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**ACTIVATION OF THE ENDOGENOUS ALPHA-GLOBIN GENE IN  
NON-ERYTHROID CELLS BY HERPES SIMPLEX VIRUS**

**By**

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**A Thesis**

**Submitted to the School of Graduate Studies  
in Partial fulfillment of the Requirements  
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## **Abstract**

During lytic infection, herpes simplex virus (HSV) usurps the host cell machineries to facilitate viral gene expression, and executes viral-directed host shutoff activities to strongly inhibit expression of endogenous cellular genes. Our laboratory has previously observed that, in contrast to the fate of the majority of host genes, the  $\alpha$ -globin gene in non-erythroid cells is activated upon HSV infection. This finding is intriguing not only because this cellular gene escapes the viral host shutoff functions, but also because this tissue-specific gene is normally repressed in non-erythroid cells. The series of studies presented in this thesis document the characterisation and analysis of this phenomenon. HSV immediate-early proteins ICP0 and ICP4 were each found to be capable of inducing expression of the previously silent  $\alpha$ -globin gene in non-erythroid cells. ICP4 was the dominant viral transactivator responsible for transcriptional activation of the  $\alpha$ -globin gene during infection, whereas ICP0 may indirectly activate expression of this gene by disrupting its tissue-specific regulation. Efficient accumulation of  $\alpha$ -globin transcripts in HSV-infected cells also required the presence of ICP22 which appeared to regulate expression of the  $\alpha$ -globin gene at a post-transcription level. In addition, ICP27 induced accumulation of unspliced  $\alpha$ -globin premRNA. Analysis of the chromatin structure of the endogenous  $\alpha$ -globin gene showed that an array of nucleosomes are positioned along the coding region of this gene. Furthermore, activation of the  $\alpha$ -globin gene by HSV resulted in subtle alteration of the positioning of the nucleosome located at the TATA box and transcription start site of the gene, suggesting the positioning of this nucleosome may be involved in the repression of this gene in non-erythroid cells. Taken together, the results from this thesis project showed that at least four viral proteins are involved in the activation and expression of the  $\alpha$ -globin gene in HSV-infected cells and this activation may be mediated by alteration of the chromatin structure of the endogenous  $\alpha$ -globin locus.

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## **List of abbreviations**

<b>AMP</b>	<b>adenosine mono-phosphate</b>
<b>aniso</b>	<b>anisomycin</b>
<b>aph</b>	<b>aphidicolin</b>
<b>ATP</b>	<b>adenosine tri-phosphate</b>
<b>bp</b>	<b>base pair</b>
<b>C</b>	<b>Celsius</b>
<b>ca.</b>	<b>circa</b>
<b>CAT</b>	<b>chloremphenicol acetyl transferase</b>
<b>CHX</b>	<b>cycloheximide</b>
<b>CPM</b>	<b>counts per minute</b>
<b>CsCl</b>	<b>cesium chloride</b>
<b>CTD</b>	<b>carboxy-terminal domain</b>
<b>CTP</b>	<b>cytosine tri-phosphate</b>
<b>DMSO</b>	<b>dimethylsulfoxide</b>
<b>DMS</b>	<b>dimethylsulfate</b>
<b>DNA</b>	<b>deoxyribonucleic acid</b>
<b>DNase</b>	<b>deoxyribonuclease</b>
<b>ds</b>	<b>double-stranded</b>
<b>DTT</b>	<b>dithiothreitol</b>
<b>E</b>	<b>early</b>
<b>EDTA</b>	<b>Ethylenediaminetetra-acetic acid</b>
<b>EGTA</b>	<b>Ethylene bis (oxyethylenitrilo) tetra-acetic acid</b>
<b>EtBr</b>	<b>ethidium bromide</b>
<b>EtOH</b>	<b>ethanol</b>
<b>Fig.</b>	<b>figure</b>
<b>GTP</b>	<b>guanosine tri-phosphate</b>
<b>h</b>	<b>hour(s)</b>
<b>hpi</b>	<b>hours post infection</b>
<b>HS</b>	<b>hypersensitive</b>
<b>HSV</b>	<b>herpes simplex virus</b>
<b>ICP</b>	<b>infected cell protein</b>
<b>IE</b>	<b>immediate-early</b>

<b>kb</b>	<b>kilobase</b>
<b>kD</b>	<b>kilodalton</b>
<b>L</b>	<b>late</b>
<b>LAT</b>	<b>latency associated transcript</b>
<b>LCR</b>	<b>locus control region</b>
<b>MEL</b>	<b>mouse erythroleukemia</b>
<b>mg</b>	<b>milligram</b>
<b>μg</b>	<b>microgram</b>
<b>M</b>	<b>molar</b>
<b>min</b>	<b>minute(s)</b>
<b>ml</b>	<b>milliliter</b>
<b>MOI</b>	<b>multiplicity of infection</b>
<b>mM</b>	<b>millimolar</b>
<b>MNase</b>	<b>micrococcal nuclease</b>
<b>mRNA</b>	<b>messenger RNA</b>
<b>ng</b>	<b>nanogram</b>
<b>nt</b>	<b>nucleotide</b>
<b>oligo</b>	<b>oligonucleotide</b>
<b>ORF</b>	<b>open reading frame</b>
<b>PAA</b>	<b>phosphonoacetic acid</b>
<b>PBS</b>	<b>phosphate buffered saline</b>
<b>PCR</b>	<b>polymerase chain reaction</b>
<b>PFU</b>	<b>particle forming unit</b>
<b>PMSF</b>	<b>phenylmethylsulfonyl fluoride</b>
<b>RNA</b>	<b>ribonucleic acid</b>
<b>RNAP</b>	<b>RNA polymerase</b>
<b>RNase</b>	<b>ribonuclease</b>
<b>RT</b>	<b>reverse transcriptase</b>
<b>SDS</b>	<b>sodium dodecyl sulfate</b>
<b>ss</b>	<b>single-stranded</b>
<b>TE</b>	<b>Tris/EDTA</b>
<b>temp</b>	<b>temperature</b>
<b>TK</b>	<b>thymidine kinase</b>
<b>ts</b>	<b>temperature sensitive</b>

<b>TTP</b>	<b>thymidine tri-phosphate</b>
<b>U<sub>L</sub></b>	<b>unique long</b>
<b>U<sub>S</sub></b>	<b>unique short</b>
<b>UV</b>	<b>ultraviolet light</b>
<b>vhs</b>	<b>virion host shutoff</b>
<b>w/v</b>	<b>weight/volume</b>

## **Preface**

Before diving into the text of this thesis, there are a few explanatory notes for each chapter that I wish to convey to the reader. Chapter one is a general introduction to herpes simplex virus and chapter two contains a number of commonly used laboratory protocols. The next three chapters are written in the form of manuscripts each with their own introductions, materials and methods, results, discussions, and reference sections. Chapter three is a paper published in Journal of Virology in 1997 which has been formatted to comply with the thesis format regulations. Even though the authors listed in this paper include myself, Barbara Panning, and Jim Smiley, the inclusion of this paper in my thesis is entirely appropriate since I performed all the experiments described in this paper and wrote the first draft of the paper. Barbara was the student who first found that the  $\alpha$ -globin gene in HeLa cells was activated upon HSV infection, and Jim made the revisions for the final draft of this paper. In chapter four, I have presented a number of experiments that have not been published, and further experiments may be required before these results are ready to be published. The introduction for this chapter is meant to be an overview of current research on the regulation of both  $\alpha$ - and  $\beta$ - globin genes, and is more in-depth than the mini-introductions usually associated with journal papers. Chapter five is written as a manuscript to be submitted to Journal of Virology. I have performed all the experiments described and have written the current version with some editorial help from Jim. Finally, chapter six is an overall discussion for the thesis project and chapter seven contains the references for the general introduction, materials and methods, and the final discussion sections.



## **Chapter 1: Thesis Introduction**

### **1.1 The Herpesviridae family**

*Herpesviridae* is a large family of double-stranded (ds) enveloped DNA viruses that replicates in the nucleus of infected mammalian cells. Members of this family of viruses are characterised by their distinctive patterns of gene expression during productive infection, and their abilities to establish and maintain latent infections. Over 80 types of herpesviruses have been identified to date, and eight of these are known to infect humans (reviewed in Roizman and Sears, 1996 and Wagner, et al., 1995). Human herpesviruses are the cause of a variety of diseases: infection with herpes simplex virus (HSV) types 1 and 2 results in facial and genital herpetic lesions respectively, and varicella-zoster virus (VZV) causes chicken pox and shingles. Epstein-Barr virus (EBV) is the infectious agent responsible for mononucleosis, and has also been linked to some human cancer diseases. Human cytomegalovirus (HCMV) and human herpesviruses-6 and-7 (HHV-6, HHV-7) are often associated with clinical problems in immunocompromised patients such as those suffering from AIDS. Finally, recent studies have identified a novel herpesvirus found in Kaposi's sarcoma (KS) specimens (reviewed in Ganem, 1997). The presence of this virus, designated as human herpesvirus 8 (HHV-8) or KS-associated herpes virus (KSHV),

correlates well with KS tumours, and its putative causative role is currently under investigation

Herpesviruses are generally classified into three distinct groups ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) based on their tissue-tropism and their growth characteristics (Wagner, et al., 1995). HSV types 1 and 2, as well as VZV, are typical examples of  $\alpha$ -herpesviruses. These viruses are neurotropic but can also replicate in a broad range of host cells. They are characterised by rapid replication cycles which produce high titres in cell cultures. The  $\beta$ -herpesviruses, including HCMV and HHV6, have more restricted host range, and are characterised by lengthy replicative cycles and low titre yields in culture. The  $\gamma$ -herpesviruses, as represented by EBV and KSHV, are lymphotropic, and these two particular viruses have been linked to human cancers.

As noted earlier, herpesviruses can undergo productive replication cycles to yield progeny viral particles, or remain dormant in a latent state of infection. In infected organisms, the latently infected tissues serve as reservoirs for the virus. Triggered by stress or other stimuli, these latent viruses are periodically reactivated to enter limited rounds of lytic growth and infectious viruses are shed to infect other organisms. These two distinct phases of the herpesvirus life cycle have been intensively studied by many researchers and are best understood for the herpesvirus prototype HSV. There are two distinct serotypes of HSV, type 1 and 2, with the former being more

frequently used in research. As most data described here are derived from studies using HSV type 1, a reference to "HSV" in this thesis refers to HSV type 1 unless otherwise indicated. A tremendous amount of information has been gathered over the past few decades of HSV research, and a comprehensive discussion of all topics is beyond the scope of this thesis. In this Introduction, I will provide an overview of HSV lytic and latent growth and pertinent details regarding the functions of some viral proteins.

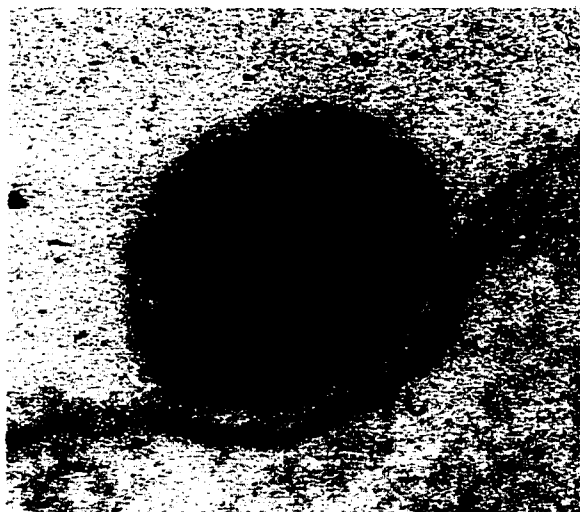
## **1.2 Herpes simplex virus lytic life cycle**

### **1.2.1 HSV virion structure**

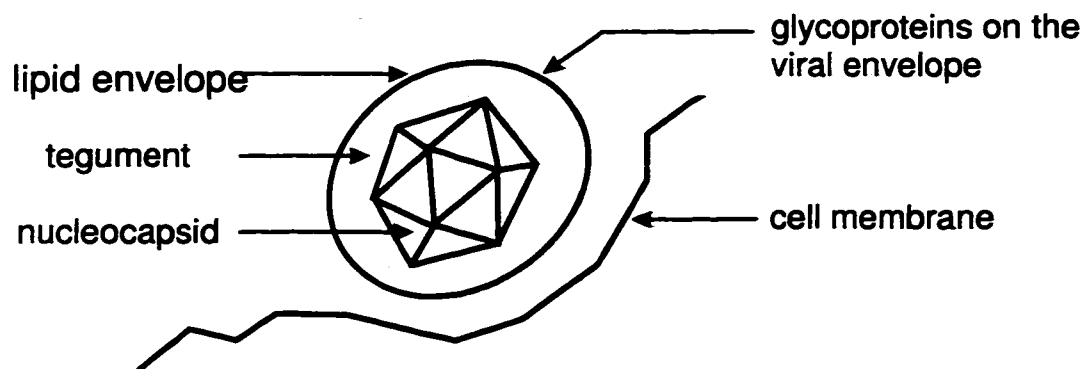
The HSV virion is comprised of four separate elements: the viral DNA core, the proteinaceous capsid enclosing the viral DNA, the tegument surrounding the capsid, and the lipid envelope (schematically represented in Fig. 1.1) The HSV genome is a 152 kb linear ds DNA molecule which has been predicted to contain 72 genes based on sequence analysis (McGeoch, 1991). The actual number of viral proteins encoded by the genome is greater than 80, likely due to additional nested or overlapping open reading frames (ORFs). This genome consists of two unique segments, U<sub>L</sub> (108 kb) and U<sub>S</sub> (13 kb), flanked by separate repeat regions, R<sub>L</sub> and R<sub>S</sub> (Fig. 1.2, reviewed in Roizman and Sears, 1996 and Wagner, et al., 1995). The unique regions with their flanking repeats are separated by additional repeat

**Fig. 1. (A) Electron micrograph of a herpes simplex viral particle (photograph courtesy of Craig Brunetti). (B) Schematic representation of the viral particle shown in panel A illustrating the various components of an HSV particle.**

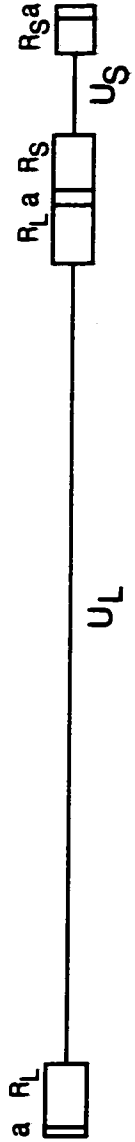
**A**



**B**



**Fig. 2. Schematic drawing representing the HSV genome and the orientations of the U<sub>L</sub> and U<sub>S</sub> regions of the four possible isomers generated by homologous recombination between the repeat sequences. U<sub>L</sub>, unique long region; U<sub>S</sub>, unique short region.**



HSV genome



orientation of the  
4 different isomers



sequences, termed  $\alpha$  sequence, which are involved in the packaging of viral DNA. This organisation of the viral genome and the flanking repeat sequences allow the  $U_L$  and  $U_S$  regions to invert relative to each other, resulting in four possible isomers of the HSV genome which can be found in equimolar ratios in a given virus population.

Early studies on purified virions showed that each virion contains greater than 33 proteins designated as virion polypeptides (VPs) and numbered based on their relative positions on denaturing polyacrylamide gels (Heine, et al., 1974; Spear and Roizman, 1972). Among these proteins are the capsid components that make up the nucleocapsids, a number of tegument proteins that are brought into the infected cell to help initiate viral infection, and a series of glycoproteins present on the viral envelope which mediates interactions between the infectious viral particles and the target host cells. These glycoproteins, some of which are involved in the entry of virus into host cells, are probably the most antigenic components of the virions since they are exposed to the outside environment. In fact, two of these glycoproteins, gE and gI, together form Fc receptors which function to protect the virus from neutralising antibodies by misorienting their binding to the viral envelope (Frank and Friedman, 1989; Johnson, et al., 1988).

### **1.2.2 Viral entry**

Entry of HSV into infected cells is a multistep process involving general adsorption of viral particles to cell surface, attachment of viral proteins to specific cell surface receptors, and fusion of viral envelope with host plasma membrane (reviewed in Spear, 1993). Viral adsorption is primarily mediated by interactions between HSV envelope glycoproteins gB and gC with cell surface heparan sulfate. gC appears to be the principal glycoprotein involved in this process as demonstrated by the findings that antibodies directed against gC can inhibit adsorption of HSV particles to cells (Fuller and Spear, 1985), that purified gC can also bind to cells (Svennerholm, et al., 1991), and that mutants lacking gC adsorb to cells less efficiently than do wild type particles (Herold, et al., 1991; Sears, et al., 1991). However, in the absence of gC, gB can substitute for its function in mediating interaction with cell surface proteoglycans (Herold, et al., 1994). Other studies have demonstrated that heparan sulfate is critical for the initial interaction with HSV particles. First, removal of heparan sulfate from the cell surface either by enzymatic treatment or genetic mutations greatly reduces binding of HSV particles to cells and renders them partially resistant to infection (WuDunn and Spear, 1989; Gruenheid, et al., 1993). Second, exogenously added heparin (a molecule structurally similar to heparan sulfate) can interfere with binding of viral particles to cell surface (WuDunn and Spear, 1989). Third, purified gC and gB molecules from infected cells have been

shown to directly bind to immobilized heparin in affinity column assays (Herold, et al., 1991). Incidentally, cells that lack heparan sulfate are still infected by HSV, albeit at much reduced levels. In its absence, gC and gB can interact with other cell surface proteoglycans such as chondroitin sulfate or dextran sulfate to initiate contact (Banfield, et al., 1995; Dyer, et al., 1997).

