prove useful in the more specific identification of the protein or proteins involved, and the mechanism by which gene regulation in HSV-1 is brought about.

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## LEGENDS FOR THE FIGURES

FIG. 1. Fractionation of whole cell extracts. HEp-2 cell extracts were fractionated as outline above (and described in the Materials and Methods section) by the method of Jones et al. (). The fractions eluted from the DEAE-Sepharose CL-6B columns were used in transcription assays with linearised DNA containing HSV-1 of different temporal classes. The mock-infected cell fractions are designated as M.1, M.2 and M.5. The corresponding fractions from 8h HSV-1 infected cells are designated as H.1, H.2 and H.5.

FIG. 2. Effect of the DEAE-Sepharose CL-6B fractions on transcription in vitro from the α47 promoter. The 1.9-kbp Bam HI-Z template containing the IE-5 (α47) promoter was transcribed in vitro with Pol II-M to which CL-6B fractions from mock-infected (Lanes M.1, M.2, M.5) and infected (lanes H.1, H.2, H.5) were added. Lanes Pol II-M and Pol II-H show transcription of the α47 promoter with these polymerase II preparatio[ns. Lanes show 5' $^{32}$P-labelled Hind III-restricted Lambda DNA used as markers.

FIG. 3. Effect of DEAE-Sepharose CL-6B (CL-6B) Fractions on transcription from the αO promoter. The 2.9-kbp Eco RI-Sal I template containing the IE-1 (ICP0) promoter was transcribed as described in Figure 2. Lanes M.!, M.2 and M.5 show transcription products produced by the addition of mock-infected cell CL-6B fractions to Pol II-M. Lanes H.1, H.2 and

H.5 show transcription products produced in presence of the corresponding infected cell CL-6B fractions added to Pol II-M. Lanes Pol II-M and Pol II-H show transcription of the αO template with partially purified polymerase II preparations from mock-infected and HSV-1 infected cells, respectively. $^{32}$P-labelled Hind III-restricted lambda DNA which were used as markers (Lane ). The sizes of the template and transcription products are given in kb.

FIG. 4. Effect of the DEAE-Sepharose CL-6B (CL-6B) fractions on transcription from the ßgB promoter. The 2.7-kbp Sal I-Kpn I template containing the ßgB (VP7) promoter was transcribed with partially purified polymerase II prepared from mock-infected (Pol II-M) and 8h HSV-1 infected (Pol II-H) cells. Transcription from the ßgB promoter was also analysed using Pol II-M supplemented with CL-6B fractions from mock-infected cells (Lanes M.1, M.2 and M.5) and 8h HSV-1 infected cells (Lanes H.1, H.2 and H.5). Lanes contain $^{32}$P-labelled Hind III-restricted Lambda DNA. Sixe of the template and transcription products are given in kb.

FIG. 4. Effect of the DEAE-Sepharose CL-6B (CL-6B) fractions on transcription from the ßτ5 promoter. The 1.7-kbp Sal I template containing the ßτ5 (ICP5) promoter was used in transcription assays with the CL-6B fractions as described for Figures 2-4. Lanes M.1, M.2 and M.5 show transcription from the ßτ5 promoter using Pol II-M supplemented with the CL-6B fractions from mock-infected cells. Lanes H.1, H.2 and H.5

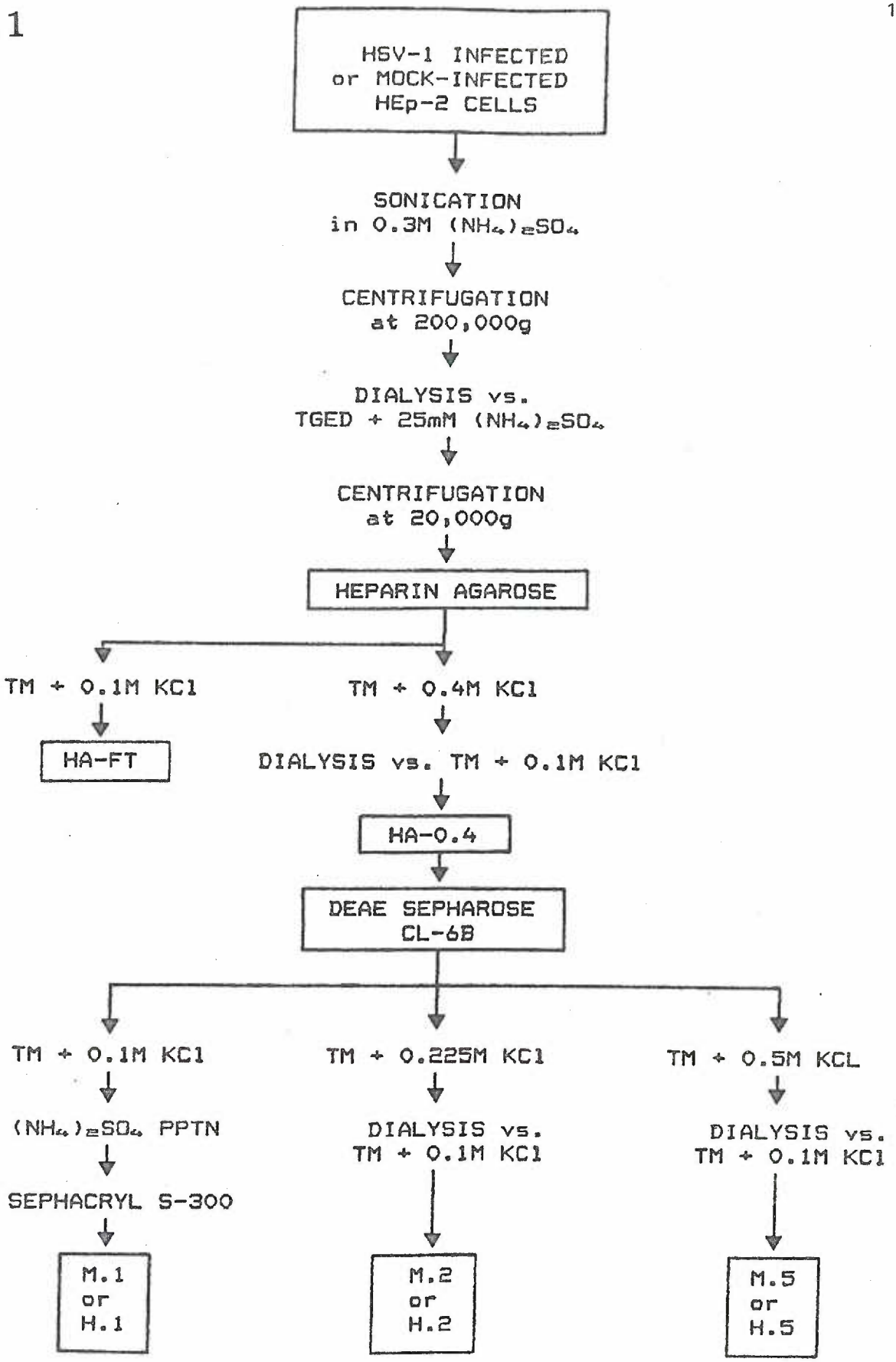
show transcription of this template by Pol II-M supplemented with the corresponding CL-6B fractions from 8h HSV-1 infected cells. Lanes Pol II-M and Pol II-H show transcription from the βτ5 template with partially purified polymerase II from mock-infected and HSV-1 infected cells, respectively.

FIG. 6. Western Blot with the rabbit hyperimmune antiserum F69. Partially purified polymerase II preparations, heparin-agarose 0.1M KCl fractions and the transcriptionally active CL-6B fractions from both mock-infected and HSV-1 infected cells were analysed immunologically with antiserum to 7h HSV-1 infected cell polypeptides. lanes 8h and CR show the $^{35}$S-labelled protein extracts from 8h infected and cycloheximide reversed infected HEp-2 cells.

FIG. 7. Western Blot with monoclonal antibody Aα005. Partially purifed polymerase II preparations, whole cell extracts and subfractions from mock-infected and 8h HSV-1 infected cells were analysed immunologically with monoclonal antibody to the HSV-1 IE protein ICP4. Lanes CR and 8h show $^{35}$S-labelled protein extracts from cycloheximide and normally 8h HSV-1 infected HEp-2 cells.

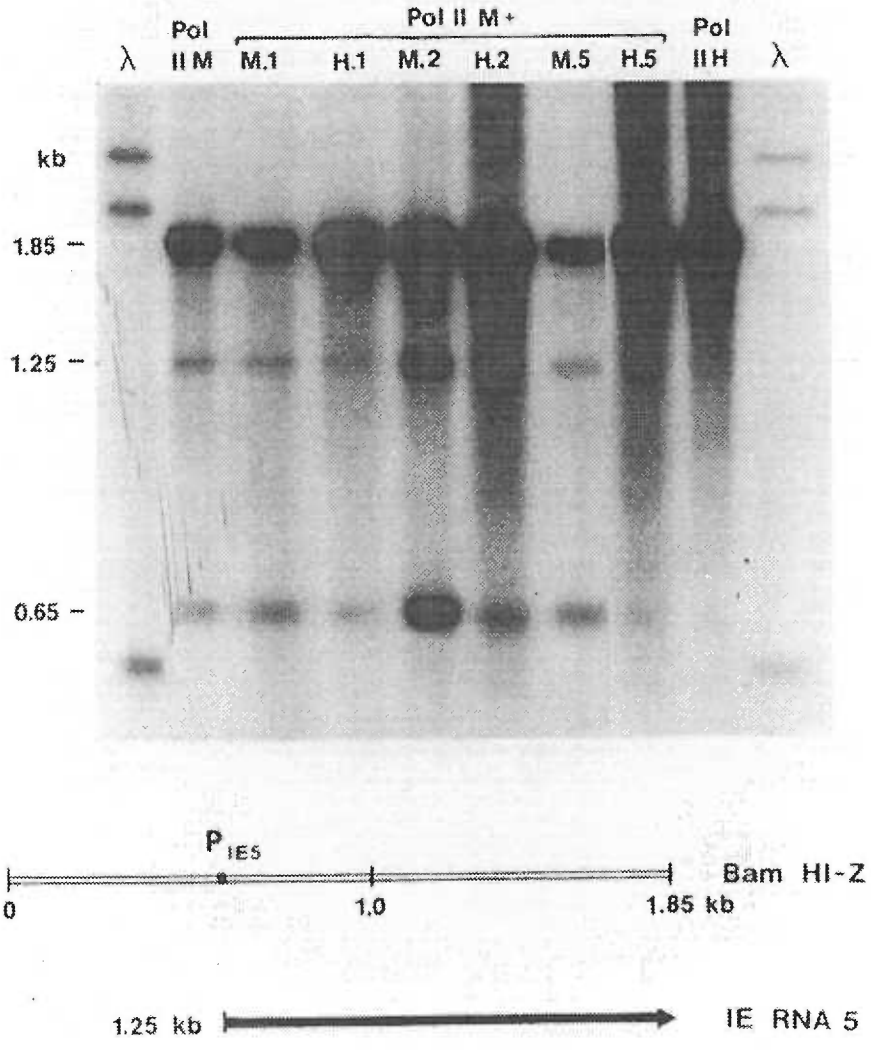
FIG. 8. Western Blot with monoclonal antibody H1083. partially purified polymerase II preparations and the transcriptionally active CL-6B fractions from mock-infected and HSV-1 infected cells were analysed with monoclonal antibodies to the HSV-1 IE protein ICP0. Lanes CR and 8h show $^{35}$S-labelled protein extracts from cycloheximide and normally 8h HSV-1 infected HEp-2 cells.