Following non-specific adsorption, a second attachment step involving HSV gD and specific cell surface receptor molecules is required before viral entry can occur. Indeed, certain cell lines are resistant to HSV infection, presumably due to the lack of such gD receptors, even though viral particles adsorb to their cell surface efficiently (Shieh, et al., 1992; Subramanian, et al., 1994). Involvement of gD in this process is illustrated by the fact that incubation of susceptible cells with soluble gD can block HSV infection (Johnson, et al., 1990). Furthermore, transfected cells expressing gD are also resistant to HSV infection (Campadelli-Fiume, et al., 1988; Johnson and Spear, 1989), likely due to sequestration of cell surface receptor by the endogenously expressed gD molecules. As this attachment step is required for productive infection, considerable efforts have been made to find this elusive gD-receptor. To date, two candidates, the mannose-6-phosphate receptor (Brunetti, et al., 1994), and the novel receptor molecule named HVEM (herpesvirus entry mediator, Montgomery, et al., 1996) have been identified. The mannose-6-phosphate receptor was identified by biochemical assays as a cellular protein that specifically

interacts with soluble gD molecules, whereas HVEM was isolated by selecting for Chinese hamster ovary (CHO) cell clones transfected with HeLa cell cDNA library that become permissive to HSV infection. A recent study found that HVEM does indeed bind to gD molecules (Whitbeck, et al., 1997), suggesting that it is a bona-fide gD receptor. Their roles in the viral entry process are still under investigation; however, productive HSV infection can occur in cells lacking mannose-6-phosphate receptors or in HeLa cells incubated with HVEM antiserum (Brunetti, et al., 1995; Montgomery, et al., 1996). These data therefore suggest that HSV utilizes multiple cellular receptor molecules for viral entry which perhaps explains HSV's ability to infect a broad range of cell types.

Viral entry occurs in a pH-independent manner (Koyana and Uchida, 1984; Wittels and Spear, 1989), and therefore does not involve the endocytic pathway. Instead, the current hypothesis suggests that attachment of gD to cell surface receptors brings the viral envelope and host plasma membranes into close proximity allowing membrane fusion to occur. Studies using viral mutants have shown that glycoproteins gB, gD, gH, and gL are essential for the membrane fusion process (Cai, et al., 1988; Ligas and Johnson, 1988; Forrester, et al., 1992; Roop, et al., 1993). Their involvement is further supported by the observations that antibodies directed against these glycoproteins do not block attachment of viral particles but prevent subsequent viral entry (Highlander, et al., 1988; Fuller and Spear, 1987; Fuller, et al.,

1989). At present, the role of each glycoprotein and the mechanisms involved in this membrane fusion process are not fully elucidated.

### **1.2.3 Delivery of the HSV genome into infected cell nucleus**

Electron and immunofluorescence microscopy analyses indicated that after fusion of viral and cellular membranes, nucleocapsids are separated from the tegument layers and are transported to the nuclear rim through the cellular microtubule network (Sodeik, et al., 1997). The subsequent steps leading to nucleocapsid uncoating and release of viral DNA into the nucleoplasm are not well defined. It appears that a viral function is required for these processes since a temperature mutant, (HFEM)*tsB7*, accumulates at nuclear pores at non-permissive temperature, and releases the viral DNA into the nucleoplasm only after a shift down to the permissive temperature (Batterson, et al., 1983). This temperature sensitive mutation has been mapped to the HSV-1 U<sub>L</sub>36 gene (Batterson, et al., 1983) which encodes a tegument protein, ICP1/2 (also designated as VP1/2), that is found to be tightly associated with nucleocapsids (McNabb and Courtney, 1992). This protein appears to be involved in the cleavage and/or packaging of newly synthesized DNA in progeny virus (Chou and Roizman, 1989) as well as in the release of the viral genome from nucleocapsid; however, its specific roles in these processes are not well defined.

#### **1.2.4 Initiation of HSV lytic replication cycle**

Upon entry into the host cell nucleus, the linear viral genome circularises, and at least two of the virion tegument proteins, VP16 and vhs (virion host shutoff), help initiate the viral lytic replication cycle (reviewed in Roizman and Sears, 1996; and Wagner, et al., 1995). VP16 (also known as  $\alpha$ TIF or Vmw65) is a transcription activator brought into the nucleus to induce expression of viral immediate-early (IE) genes. Viral gene expression occurs in a tightly regulated cascade which involves sequential expression of the IE, early (E), and late (L) classes of viral genes (Hones and Roizman, 1974). Concomitant with the activation of viral gene expression, the tegument vhs proteins induce disruption of cellular polyribosomes, and non-specific degradation of cytoplasmic mRNA, resulting in a general cessation of host gene expression (reviewed in Fenwick, 1984). This activity not only promotes viral gene expression by minimizing competition from cellular RNA pools, but since the vhs-induced RNA degradation does not distinguish between cellular and viral mRNA (Kwong and Frenkel, 1987), also allows for rapid transition from one phase of viral gene expression to the next.

#### **1.2.5 Viral gene expression cascade and viral DNA replication**

Viral gene expression during the HSV lytic replication cycle is tightly regulated. This expression cascade starts with the

transcription and synthesis of a small number of viral regulatory proteins, the IE gene products, which in turn direct progression of the viral replication cycle. IE genes are defined as viral genes that are expressed in the absence of *de novo* viral protein synthesis due, in part, to their induction by the virion transactivator protein VP16 (Roizman and Sears, 1996; Wagner, et al., 1995). Of the five IE gene products, infected cell protein 47 (ICP47) is the only one not involved in regulation of gene expression. Recent studies showed that it interferes with the cellular peptide transport system, preventing MHC class I presentation of viral antigens on the cell surface of the infected cells (Hill, et al., 1995; York, et al., 1994). The other four IE gene products, ICP0, ICP4, ICP22, and ICP27, are involved in regulating viral gene expression at the transcription or post-transcription levels (see below). E genes are expressed upon activation by IE gene products, and they encode products that are either directly or indirectly involved in viral DNA replication. Studies using viral mutants demonstrated that seven of the E gene products are essential for the replication of viral DNA during HSV infection (reviewed in Knipe, 1989; Weller, 1991). These proteins are the helicase-primase components UL5, UL8, and UL52, the origin DNA-binding protein UL9, the viral DNA polymerase UL30, the polymerase accessory protein UL42, and the single-stranded DNA-binding protein UL29 (or ICP8). Expression of these core components in transiently transfected cells is sufficient for the formation of functional complexes that can replicate co-transfected reporter constructs containing HSV origins of replication (Challberg,

1986; Wu, et al., 1988). In addition to these essential replication proteins, other HSV E gene products are expressed to enhance efficiency of viral DNA replication. These accessory proteins include thymidine-kinase and two separate components of the ribonucleotide reductase which are involved in increasing the deoxyribonucleotide pools in the infected cell, as well as uracil DNA-glycosylase and alkaline exonuclease which are enzymes utilised for repair of newly-synthesized viral DNA. Once sufficient amounts of these E gene products are made, viral DNA replication ensues in the infected-cell nucleus. It has been suggested that HSV DNA replication initially occurs by a theta mechanism and subsequently switches to a rolling-circle mechanism (reviewed in Boehmer and Lehman, 1997); however, the actual mechanisms involved are likely more complex. Early studies demonstrating the presence of HSV genomes without free ends late in infection suggested that newly synthesized viral DNA is generated by a rolling-circle mechanism (Becker, et al., 1978; Jacob, et al., 1979; Jongeneel and Bachenheimer, 1981). This hypothesis is supported by the detection of head-to-tail concatemeric DNA synthesized from reporter plasmids containing HSV origins of replications (Stow, 1982). However, further analyses of the newly-replicated HSV genomes by partial digestion, pulse field gel electrophoresis, and 2-dimensional gel electrophoresis showed that the concatemeric HSV genomes are highly branched, and these complex structures cannot be generated by a rolling-circle replication mechanism alone (Severini, et al., 1994; Zhang, et al., 1994; Severini, et



al., 1996). These branched structures may be generated by a combination of DNA replication and recombination processes. Indeed, examination of the concatemeric DNA demonstrated that consecutive HSV genomes have varying orientations of the  $U_L$  and  $U_S$  regions (Severini, et al., 1994; Zhang, et al., 1994). Insofar as inversion of these regions within the HSV genome is thought to occur by homologous recombination (Delius and Clements, 1976; Hayward, et al., 1975), these findings suggest that HSV DNA replication and recombination occur concomitantly, and these processes may be functionally linked. While hypothetical models have been proposed to explain the generation of the branched concatemeric genomes (Severini, et al., 1996), full understanding of the viral DNA replication mechanism will require further experimental analyses.

During progression of the HSV lytic cycle, viral DNA replication triggers expression of L genes, and expression of these genes requires the functions of ICP4, ICP22, ICP27 (DeLuca, et al., 1985; Sears, et al., 1985; McCarthy, et al., 1989). This class of viral genes can be further divided into two sub-classes: the early-late (EL) and true late (L) genes. EL genes are expressed at low levels prior to viral DNA replication and their expression is greatly augmented upon DNA replication, whereas L genes are transcribed only after viral DNA synthesis has occurred. Although it is not known how viral DNA replication regulates expression of the L genes, it may augment L gene expression by increasing the number of HSV genome templates. In addition,

immunofluorescence studies have demonstrated that viral DNA replication occurs in discrete foci called replication compartments and viral transactivators ICP4 and ICP27 are recruited to these replicative foci at late times during infection (de Bruyn Kops and Knipe, 1988; Knipe and Smith, 1986; Zhong and Hayward, 1997). Therefore, such colocalization of viral DNA and IE transactivators may be required for the efficient expression of L genes. In all, over 30 virion structural components are expressed from the L genes towards the end of the HSV lytic cycle. Once viral DNA and structural proteins are synthesized, viral assembly occurs in the infected-cell nucleus and the progeny viral particles are released from the infected cell to initiate subsequent rounds of HSV infection.

### **1.2.6 Viral capsid assembly and egress**

The capsid of HSV is approximately 125 nm in diameter and is made up of 162 capsomers (reviewed in Newcomb, et al., 1996). It is icosahedral in shape with twelve penton vertices (each made up of 5 capsomers in a 5-fold symmetry arrangement), and twenty faces (each made up of 6 capsomers in a 6-fold symmetry arrangement). Recent developments in the use of recombinant baculovirus expressing HSV capsid components have defined the minimal requirement for this process as expression of six L genes: U<sub>L</sub>19 which encodes viral protein 5 (VP5), U<sub>L</sub>18 which encodes VP23, U<sub>L</sub>38 which encodes VP19C, U<sub>L</sub>35 which encodes VP26, U<sub>L</sub>26 which encodes VP21 and VP24, and U<sub>L</sub>26.5

which encodes VP22a (Newcomb, et al., 1994; Thomsen, et al., 1994). VP5, 19C, 23, and 26 are the structural components that make up viral capsids (reviewed in Newcomb, et al., 1996 and Trus, et al., 1996). VP5 is the major component of capsomers which are physically linked in groups of threes by association with trigonal structures called triplexes (each triplex is, on average, made up of one VP19C and two VP23 molecules). Finally, VP26 molecules are found at the tips of hexons with one VP26 molecule bound to each hexon-associated VP5 molecule. In addition to these structural components, formation of viral capsids requires the scaffold protein encoded by the U<sub>L</sub>26.5 gene, and the maturational protease encoded by the U<sub>L</sub>26 gene. These gene products are first expressed as full length proteins which are cleaved during or subsequent to capsid assembly by the proteolytic activity associated with the amino-terminal of the U<sub>L</sub>26 gene product. Cleavage of the U<sub>L</sub>26 protein gives rise to VP21 and VP24, and cleavage of the U<sub>L</sub>26.5 protein removes the last 25 amino acids of its carboxy-terminal to generate VP22a. Examination of the intracellular location of capsid proteins expressed individually or in combination by transfection or infection with recombinant vaccinia virus showed that VP5 associates with pre-VP22a (the full length product of U<sub>L</sub>26.5) in the cytoplasm and transport of VP5 into the nucleus is dependent on this association with the nuclear-localising pre-VP22a (Matusick-Kumar, et al., 1994; Nicholson, et al., 1994). Combining this information with data from studies using recombinant baculovirus expressing HSV capsid proteins, Thomsen *et al.* proposed a model of

capsid assembly which suggests that once inside the nucleus, the VP5/pre-VP22a complexes polymerise into capsid structures through self-association of pre-VP22a proteins (Thomsen, et al., 1994). The pre-VP22a proteins also serve as scaffolds for proper assembly of the capsid outer shell, and triplexes made up of VP19C and VP23 are brought in to stabilise the assembling capsid shell. The U<sub>L</sub>26 gene products are also recruited into the capsid structure to mediate self-cleavage and processing of pre-VP22a proteins during capsid maturation. Finally, newly synthesized viral DNA are packaged into the empty capsids and during this process, VP22a and VP21 proteins are removed from the complete virions.

As mentioned earlier, newly synthesized viral DNA is concatemeric and highly complex in structure and genetic studies suggested that the HSV encoded alkaline nuclease is involved in the resolution of these branched DNA intermediates (Martinez, et al., 1996). While the precise mechanism responsible for viral DNA cleavage and packaging is still under investigation, the sequence of events for this process has been suggested to involve protein complexes that bind to specific pac sites located within the  $\alpha$  sequence of HSV genomes (Deiss, et al., 1986). This complex links the concatemeric HSV DNA to the awaiting capsid and allows spooling of the viral DNA into the capsid lumen. This packaging machinery is believed to scan for the next  $\alpha$  sequence of like polarity, and direct cleavage and repair of the  $\alpha$  sequences to generate one linear HSV genome per virion

capsid. Once DNA encapsidation is complete, the full capsids are exported out of the nucleus by budding through the inner-nuclear membrane. Through this process, the virions acquire their lipid envelopes and enveloped viral particles can be observed by electron microscopy to accumulate in the perinuclear space between the inner and outer nuclear membranes when viral egress is blocked by genetic mutations or chemical treatments (Baines, et al., 1991; Cheung, et al., 1991). Two contrasting models have been proposed to describe the egress of viral particles from the perinuclear space to the extracellular medium. Johnson and Spear found that chemicals that inhibit glycoprotein processing inhibited egress of viral particles and suggested that the enveloped viral particles physically travel through the cellular secretory pathway (the endoplasmic reticulum (ER) and Golgi apparatus) before being released from the infected cells (Johnson and Spear, 1982). In contrast, based on electron microscopy data, Jones and Grouse proposed that enveloped viral particles at the perinuclear space de-envelope as they fuse with the outer-nuclear membrane and are released into the cytoplasm as naked virion particles (Jones and Grose, 1988). These particles then re-acquire the viral envelope as they bud into the trans-Golgi network prior to their release to the extracellular space. Related to these two models is the central but still unresolved question: are viral glycoproteins processed as the entire viral particle travels through the ER - Golgi network or are they processed as individual glycoproteins prior to viral envelopment at the trans-Golgi membranes. Although supporting

evidence has been reported for both models, neither hypothesis has been definitively proven.

### **1.2.7 Viral proteins involved in regulation of gene expression**

The progression of the HSV lytic cycle is dependent on the host transcription, processing, and translation machinery (reviewed in Roizman and Sears, 1996; Wagner, et al., 1995). HSV promoters are similar to eucaryotic promoter sequences and are transcribed by host RNA polymerase II to produce viral transcripts that are 5' capped and 3' polyadenylated. In addition, at least four lytic-cycle transcripts, ICP0, ICP22, ICP47, and U<sub>L</sub>15 have intron sequences which are dependent on the host splicing machinery for their processing. During the course of infection, HSV produces a small number of viral proteins which regulate viral gene expression at both transcriptional and post-transcriptional levels. Among these HSV regulators, VP16 is perhaps the best known since it has been well studied and widely used as a general transcription activator. In the context of HSV infection, VP16 is both a structural protein as well as a transcription activator. It is brought in as part of the infecting virion to induce expression of the IE genes through the consensus sequence TAATGARAT (where R represents any purine nucleotide) present on all IE promoters (Campbell, et al., 1984; Gaffney, et al., 1985; Kristie and Roizman, 1984; Mackem and Roizman, 1980). By itself, VP16 only has low affinity for this recognition sequence (Kristie and Sharp, 1990); however, when

associated with cellular factors Oct-1 and HCF (host cell factor), this complex avidly binds to the consensus sequence and activates transcription of the target promoter (Gerster and Roeder, 1988; O'Hare and Gooding, 1988; Preston, et al., 1988; Wilson, et al., 1993; Xiao and Capone, 1990). Mutational analysis of VP16 showed that its transactivation activity is mediated by its acidic C-terminal domain, and that the net negative charge of this region contributes to, but is not sufficient for, transactivation (Triezenberg, et al., 1988; Cress and Triezenberg, 1991). Although the precise mechanism for transactivation by VP16 is not known, it likely involves interaction with the transcription initiation complex since experimental evidence indicates that VP16 can bind to TATA box binding factor TFIID, general transcription factor TFIIB, as well as the co-activator protein ADA2 (Stringer, et al., 1990; Lin and Green, 1991; Gupta, et al., 1996; Kelleher, et al., 1990). In addition, VP16 has been shown to physically interact with the transcription factor TFIIH and a cyclin-dependent kinase complex containing CDK8, both of which are involved in the phosphorylation of the carboxy-terminal domain (CTD) of the largest subunit of the RNA polymerase II (Gold, et al., 1996; Xiao, et al., 1994). Insofar as phosphorylation of the CTD has been suggested to be associated with promoter clearance and transcription elongation (Payne, et al., 1989; O'Brien, et al., 1994), VP16 may also enhance transcription of target genes by stimulating the transcription elongation step (Blau, et al., 1996).

VP16-null mutants are non-viable unless cultured in complementing cell lines (Weinheimer, et al., 1992). Interestingly, it seems that the essential function(s) of VP16 during infection is not related to its transactivation ability since HSV mutants that encode full length but transcriptionally deficient VP16 molecules are viable (Ace, et al., 1989). Instead, as demonstrated by Lam *et al.*, VP16 performs two other essential functions at late times post-infection (Lam, et al., 1996). The authors confirmed that VP16-null mutants can initiate viral infection but viral protein synthesis declines rapidly at intermediate times post infection. Furthermore, they demonstrated that cessation of protein expression is due to unregulated activity of newly synthesized vhs molecules in the absence of VP16 by showing that VP16-vhs double mutants have normal levels of viral protein expression throughout infection. These double mutants, however, still fail to propagate without provision of exogenous VP16, indicating that in addition to its roles as a transcription activator and inhibitor of vhs activity, VP16 likely also plays a structural role that is essential for the assembly of progeny virions.

ICP4 is another viral transactivator that is essential for HSV replication. Viral mutants lacking ICP4 are not viable and only express IE proteins, demonstrating that ICP4 is critical for the expression of E and L genes (DeLuca, et al., 1985). Transient transfection studies confirmed that ICP4 can transactivate a variety of HSV and cellular promoters (Everett, 1984; Everett, 1985; O'Hare and



Hayward, 1985), and suggested that this function relies on binding of ICP4 molecules to target promoters since mutations in the DNA-binding domain of ICP4 almost invariably eliminate its transactivation ability (Paterson and Everett, 1988; Shepard, et al., 1989). ICP4 can also repress gene expression which functions to autoregulate its own and other IE gene expression during infection (Michael and Roizman, 1989; Roberts, et al., 1988). Mutation analyses of ICP4 binding sites showed that the repression activity of ICP4 is mediated through high affinity binding sites located close to the TATA box (Leopardi, et al., 1995; Michael and Roizman, 1993), whereas its activation function involves non-specific contacts between ICP4 and DNA (Gu and DeLuca, 1994; Smiley, et al., 1992). Biochemical studies demonstrated that purified ICP4 can physically interact with cellular transcription factors TBP, TFIIB, and TAF250 (Smith, et al., 1993; Carrozza and DeLuca, 1996). While these interactions may be involved in the transactivation function of ICP4, the formation of an ICP4-TBP-TFIIB tripartite complex has been more definitely determined as a requirement for the repression function of ICP4 (Kuddus, et al., 1995).

ICP0 is a promiscuous transactivator which can activate viral and cellular promoters that exhibit basal levels of expression in transient transfection assays (Everett, 1986; Everett, 1987; Gelman and Silverstein, 1985; Mosca, et al., 1987; O'Hare and Hayward, 1985). Unlike ICP4, it does not appear to activate target promoters by direct binding. ICP0-deletion mutants replicate efficiently when cells are

infected at high multiplicity of infection (MOI), suggesting that it is dispensible in cell culture. However, at low MOI, which may be more similar to *in vivo* situations, these mutants are severely defective due to poor expression of E and L genes (Cai and Schaffer, 1992; Chen and Silverstein, 1992). Notably, ICP0 null mutants grown on Vero cells at 6 - 8 h post release from growth arrest or on U2OS cells are significantly enhanced in their plating efficiencies, suggesting that ICP0's function may be substituted by cell cycle- or cell type-dependent activities (Yao and Schaffer, 1995; Cai and Schaffer, 1991). Further analysis of these complementing cellular functions may help in understanding ICP0's mode of action.

While VP16, ICP4 and ICP0 act by affecting the rate of transcription (Beard, et al., 1986; Imbalzano and DeLuca, 1992; Ingles, et al., 1991; Jordan and Schaffer, 1997; Kristie and Roizman, 1984; O'Hare and Hayward, 1985; O'Hare, et al., 1988; Smith, et al., 1993), HSV also encodes proteins that regulate gene expression at the post-transcriptional level. For example, ICP27 has been shown to activate or repress gene expression by affecting the cellular polyadenylation and splicing machineries (see Chapter 5 for a more thorough discussion) (Rice and Knipe, 1988; Su and Knipe, 1989; McLauchlan, et al., 1992; McGregor, et al., 1996; Sandri-Goldin and Mendoza, 1992; Hardy and Sandri-Goldin, 1994; Brown, et al., 1995). Also, ICP22 has been shown to affect the stability of specific mRNAs (Chapter 5, Carter and Roizman, 1996). Interestingly, HSV has been shown to alter the

phosphorylation status of the cellular RNA polymerase II by an ICP22-dependent process (Rice, et al., 1994; Rice, et al., 1995). In addition, a recent paper showed that HSV U<sub>L</sub>13 kinase is involved in the hyperphosphorylation of the translation elongation factor 1delta (Kawaguchi, et al., 1998). Collectively, these findings suggest that HSV not only expresses regulatory factors to regulate viral gene expression, but it also reprograms the cellular transcription and translation machinery to promote viral gene expression.

#### **1.2.8 Functional and physical interaction among ICP0, ICP4 and ICP27**

In addition to their individual regulatory functions, a growing body of evidence indicates that ICP0, ICP4 and ICP27 also interact with and influence the activities of one other. A number of co-transfection assays have demonstrated that ICP0 and ICP4 in combination can activate reporter gene expression to a much greater extent than on their own, suggesting that these two viral proteins can act in synergy (Everett, 1984; O'Hare and Hayward, 1985; Quinlan and Knipe, 1985). This functional cooperation is supported by biochemical data showing that ICP0 and ICP4 physically interact with each other (Yao and Schaffer, 1994). Transfection experiments also showed that ICP27 further positively or negatively modulates expression of reporter genes already activated by ICP0 and ICP4 (Sekulovich, et al., 1988; Sandri-Goldin and Mendoza, 1992). Indeed, ICP4-ICP27 complexes

have been detected in HSV-infected cell extracts (Panagiotidis, et al., 1997), and gel shift assays showed that these complexes bind to reporter TK genes through ICP4 binding sites, a finding which is consistent with the observation that full expression of TK during HSV infection requires the presence of both ICP4 and ICP27 (Samaniego, et al., 1995). During infection, ICP0, ICP4, and ICP27 are predominantly, although not exclusively, found in the nuclei of infected cells where they function to regulate gene expression. Several reports suggested that the activity of these proteins can be indirectly regulated by altering their location within the infected cell. Knipe and Smith first showed that a temperature-sensitive mutant form of ICP4 can inhibit entry of ICP0 into the nucleus (Knipe and Smith, 1986). Also, Gelman and Silverstein found that co-transfection of ICP4 alters the nuclear distribution of ICP0 (Gelman and Silverstein, 1986). In a recent series of reports, Zhu *et al.* used indirect immunofluorescence assays to demonstrate that nuclear localisation of ICP0 is determined by the combined effects of ICP4 and ICP27 in HSV-infected cells (Zhu, et al., 1994; Zhu and Schaffer, 1995; Zhu, et al., 1996). In brief, the authors found that ICP4 promotes the nuclear residence of ICP0, whereas ICP27 inhibits the nuclear localisation of both ICP0 and ICP4. For example, in cells infected with ICP4 defective mutants, ICP0 was found exclusively in the cytoplasm, and, as the authors indicated, this observation may explain the lack of transactivation of viral gene expression by ICP0 in these HSV-infected cells. Therefore,

the directed localisation of these viral transactivators may be an important means of regulating their functions during HSV infection.

### **1.2.9 Alteration of the nuclear architecture by HSV**

Immunofluorescence (IF) studies showed that ICP0 displays a punctate intranuclear staining pattern which, at early times during infection, coincides with pre-existing nuclear structures called nuclear domain (ND) 10 sites (also known as PML nuclear bodies or PML oncogenic domains) (Maul, et al., 1993; Maul and Everett, 1994). These sites, of which there is an average of 10 - 30 per cell, are disrupted upon infection by several DNA viruses including HSV, HCMV, EBV, adenovirus 5, and SV40 (Ascoli and Maul, 1991; Maul, et al., 1993; Doucas, et al., 1996). The cellular function of ND 10 sites is unknown; however they are found to be related to the disease promyelocytic leukemia (PML) (Dyck, et al., 1994), a condition caused by a chromosomal translocation event which results in the synthesis of a novel fusion protein. This protein consists of the N-terminal domain of a protein now called PML protein and the retinoic acid  $\alpha$  receptor (RAR $\alpha$ ) (Dyck, et al., 1994; Koken, et al., 1994). PML protein is normally localised in ND 10 sites, but in promyelocytic leukemic cells, the PML-RAR $\alpha$  fusion protein, along with other ND 10 site resident proteins such as Sp100 and NDP52, are dispersed into hundreds of smaller structures found in both cytoplasm and nuclei of these cells. In HSV infected cells, *in situ* hybridisation and IF studies respectively

showed that input HSV genomes and newly synthesized ICP0 are recruited to the ND 10 sites prior to the viral-induced dispersal of these sites (Maul, et al., 1996). Moreover, studies using mutants lacking ICP0 demonstrated that this viral protein is required for the redistribution process (Maul, et al., 1993), and expression of ICP0 alone, by transfection or by infection with an ICP0-expression adenovirus recombinant, is sufficient to disrupt the ND 10 sites (Everett and Maul, 1994; Maul and Everett, 1994). Two possible explanations have thus far been proposed to explain the link between HSV infection and these intranuclear structures (Maul, et al., 1996). ND 10 sites may contain high concentrations of factors necessary for viral gene expression and replication, and hence several different viruses target to these cellular structures upon entry into cells; or alternatively, ND 10 sites may be part of a cellular defense mechanism which gathers and disables incoming foreign DNA. Consistent with the latter hypothesis, interferons which have anti-viral properties up-regulate expression of ND 10-associated proteins (Guldner, et al., 1992). Furthermore, overexpression of PML protein has been shown to result in suppression in cell growth (Mu, et al., 1994). As implied by Maul *et al.* (Maul, et al., 1996), ICP0 may counteract this cellular defense mechanism. Indeed, mutants lacking ICP0 are defective in viral growth when low MOI of this virus is used to infect cells (Cai and Schaffer, 1992; Chen and Silverstein, 1992). At high MOI, these mutants replicate as efficiently as wild type viruses, and therefore,

this putative defense system may be limited in its capacity to handle large amounts of incoming virus.

It should be noted that these two models are not mutually exclusive. Even though HSV disperses ND 10-associated cellular proteins, it appears to remain associated with the residual nuclear structures as it progresses through early rounds of transcription and replication. Phelan *et al.* found that viral RNA is associated with ND 10 sites, and suggested that transcription of the viral genome takes place at these sites (Phelan, et al., 1997). Also, as mentioned earlier in this Introduction, viral DNA replication occurs at discrete foci within infected cell nuclei, and two recent studies have suggested that these replication compartments are linked to ND 10 structures. First, Maul *et al.* examined the replication of ICP0-defective mutants which do not disperse ND 10 resident proteins, and found that newly replicated viral DNA accumulates next to ND 10 sites (Maul, et al., 1996). Second, Lukonis and Weller used a cotransfection system previously established by Challberg *et al.* (Challberg, 1986) to reproduce these replication compartments in transfected cells and demonstrated these foci preferentially assemble adjacent to ND 10 sites (Lukonis and Weller, 1997). In wild type HSV infection, ND 10 sites are dispersed prior to formation of the replication compartments, so it is not possible to determine whether replication foci are associated with the remnants of the ND 10 sites. However, based on the available data, it is

likely that HSV can usurp pre-existing nuclear structures and use them as centres for viral transcription and replication.

### **1.3 Herpes simplex virus latency**

#### **1.3.1 HSV primary and latent infection**

The ability to establish latent infection in neuronal tissue is one of the hallmarks of HSV infection. In humans, primary infection by HSV is initiated through contact with individuals experiencing active HSV infection, and often occurs in mucosal epithelial cells around the lips (HSV-1) or the genital area (HSV-2). Viral infection proceeds through several rounds of lytic replication and produces cytopathic lesions in the surrounding tissue until the host immune system is activated to limit and reverse the spread of disease. During this period of productive growth, infectious viruses are spread to sensory neurons, such as the trigeminal ganglia, where HSV enters a quiescent state called latency. Following the initial infection and recovery, the latent genomes lie dormant until they are reactivated by a variety of external stimuli. Throughout the lifetime of the infected individual, reactivation or recrudescence can occur repeatedly. Since HSV reactivation does not appear to cause significant, if any, neuronal cell death, this suggests that latent HSV replicates only to a very limited extent in neurons such that the survival of these cells is not



threatened, but is sufficient to spread infectious particles to adjacent epithelial tissues where full-blown lytic infections occur.

### **1.3.2 Animal and *in vitro* models for studying HSV latency**

Due to the nature of latency, this viral process is best examined by animal studies. Several animal models have been developed, but none of them mirror all aspects of the human disease (reviewed in Roizman and Sears, 1996; Wagner and Bloom, 1997). The models closest to the human condition are those using rabbits and guinea pigs. Acute infection of the corneal epithelial tissues of rabbit eyes by HSV results in establishment of latency in trigeminal ganglia of infected rabbits (Nesburn, et al., 1972). After recovery from the initial infection, latent HSV reactivates spontaneously, albeit with low frequency, or can be efficiently induced by epinephrine iontophoresis (Berman and Hill, 1985; Hill, et al., 1987; Kwon, et al., 1981). Reactivated viruses are harvested by eye swabs and examined by culturing on feeder cells. Vaginal inoculation of guinea pigs with HSV has also been employed as a model, although reactivation of latent infection cannot be reliably induced (Bratcher, et al., 1993). Unfortunately, both these models involve significant costs which prohibit their widespread usage.

More economical models using mice have been developed and are commonly used for studying HSV latency. Corneal scarification is

used to infect mice which results in establishment of HSV latent infections in their trigeminal ganglia (Rock and Fraser, 1983; Spivack and Fraser, 1988). *In vivo* reactivation cannot be achieved with this model, but *in vitro* reactivation is efficiently induced by cocultivation of explanted ganglion tissue with feeder cells. To more closely mimic the *in vivo* reactivation process, Sawtell and Thompson devised a procedure involving exposing latently infected mice to hyperthermia (Sawtell and Thompson, 1992). The extent of reactivation is then examined directly on explanted trigeminal ganglia tissues by immunohistochemistry or PCR methods. These different animal models have provided a wealth of information regarding characteristics and viral genes involved in the latency process. However, these different model systems sometimes produce contrasting data, and therefore one must be aware that some observations may be model specific.

As alternatives to animal studies, a number of *in vitro* models using cultured epithelial or neuronal cells have also been developed (O'Neil, et al., 1972; Wigdahl, et al., 1984; Wigdahl, et al., 1984; Harris, et al., 1989). All of these experimental systems rely on inhibiting progression of HSV lytic infection by using viral mutants or through chemical treatments. These "induced" states of latency may be viewed by some as artificial; however, their ease of manipulation and better controlled conditions provide significant advantages over animal

models. A combination of both *in vitro* and *in vivo* studies may be the best way to investigate the molecular details involving HSV latency.