Fig.1




:k & Millette (1982) JBC 257 12780
:en & Tjian (1983) Cell 32 669

# Effect of CL-6B Fractions on α47 Transcription



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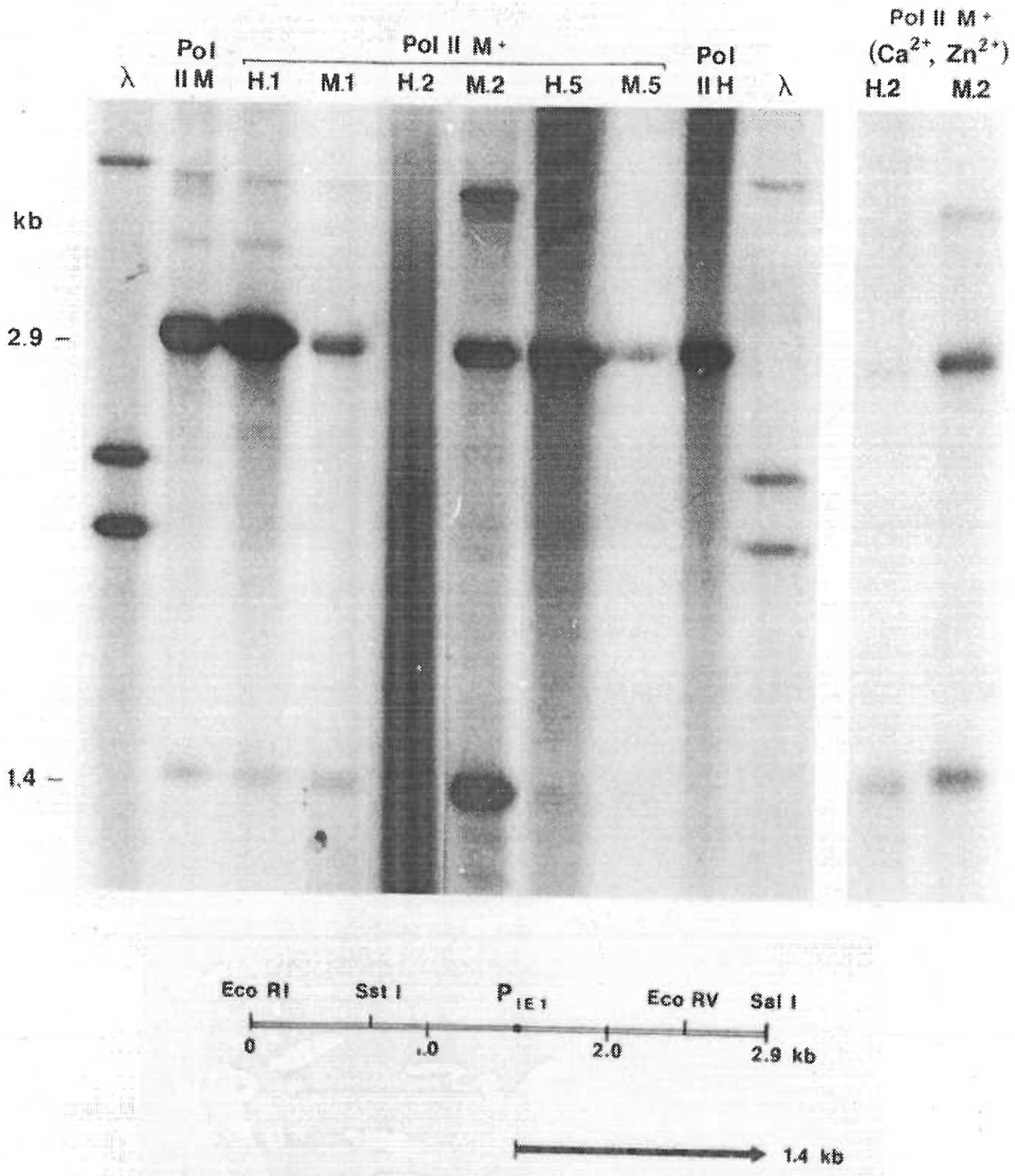
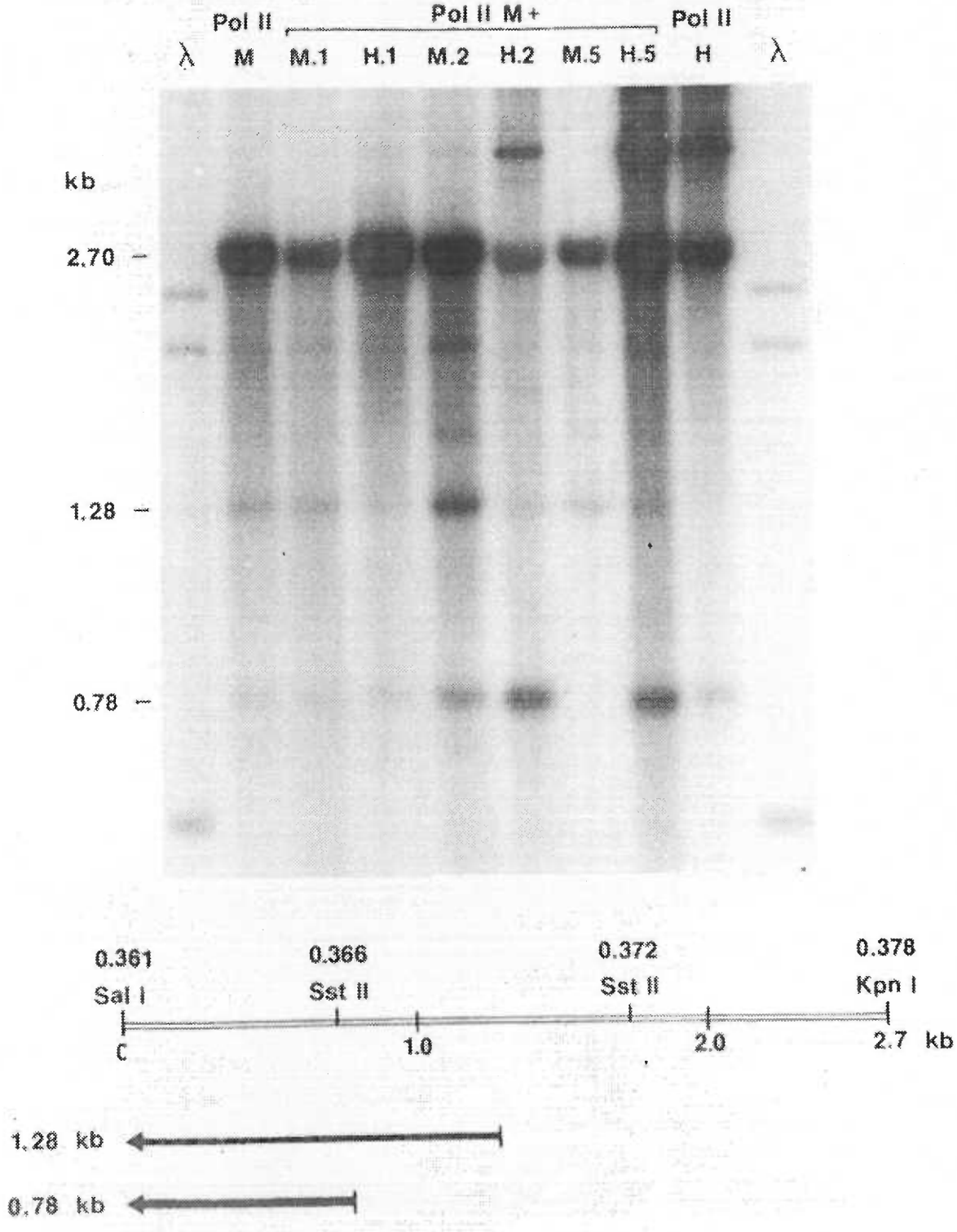
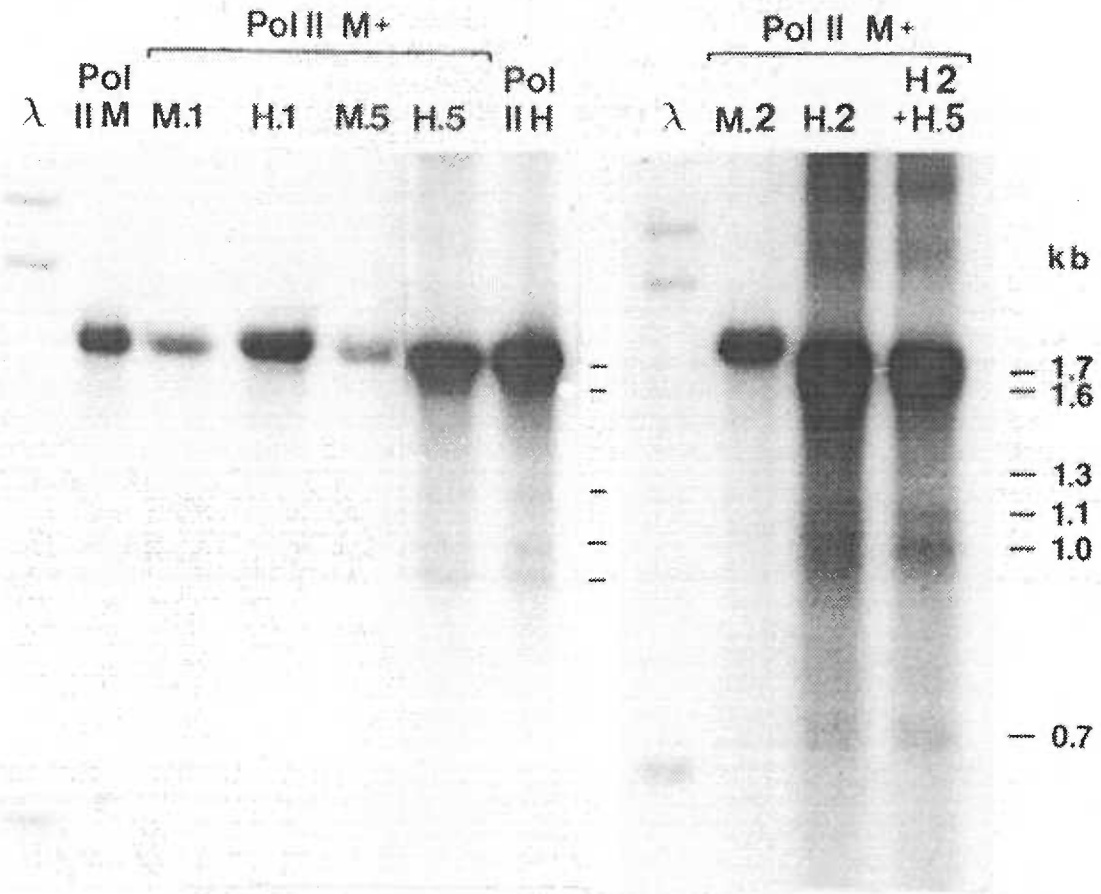
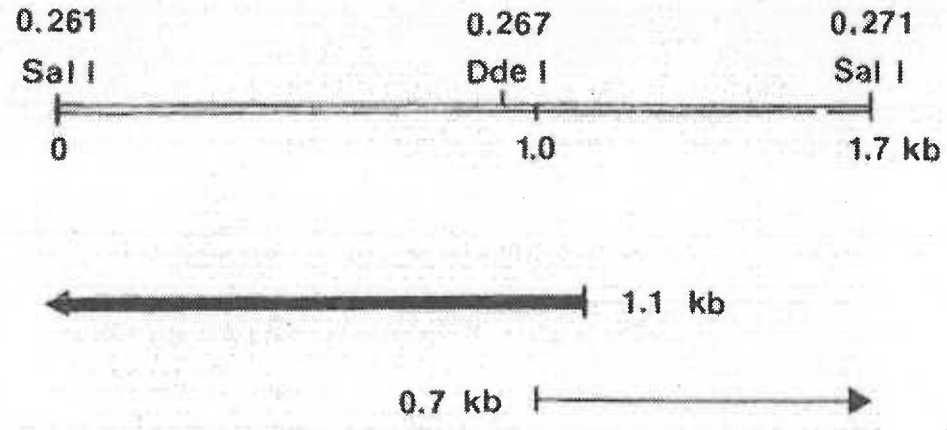


# Effect of CL-6B Fractions on gB (β) gene Transcription



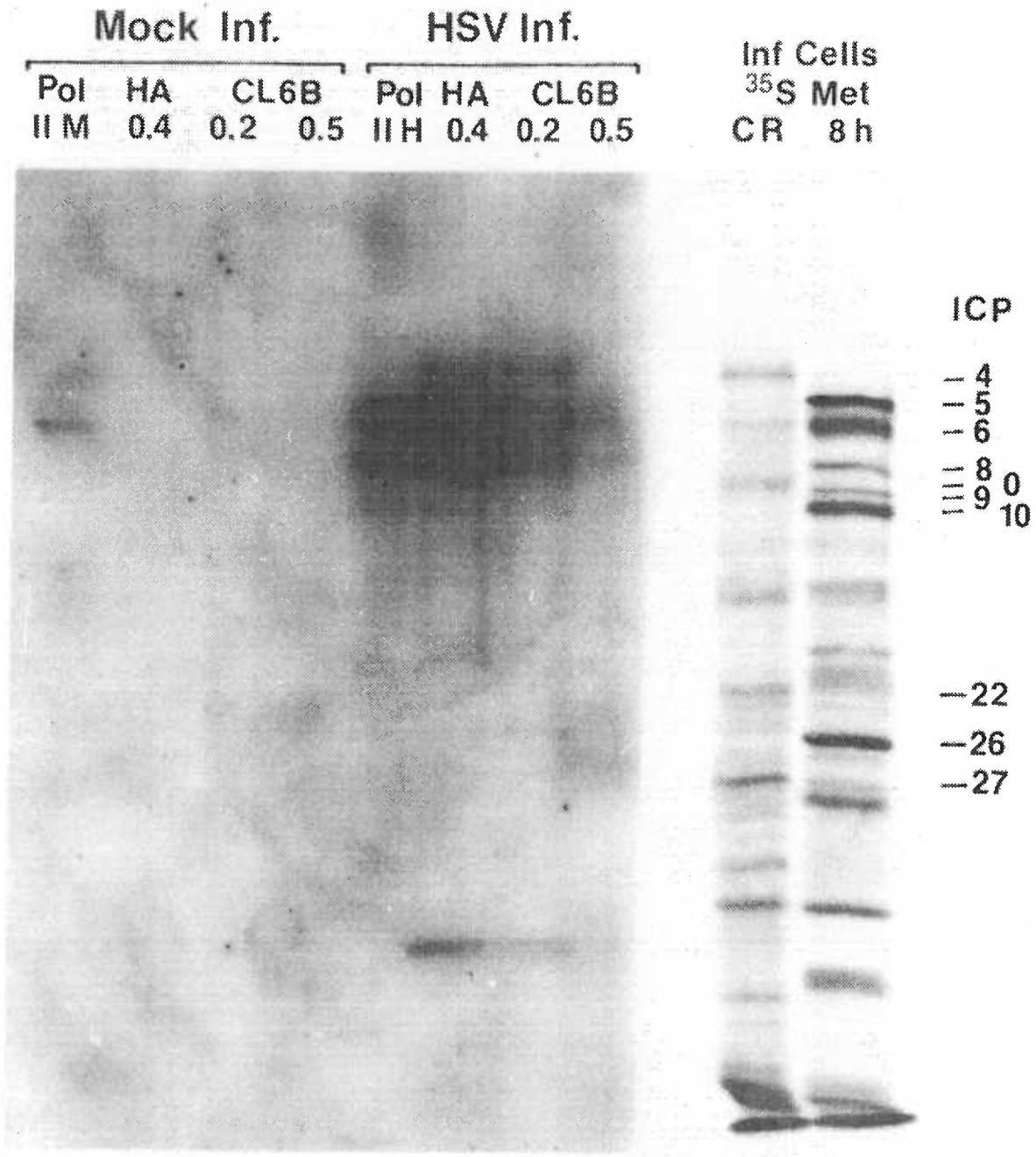
# Effect of CL-6B Fractions on βγ5 Transcription