### **1.3.3 Establishment and maintenance of latency**

During latency, viral gene expression from the HSV genome is severely restricted, and the only viral transcripts consistently detected in latently infected cells are the latency-associated transcripts (LATs)(Feldman, 1991). In simplistic terms, HSV latency may be viewed as a failure to enter the lytic replication cascade. This idea is supported by *in vitro* studies which showed that restriction of IE gene expression can lead to a latency-like state of infection. For example, Preston and co-workers found that at low MOI, the VP16 transactivation-defective mutant in1814 is unable to initiate efficient productive infection (Ace, et al., 1989; Harris and Preston, 1991). Under such conditions, only 0.1 - 1 % of infected cells progress through the lytic cycle, whereas the vast majority of infected cells retain the in1814 genome in a quiescent state termed by the authors as *in vitro* latency. Once such "latency" is established, the genome is no longer responsive to subsequent provision of VP16 or to activation by hexamethylene bisacetamide (HMBA) which has previously been shown to complement the in1814 VP16 transactivation defects. These genomes can be recovered by superinfection with HSV which indicates that they are in a silenced but not permanently inactivated state. In the *in vivo* situation, wild type HSV establishes latency only in

neurons. Since neuronal cell cultures can support lytic infection by HSV (Doerig, et al., 1991; Wigdahl, et al., 1984), additional factors must be involved in the establishment of latency *in vivo*. One early theory postulated that VP16 may be lost while the infecting virion is delivered from the peripheral nerve endings to the neuronal nucleus, and therefore become defective in entering the lytic replication cycle (Roizman and Sears, 1987). This hypothesis, however, is no longer in favour since inducing expression of VP16 in latently infected neurons cannot activate latent genomes, nor do transgenic mice expressing VP16 become resistant to latent infection (Roizman and Sears, 1996). At present, it is not known what factors are involved in inducing latency; however, *in vivo* models showed that replication defective HSV mutants can still establish latency (Katz, et al., 1990; Sedarati, et al., 1993; Steiner, et al., 1990), suggesting that viral gene expression and productive replication are not essential for this process.

In latently infected neurons, the HSV genomes are maintained as non-integrated, nucleosome-bound, circular episomes (Mellerick and Fraser, 1987; Deshmane and Fraser, 1989). It appears that no specific viral function is required for maintaining these quiescent HSV genomes in neurons. This is in contrast to another well-characterised herpesvirus, EBV, which persists in lymphatic cells. Latent EBV must replicate its genome in keeping with host cell division, but at the same time limit its gene expression so as not to damage the host. It has therefore developed mechanisms for replicating its viral genome

in synchrony with latently infected cells, and expresses viral proteins that prevent host cells from entering apoptosis (Silins and Sculley, 1995; Sugden, 1994). HSV genomes have had no need to develop such elaborate mechanisms since they reside in relatively inert, non-dividing neurons. By most accounts, maintenance of HSV latency in neurons appears to be a passive process.

#### **1.3.4 Latency-associated transcript**

In this dormant state, viral gene expression is almost completely shut off with the exception of the transcription of latency-associated transcripts (LATs) (Krause, et al., 1988; Rock, et al., 1987; Stevens, et al., 1987; Wagner, et al., 1988). There are two partially colinear LAT species, the 2.0 kb and 1.5 kb LATs, that are easily detected by Northern blot analysis of RNA extracted from latently infected neurons. It has been suggested that the 1.5 kb LAT is a spliced version of the larger LAT since their sequences are almost identical except for an approximately 500 bp region that is missing in the smaller transcript (Spivack, et al., 1991). These LATs map to the repeat region of the HSV genome and are transcribed in the opposite orientation relative to the nearby ICP0 gene. In fact, their 3' ends overlap the 3' end of ICP0 transcripts in the antisense orientation. It is important to note that the 2.0 kb LAT is detected in both latently and productively infected cells (Spivack and Fraser, 1987; Spivack and Fraser, 1988; Wagner, et al., 1988). However, while LATs are the only

transcripts found in the former situation, the 2.0 kb LAT is one of many viral transcripts expressed during the HSV lytic cycle. At present, the function of this transcript in the lytic replication cycle is not known.

Several lines of evidence suggest that the 2.0 kb LAT itself is a stable intron. First, LATs are found in the nucleus of latently infected cells and do not appear to encode any viral proteins (Stevens, et al., 1987). Second, they are neither 5' capped nor 3' polyadenylated (Deatly, et al., 1987; Devi-Rao, et al., 1991). Third, precise mapping of the isolated 2.0 kb LAT by S1 nuclease and RNase protection assays showed that the 5' and 3' ends of this RNA map to canonical splice sites (Wagner, et al., 1988; Wagner, et al., 1988). Fourth, structural analysis of the 2.0 kb LAT showed that it is an endless RNA similar to intron lariats (Wu, et al., 1996; Zabolotny, et al., 1997). Fifth, Farrell *et al.* expressed the primary LAT transcription unit in transfected cells and showed that a stable 2.0 kb intron fragment is generated from the splicing process (Farrell, et al., 1991). At present, some researchers still question whether the 2.0 kb LAT is derived from splicing reactions (reviewed in Fraser, et al., 1992). One nagging concern is that the corresponding mRNA cannot be detected in latently infected cells. Also, skeptics are uneasy with the possibility that the 1.5 kb LAT is an intron within another intron. In spite of these concerns, it is now widely accepted that the 2.0 kb LAT is a stable intron RNA.

It is believed that LATs are derived from 8.3 kb primary transcripts designated as minor LATs (mLATs) in reference to their low abundance in latently infected cells (Mitchell, et al., 1990). This poly (A)<sup>+</sup> transcript was identified by Northern blot analysis in productively infected cells (Dobson, et al., 1989); however, in latently infected cells, its presence can only be detected using more sensitive techniques such as *in situ* hybridisation (Mitchell, et al., 1990). The difficulty in detecting the primary transcript in latently infected ganglia is likely due to the fact that only a portion of neurons harbour latent HSV genomes, and of those cells, only 10 - 30% of them express the stable intron LATs. Mapping of the 5' end of the primary transcript isolated from productively infected cells showed that a TATA box is located 25 nt upstream of the start site (Krause, et al., 1991). Deletion of 200 bp surrounding this TATA box abolishes expression of the 2.0 kb LAT in latently infected cells (Devi-Rao, et al., 1994; Dobson, et al., 1989), supporting the hypothesis that the 2.0kb LAT is derived from the 8.3 kb mLAT. This LAT promoter, also known as latency-associated promoter 1 (LAP1), is located approximately 700 bp upstream of the 5' end of the 2.0 kb LAT, and replacement of the primary LAT sequences with a  $\beta$ -galactose reporter gene showed that this promoter is indeed active during latency (Dobson, et al., 1989). A second TATA-less promoter, designated as LAP2, has also been found just upstream of the 2.0 kb LAT (Goins, et al., 1994). Although this putative promoter can direct transcription of reporter genes in transfection assays, recent studies indicated that it is

only active during productive infection but not in latently infected cells (Chen, et al., 1995).

### **1.3.5 LAT expression and reactivation**

Being the only viral transcripts detected in latently infected cells, many studies have examined whether LATs play a role in HSV latency. The consensus from these studies is that LAT expression is required for efficient reactivation of the latent HSV genome. Viral mutants that have deletions in the LAT promoter and surrounding sequences have repeatedly been shown to be competent in establishing latency in rabbits and mice, but are defective in reactivation upon induction (Block, et al., 1993; Leib, et al., 1989; Hill, et al., 1990; Perng, et al., 1994; Trousdale, et al., 1991). In search of a functional link between LAT expression and reactivation, some researchers initially proposed that LATs may function as antisense inhibitors of ICP0 since the 3' end of LAT introns is partially complementary to the ICP0 transcript, and therefore LATs may regulate expression of ICP0 which could initiate lytic replication in latently infected neurons (Stevens, et al., 1987). However, enthusiasm for this model has greatly diminished because recent experimental data suggested otherwise. For example, LAT-null mutants express wild type levels of ICP0 during productive infection, demonstrating that antisense LATs do not affect expression of ICP0 (Javier, et al., 1988). In addition, two independent studies have shown that mutant viruses that express



truncated primary mLATs, but not LAT introns, are still capable of establishing latency and reactivate with the same efficiency as wild type viruses (Bloom, et al., 1996; Perng, et al., 1996). Therefore, the absence of LAT introns apparently has no effect on HSV latency, but the transcription of the LAT promoter appears to be critical for reactivation.

Bloom *et al.* showed that the viral mutant 17 $\Delta$ 348, which contains a 348 bp deletion starting from 217 nt downstream of the transcription start site of the primary mLAT, is proficient at establishing latency in the rabbit eye model, but is greatly deficient in reactivation after epinephrine iontophoresis (Bloom, et al., 1996). Interestingly, in latently-infected neurons, this mutant expresses primary mLAT and the 2.0 kb LAT intron in amounts comparable to wild type HSV, indicating that LAT expression from this reactivation-deficient mutant is not compromised. Therefore, LAT transcription alone may not be sufficient to insure HSV reactivation. Similar findings were obtained in other studies which showed that several viral mutants that express wild type levels of LAT are nevertheless defective in reactivation in the *in vivo* rabbit eye model (Hill, et al., 1997; Bloom, et al., 1997). From these studies, it appears that reactivation may not only require LAT transcription, but also involve an increase in the transcription rate in response to the reactivation signal.

### 1.3.6 Signals that trigger HSV reactivation

That reactivation may be associated with a shift in the rate of LAT transcription is supported by the fact that cyclic AMP (cAMP) response elements (CREs) present in the LAT promoter appear to be important for the reactivation process. Two upstream CREs are present within 85 nts of the mLAT transcription start site (Leib, et al., 1991; Kenny, et al., 1994), and earlier studies showed that the CRE at position -43 is critical for expression of LAT in transient transfection assays (Leib, et al., 1991). Bloom *et al.* recently found that the reactivation frequency of an HSV mutant containing a 7 bp deletion in this CRE is significantly reduced compared to wild type virus in the rabbit eye model (Bloom, et al., 1997). These data therefore suggest that cAMP levels may enhance transcription level at the LAT promoter to trigger reactivation of the latent HSV genome. Insofar as recrudescence of HSV infections in humans is often triggered by stress, this hypothesis is particularly attractive since it offers a possible link between cellular stress signals, as mediated by a change in cAMP levels, and reactivation of HSV. However, it should be noted that a similar mutant used in the mouse trigeminal ganglia explant model showed only a modest reduction in reactivation frequency (Rader, et al., 1993). It appears that HSV reactivation in the rabbit eye and mouse ganglia explant models may have different requirements. Indeed, the previously described reactivation-deficient phenotype of the 17Δ348 mutant is also specific to the rabbit eye model since this mutant

apparently reactivates efficiently in mouse explants (Bloom, et al., 1996). It is possible that the trauma of explanting ganglia tissues may produce additional stress to reactivate the HSV latent genomes. Further analysis will be required to elucidate the differences in HSV reactivation in the two animal models.

### **1.3.7 ICP0 and HSV latency**

In addition to LAT, the IE gene product ICP0 has also been suggested to play a role in HSV latency. Harris *et al.* first described a requirement for ICP0 in HSV reactivation using *in vitro* latent cultures established by infecting human fetal lung cells with low MOI of HSV type 2 at the supraoptimal temperature of 42°C (Harris, et al., 1989). They found that superinfection with HSV-1 can reactivate latent HSV-2 genomes only if the HSV-1 strains express functional ICP0. Furthermore, recombinant adenoviruses expressing HSV ICP0, but not the control adenoviruses, can also reactivate the latent HSV-2. Therefore, these results strongly argue that ICP0 is required for the reactivation of latent HSV genomes, at least in *in vitro* latently infected cultures. Complementing these findings, Leib *et al.* found that ICP0-null mutants are able to establish latency in mouse trigeminal ganglia, albeit at significantly reduced efficiencies compared to wild type HSV (Leib, et al., 1989). However, these ICP0 defective latent genomes do not readily reactivate from explanted ganglia unless they are treated with DMSO or superinfected with HSV, suggesting that

they are defective in the reactivation process. Cai *et al.* showed similar results using ICP0-defective mutants that retained LAT expression (Cai, et al., 1993), and demonstrated that the defects in latency establishment and reactivation seen with the ICP0-null mutants are not due to secondary deletions in the LAT transcription unit.

In order to efficiently establish latency *in vivo*, HSV must be able to replicate in peripheral tissues in order to spread to neuronal cells. For example, viral mutants lacking ICP4 or ICP27 are unable to complete their viral replication cycles and are severely defective in establishing latency in mouse trigeminal ganglia (Leib, et al., 1989). The need for ICP0 in the establishment of latency most likely reflects its ability to promote efficient replication of HSV during lytic infection, especially in *in vivo* conditions where cells are likely exposed to low MOI of viruses. ICP0-null latent genomes also have defects in their ability to reactivate. At present, it is not clear how ICP0 facilitates this viral process. It may be required for initiating the lytic cycle during reactivation since, in the absence of VP16, ICP0 becomes essential for initiating expression of IE and other viral genes. Such a requirement for ICP0 is seen when infectious HSV genomes are transfected into tissue culture cells. In this situation, VP16, which is normally delivered as part of the virion, is absent, and ICP0-null genomes are severely deficient in initiating viral replication (Sacks and Schaffer, 1987; Cai and Schaffer, 1992). ICP0 may play a second role in reactivation of HSV by disrupting the silencing mechanism imposed

upon the latent genome. In *in vitro* models, once a latent state has been established, HSV genomes do not respond to exogenously added VP16, indicating that they are silenced and maintained in a repressed state. The specific requirement for ICP0 in reactivation of these silenced genomes suggests that ICP0 can alleviate the latent viral genome from a state of repression. Indeed, data presented in this thesis show that ICP0 can bypass the tissue-specific silencing of the cellular  $\alpha$ -globin gene and activate its expression in non-erythroid cells. Therefore, ICP0 may have a general ability to activate genes that are normally under repression.

#### **1.4 Activation of cellular genes by HSV**

The work presented in this thesis follows earlier studies from this laboratory which examined how HSV distinguished between cellular and viral genes. During lytic infection, expression of cellular genes is largely inhibited whereas viral genes are preferentially activated in a cascade manner. However, when taken out of the chromosomal context, cellular promoters are in fact responsive to viral transactivators. Early studies showed that transfected copies of the rabbit  $\beta$ -globin gene are readily activated by HSV IE regulators (Everett, 1984; Everett, 1985). Furthermore, Smibert *et al.* elegantly showed that, while the endogenous  $\beta$ -globin gene in HSV-infected mouse erythroleukemia (MEL) cells is suppressed, a recombinant rabbit  $\beta$ -globin gene present as part of the infecting HSV genome is

robustly expressed and regulated as an HSV E gene (Smibert and Smiley, 1990). This finding strongly argues that HSV does not distinguish between cellular and viral promoters based on DNA sequences, and that the context surrounding a cellular promoter dictates whether it is activated or repressed by HSV.

Experiments using the human  $\alpha$ -globin gene showed that when recombined into the viral genome, this gene is also activated and expressed in an ICP4-dependent manner during HSV infection (Panning and Smiley, 1989; Smiley and Duncan, 1992). Through this series of experiments, it was unexpectedly discovered that HSV infection can activate expression of the endogenous  $\alpha$ -globin gene in human non-erythroid cells. This finding is intriguing since the expression of this endogenous gene during HSV infection is in striking contrast to the fate of most cellular genes which are non-specifically inhibited. Furthermore, the  $\alpha$ -globin gene is normally silent in non-erythroid cells, and its activation upon HSV infection suggests that viral infection must somehow disrupt the tissue-specific regulation of this gene. This thesis documents my studies characterising this phenomenon. In brief, I found that viral transactivators ICP0 and ICP4 are each capable of activating expression of the silenced  $\alpha$ -globin gene, and that they likely do so by distinctly different mechanisms. In addition, structural analyses of the  $\alpha$ -globin genes in non-erythroid cells indicated that HSV does not alter the methylation status of this gene, but appears to affect the accessibility of DNA sequences around

transcription start site. Combined with the finding that a nucleosome is positioned over the TATA box and transcription start site of this gene, these results suggest that transcription of the  $\alpha$ -globin gene in non-erythroid cells is normally restricted by its chromatin conformation. Finally, analyses of the expression of  $\alpha$ -globin RNA in HSV infected cells revealed that ICP22 is required for maximal accumulation of globin transcripts during infection, possibly by affecting the stability of the globin RNA, and that ICP27 induces accumulation of unspliced  $\alpha$ -globin transcripts during infection. These results show that expression of  $\alpha$ -globin in HSV-infected cells is regulated by multiple viral factors. In sum, analysis of the HSV-induced expression of the  $\alpha$ -globin gene not only served as a useful tool for examining the effects of HSV transactivators on cellular genes, it also provided a novel approach to investigate the mechanisms involved in regulating expression of this erythroid-specific gene.

## **Chapter 2: Materials and Methods**



## **2.1 Cell culture and growth of HSV**

### **2.1.1 Maintenance of mammalian cell cultures**

All tissue-culture cells were maintained in humidified incubators at 37°C with 5% CO<sub>2</sub>, and were grown in various growth media supplemented with 1.0% L-glutamine and 1.0% penicillin-streptomycin, and 5 or 10% fetal bovine serum (FBS).

For adherent cells (HeLa, MRC5, Vero, E5, 3-3), monolayers were grown on Corning tissue-culture flasks or on Nunc tissue-culture plates. HeLa cells were grown in alpha-Minimal Essential Medium ( $\alpha$ MEM) with 10% FBS. MRC5 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS. Vero, E5, and 3-3 cells were grown in  $\alpha$ MEM with 5% FBS. To passage adherent cells, confluent monolayers were washed once with phosphate buffered saline (PBS) and were trypsinized with trypsin-EDTA (Gibco) to disrupt cell-cell attachments and to dislodge cells from the tissue-culture plates. Pre-warmed growth medium was then added to trypsinized cells to neutralise the trypsin activity as well as to dilute the cells in a larger volume. The cell density was determined by counting an aliquot of the cell suspension with a haemocytometer, and the appropriate number or dilution of cells was then added to fresh growth medium in new tissue-culture plates. The freshly diluted cell

cultures were then returned to the incubator to allow for attachment and growth.

Suspension cells such as K562 and mouse erythroleukemia (MEL) cells were grown in Corning tissue-culture flasks in RPMI and  $\alpha$ MEM supplemented with 1.0% L-glutamine, 1.0% penicillin-streptomycin, and 10% FBS respectively. The cell densities of the cultures could be monitored by counting an aliquot of well suspended cells with a haemocytometer. To passage suspension cells, the appropriate number or volume of cells was diluted in pre-warmed fresh medium in new tissue-culture flasks and returned to the incubator for growth. To induce K562 and MEL cells for differentiation, 50  $\mu$ M of Hemin and 5 mM of HMBA respectively were added to these cultures at a cell density of  $4 \times 10^5$  cells per ml and cells were left to grow for 3 days before use.

To prepare frozen aliquots of tissue-culture cells for storage, cells from subconfluent, actively growing cultures were harvested and diluted to a density of approximately  $2 \times 10^6$  cells per ml of storage medium, aliquoted to 1 ml samples in Nunc cryovials, and kept overnight at  $-70^{\circ}\text{C}$  before placement into the liquid nitrogen tank. For all cells, the storage medium consisted of 50% FBS, 43% growth medium, and 7% DMSO.

To start cell cultures from frozen aliquots, the aliquots were removed from liquid nitrogen and briefly thawed at 37°C. The thawed cell suspensions were diluted in 10 ml of the appropriate growth medium, and pelleted at low speed to wash away the DMSO. After centrifugation, cell pellets were resuspended in the appropriate growth medium and plated out on tissue-culture plates or flasks.

### **2.1.2 Preparation and storage of HSV stocks**

Most HSV strains were propagated on Vero cells. ICP4 and ICP27 are essential HSV proteins, and mutants lacking ICP4 (d120) or ICP27 (5dl1.2) have to be propagated on complementing cell lines such as E5 (for ICP4) and 3-3 (for ICP27). In all cases, ten 150 mm tissue-culture plates of Vero, E5 or 3-3 cells were grown to subconfluency and infected with the desired HSV strain at an MOI of 0.1. When most cells exhibited extensive cytopathic effects (CPE), infected cells were collected, pooled, and pelleted at 1200 rpm for 5 min in a bench top centrifuge at 4°C. Infected cells were washed once with PBS, pelleted again, and resuspended in 5 ml of  $\alpha$ MEM without FBS. Infected-cell suspensions were either transferred to a 15 ml Corning tube and disrupted by three 30 sec pulses with a sonicator probe, or transferred to a 50 ml Corning tube and disrupted by 5 cycles of freeze/thaw treatment. These samples were pelleted at 2000 rpm for 5 min to remove cellular debris, and the supernatants containing the released

HSV particles were aliquoted into 400  $\mu$ l samples and stored in cryovials at  $-70^{\circ}\text{C}$ .

### **2.1.3 Titration of HSV stocks**

20  $\mu$ l of a HSV stock was first diluted in 2 ml of  $\alpha$ MEM without FBS. This 1:100 dilution of the virus stock was then serially diluted by adding 200  $\mu$ l of the diluted virus to 1.8 ml of  $\alpha$ MEM (each step representing a 10 fold dilution). 800  $\mu$ l of each virus sample at a dilution factor of  $1 \times 10^{-4}$  to  $1 \times 10^{-9}$  was used to infect Vero cells grown to just under confluency in 6 well plates. After 1.5 h of adsorption, 2 ml of  $\alpha$ MEM containing 0.1% human immune serum (HIS) was added to each well of the 6 well plates to prevent secondary spreading of the virus. Plaques were visible after 2 to 3 days of growth. At that time, the growth medium was removed and cell monolayers were stained with crystal violet fixative solution (70% EtOH, 10% formalin, 5% acetic acid, and 1.2% crystal violet). Excess stain was removed by rinsing the plates with  $\text{H}_2\text{O}$  and the plates were air dried briefly before being counted under the microscope. For statistical accuracy, only the dilutions that gave 20 - 200 plaques were used to calculate the virus titre of the stock.

#### **2.1.4 Infection of mammalian cells with HSV**

In most experimental procedures, cells to be infected were grown in 100 or 150 mm tissue-culture plates to subconfluent density. To infect cells, growth medium from the plates was removed and replaced with a small volume of inoculum. Depending on the desired multiplicity of infection (MOI), the appropriate amount of virus stock was diluted in 2 or 5 ml of  $\alpha$ MEM and used to infect a 100 or 150 mm plate of cells respectively. After 1.5 hours of adsorption, fresh medium containing 10% FBS was added to the infected cells and the infection was allowed to proceed until the desired time for harvest.

#### **2.1.5 Preparation of viral capsid DNA**

Viral capsid DNA was extracted from HSV-infected cells and used to construct the ICP22 expression clone. Five 150 mm tissue-culture plates of Vero cells were infected with HSV-1 strain 17 at an MOI of 10 as described in the previous section. After 24 h when 95% of the cells exhibited CPE, infected cells were harvested by scraping into the growth medium, pelleted by centrifugation at 2,500 rpm, at 4 °C, for 10 min, and washed once with ice-cold PBS. The cells were repelleted by centrifugation and the cell pellet was resuspended in 1.5 ml of viral cell lysis buffer I (0.1 M Tris, pH 7.8, 10 mM EDTA). 1.5 ml ice-cold viral cell lysis buffer II (0.1 M Tris, pH 7.8, 10 mM EDTA, 0.2% NP-40) was added to the cell suspension, mixed gently, and the cells

were disrupted using a type A cell homogenizer on ice. The cell lysate was transferred to a 15 ml Corex tube and the nuclei were pelleted by centrifugation at 5,000 rpm at 4°C for 5 min. The supernatant was carefully transferred to a new 15 ml Corex tube and the nucleocapsids were pelleted by centrifugation at 12,000 rpm at 4°C for 30 min. The nucleocapsid pellet was resuspended in 0.5 ml of viral DNA extraction buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 150 mM NaCl), vortexed briefly and transferred to an eppendorf tube. 25 µl of RNase A (10 mg/ml stock) was added and the nucleocapsid suspension was incubated at room temp for 10 min. The viral capsids were disrupted by the addition of 25 µl of 10% SDS, and the capsid DNA suspension was mixed gently by inversion. Viral capsid proteins were removed by two successive phenol-CIA (chloroform with isoamylalcohol in a 24:1 volume ratio) extractions. The final aqueous phase was dialysed against TE buffer overnight at 4°C with 3 changes of the buffer over the dialysis process. The dialysed viral DNA was quantified by spectrophotometry (see section 2.2.4) and stored at 4°C. For construction of the ICP22 expression clone, the intact viral genome DNA was digested with the restriction enzymes Kpn I and Bcl I, and separated on a 1 % agarose gel. The gel area containing DNA fragments from approximately 2 - 4 kb was excised and the viral DNA was recovered (see section 2.2.6) and cloned into the pUC18 vector. The details for the construction of the ICP22 expression clone is further described in the Materials and Methods section of Chapter 3.

## **2.2 Molecular biology protocols I: production and manipulation of plasmid DNA**

The following protocols are adapted from protocols published in Current Protocols of Molecular Biology or in Molecular Cloning, a laboratory manual (Ausubel, et al., 1989; Sambrook, et al., 1989).

### **2.2.1 Growth and maintenance of bacterial cultures**

All double-stranded DNA plasmids were amplified and maintained in the DH5 $\alpha$  strain of *Escherichia coli*. Since all plasmids used contained the ampicillin-resistance gene, *E. coli* containing the recombinant plasmids were grown in growth medium containing 100  $\mu$ g/ml of ampicillin (amp) for selection purposes. For small scale plasmid preparations and for general 5 ml overnight cultures, bacterial cultures were grown in Luria-Bertani broth (LB - 1% w/v bacto-tryptone, 0.5% w/v yeast extract, and 1% w/v NaCl) with amp. For large scale CsCl preparations, bacterial clones were grown in Terrific Broth (TB - 1.2% w/v bacto-tryptone, 2.4% w/v yeast extract, 0.4% glycerol, 17 mM KH<sub>2</sub>PO<sub>4</sub>, and 72 mM K<sub>2</sub>HPO<sub>4</sub>) with amp. Bacterial liquid cultures could be streaked out and grown on LB agar plates (LB containing 1.5% w/v bacto-agar, and 100  $\mu$ g/ml of amp) to isolate individual colonies. For long term storage, sterile glycerol was added to freshly grown *E. coli* cultures to a final concentration of 30%, mixed, aliquoted into 1 ml samples, and stored at -70°C in cryovials.

### **2.2.2 Small scale preparation of recombinant DNA**

A single bacterial colony was used to inoculate a 5 ml culture and grown overnight to saturation in the shaking incubator at 37°C. 1.5 ml of the bacterial culture was transferred to an eppendorf tube and pelleted in an eppendorf microfuge for 30 sec. The supernatant was removed and the bacterial pellet was resuspended in 200 µl of P1 solution (50 mM Tris, pH7.5, 10 mM EDTA) by vortexing. The bacterial suspension was lysed by addition of 200 µl of P2 solution (200 mM NaOH, 1% SDS), and cellular debris were precipitated by addition of 200 µl of ice-cold P3 solution (3.0M KOAc, pH 5.5) and incubation on ice for 10 min. The precipitated chromosomal DNA and cellular debris were removed by centrifugation at 4°C for 10 min and the supernatant containing plasmid DNA was transferred to a fresh eppendorf tube. This supernatant was extracted once with an equal volume of phenol-CIA, and the aqueous phase containing the plasmid DNA was removed to another eppendorf tube. Plasmid DNA was precipitated with two volumes of ice-cold 95% EtOH and pelleted by centrifugation. The harvested DNA sample was dried briefly in the speedvac, resuspended in 60 µl of TE, and 5-10 µl of the sample was analysed by restriction enzyme digestion or by dideoxy-DNA sequencing.



### **2.2.3 Large scale preparation of plasmid DNA**

All large scale DNA preparations were done using CsCl banding method. One ml of freshly grown starter culture was used to inoculate 250 ml of TB containing 100 µg/ml amp which was then grown overnight in a 2L Erlenmeyer flask in a shaking incubator at 37°C. The grown culture was pelleted at 8000 rpm for 5 min in a Beckman JA-10 rotor, and the bacterial pellet was resuspended in 9 ml of P1 containing 10 mg/ml of lysozyme (see section 2.2.3 for recipes for P1, P2, and P3). The bacterial suspension was lysed by adding 10 ml of P2 and incubated on ice for 10 min. 10 ml of ice-cold P3 solution was then added to the bacterial lysate, mixed by inversion, and incubated on ice for 15 min. Cellular debris and chromosomal DNA were pelleted by centrifugation at 15,000 rpm at 4°C for 15 min in a Beckman JA-20 rotor. The supernatant was transferred to a new tube and repelleted to remove any residual precipitates. 0.6 volume of isopropanol was added to the cleared lysate and incubated at room temp for 30 min to precipitate the nucleic acids. The precipitate was pelleted by centrifugation, resuspended in 9.5 ml TE, and transferred to a 35 ml centrifuge tube. 10 g of CsCl was added to this suspension and dissolved by incubation at 37°C for 10 min. One ml of 10 mg/ml of ethidium bromide (EtBr) was then added to the CsCl/nucleic acid solution, mixed by inversion, and centrifuged at 10,000 rpm at room temp for 10 min. The supernatant was transferred to a 5/8 X 3 in. polyallomer seal cap tube, and the sample was heat sealed and

centrifuged in a Beckman VTi65.1 ultracentrifuge rotor at 55,000 rpm at 14°C for 16 hr. After centrifugation, the plasmid DNA band was collected and washed 3 times with 3 ml of Cesium saturated isopropanol (to remove all EtBr). The DNA was then diluted in 12 ml of dH<sub>2</sub>O and precipitated with 30 ml of ice-cold 95% EtOH at -20°C. The precipitated DNA was pelleted by centrifugation at 3200 rpm at 4°C for 45 min and resuspended in 300 µl of TE buffer. The DNA was precipitated once more with 1 ml of ice-cold 95%EtOH, and pelleted in an eppendorf centrifuge at 4°C. The DNA pellet was washed once with 70% EtOH, air dried briefly in the speedvac and resuspended in the desired volume of dH<sub>2</sub>O. A small aliquot of this DNA suspension was then used to quantify the yield of the plasmid preparation.

#### **2.2.4 Quantification of DNA and RNA**

All DNA and RNA samples were quantified by measuring their absorbance at 260 nm with the Ultrospec spectrophotometer. An appropriate amount of DNA or RNA was diluted in 1 ml of dH<sub>2</sub>O, and transferred to a quartz cuvette, and its absorbance was measured. The concentration of DNA or RNA was calculated by using the conversion factor of 1 absorbance unit at 260 nm is approximately equal to 50 mg/ml of double stranded DNA or 40 mg/ml of RNA. For DNA preparations, the ratio of their absorbance at 260nm and 280 nm was used as a measure of DNA purity. Generally a ratio of 1.7 - 2.0 is considered acceptable.

### **2.2.5 Restriction enzyme digestion and enzymatic modification of the digested DNA**

All restriction and modifying enzymes were purchased from New England Biolabs and used according to the manufacturers's recommended protocols. Typical digestion reactions were done in a volume of 20-30  $\mu\text{l}$  with the amount of restriction enzymes used never exceeding 10% of the total volume. Double digestions were usually done in buffers that offered maximal activity for both enzymes. If this could not be achieved, digestion was first done with the enzyme requiring low-salt conditions, followed by the enzyme requiring high-salt conditions with the appropriate adjustment of the buffering conditions between the two digestions. Partial digestions were done by preparing 60 - 80  $\mu\text{l}$  restriction reactions and sequentially removing 10  $\mu\text{l}$  aliquots at 5 min intervals during digestion and were transferred to fresh eppendorf tubes containing gel loading buffer with EDTA to stop the reactions.

To generate blunt-ended DNA fragments after restriction enzyme digestion, Klenow fragment was used for filling-in 3' overhangs, or T4 DNA polymerase was used if the restricted DNA fragments contained both 3' and 5' overhangs. In both cases, modifying enzymes could be added directly to the restriction enzyme reactions once digestion was completed. For a typical 30  $\mu\text{l}$  digestion reaction, 1  $\mu\text{l}$  of dNTP (100 mM stock) was added along with 2  $\mu\text{l}$  of

Klenow or T4 DNA polymerase, 2  $\mu$ l of 10 X buffer, and 15  $\mu$ l of dH<sub>2</sub>O to make up the reaction volume to 50  $\mu$ l. The reactions were allowed to proceed at 37°C for 30 min, after which the samples were either extracted with CIA or directly separated on agarose gel and purified from agarose gel slices.

To dephosphorylate the restriction digested DNA fragments, 2  $\mu$ l of calf intestine phosphatase (CIP) was directly added to the typical 30  $\mu$ l restriction enzyme digestion after digestion has completed, with 2  $\mu$ l of 10 X buffer and 18  $\mu$ l of dH<sub>2</sub>O, and incubated at 37°C for 60 min. CIP-treated samples could either be extracted with CIA or directly separated on agarose gel and purified from agarose gel slices.

### **2.2.6 DNA agarose gel electrophoresis and purification of DNA fragments from agarose gels**

DNA fragments were separated by electrophoresis on 1% agarose gel in 1X TAE (40 mM Tris-acetate, 2 mM EDTA) or 1X TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA). Agarose gels were stained with EtBr and visualised with a UV transilluminator.