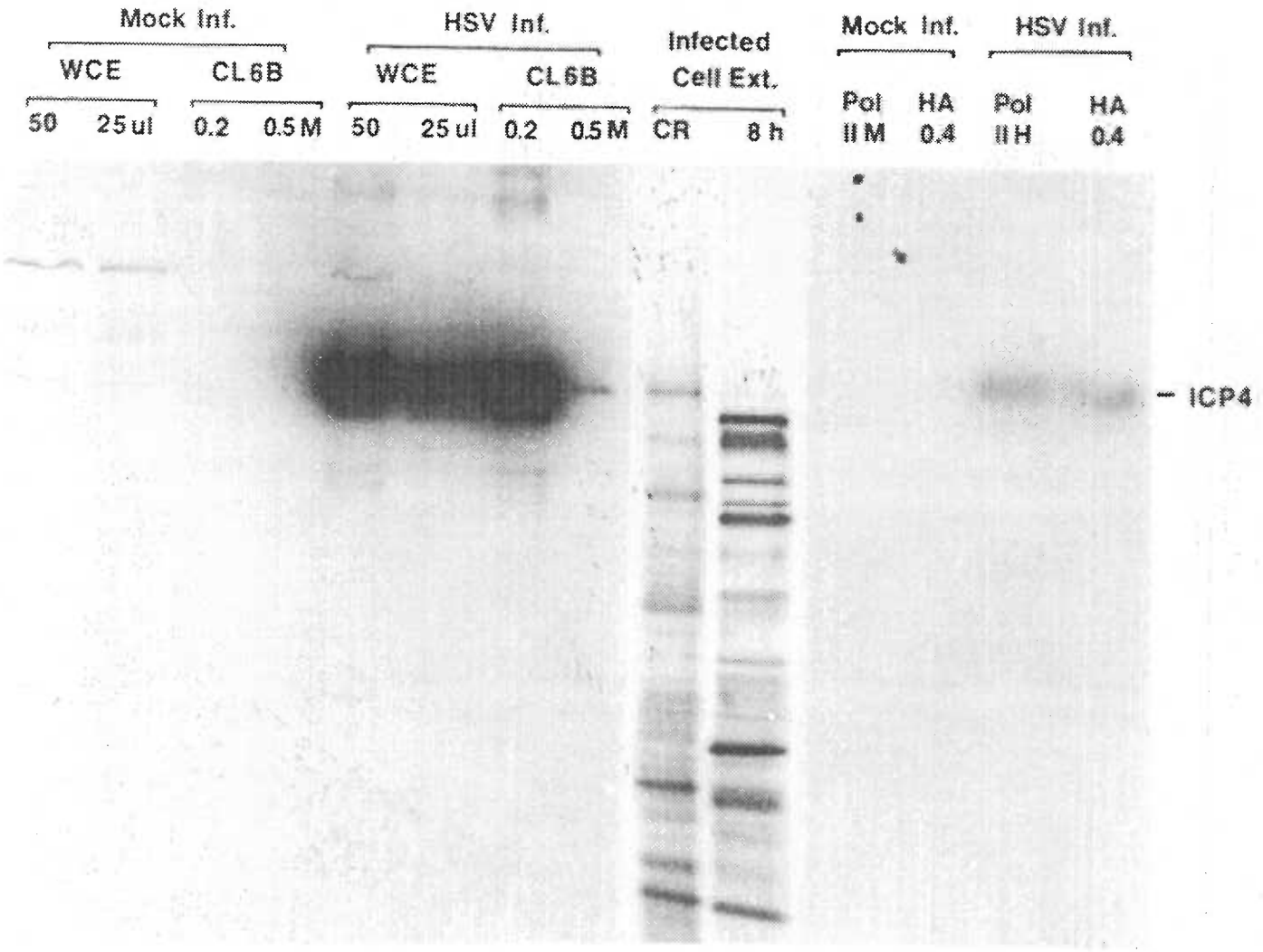
# Western Blot

## HSV-1 Antiserum

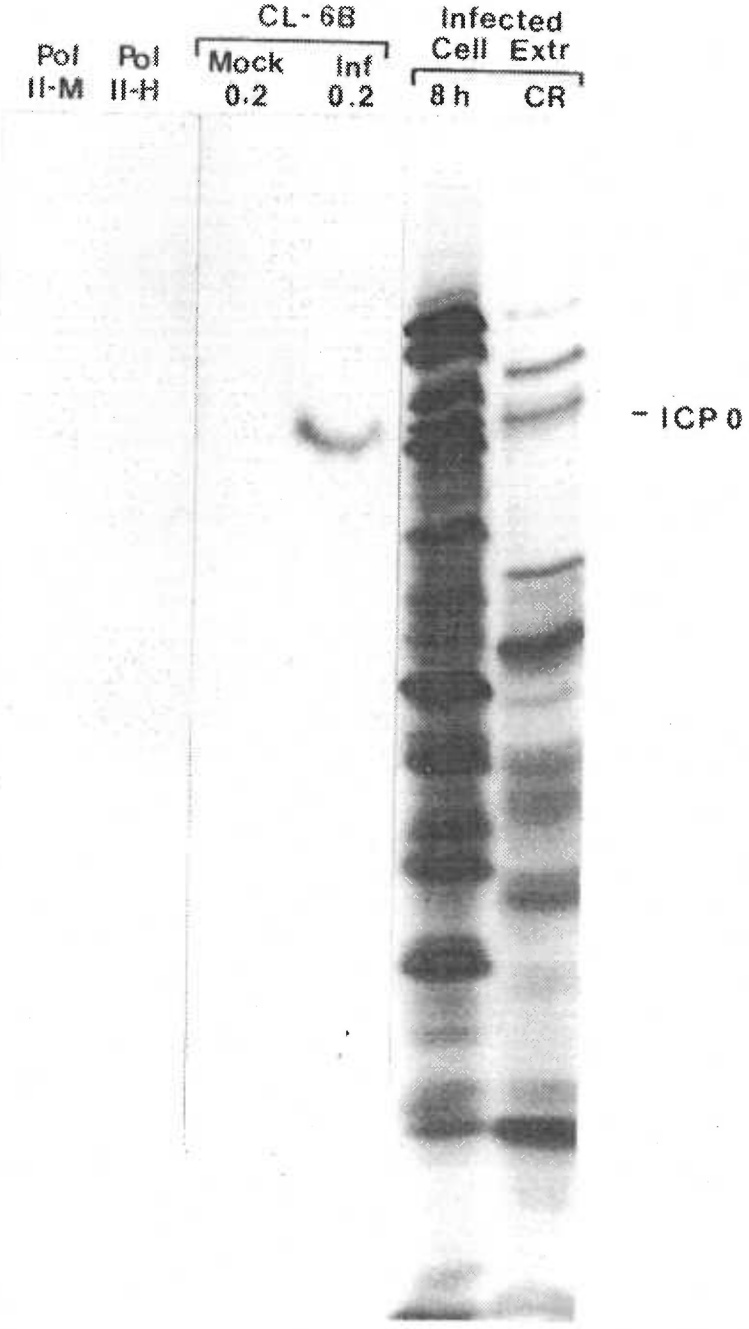


# Western Blot

## Anti ICP4



# Western Blot
# Anti ICP0



# DISCUSSIONS

## Summary of the research done in this dissertation.

In the first part of research done for this dissertation (Part II), I have demonstrated selectivity in the in vitro transcription of the different classes of herpes simplex virus type 1 (HSV-1) promoters by a partially purified preparation of RNA polymerase II from mock-infected and HSV-1 infected HEp-2 cells. I first prepared partially purified RNA Polymerase II from HEp-2 cells that either (a) do not contain any viral polypeptides, (b) contain all the viral proteins normally present in 8hrs HSV-1 infected cells, (c) do not contain a functional ICP4 protein (an HSV-1 immediate-early protein demonstrated to be important in HSV-1 gene regulation), or (d) contain all infected cell polypeptides present at 8hrs post-infection after removal of a 7hr cycloheximide block. To minimise loss of proteins during purification, in one instance, whole cell extracts (WCE) were prepared by the method of Manley et al. (184) from mouse cells that were either untransformed [Lta cells], or transformed with and constitutively express the ICP4 gene [Z4 cells]. Table 1 (Part II) gives a description of the different kinds of cells used and the transcription systems derived from them. I then used these systems for in vitro transcription assays with linearised DNA templates, each containing a specific temporal class of promoter: namely immediate-early (IE or α),

delayed-early (DE or ß) and leaky-late (LL or ßτ). The promoters used for transcriptions in this study are those for the αO (or ICPO) gene, the α47 (or ICP47) gene, the ß glycoprotein gB gene, the ß1.2-kb RNA gene (located in the Hind III-K fragment of the genome), and the ßτ gene for the major capsid protein (ICP5). The results obtained may be summarised as follows. (1) Transcription from the HSV-1 α and ß promoters occurred preferentially in transcription systems prepared from cells that either do not contain any viral proteins (Pol II-M and Lta WCE) or contain only 3 (ICP4, ICP47, Tk) of the 75 known HSV-1 proteins (Z4 WCE). Transcription from the α and ß promoters was also observed in a system made from cells that were infected at the non-permissive temperature with an α4 temperature-sensitive (ts) mutant, and thus lacked functional ICP4 protein (Pol II-ts 39). (2) Transcription from the ßτ5 promoter, however, occurred preferentially in a system prepared from cells that contain proteins normally present at 8hrs post-infection (Pol II-H). Transcription from this ßτ promoter was also observed in systems prepared from cells infected with the α4 ts mutant at the permissive temperature (Pol II-ts32). (3) A transcription system prepared from cells containing all viral proteins normally produced following removal of a translation block due to cycloheximide (Pol II-CR), however, transcribed well from the α, ß and the ßτ5 promoters.

Thus, using an in vitro system I have simulated differences that exist in preferences for transcription from

the different classes of HSV-1 promoters, by the cellular transcription machinery, during HSV-1 infection.

To test whether the promoter selectivity demonstrated by the transcription systems prepared from mock-infected and 8 hrs HSV-1 infected cells (Pol II-M and Pol II-H, respectively) used in Part II was due to specific proteins, I have carried out a preliminary biochemical dissection of these transcription systems (Part III). WCEs from mock-infected and HSV-1 infected cells were chromatographically fractionated by the method of Jones et al. (150) over heparin-agarose, DEAE Sepharose-CL6B and Sephacryl S-300 columns. The fractions isolated were then added individually to Pol II-M, and assayed for their ability to confer on Pol II-M the transcriptional features observed with Pol II-H.

From such assays, infected cell fractions eluted at 0.225M and 0.5M KCl from the DEAE Sepharose CL-6B column (fractions H.2 and H.5) were identified as the fractions able to inhibit transcription from the α and ß promoters by Pol II-M, giving a transcriptional pattern similar to that observed with Pol II-H on these templates. These fractions were also shown to activate transcription from the ßτ5 promoter by Pol II-M, in a manner similar to that observed with Pol II-H. Since these activities could not be demonstrated with the corresponding fractions from mock-infected cells (namely fractions M.2 and M.5), it may be inferred that the Pol II-H-like properties conferred upon Pol II-M by fractions H.2 and H.5 are due to specific infected cell polypeptides.

These active infected cell fractions were then analysed by immunoblotting to determine the HSV-1 proteins present in them. Using monoclonal antibodies, the HSV-1 immediate-early proteins ICP0 and ICP4 were identified in fractions H.2 and H.5. By using an antiserum made to 7hr HSV-1 infected cell polypeptides, the early proteins ICP6 and ICP8 were also demonstrated in these fractions.

The cumulative results indicate that 8 hr HSV-1 infected cell proteins can specifically inhibit transcription from α and ß promoters, and activate transcription from the ßγ5 promoter. Furthermore, three of the presently hypothesised HSV-1 regulatory proteins - ICPs 0, 4 and 8 (193) - along with ICP6 have been identified in the active infected cell fractions.

**Utility of In Vitro Transcription systems.**

In vitro transcription has been developed over the last few years as an alternative method to in vivo gene transfer methods for studying gene regulation. The success of such systems was first observed in the study of adenoviruses gene expression. By using a crude WCE prepared from uninfected cells Weil et al. (329) and Manley et al. (184) acheived successful run-off transcriptions from linearised templates containing the adenovirus major late promoter. The developement of these in vitro transcription systems has resulted in great advances in the field of gene expression, leading to the defining of control regions and transcriptional

factors that are necessary for accurate and selective in vivo and in vitro transcription from a variety of cellular and viral promoters (20, 62, 126, 188, 189, 200, 254, 271, 276, 331). However, attempts to observe regulated in vitro transcription of genes inducible or repressible in vivo have not proved very successful. For example, the whole cell extract transcription systems prepared from cells which do not express globin genes at all, transcribe the embryonic, foetal and adult globin genes with nearly equal efficiencies. Furthermore, although the SV40 late promoters are detectably expressed only after infection (158, 160), Manley systems prepared from uninfected cells utilise both the early and late SV40 promoters with equal efficiency (254). Therefore, although the systems of Manley and Weil can correctly initiate transcription from gene promoters, they do not project a true image of gene expression as it occurs in vivo.

Attempts to use the Manley and Weil transcription systems to demonstrate selective transcription from the different classes of HSV-1 promoters (as observed in vivo during lytic infection), have not proved very successful. The WCE transcription systems prepared from uninfected cells by the method of Manley (184) , or the nuclear extract transcription system prepared by the method of Dignam (71), all show a strong usage of IE (e.g., α22) and DE(e.g., ßgB, ßgD and the ß1.2-kb RNA) promoters (102, 150, 229, 249). However, these systems have also been shown to transcribe well from the LL (e.g., ßτ5) and true-late (τgC) promoters (34, 229, 249).

**Relating the data presented here with current observations on HSV-1 gene transcription.**

A partially purified in vitro transcription system developed by Thomas Beck in this laboratory (17) has, however, shown promoter selectivity of HSV-1 genes, akin to that observed during infection in vivo (18). The initial studies done with this system have shown preferential transcription of the HSV-1 genome repeat regions [which contain the α genes(5, 153, 179, 208, 209, 244, 326, 328)] by a partially purified transcription system from mock-infected cells [Pol II-M] (18). In this dissertation, I have extended these findings to show differential effects on the different classes of HSV-1 promoters by cellular polymerase II (Pol II) made from mock-infected and HSV-1 infected cells. In addition, I have carried out a preliminary chromatographic fractionation of the system, to identify the effectors of this observed promoter selectivity.

The α promoters are known to be transcribed soon after infection by the existing cellular transcription system (168, 169, 233). The fact that transcription of the α genes occurs even in the absence of de novo protein synthesis (40, 144, 153, 180), indicates that the cellular transcription system does not have to be modified by viral proteins for α gene transcription. Numerous experiments with chimaeric genes [containing α promoters recombined with the coding sequences of various viral or cellular genes] have also shown efficient transcription from the α promoters following their

transfection into uninfected cells (232). Transcription from the α promoters was shown to occur in uninfected cells regardless of whether they were part of the viral or cellular genome (127, 181-183, 234).

The results presented here show preferential transcription from the α47 and α0 promoters by a partially purified Pol II preparation from mock-infected cells (Pol II-M). A similar system from 8 hr HSV-1 infected cells (Pol II-H), however, showed negligible transcription from these α promoters. The results of in vitro transcription presented here, therefore, correlate well with the in vivo observations on α gene expression. Transcription in vivo from the α promoters has, however, been shown to be enhanced in the presence of a virion protein VP16 (13, 233). Whether a similar enhancement will be observed by a partially purified transcription system containing this virion protein remains to be seen.

The transcription results presented here also demonstrated an inhibition of transcription from α and ß promoters by Pol II-H (Part II). This lack of transcription from the α and ß promoters could have been due to inactivation of the cellular transcription system in infected cells. However, the efficient transcription from the ßτ5 promoter by the infected cell system observed (Figure 3, Part II) negates this idea. The fact that transcription from the α and ß promoters was specifically inhibited by the addition of infected cell fractions eluted at 0.225M and 0.5M KCl from the

DEAE-Sepharose CL-6B columns at (fractions H.2 and H.5, respectively) to Pol II-M, suggests a specific inhibition in transcription from these promoters by specific infected cell proteins. At late times in infection (7-10 hrs post-infection), transcription from the α and ß promoters is known to be decreased (119). The inhibition of α and ß promoter transcription observed in vitro, either in the presence of 8 hr infected cell polypeptides (Part III), or by a partially purified system from 8 hr infected cells (Part II), correlates well with this in vivo observation.

The ßγ promoters are transcribed at low levels before DNA synthesis, and at high levels following DNA replication (128). Dennis and Smiley (68) have demonstrated that the transfected promoter for the major capsid protein (ICP5 or ßγ5) is activated in <u>trans</u> when the cells are infected with a fully expressive virus. This observation has been extended by Persson et al. (228) who demonstrate a need for viral proteins in addition to ICP4, for optimal ßγ5 transcription. Persson et al. (228) have used a mouse cell line (Z4) which has been transformed with and therefore constitutively expresses the product of the IE gene ICP4 as host cells. In the presence of a cycloheximide block they demonstrated that transcription of the ßγ5 gene was marginal in Z4 cells despite the presence of preexisting ICP4. Removal of the translation inhibitor, however, resulted in translation of the accumulated α, αß and some ß mRNA, and good transcription from the ßγ5 promoter. This demonstrates that ICP4 protein alone is incapable of

optimally activating the ßγ5 promoter. This result also suggests the probable involvement of other ICP4 induced viral or cellular genes (whose products are not made in the presence of cycloheximide) in the activation of ßγ transcription. Cells infected with HSV-1 in the presence of cycloheximide are known to be incapable of expressing many ß proteins. Therefore, the virus in such cells does not undergo replication, an event which requires many ß proteins (120, 264). The marginal transcription from the ßγ5 promoter observed in cycloheximide treated Z4 cells may, therefore, be due to the lack of viral replication in these cells.

The results obtained with the ßγ5 promoter in this dissertation, are in good agreement with all of the aforementioned published in vivo observations on ßγ5 transcription. This study has shown that transcription from the ßγ5 promoter occurs in partially purified systems prepared from cells containing HSV-1 proteins present at 8 hrs post-infection (Pol II-H, Pol II-ts32) or HSV-1 proteins present at 8 hrs post-infection following removal of a 7hr cycloheximide block (Pol II-CR). Pol II-M from uninfected cells, however, showed marginal transcription from the ßγ5 promoter. In vitro systems prepared from cells containing mainly IE proteins, but lacking a functional ICP4 (Pol II-ts39) or containing only the ICP4 and ICP47 proteins (Z4 WCE) also showed no detectable transcription from the ßγ5 promoter. Furthermore, Pol II-M was shown to be able to transcribe from the ßγ5 promoter only upon the addition of 8 hr infected cell fractions H.2 and H.5

(Figure 5, Part III). This demonstrated that the ßτ5 promoter was trans-activated by proteins present in 8 hr HSV-1 infected cells. Therefore, the in vitro transcription results on ßτ5 presented here support the data on regulation of that gene promoter obtained from in vivo studies.

There are, however, two results obtained in the in vitro studies done here, which contradict the results obtained from studies done in vivo.

(1) During normal lytic infection the ß genes (e.g., Tk, gB, gD and ICP8) are expressed 5-7hrs post-infection, and after transcription and translation of the viral α genes have taken place (72, 132, 133, 145, 174, 175, 270). However, as shown in this dissertation, the ß genes were transcribed like the α genes, by Pol II-M and Pol II-ts39. Pol II-H and Pol II-ts32 showed only marginal transcription from the ß promoters. Pol II-CR, however, appeared to transcribe well from the ß promoters. Transcription from the ßTk promoter has also been demonstrated in vitro with Manley transcription systems prepared from uninfected cells (102, 249). Recently, using an uninfected nuclear extract system, Pizer et al. (229) have demonstrated transcription from the ßgD promoter. Therefore, it appears that in vitro the ß genes are transcribed like the α genes.

Numerous transfection studies have shown that expression of ß promoters can also occur in uninfected cells. Chimeric constructs bearing the ßTK, ßgD or the ßICP8 promoter have been shown to be efficiently transcribed in cells that do not

contain any viral proteins (8, 84, 171, 194-199, 218, 251, 331). However, in all cases a marked stimulation in transcription from the transfected ß promoter was observed in the presence of a fully expressive superinfecting HSV-1, or the IE proteins ICP0 and ICP4 supplied in trans (80, 85, 218-221). This, therefore, implies that ß promoters although transcribed by the cellular unmodified Pol II, are further stimulated in the presence of viral IE proteins. The enhanced ß transcription observed with Pol II-CR (which was made from cells that contain proteins made immediately following a cycloheximide block reversal) in this study, supports this idea.

(2) From the 1.9-kbp Bam HI-Z template [which contains the α47 promoter (326)], a smaller 0.65-kb product is transcribed preferentially by Pol II-M, rather than by Pol II-H (Figure 1 Appendix, Figure 2 Part III). This 0.65-kb transcription product has been named "RNA B" (256), and shown kinetically to originate from a τ promoter (US11) situated downstream from the α47 promoter (148). Based on the success observed with our in vitro transcription system on mimicing in vivo gene expression from α and the ßτ5 promoters, this τ promoter would be expected to be transcribed preferentially by Pol II-H rather than than by Pol II-M. Furthermore, as observed with the α and ß promoters, transcription from the 0.65-kb promoter by Pol II-M is inhibited by the addition of infected cell fractions H.2 and H.5. Therefore, the in vitro transcription results argue against the 0.65-kb RNA

originating from a downstream τ promoter on the Bam HI-Z template. Since there is an AT-rich region in the vicinity of the US11 promoter (209, 326), the transcription results presented here predict a termination of transcription from the α47 promoter at this region. However, Pol II-M initiating at the upstream α47 promoter may also reinitiate competently at the downstream τUS11 promoter. Whether the 0.65-kb RNA observed in transcription from the 1.9-kbp Bam HI-Z template is a α47 termination product or an aberrantly transcribed τUS11 product however, awaits resolution by further transcriptional assays.