Purification of DNA fragments from agarose gels were done using Quiex kits (Qiagen) according to the manufacturer's directions. Briefly, the desired DNA fragment was excised from the agarose gel by razor blade and transferred to a pre-weighed eppendorf tube. The

weight of the agarose gel slice was determined and QX1 buffer was added in a ratio of 300  $\mu$ l of buffer to 100 mg of agarose. 10  $\mu$ l of Quiex beads was also added and the mixture was incubated at 50°C for 10 min to dissolve the agarose. The DNA bound beads were pelleted by centrifugation for 30 sec, washed once with 500  $\mu$ l of QX1 buffer, and twice with 500  $\mu$ l of QX2 buffer each time. The pelleted beads were dried briefly in the speedvac, resuspended in 20  $\mu$ l of dH<sub>2</sub>O, and incubated at room temp for 5 min. The beads were pelleted by centrifugation and the supernatant containing the eluted DNA was transferred to a new eppendorf tube. The beads were eluted a second time with another 20  $\mu$ l dH<sub>2</sub>O, pelleted by centrifugation again and the second eluant was pooled with the first.

### **2.2.7 Ligation of DNA**

All DNA fragments used for ligations were purified from agarose gels using Quiex beads, and vector DNA fragments were sometimes dephosphoryated by CIP treatment to reduce the frequency of self-ligation. For all ligations, the vector to insert ratio was maintained at 1:3 or 1:5, and a reaction volume of 20  $\mu$ l containing 2  $\mu$ l of T4 DNA ligase, 2  $\mu$ l of 10 X buffer and the DNA fragments was used. The ligation reactions were incubated at room temp for 2 h and then at 4°C overnight. 10  $\mu$ l of each reaction was then used to transform competent bacteria.

### **2.2.8 Preparation of competent bacteria**

A single colony of DH5 $\alpha$  was used to inoculate 2.5 ml of LB and grown overnight with shaking at 37°C. The culture was then subcultured 1:100 in 250 ml of LB + 20 mM MgSO<sub>4</sub> and grown to an OD<sub>595</sub> of 0.4 - 0.6 (approx. 2 - 3 h). The grown culture was centrifuged at 5,000 rpm at 4°C for 5 min and the supernatant was removed. The bacterial pellet was resuspended in 100 ml (0.4 volume of the original volume) of ice-cold TFBI (30 mM KOAc, 100 mM RbCl, 10 mM CaCl<sub>2</sub>, 50 mM MnCl<sub>2</sub>, 15% glycerol, at pH 5.8), and incubated on ice for 5 min. This bacterial suspension was centrifuged again and the bacterial pellet was resuspended in 10 ml (1/25 of the original volume) of ice-cold TFBII (10 mM MOPS, 75 mM CaCl<sub>2</sub>, 10 mM RbCl, 15% glycerol, at pH 6.5). This mixture was incubated on ice for 60 min and aliquoted into 400  $\mu$ l aliquots and frozen and stored at -70°C until use.

### **2.2.9 Transformation of competent bacteria**

10  $\mu$ l of plasmid DNA (20 - 200 ng) or ligation mixture was added to 100  $\mu$ l of competent cells in 6 ml Falcon tubes and incubated on ice for 30 min. The DNA plus competent cells were then heat shocked at 42°C for 45 sec, and incubated on ice for 2 min. 1 ml of LB (without amp) was added to the competent cells and grown for 1 h at 37°C in the shaking incubator. 50 - 250  $\mu$ l of the 1 ml culture was spread-plated on LB plates with 100  $\mu$ g/ml amp, and grown overnight at 37°C.

### **2.2.10 Colony hybridisation**

Colony hybridisation was used to screen for recombinant plasmids that had low efficiency of ligation between vector and insert (e.g. for blunt end ligations). Individual amp resistant colonies grown from transformed competent bacteria were picked using sterile toothpicks, replica plated onto two new LB amp plates and grown overnight at 37°C. The plates were marked so that their orientations could be identified and matched. For each set of replica plates, one was used for colony lift and the other was kept as stock for the bacterial clones. A nitrocellulose disk was marked with asymmetrical notches and was carefully placed over colonies of one of the replica plates. The nitrocellulose disk was lifted from the plate and placed on 3 mm Whatman filter paper soaked with 0.5 M NaOH with the colony side up. After 30 min of lysis, the disk was neutralised on 3 mm Whatman filter paper soaked in a solution of 1.0 M Tris, pH7.0 and 1.5 M NaCl for 30 min, and then UV cross-linked using the Stratalinker 2400 (Stratagene). The cross-linked disks were prehybridised at 65°C for 30 min in 10 ml of Church buffer (250 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, 7% SDS, 1 mM EDTA, 1% BSA). A random-primer labelled DNA probe or end-labelled oligonucleotide probe complementary to the desired insert sequences was added directly to the prehybridisation mixture and mixture was left to hybridize at 65°C overnight in a Techne rotating hybridisation oven. Following hybridisation, the disks were washed twice with 250 ml of wash buffer I (2X SSC, 0.1% SDS [1X SSC = 150

mM NaCl, 15 mM Na-Citrate, pH 6.35]), at 65°C, and then twice with wash buffer II (0.1X SSC, 0.1% SDS) at 65°C. The washed disks were gently blotted to remove excess wash buffer, placed between saran wrap, and exposed to X-AR (Kodak) film for 1 - 4 h.

### **2.2.11 Random primer labelling of DNA fragments**

Internally labelled DNA fragments were often used as probes in colony lifts, Southern blot, and Northern blots. Approximately 50 - 200 ng of double stranded DNA (usually agarose gel purified DNA fragments) was added to 5 mM random hexamer primers (Pharmacia) in a volume of 38  $\mu$ l and was boiled for 5 min. The boiled sample was snap cooled in a stratacooler previously kept at -20°C for 5 min. The reaction was made up to 60  $\mu$ l with 50  $\mu$ M (final concentration) of each dNTP except for dCTP, 1X Klenow buffer, 50  $\mu$ Ci of  $\alpha^{32}$ P-dCTP (Dupont/NEN, 10 mCi/ml, 3000 Ci/mmol stock), and 6 units of Klenow. This labelling reaction was incubated at room temp for 1 h. After labelling, the reaction was diluted to 200  $\mu$ l with dH<sub>2</sub>O and loaded onto a 1 ml sephadex G50 spin column to remove unincorporated  $\alpha^{32}$ P-dCTP. This column was prepared by plugging the end of a 1.0 ml syringe with siliconized glass wool and packed with sephadex G50 beads pre-swollen in TE. The column with the labelling reaction was centrifuged at 2000 rpm for 4 min and the flow-through material which contained the labelled DNA fragment was collected,



boiled for 5 min to denature the double-stranded DNA, and then added to the prehybridised filters.

#### **2.2.12 End-labelling of single-stranded oligonucleotide DNA**

End-labelled oligonucleotides were used in primer extension analysis or as probes in colony lifts, Southern, and Northern blots. In general, 1  $\mu$ l of a 1 OD/ml stock of oligonucleotide was used per labelling reaction in a total volume of 50  $\mu$ l containing 1X Kinase buffer, 50  $\mu$ Ci of  $\gamma^{32}$ PdATP (Dupont/NEN, 10 mCi/ml), 1X Kinase buffer, and 2  $\mu$ l T4 Polynucleotide Kinase. The labelling reaction was incubated at 37°C for 1 - 2 h.

For usage as primers in primer extension analysis, the labelled primers were purified from sequencing gels to obtain primers of uniform-length. After labelling, 200  $\mu$ l of 2.5 M  $\text{NH}_4\text{OAc}$  was added to the 50  $\mu$ l reaction mixture followed by the addition of 5  $\mu$ l of carrier tRNA, and 1 ml of ice-cold 95% EtOH to precipitate the oligonucleotide DNA. The labelled oligonucleotide was pelleted by centrifugation at 4°C for 15 min and resuspended in 3  $\mu$ l  $\text{dH}_2\text{O}$ . 9  $\mu$ l of sequencing gel loading buffer (deionized formamide containing 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue) was added and the mixture was loaded onto a pre-warmed 8% polyacrylamide sequencing gel (7.72% Acrylamide, 0.28% Bis-acrylamide, 7 M Urea, 0.04% ammonium persulfate [APS], 1X TBE), and electrophoresed at 100 W

for 1 h. At the end of electrophoresis, the gel plates were separated and the gel resting on one plate was covered with Saran wrap, and exposed to X-AR film for 3 min. The film was developed and superimposed onto the actual gel to identify the portion of the gel containing the labelled primer. This gel region was excised with a razor blade, and eluted in 300  $\mu$ l elution buffer (300 mM  $\text{NH}_4\text{OAc}$ , 10 mM  $\text{MgOAc}$ , 1 mM EDTA, 0.1% SDS) by incubation overnight at 37°C. The supernatant containing the eluted labelled oligonucleotide was removed, spun through a Spin-X tube (Co-star) to remove any residual polyacrylamide, and transferred to 1.5 ml eppendorf tube. The gel slice was washed once with 200  $\mu$ l elution buffer and this supernatant was also spun through the Spin-X tube and pooled together with the first supernatant. 1 ml of ice-cold 95% EtOH was added to the supernatant with 5  $\mu$ l of carrier tRNA and the mixture was precipitated at -20°C for 2 h. The labelled DNA primer was then pelleted by centrifugation, washed in 70% EtOH, dried in speedvac, and resuspended in 60  $\mu$ l of TE. 1  $\mu$ l of this labelled primer was counted using a scintillation counter. Approximately 50,000 Cerenkov counts of the labelled oligonucleotide was used for each sample in primer extension analysis.

### **2.2.13 DNA sequencing**

All DNA sequencing reactions were done using the  $\text{T}^7$  Sequencing kit (Pharmacia) based on the dideoxy chain termination

method. Double-stranded DNA from small and large scale plasmid preparations as well as single-stranded DNA from M13 clones were used as templates, and various oligonucleotides and the Universal primer were used as primers for the sequencing reactions. The protocol for the sequencing reactions was performed as instructed in the manufacturer's guide. The sequencing reactions were separated on 8% polyacrylamide sequencing gels and exposed to X-AR films for 2 - 6 h.

#### **2.2.14 Introduction of plasmid DNA into mammalian cells**

Several expression clones of HSV regulatory proteins were introduced into mammalian cells by transient transfection to examine their effects on gene expression. All transfections were done using the calcium phosphate method. One day prior to the transfection procedure, approximately  $3 \times 10^6$  HeLa cells were seeded onto each 100 mm tissue-culture plate and were allowed to attach and grow overnight. 3 h prior to transfection, the spent growth medium was aspirated and replaced by exactly 9 ml of fresh  $\alpha$ MEM containing 10% FBS. For each transfection sample, 2 separate solutions were prepared in sterile eppendorf tubes: in one tube, 5 - 30  $\mu$ g of the plasmid of interest diluted in 450  $\mu$ l of transfection quality dH<sub>2</sub>O was mixed with 50  $\mu$ l of 2.5 M CaCl<sub>2</sub>, and in the second tube, 500  $\mu$ l of 2X HEBS (62 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES], 225 mM NaCl, 2 mM NaHPO<sub>4</sub>, pH 7.05) was dispensed. The DNA solution

in the first tube was added to the 2X HEBS in the second tube in a dropwise fashion while the mixture was gently mixed by the bubbling action of a pasteur pipette attached to a mechanical pipettor (see *Current Protocols in Molecular Biology* for an illustration of the setup). The DNA-CaCl<sub>2</sub>-HEBS mixture was incubated at room temp for 30 min, and the precipitated DNA was gently mixed and added to the HeLa cell culture. The cells were incubated with the DNA precipitate for 16 h, and were then washed 3 times each with 5 ml of fresh  $\alpha$ MEM. After the DNA precipitates were washed away, 10 ml of fresh  $\alpha$ MEM with 10% FBS was added and the cells were allowed to recover for another 24 h before total RNA harvested as described in 2.4.1.

## **2.3 Molecular biology protocols II: Generation of single-stranded (ss) DNA**

### **2.3.1 Strand separation of double-stranded DNA**

Double-stranded (ds) DNA fragments were separated into (+) and (-) strands by electrophoresis for isolation of ss DNA probes used in S1 nuclease protection assays. This protocol was adapted from one obtained from Dr. D. Chui's laboratory. 10  $\mu$ g of plasmid DNA was digested with restriction enzymes that flank the desired DNA sequence to generate linear DS DNA fragments. The restriction enzymes were chosen to generate 5' overhangs so that they could be radioactively labelled by Klenow fill-in reactions. The digested DNA fragments were

separated on 1% agarose gel, and the fragment containing the desired DNA sequence was excised from the agarose gel and eluted using the Quiex gel elution kit as described in section 2.2.6. The eluted DNA fragment was EtOH precipitated at  $-20^{\circ}\text{C}$ , pelleted by centrifugation, and resuspended in  $10\ \mu\text{l}$  of  $\text{dH}_2\text{O}$ . This DNA fragment was radioactively labelled by adding  $5\ \mu\text{l}$  of  $\alpha^{32}\text{P}$ -dCTP (Dupont/NEN,  $10\ \text{mCi/ml}$ ,  $3000\ \text{Ci/mmol}$ ),  $5\ \mu\text{l}$  of 10X Klenow buffer,  $1\ \mu\text{l}$  each of dATP, dGTP, dTTP ( $100\ \text{mM}$  stock),  $2\ \mu\text{l}$  of Klenow large fragment, and  $25\ \mu\text{l}$   $\text{dH}_2\text{O}$ . The  $50\ \mu\text{l}$  reaction was incubated at  $37^{\circ}\text{C}$  for 1 h, diluted to  $200\ \mu\text{l}$  with  $\text{dH}_2\text{O}$  at the end of the labelling reaction, and passed through a sephadex G50 column (described in section 2.2.12) to remove the unincorporated radioactive nucleotides. The flowthrough from the spin column containing the labelled DNA fragment was EtOH precipitated, pelleted by centrifugation, washed once with 70% EtOH, dried briefly in the speedvac, and resuspended in  $36\ \mu\text{l}$   $\text{dH}_2\text{O}$ .  $4\ \mu\text{l}$  of 10X Everett's TBE ( $1.25\ \text{M}$  Tris,  $0.4\ \text{M}$  Boric acid, and  $25\ \text{mM}$  EDTA) was added to the DNA and  $5\ \mu\text{l}$  of the mixture was removed and added to  $10\ \mu\text{l}$  of sequencing gel loading buffer as the control sample without denaturation. To the rest of the mixture,  $20\ \mu\text{l}$  of DMSO and  $10\ \mu\text{l}$  of sequencing gel loading buffer were added, and the entire mixture was incubated at  $65^{\circ}\text{C}$  for 15 min, heated to  $90^{\circ}\text{C}$  for 5 min, and snap cooled on ice. This denatured sample as well as the control sample without DMSO were then loaded onto a 5% polyacrylamide strand separation gel made with 1X Everett's TBE and electrophoresed for 16 h at 150 V. At the end of electrophoresis, the gel apparatus was dismantled, the

polyacrylamide gel was covered by Saran wrap, and exposed to X-AR film for 3 min and developed with the automatic developer. By comparing the DMSO treated sample to the untreated control sample, the DMSO treated sample should yield two additional bands representing the (+) and (-) ss DNA derived from the ds DNA fragment. The gel regions containing the separated strands (as shown on the film) were separately excised and the ss DNA from each gel slice was eluted by the same method used to elute  $^{32}\text{P}$ -labelled oligonucleotides as described in section 2.2.12. The two ss DNA samples were hybridised to control mRNA and examined by S1 nuclease protection assay to determine which strand was complementary to the mRNA.

### **2.3.2 Transformation of M13 RF vector into competent bacteria**

DNA fragments were cloned into M13 replicative form (RF) vector for generation of ss DNA probes used in nuclear run-on assays. Identical to the ligation protocol described in 2.2.7, agarose gel purified insert and vector DNA fragments were ligated overnight in a 20  $\mu\text{l}$  reaction volume. 10  $\mu\text{l}$  of this ligation mixture was then used to transform competent DH5 $\alpha$ F' bacteria (Gibco, BRL). In preparation for this transformation, a 5 ml DH5 $\alpha$ F' culture was grown overnight in LB and used as lawn bacteria. Also, LB top agar (LB plus 7.5 g/L bactoagar) was melted and dispensed into 3 ml aliquots in 15 ml Falcon tubes and incubated at 42°C to maintain fluidity. 10  $\mu\text{l}$  of the

ligation reaction was mixed with 50  $\mu$ l of competent bacteria and incubated on ice for 30 min. This mixture was heat shocked at 42°C for 45 sec and was snap cooled on ice for 2 min. 100  $\mu$ l of lawn bacterial, X-gal and IPTG was added to and mixed with the competent bacteria and 10 - 30  $\mu$ l of this mixture was diluted in 3 ml of LB top agar and poured over prewarmed (37°C) LB agar plates. Once the top agar solidified, the plates were grown upside down at 37°C for 8 - 12 h and white plaques were picked and processed to obtain ss DNA (see next section).