**Probable Mechanisms for Gene Expressions in Eukaryotic Systems.**

The study of transcriptional regulation has been an important area of research in the last few years. A eukaryotic system that has been widely used for the study of gene regulation is that of the adenovirus. The adenovirus has been particularly useful for such studies because the regulatory gene (E1A) that mediates major regulation of adenovirus transcription is identifiable and available (151). Furthermore, besides the early and late adenovirus genes, several heterologous viral and cellular promoters are responsive to regulation by E1A (104, 154, 158, 211, 295, 303). Therefore, the E1A-regulated transcription in adenovirus has been an attractive and useful system for study of gene expression.

Correlation of HSV-1 Gene Regulation with that in Adenovirus, and Homology between the ICP4 and E1A Proteins.

The HSV-1 transcriptional regulatory circuits have many similarities with those present in adenovirus. The genome of both viruses is temporally expressed. In both cases, at the onset of infection only a small segment of the viral genome is expressed (54, 79, 169, 318). The products of these genes (the Ad E1A protein and the HSV-1 IE proteins) are important in the transcriptional activation of the other viral genes (29, 54, 72, 95, 133, 176, 213, 267). These proteins are also involved in the repression of transcription from their own and other genes belonging to temporally similar classes (29, 133, 225). In other words, these proteins mediate both the positive and negative regulations observed during the coordinated expression of their genome. The regulatory proteins of the two viruses are therefore, quite similar in function. The E1A protein can also be replaced in its regulatory functions by the IE protein of Pseudorabies virus and the ICP4 protein of HSV-1 (89, 140, 310). The E1A protein can, therefore, be termed as the adenovirus equivalent of the HSV-1 IE protein ICP4. Thus, though not obligatory, the ICP4 mode of action (as yet unknown) may be similar to one or more of those suggested for E1A. Therefore, models hypothesised for E1A activity may be extrapolated to accomodate ICP4 activity keeping in mind, however, the genetic unrelatedness between the two viruses.

Models for HSV-1 gene regulation.

The E1A protein has been shown to be replacable in function by the PRV IE protein and the HSV-1 IE protein ICP4 (89, 140, 310). Despite the similarity in their functions, it is difficult to envision common target sequences for the adenovirus and herpesvirus proteins existing in the promoters activated by them. It is equally difficult to envision similarities in specificity existing among the regulatory proteins of such genetically unrelated viruses. These points, therefore, argue against a direct DNA-protein interaction for the activation of transcription from the various promoters by these different viral regulatory proteins. It does instead indicate the involvement of pre-existing cellular factors in transcription from promoters apparently transactivated by viral proteins.

Suggestions of cellular factors being involved in the transcription of viral genes, is supported by recent studies showing that regions in the E2A gene promoter known to be critical for E1A induction (139, 141), are also required for basal transcription from this promoter, and are bound by a cellular factor. Loeken et al. (178) have demonstrated that this site contains a pair of perfect repeats flanking the pentanucleotide upstream CCAAT, box found in the upstream region of many eukaryotic genes (70), which also binds a specific cellular factor (150). This predicts the interaction between different cellular factors bound to separate regions on the promoter, for efficient transcription to occur.

Sawadoga and Roeder (276, 277) have also shown that for transcription (both in vivo and in vitro) from the adenovirus major late promoter (MLP), uninfected cell factors USF and TFIID (190) must bind to an upstream sequence (US) and the TATA box, respectively. However, since correct transcription from the MLP occurs only late in infection and requires E1A, Sawadoga and Roeder (278) postulate that E1A (directly or indirectly) causes an increase in the amount of pre-existing transcription factors, or a modification of the proteins. Either way, this ensures a better binding of these cellular factors to their respective sequences, resulting in either a conformational change in the DNA or in one or more of the DNA-bound cellular factors. Either of these modifications creates a better signal for binding of Pol II or interaction of the transcription complex with another transcriptional factor.

Recent studies with eukaryotic promoters have demonstrated that the promoter elements required for basal Pol II transcription correspond to DNA binding sites for cellular factors (62, 76, 77, 108, 150, 192, 271). The data from E2A gene transcription studies, therefore, suggests that sequences (and perhaps the factors) required for basal and induced transcription may be the same. Induction would be due to facilitation of a transcriptional step or factor that is rate-limiting.

(a) A factor required for viral gene transcription may be present in host cells in limiting concentrations, thus allowing basal levels of transcription to occur from the

promoter. In the presence of the inducing protein, this factor may be increased to amounts required for optimal transcription, resulting in induced levels of transcription from that promoter.

(b) A cellular transcriptional complex may be inefficiently assembled on a viral promoter, thus allowing low levels of transcription to occur. The inducing protein may modify the factor(s), and by increasing its affinity for DNA or another factor in the complex, cause a more efficient complex to be formed. This efficient complex may then induce higher transcription from that same promoter.

Studies on SV40 late (L) gene expression demonstrate that efficient transcription from L promoters requires binding of cellular factors to two cis control regions, that must be critically spaced apart (158, 178, 255). Therefore, this also appears to support the model for cooperative action between different DNA-bound proteins for efficient transcription.

The same postulate may hold for ICP4 mediated activation of ß and ßτ genes. The HSV-1 ßTk gene is an ICP4-inducible gene (105, 221, 228). However, the control regions of Tk do not bind ICP4 (80, 96). The Tk control region do, however, bind the general transcription factor Sp1 [which binds to a hexanucleotide sequence GGGCGG (76, 108)] and the CCAAT Transcription Protein or CTF [which binds to the CCAAT box (150)]. The ßτ5 promoter, also inducible by infected cell proteins, contains sequences which could theoretically bind the CTF and Sp1 factors (317). Both gene promoters contain a

TATA box (197, 317, 320) and may possibly bind a TATA-specific factor (62, 190, 271). Purified ICP4 thus far has not been demonstrated to bind to either of these control regions (96). It may, therefore, be suggested that in the cell sequences within the ß and ßτ transcription control regions are bound by pre-existing cellular transcription factors resulting in basal level transcription from these promoters. However, in the presence of HSV-1 IE protein(s), these cellular factors may be modified such that their binding to the specific DNA sequences is enhanced. Alternatively, the HSV-1 proteins may themselves bind to the DNA-bound cellular proteins by a protein-protein interaction, thereby improving the binding of the cellular transcription assembly to the DNA. The improved and more stable binding of these cellular transcription factors may then result in a conformational change in the DNA, allowing Pol II or other required transcription factors to bind better. Data obtained from band competition studies on the ßτ5 promoter with WCEs or DEAE-Sepharose CL-6B 0.2M fractions from mock-infected and infected cells (D. Tomar, J. Lahiri, K. E. Sampson and R. L. Millette, manuscript in preparation) support an involvement of cellular proteins in the transcription of the ßτ5 gene. In these studies a ßτ5-specific complex of similar elecrophoretic mobility was produced with proteins from both mock-infected and infected cells. This suggests the formation of a ßτ5-specific transcription complex formed by pre-existing cellular proteins. Activation in infected cells would then occur via a

quantitative increase or a virally induced enhanced specificity of the cellular factors for the ßT5 DNA.

The HSV-1 α genes have also been shown to be involved in repression of the IE genes. By competition assays on chimeric constructs that rely on enhancer elements (69) for their activation, Borrelli et al. (24) have provided evidence that the enhancer element [known to be present in multiple copies in the 5' untranscribed region of the E1A gene (22, 139)] is the target for E1A repression. The HSV-1 α genes have been shown to contain enhancer sequences in their upstream regions (183). If indeed the mode of ICP4 action proves to be similar to that of E1A, it may also be postulated that the repression of IE gene by ICP4 is via these enhancer regions.

**Conclusions.**

From the in vitro transcription results obtained using both partially purified RNA Polymerase II preparations and WCEs (Part II) the following conclusions may be drawn.

(1) No viral gene products are required for in vitro transcription from α and ß promoters.

(2) Viral proteins in addition to ICP4 are required for optimal transcription from the ßτ5 promoter.

(3) A functional ICP4 protein is, however, required for transcription from the ßτ5 promoter.

(4) Proteins present in 8 hr HSV-1 infected cells and in cycloheximide reversed infected cells are necessary for transcription from the ßτ5 promoter.

(5) Proteins present in 8 hr infected cells are involved in the repression of transcription from α and ß promoters.

Chromatographic fractionation of mock-infected and 8 hr HSV-1 infected cell extracts has led to the identification of protein fractions from infected cells that are involved in the regulation of in vitro transcription from α, ß and the ßτ5 promoters.

(1) An infected cell fraction was identified that allowed optimal transcription from the ßτ5 promoter by a partially purified Polymerase II preparation from mock-infected cells (Pol II-M) in in vitro transcription stimulation assays.

(2) By similar transcription assays an active component required for the shut-off of transcription in vitro by Pol II-M from α and ß promoters was identified in infected cell fractions.

(3) Immunoblotting of these infected cell fractions has identified ICP0, 4, 6 and 8 in these active fractions.

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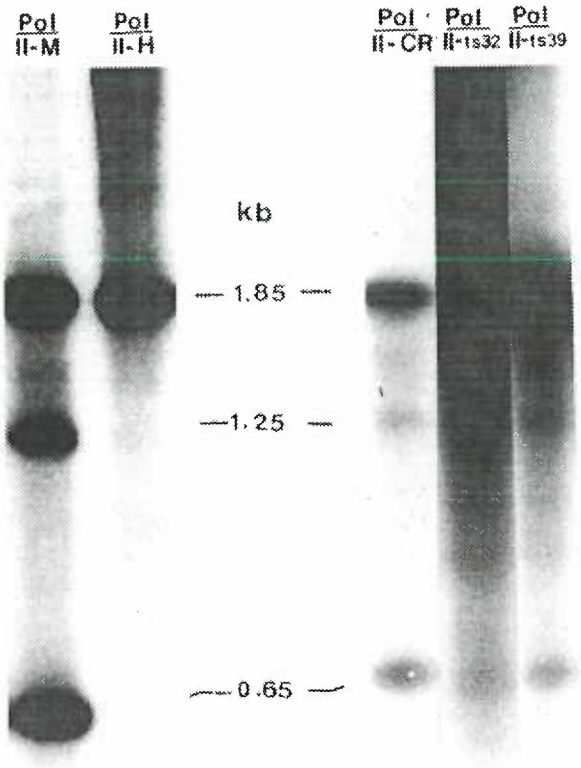
# APPENDIX I (to Part II)

**FIGURE A. In vitro transcription of IE-5 (ICP47) RNA.** The 1.9-kbp Bam HI-Z template containing the IE-5 (ICP47) promoter was transcribed in vitro with partially purified RNA polymerase II preparations from HEp-2 cells that were mock-infected (Pol II-M), infected with HSV-1 for 8 hrs (Pol II-H), infected with HSV-1 for 8 hrs with cycloheximide present for 0-7h postinfection, infected for 8 hrs with the ICP4 temperature-sensitive mutant tsLB2 at the permissive temperature (Pol II-ts32) or at the non-permissive temperature (Pol II-ts39). Relative position of the IE RNA-5 on the Bam HI-Z fragment (inverted prototypic orientation) is shown at the bottom. Size of the DNA template and the transcription products are given in kb.

# Transcription of IE-5 (ICP47) RNA



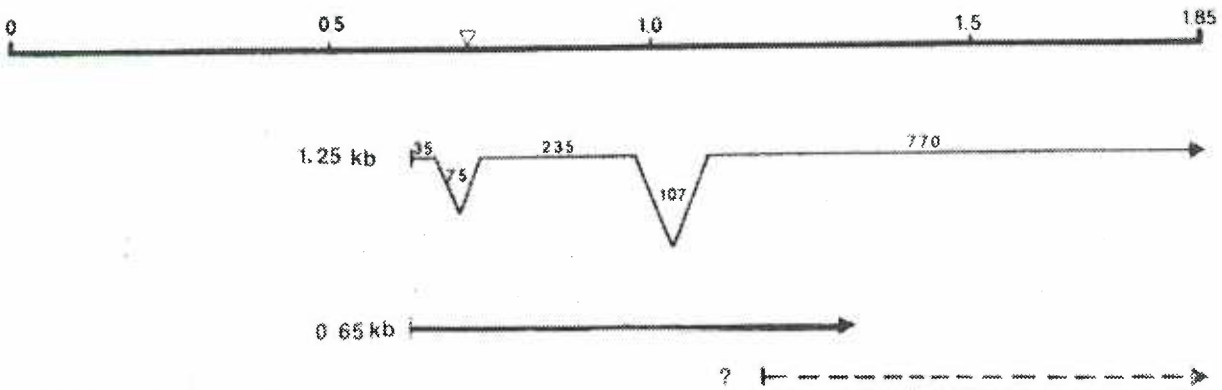




Fig.A

**FIGURE B. In vitro transcription of ß1.2-kb RNA.** The 0.8-kbp Hind III-Bgl II template (m.u. 0.587-0.592) containing the ß1.2-kb RNA promoter, was transcribed with partially purified RNA polymerase II from HEp-2 cells that were infected in the presence of a cycloheximide block which was removed 1h before harvesting (Pol II-CR), mock-infected cells (Pol II-M), 8 hrs HSV-1 infected cells (Pol II-H), and cells infected with the α4 temperature-sensitive mutant at either the permissive temperature (Pol II-ts32) or at the non-permissive temperature (Pol II-ts39). The mapping of the ß1.2-kb RNA on the Hind III-Bgl II template (prototypic orientation) is shown below. The size of the template and the transcription products is given in kb.

# Transcription of β1.2 kb RNA

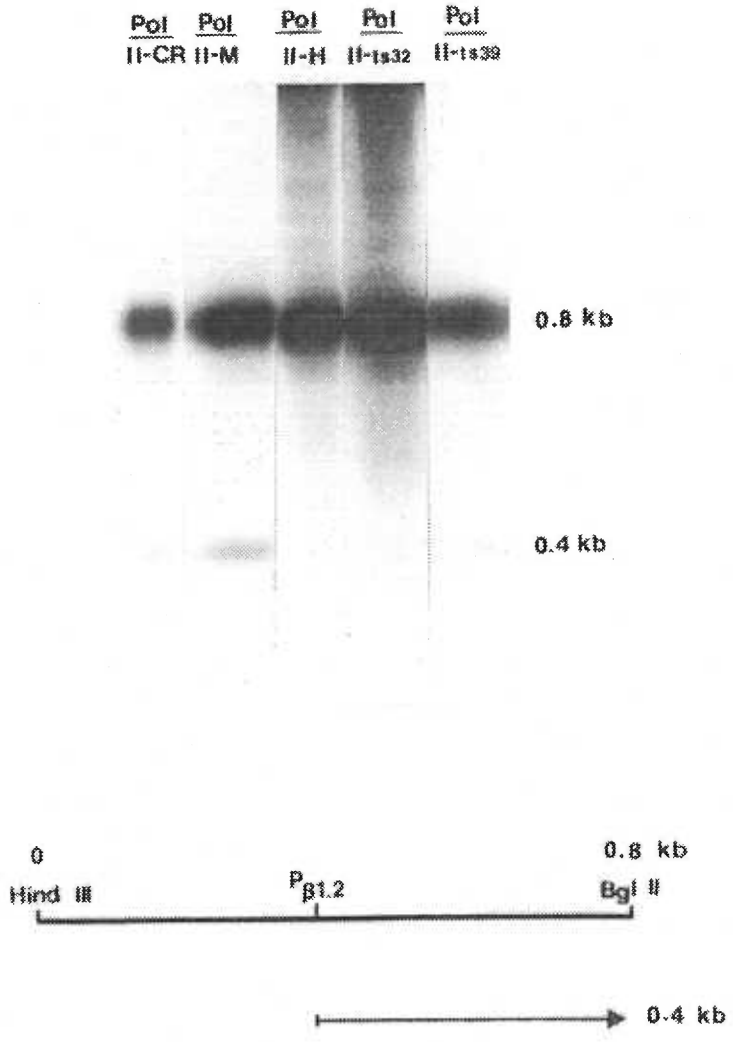
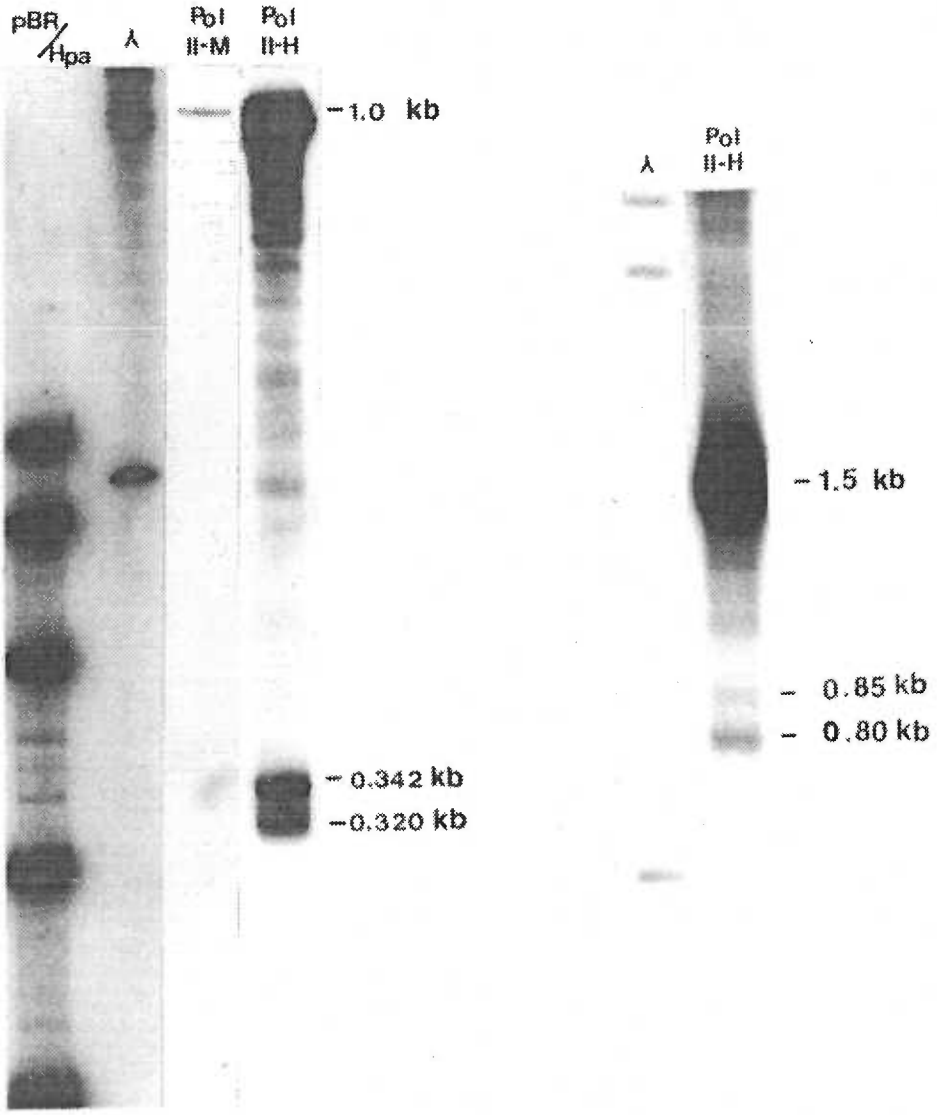



Fig.B

**FIGURE C. Transcription from the βτ promoter on truncated templates.** The 1.7-kbp Sal I template containing the βτ5 promoter (Part II, Figure 3) was restricted with the restriction enzymes Hpa I and Hind III (to give a 1.2-kbp fragment) or with Sal I and Pvu II (to generate a 1.0-kbp fragment). Both truncated DNAs were used in in vitro transcriptions with partially purified RNA polymerase II prepared from mock-infected (Pol II-M) and 8 hrs HSV-1 infected (Pol II-H) cells, to confirm the start sites of the 1.0-kb and 1.1-kb products transcribed from the 1.7-kbp Sal I template. The mapping of the βτ5 transcript as published (), is shown below. Template and transcription product sizes are given in kb.