### **2.3.3 Small scale preparation of ss DNA from M13 plaques**

50 ml of 2XYT medium (1.6% w/v bactotryptone, 1.0% w/v yeast extract, and 0.5% w/v NaCl) was inoculated with 0.5 ml (1:100 dilution) of an overnight culture of DH5 $\alpha$ F' and grown at 37°C for 1 h. After the 1 h of growth, this culture was dispensed into 2 ml aliquots in 15 ml Falcon tubes. White plaques derived from the transformed competent bacteria were picked using a P1000 pipettman, and used to inoculate the dispensed 2 ml cultures. These cultures were grown at 37°C in the shaking incubator with high aeration (350 rpm) for 5 - 6 h. At the end of the growth period, 1.5 ml of each culture was transferred to eppendorf tubes and the bacteria were pelleted by centrifugation at 14,000 rpm at room temp for 5 min. 1 ml of the supernatant was carefully extracted without disturbing the bacterial pellet, and transferred to a fresh eppendorf tube, whereas the rest of the

supernatant was kept and later used as inoculum for large scale preps. 250  $\mu$ l of 20% PEG / 2.5 M NaCl was added to the supernatant, inverted to mix, and incubated at room temp for 10 min. The precipitated M13 phage was pelleted by centrifugation at 14,000 rpm at room temp for 5 min. The supernatant was aspirated and the pellet was respun to remove the residual supernatant. The phage pellet was then resuspended in 200  $\mu$ l TE (pH 7.6), extracted with equal volume of phenol/CIA, and centrifuged at 14,000 rpm at room temp for 5 min to separate the phases. 175  $\mu$ l of the aqueous phase was carefully extracted and transferred to a fresh eppendorf tube. 17.5  $\mu$ l of 3 M NaOAc, pH 5.2, and 350  $\mu$ l of ice-cold 95% EtOH was added and the phage DNA was precipitated at -20°C for several hours. The precipitated DNA was pelleted by centrifugation, washed once with 70% EtOH, dried briefly in the speedvac, and resuspended in 40  $\mu$ l of TE. 10  $\mu$ l of the sample was examined by agarose gel electrophoresis to determine the purity of the ss DNA. Another 10  $\mu$ l of the sample was used in sequencing reactions (described in 2.2.13) to determine the identity of the clones and the orientation of the insert relative to the vector. Once the appropriate clones were obtained, the saved phage inoculum could then be used for large scale ss DNA preparations.

#### **2.3.4 Large scale preparation of ss DNA from M13 clones**

25 ml of 2XYT was inoculated with 1 ml of a 5 ml overnight culture of DH5 $\alpha$ F'. This new culture was grown for 4 - 6 h at 37°C in a



shaking incubator set at 350 rpm. 5 ml of this culture was then used to inoculate 500 ml of 2XYT (1:100 dilution) in conjunction with 0.5 ml of the supernatant (phage stock) saved from the small scale preparation (see previous section), and this phage/bacteria culture was grown for 12 h at 37°C in a shaking incubator. The freshly grown culture was centrifuged at 8,000 rpm for 15 min to pellet the bacteria, and the supernatant was carefully removed and saved. 10 ml of the supernatant was saved as phage stock for future use, and 125 ml (1/4 of supernatant volume) of 20% PEG/2.5 M NaCl was added to the remaining supernatant, mixed, and incubated at room temp for 15 min. The precipitated phage was pelleted by centrifugation at 6,000 rpm, at room temp for 30 min, and resuspended in 16 ml of suspension medium I (10 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA). 2 ml (1/8 of the phage suspension volume) of 20% PEG/2.5 M NaCl was added and incubated at room temp for 15 min to reprecipitate the resuspended phage. This solution was transferred to 30 ml Corex tubes and pelleted by centrifugation at 10,000 rpm at room temp for 30 min. The phage pellet was resuspended in 2.5 ml of suspension medium II (100 mM NaCl, 17 mM MgSO<sub>4</sub>, 50 mM Tris, pH 7.5, and 1.0% w/v gelatin), and 186.5 mg of KCl was added so that the suspension contained a final concentration of 1 M KCl. This solution was incubated on ice for 30 min and then centrifuged at 15,000 rpm, at 4°C, for 15 min. The supernatant was saved and an equal volume of urea/SDS (7 M urea, 0.35 M NaCl, 10 mM Tris, pH 7.8, 10 mM EDTA, 1% SDS) was added. This mixture was then extracted 2 - 3 times with

equal volume of phenol/CIA until the aqueous phase was clear of any precipitate. NaCl was added to the extracted aqueous phase to make a solution with a final concentration of 0.15 M NaCl (approximately 155  $\mu$ l of 5 M NaCl per 5 ml volume), and 2 volumes of ice-cold 95% EtOH was added to precipitate the ss DNA. The ss DNA was pelleted by centrifugation at 12,000 rpm at 4°C for 20 min, and resuspended in 250  $\mu$ l TE. The ss DNA sample was transferred to an eppendorf tube, and reprecipitated with the addition of NaCl and EtOH. The precipitated ss DNA was pelleted by centrifugation at 14,000 rpm at 4°C for 30 min and resuspended in 250  $\mu$ l TE. The ss DNA sample was then quantified by measuring the absorbance at 260 nm (1 A unit is approximately 33  $\mu$ g of ss DNA) and a small aliquot of this ss DNA was also examined by agarose gel for determination of the purity of the DNA. Approximately 1  $\mu$ g of each ss DNA probe was used for each nuclear run-on reaction.

#### **2.4 Molecular biology protocols III: Isolation and manipulation of cellular RNA**

Where possible, all solutions used for RNA work, with the exception of Tris and TE, were treated with 0.1% DEPC (diethylpyrocarbonate) to inactivate any contaminating RNases. The DEPC treated solutions were autoclaved before usage to inactivate the DEPC.

### **2.4.1 Harvesting of total cellular RNA**

Total cellular RNA was harvested from HSV-infected or mock-infected mammalian cell cultures using the Trizol reagent (Gibco/BRL) according to the manufacturer's instructions. Briefly, mammalian cells grown on 100 mm tissue-culture plates were scraped to one section of the plate using a sterile cell scraper. 1 ml of Trizol was added to the tissue-culture plate directly, and pipetted up and down to lyse the cells. The cell lysate was transferred to an Eppendorf tube, mixed with 200  $\mu$ l of CIA by vortexing, incubated on ice for 5 min, and centrifuged at 14,000 rpm, at 4°C for 15 min. The aqueous phase was removed and extracted once more with an equal volume of CIA. The mixture was centrifuged again, the aqueous phase was transferred to a fresh eppendorf tube, and 600  $\mu$ l of isopropanol was added to precipitate the RNA at -20°C. The RNA was pellet by centrifugation, resuspended in 250  $\mu$ l of 0.3 M NaOAc (DEPC-treated), and reprecipitated with 1 ml of ice-cold 95% EtOH. The reprecipitated RNA was pelleted by centrifugation, washed once with 70% EtOH, dried in the speedvac, and resuspended in 500  $\mu$ l of DEPC-treated dH<sub>2</sub>O. RNA samples were quantified by measuring the sample's absorbance at 260 nm. In general, 10  $\mu$ g of total RNA was used per sample for primer extension, S1 nuclease protection, or Northern blot analysis.

#### **2.4.2 Removal of poly (A) sequences or sequence directed cleavage of cellular RNA by RNase H treatment**

RNase H preferentially cleaves DNA-RNA heteroduplexes, and was used in conjunction with poly dT oligonucleotides (Pharmacia) to remove poly (A) sequences from mRNA transcripts, or in conjunction with specific oligonucleotides (synthesized by the Central Facility of MOBIX, McMaster University) to direct sequence-specific cleavage of selected mRNA. The RNase H cleavage protocol is described in the Materials and Methods section of Chapter 5.

#### **2.4.3 Primer extension analysis**

Primer extension analysis was used to detect and quantify the steady-state of amount  $\alpha$ -globin and several HSV RNA in HSV-infected cells. In general, 25mer oligonucleotides complementary to the desired transcripts (Central Facility, MOBIX) were 5' end labelled as described in 2.2.12 and used to prime synthesis of ss cDNA from the population of total RNA using AMV (Life Sciences) or Superscript (Gibco/BRL) reverse-transcriptase. The detailed protocol for primer extension analysis is described in the Materials and Methods section of Chapter 3.

#### **2.4.4 S1 nuclease protection assay**

S1 nuclease protection assay was used to distinguish between  $\alpha 2$  and  $\alpha 1$  RNA (Chapter 3) as well as to assay for the presence of intron containing  $\alpha$ -globin transcripts (Chapter 5). In general, 3' end labelled ss DNA probes (described in 2.3.1) were hybridised to 10  $\mu$ g of total RNA and then treated with S1 nuclease. The RNA protected regions of the  $^{32}$ P-labelled ss DNA were then analysed by resolving the resulting products on sequencing gels. The detailed protocol for S1 nuclease protection assay is described in the Materials and Methods section of Chapter 3.

#### **2.4.5 Northern blot analysis**

Northern blot analysis was used to detect and quantify the presence of specific transcripts within the total cellular RNA. 10  $\mu$ g of total RNA was fractionated on a formaldehyde/agarose gel (6%/1.5%) by electrophoresis, after which, the gel was soaked in 1L of dH<sub>2</sub>O for 20 min, 1 L of 50 mM NaOH, 10 mM NaCl for 15 min, 1 L of 100 mM Tris, pH 7.5 for 15 min and finally rinsed in 1 L dH<sub>2</sub>O for 10 min. The RNA in the gel was transferred to positively charged Nytran Plus membrane (S&S) by the standard capillary method (described in *Molecular Cloning, a Laboratory Manual*) or with a PosiBlot pressure blotter (Stratagene) according to the manufacturer's instructions. The RNA was UV-cross linked to the Nytran Plus membrane with the

Stratalinker (Stratagene) and prehybridised in 10 ml of Church buffer (described in section 2.2.10) at 65°C for 1 h. After prehybridisation, the used Church buffer was discarded and 10 ml of fresh Church buffer containing the random priming labelled probe (described in section 2.2.10) was added to the membrane filter and hybridised at 65°C for 12 - 16 h. The filter was washed as described in section 2.2.10 and exposed to X-AR film for 1 - 3 days.

## **2.5 Extraction and manipulation of genomic DNA**

### **2.5.1 Extraction of genomic DNA**

Genomic DNA from HSV- or mock-infected cells was harvested and its methylation status was examined by digestion with the restriction enzymes HpaII and MspI. The extraction method is adapted from the protocol published by Laird *et al.* (Laird, et al., 1991), and the protocol is described in the Materials and Methods section of Chapter 4.

### **2.5.2 Harvesting mammalian cell nuclei**

Two separate methods were used to harvest nuclei from HSV- or mock-infected HeLa cells. One procedure involved centrifugation over a sucrose cushion which produced nuclei samples that clumped to a lesser extent which was useful for experiments that involved

aliquoting and preparing multiple samples containing equal number of nuclei. The second procedure was simpler and was used for nuclear run-on assays which used the entire nuclei sample per reaction.

#### **2.5.2a Method I**

This method involved ultracentrifugation over a sucrose cushion and was used to examine the sensitivity of nuclei DNA to micrococcal nuclease (MNase) and DNase I. The protocol was described in detail in the Materials and Methods section of Chapter 4.

#### **2.5.2b Method II**

This method was used to prepare nuclei for nuclear run-on assay. Since this assay required a large amount ( $1.5 - 2 \times 10^7$ ) of nuclei per sample, in general, nuclei from four 150 mm dishes of HeLa cells were collected and pooled as one single sample for nuclear run-on analysis. One day prior to the nuclei harvest, approximately  $5 \times 10^6$  HeLa cells were seeded onto each 150 mm tissue-culture plates and were grown overnight. Cells were mock-infected or infected with various HSV strains and the infections were allowed to proceed for 6 h. The growth medium from each plate was collected into 50 ml Conical tubes, and the cells were rinsed once with ice-cold PBS. The cells were then scraped into 10 ml of ice-cold PBS and collected into a separate 50

ml conical tube. These samples were centrifuged at 1000 rpm at 4°C for 10 min and the cell pellets from the same samples were pooled and resuspended in 20 ml of ice-cold RSB (10 mM NaCl, 10 mM Tris, pH 7.4, 5 mM MgCl<sub>2</sub>). 30 µl of this cell suspension was removed and counted using a haemocytometer to provide an estimate of the number of nuclei harvested. The remaining cell suspension was centrifuged again at 1000 rpm and the pelleted cells were first resuspended in 2 ml of ice-cold RSB, and then mixed with 18 ml of ice-cold RSB containing 0.5% NP-40. The cellular membrane was stripped from the cells by vigorous pipetting and the nuclei were pelleted by centrifugation at 2100 rpm, at 4°C for 5 min. The supernatant was removed, the nuclei pellet was briefly spun again, and the residual RSB buffer was removed. The nuclei pellet was resuspended in 210 µl of nuclear freezing buffer (50 mM Tris, pH 8.0, 5 mM MgCl<sub>2</sub>, 40% sterile glycerol, 0.5 mM DTT) and stored at -70°C.

### **2.5.3 Nuclear run-on assay**

Nuclear run-on assay was used to determine the level of ongoing transcription at the time of nuclei harvest. A detailed protocol for this procedure is described in the Materials and Methods section of Chapter 5.



#### **2.5.4 Analysis of nuclei DNA by MNase, DNase, or restriction enzyme digestion**

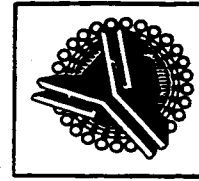
Nuclei harvested by method I were treated with MNase, DNase, or the restriction enzyme MspI. These experimental protocols are described in detail in the Materials and Methods section of Chapter 4.

#### **2.5.5 Genomic Southern blot**

Genomic DNA harvested directly from mammalian cells or from MNase, DNase, or MspI digested nuclei were analysed by Southern blot analysis. The protocol for this procedure is described in the Materials and Methods section of Chapter 4.



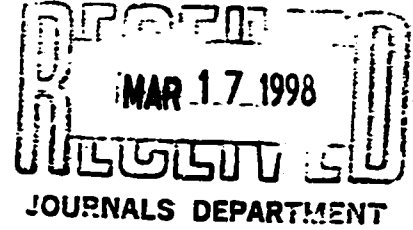
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**Chapter 3: Herpes simplex virus immediate-early proteins ICP0 and ICP4 activate the endogenous human  $\alpha$ -globin gene in nonerythroid cells**

Paper published in *J. Virol.*, 1997, 71: 1784-1793.

### **Abstract**

Globin genes are normally expressed only in erythroid cell lineages. However, we found that the endogenous  $\alpha$ -globin gene is activated following infection of human fibroblasts and HeLa cells with herpes simplex virus (HSV), leading to accumulation of correctly initiated transcripts driven by the  $\alpha$ -globin promoter. The  $\alpha 1$ - and  $\alpha 2$ -globin genes were both induced, but expression of  $\beta$ - or  $\zeta$ -globin genes could not be detected. Experiments using HSV mutants showed that null mutations in the genes encoding the viral immediate-early proteins ICP4 and ICP22 reduced induction approximately 10-fold, while loss of ICP0 function had a smaller inhibitory effect. Transient transfection experiments showed that ICP0 and ICP4 are each sufficient to trigger detectable expression of the endogenous gene, while ICP22 had no detectable effect in this assay. In contrast, the adenovirus E1a protein did not activate the endogenous  $\alpha$ -globin gene and inhibited expression of the plasmid-borne  $\alpha 2$  gene. Previous studies have led to the hypothesis that chromosomal  $\alpha$ -globin genes are subject to chromatin-dependent repression mechanism that prevents expression in non-erythroid cells. Our data suggest that HSV ICP0 and ICP4 either break or bypass this cellular gene silencing mechanism.