# βγ5 (ICP5) Truncation



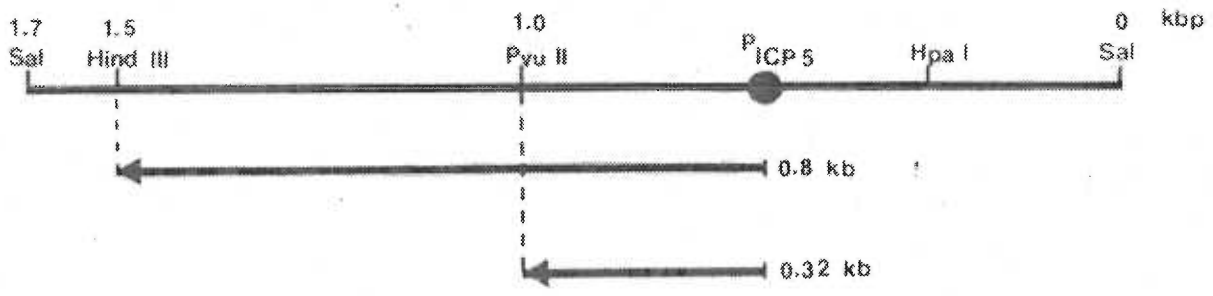
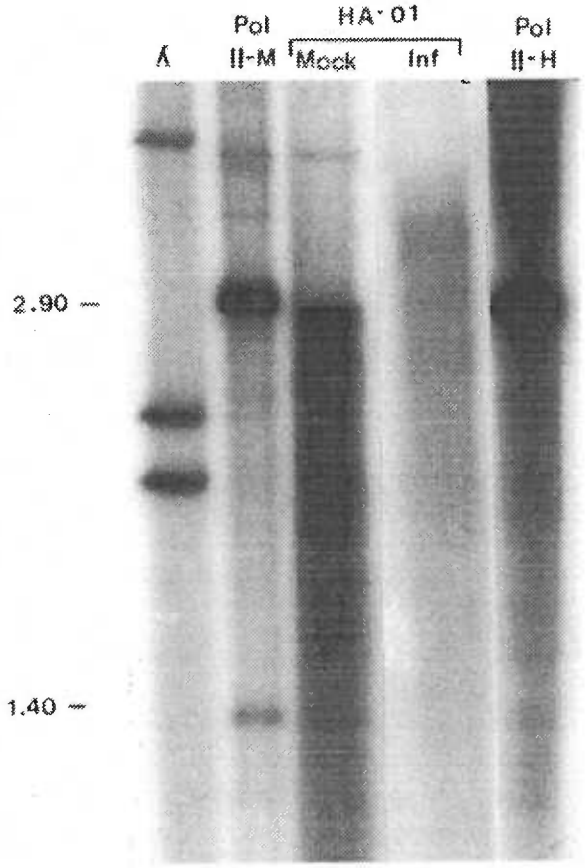




Fig.C

## APPENDIX B (to Part III)

**FIGURE A. Effect of the heparin-agarose (HA) 0.1M Fractions on αO transcription.** The 2.9-kbp Eco RI-Sal I template containing the IE-1 (ICP0/αO) promoter (Part II) was used in transcriptions with partially purified polymerase II preparations from mock-infected cells (Pol II-M) supplemented with HA 0.1M KCl fractions from mock-infected and 8 hrs HSV-1 infected HEp-2 cells. The lanes Pol II-M and Pol II-H show the transcription products from this template using unsupplemented RNA polymerase II systems from mock-infected (Pol II-M) and 8 hrs HSV-1 infected (Pol II-H) HEp-2 cells.

# Effect of HA-0.1 Fractions on αO Transcription



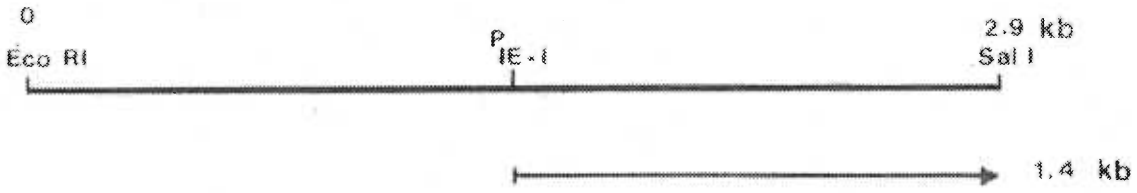




Fig.A

**FIGURE B. Effect of the DEAE-Sepharose CL-6B (CL-6B) column fractions on ß1.2-kb RNA transcription.** The 0.8-kbp Hind III-Bgl II template containing the 1.2-kb ß RNA was used in in vitro transcriptions with partially purified polymerase II from mock-infected cells (Pol II-M). Reaction mixtures were supplemented with CL-6B fractions from mock-infected (M.1, M.2, M.5) and 8 hrs HSV-1 infected (H.1, H.2, H.5) cells. Transcription with the H.2 fraction contained 1mM $CaCl_2$ and 0.5mM $ZnCl_2$ to counteract the degradative action of a DNase present in infected cell extracts. Transcription of the 0.8-kbp Hind III-Bgl II template was also carried out with partially purified polymerase II from mock-infected (Pol II-M) and 8 hrs HSV-1 infected (Pol II-H) cells. The size of the template and transcription products are given in kb.

# Effect of CL-6B Fractions on β1.2-kb RNA Transcription

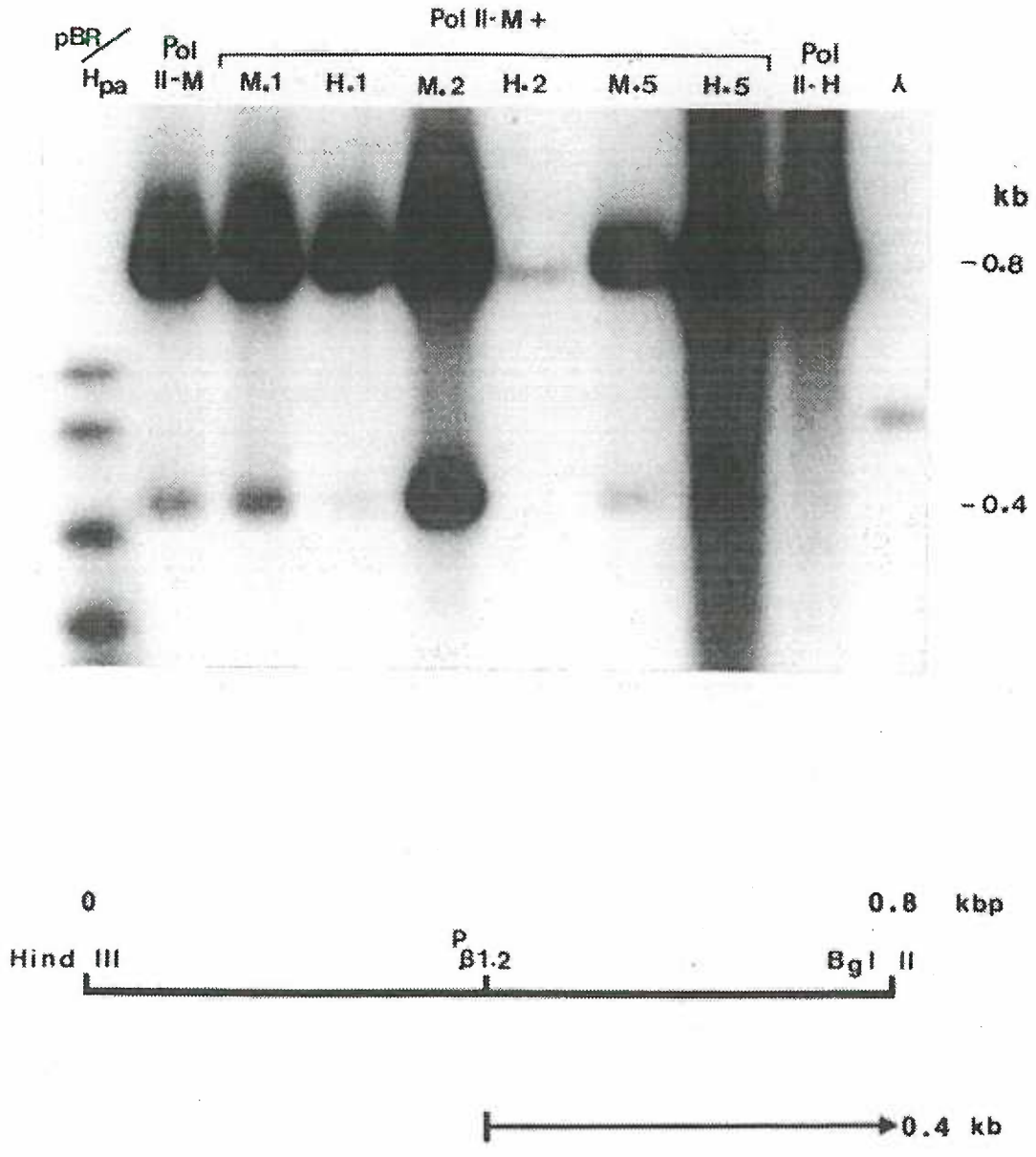



Fig.B