## **Introduction**

**Herpes simplex virus (HSV) is a large enveloped DNA virus that replicates in the nuclei of mammalian cells. HSV executes a complex genetic program encompassing a variety of controls at the transcriptional and post-transcriptional levels during lytic infection: expression of most cellular genes is strongly suppressed, and three temporally distinct classes of viral genes are sequentially activated to high levels in a cascade fashion (reviewed in references 29, 83, and 99). Five viral immediate-early (IE) genes are expressed first; four of these (ICP0, ICP4, ICP22, and ICP27) encode regulatory proteins that stimulate expression of the viral early (E) and late (L) genes. The E genes are activated next, giving rise to proteins required for replication of the viral genome. Viral DNA replication then ensues, augmenting IE-dependent expression of the L genes. This lytic cascade provides a favourable system for dissecting the mechanisms by which a limited number of regulatory proteins rapidly reprogram mammalian cells to express a new set of polymerase II-transcribed genes.**

**All systems of differential gene activation must distinguish genes destined for activation from the large excess of other genes present in the same nucleus. In the case of the HSV lytic cycle, the initial source of selectivity is a specific DNA sequence element, TAATGARATTC, which targets the VP16 molecules delivered by the infecting virion to the upstream control regions of the viral IE genes,**

resulting in selective activation of IE transcription (5, 14, 34, 58, 59, 62-64, 70, 77, 96). IE proteins then stimulate expression of the viral E and L genes. However, in contrast to VP16-induced activation of IE gene transcription, the mechanisms that target E and L genes for induction have yet to be clearly defined. E and L control regions are not marked by obviously conserved class-specific sequences analogous to TAATGARATTC, and exhaustive mutational and biochemical analyses have failed to uncover specific DNA sequence elements required for activation by IE polypeptides (17, 26, 67, 89). Indeed, minimal promoters consisting of an isolated TATA box are induced (48, 54, 56), and HSV IE proteins activate a variety of viral and cellular TATA-bearing promoters in transient co-transfection assays (25, 26, 28). Perhaps the only clear conclusion to emerge from these studies is that the precise sequence of the TATA element plays a major role in dictating the degree of induction (17, 28, 51). This observation fits in well with recent data showing that the IE protein ICP4 interacts with components of the basal transcriptional apparatus including TBP, TF IIB, and TAF250 (15, 91). Despite the relaxed sequence specificity displayed with newly introduced target genes, IE proteins do not appear to globally activate expression of endogenous cellular genes. Taken in combination, these observations raise the likelihood that features other than primary nucleotide sequence play a major role in targeting viral E and L genes for selective activation. In principle, any property that distinguishes HSV E and L genes from the majority of endogenous cellular genes could contribute to this selectivity;

examples include subnuclear localization, extrachromosomal versus chromosomal state, prior transcriptional status, or differences in higher-order packaging into chromatin.

Studies of the effects of HSV IE proteins on cellular globin genes have cast some light on the foregoing issues. Early studies by Everett and coworkers revealed that transiently transfected copies of the rabbit  $\beta$ -globin gene are activated by HSV IE proteins in non-erythroid cells, whereas the endogenous chromosomal  $\beta$ -globin gene is not induced (26, 27). Although the biological relevance of the transfection assay could be questioned, entirely analogous results were obtained when the rabbit  $\beta$ -globin gene was inserted into the intact HSV genome and analyzed during lytic infection (90). Further analysis revealed that  $\beta$ -globin genes integrated into cellular chromosomes through stable transfection are also activated (25), demonstrating that extrachromosomal location is not the critical feature that distinguishes the response of transfected templates from that of the endogenous gene. These data indicate that the  $\beta$ -globin promoter is inherently susceptible to activation by IE proteins, and imply that some aspect of the higher-order structure of the endogenous  $\beta$ -globin locus precludes induction.

Recent advances in understanding of globin gene regulation (reviewed in reference 40) provide a plausible explanation for these differential responses. The 150 kb  $\beta$ -globin gene cluster (comprising

the  $\epsilon$ -,  $G\gamma$ -,  $A\gamma$ -,  $\delta$ -, and  $\beta$ - genes) is packaged into inactive heterochromatin in non-erythroid cells; the entire locus displays a DNaseI-resistant chromatin structure, and replicates late during S phase (32, 41). Tissue-specific activation during erythroid differentiation involves binding of erythroid-specific factors to the upstream locus control region ( $\beta$ -LCR) located 5 to 11 kb upstream of the cluster, generating a set of 5 DNase I hypersensitive sites (33, 39, 92). The activated LCR then mediates global decondensation of the locus into an early-replicating open chromatin conformation (32). It seems quite likely that the tightly-packed heterochromatic nature of the  $\beta$ -globin locus in non-erythroid cells shields the  $\beta$ -globin promoter from the effects of HSV IE polypeptides.

Many other tissue-specific genes are also packaged into condensed, late replicating chromatin in non-expressing cells (9, 47). However, this is not universally the case. For example, the human  $\alpha$ -globin gene cluster (comprising the  $\zeta$ 2-,  $\alpha$ 2-,  $\alpha$ 1-, and  $\theta$  genes) is embedded in a region of open, early replicating chromatin in all cell types (18, 98). Moreover, although erythroid-specific expression of the  $\alpha$ -globin gene depends on an LCR-like DNase I hypersensitive site (HS-40) located 40 kb upstream of the cluster (45, 46), this element is not required to maintain the  $\alpha$ -globin cluster in an open conformation (12). The open conformation of the  $\alpha$ -globin cluster raises interesting questions about the mechanisms that maintain the silence of the  $\alpha$ -globin gene in non-erythroid cells. Indeed, the set of transcription



factors present in non-erythroid cells is sufficient for robust  $\alpha$ -globin promoter activity, as evidenced by the finding that transfected copies of the  $\alpha$ -globin gene are strongly expressed in a variety of cell types (11, 49, 68, 95). Taken in combination, these observations argue that the endogenous  $\alpha$ -globin gene is subject to a form of negative control that does not act on newly introduced copies of the gene (11, 16). Possible explanations include (i) local chromatin-dependent repression of the  $\alpha$ -globin promoter and (ii) packaging into a form of inactive chromatin distinct from the DNase I-resistant late-replicating variety observed at the  $\beta$ -globin and many other tissue-specific loci.

We have previously shown that the human  $\alpha$ -globin gene is activated by HSV IE polypeptides when it is delivered into nonerythroid cells by infection with an HSV recombinant (72, 88). We report here that the endogenous human  $\alpha$ -globin gene is also induced during HSV infection and that the IE proteins ICP0 and ICP4 are each sufficient to trigger this response. These data demonstrate that ICP0 and ICP4 bypass or overcome the mechanisms that maintain the silence of the endogenous  $\alpha$ -globin gene in non-erythroid cells.

## Materials and Methods

**Cells and viruses.** MRC5, HeLa, and Vero cells were grown in  $\alpha$ -minimal essential medium ( $\alpha$ -MEM) containing 10%, 10%, and 5% fetal bovine serum respectively. The following HSV-1 strains were propagated on Vero cells: wild type KOS and F; KOS PAA<sup>r5</sup> (43); hrR3 (35), an ICP6 mutant; R325tk<sup>+</sup> (76), an ICP22 mutant; and N38 (97), a mutant lacking ICP47. The ICP4 null mutant d120 (20), was grown on complementing E5 cells, and the ICP27 deletion mutant, 5dl1.2 (66), was propagated on complementing 3-3 cells. The ICP0 amber mutant *n212* (12), was grown and titrated on U2OS cells (102).

**HSV infection and RNA extraction.** Unless otherwise specified, infections were at an MOI of 10 PFU/cell. Total cellular RNA was extracted using a guanidinium-isothiocyanate-based method with the RNazol B reagent (TM Cinnax, Inc.) according to the manufacturer's protocol. In some experiments, aphidicolin (10  $\mu$ g/ml) or phosphonoacetic acid (PAA, 300  $\mu$ g/ml) added to block viral DNA replication. Where indicated, cyclohexamide was added to the growth medium one hour prior to infection and maintained continuously.

**Primer extension.** 10  $\mu$ g of RNA was annealed to the 5' - <sup>32</sup>P-labelled primer (50,000 Cerenkov cpm) in 10  $\mu$ l of 10 mM Tris, pH 8.0, 1 mM EDTA, 250 mM KCl at 62° C for 1 h. 25  $\mu$ l of 20 mM Tris, pH 8.7, 10 mM MgCl<sub>2</sub>, 5 mM DTT, containing 0.33 mM of each of dATP, dCTP, dGTP,

dTTP, 10 µg/ml actinomycin D and 0.5 U of AMV reverse transcriptase (Life Sciences, St. Petersburg, FL) was then added and the reaction was incubated at 42° C for an additional hour. Extension products were precipitated with ethanol and resolved on an 8% polyacrylamide sequencing gel. Gels were exposed to X-ray films for autoradiography, or to Phosphorimager screens (Molecular Dynamics, Sunnyvale, CA) for densitometric analysis. The following synthetic primers were purchased from the central facility of the Institute for Molecular Biology and Biotechnology, McMaster University:

α-globin: 5' AGGCGGCCTTGACGTTGGTCTTGTC 3';

β-globin: 5' ACAGGGCAGTAACGGCAGACTTCTC 3';

ζ-globin: 5' CCACATGGACACAATGATGGTCCTC 3'.

**S1 nuclease protection.** 20,000 Cerenkov cpm of a 3'-labelled 300 nt BstEII-BamHI fragment spanning the 3' end of the human α2-globin gene (labelled at the BstEII site) was hybridized with 10 µg RNA at 42° C for 3 hours in 10 µl of 0.6M NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA. The samples were cooled on ice, then 140 µl 0.28M NaCl, 30 mM Na acetate pH 4.4, 4.5 mM Zn acetate containing 66 U S1 nuclease (Boehringer Mannheim) was added and the reaction was incubated at 37 °C for 1 hour. Samples were precipitated with ethanol and resolved on an 8% polyacrylamide sequencing gel.

**Plasmids.** ICP0 and ICP4 expression vectors (driven from the HCMV IE promoter; pDR27 and pBB37) were obtained from Peter O'Hare. An HCMV promoter-driven ICP22 expression vector (pICP22) was constructed as follows: a 3.5 kb KpnI-BclI fragment of HSV1 strain 17 DNA containing the ICP22 gene was cloned between the KpnI and BamHI sites of pUC18. An EcoNI to EcoRI fragment spanning the ICP22 ORF was then subcloned into the HindIII site of pRC/CMV (Invitrogen) after filling in all ends with the Klenow fragment of *E. coli* DNA polymerase I. The Adenovirus E1a expression clone (pE1a) was derived from pKH548 (6) which contains genomic Ad5 E1a and E1b DNA sequences with a BamHI site inserted at nucleotide position 548 of the E1a gene. A 1 Kb BamHI-HpaI fragment encompassing the E1a ORF was inserted into the NotI site of the pRC/CMV vector (after filling in of the BamHI end with the Klenow fragment of DNA polymerase I) to generate pE1a. pUC $\alpha$ 2 was constructed by inserting a 4.2 kb SstI fragment spanning the human  $\alpha$ 2 gene (-2775 to +1477 relative to the transcription initiation site) into pUC18. This fragment bears the globin promoter and transcription unit but lacks the LCR-like HS -40 sequence. To facilitate detection of transcripts arising from the cloned  $\alpha$ -globin gene without interference from transcripts of the endogenous gene, a modified version of pUC $\alpha$ 2 (pUC $\alpha$ 2-NsiI) bearing a 12 bp insert at the NcoI site of the  $\alpha$ 2 gene was generated. To this end, two partially complementary single-stranded oligonucleotides ( 5' CATGGTGCTATG 3' and 5' CATGCATAGCAC 3') were annealed and inserted into the NcoI on pUC $\alpha$ 2.

**Transfection.** HeLa cells were transfected with 30  $\mu\text{g}$  of ICP0, ICP4, ICP22, or E1a expression vectors using a modification of the calcium phosphate co-precipitation method (37). Where indicated, 5  $\mu\text{g}$  of pUC $\alpha$ 2-NsiI was included. In all cases, pUC18 carrier was added to bring the total amount of DNA to 60  $\mu\text{g}$ . Briefly, CsCl purified plasmid DNA was resuspended in 500  $\mu\text{l}$  of 250 mM CaCl<sub>2</sub>, then added to an equal volume of 2X HeBs buffer (350 mM NaCl, 62 mM hepes, 1.8 mM Na<sub>2</sub>HPO<sub>4</sub>). The DNA precipitate was added to HeLa cells grown to a density of  $2 \times 10^6$  cells in 100 mm tissue culture plates. After 16 h the cells were washed three times with fresh medium. The cells were then incubated for a further 24 h. Total RNA was harvested as described before and used in primer extension assays.

## Results

**Activation of endogenous human  $\alpha$ -globin genes during HSV infection of non-erythroid cells.** We examined the effects of HSV infection on expression of the endogenous  $\alpha$ -globin gene in non-erythroid cells. Parallel dishes of HeLa and MRC5 cells (immortal cervical carcinoma cells and normal human diploid fibroblasts respectively) were infected with increasing amounts of HSV-1 KOS strain Paa<sup>r</sup>5, and total cellular RNA harvested at 6 hours post infection was scored for  $\alpha$ -globin transcripts by primer extension using a 5'-labelled 25-mer complementary to residues 65-80 of  $\alpha$ -globin mRNA (Fig. 1A). As expected, uninfected cells lacked detectable  $\alpha$ -globin RNAs. However, RNA samples extracted following infection generated a strong 80 nt. primer extension signal that comigrated with that obtained using control human reticulocyte RNA. These data demonstrate that HSV infection activates expression of the previously silent  $\alpha$ -globin gene in normal human fibroblasts and HeLa cells, giving rise to correctly initiated  $\alpha$ -globin transcripts driven from the  $\alpha$ -globin promoter. We consistently observed higher levels of globin transcripts in HeLa cells compared to MRC5 cells. In one experiment where this difference was quantified, about eightfold more globin RNA was obtained in HeLa cells (not shown). We do not yet understand the basis for this difference, although the possibility that MRC5 cells are less infectable appears to be excluded by the observation that the levels of viral ICP4 mRNA are equivalent in the two cell types (not shown).

**Fig. 1. Induction of  $\alpha$ -globin transcripts in HSV-infected HeLa and MRC5 cells. HeLa and MRC5 cells were infected with HSV-1 KOS strain PAA<sup>r</sup>5 at the indicated multiplicities of infection (PFU/cell). Total cellular RNA extracted at 6 h postinfection was then analyzed for  $\alpha$ -globin transcripts by primer extension (A) and S1 nuclease protection (B). (A) Primer extension. Ten- microgram samples of RNA were analyzed with a 5'-<sup>32</sup>P-labeled oligonucleotide complementary to positions +65 to +80 of  $\alpha$ -globin mRNA, and extension products were resolved on an 8% polyacrylamide sequencing gel. Size of markers (M), HpaII fragments of pBR322 DNA, are indicated in nucleotides on the left. C, control RNA extracted from human blood. (B) Detection of  $\alpha$ 1 and  $\alpha$ 2 transcripts by S1 nuclease protection analysis. RNA samples (10  $\mu$ g) were hybridized to a 3'-labeled single-stranded DNA complementary to the 3' end of  $\alpha$ 2 mRNA (diagrammed in panel C), and then hybrids were digested with S1 nuclease. The protected portions of the probe were analyzed on an 8% sequencing gel. Signals at 212 and ca. 120 nt arise from  $\alpha$ 2 and  $\alpha$ 1 transcripts, respectively. (C) Schematic diagram of the 3'-labeled probe used in panel B. SSDNA, single-stranded DNA.**

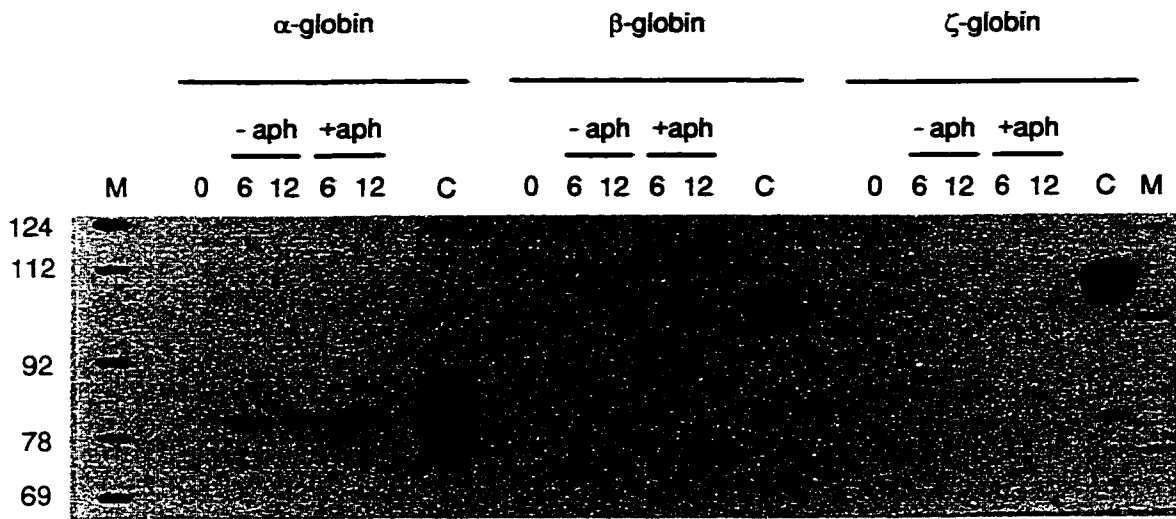




The human genome contains two tightly linked  $\alpha$ -globin genes,  $\alpha 1$  and  $\alpha 2$ , which differ only in their 3' untranslated regions. To determine whether both genes were activated, we used S1 nuclease protection analysis to differentiate between  $\alpha 1$  and  $\alpha 2$  transcripts. The 3' -labelled single-stranded probe used was derived from  $\alpha 2$  genomic sequences; as diagrammed in Fig. 1C,  $\alpha 2$  transcripts are predicted to protect 212 nt of the probe from S1 nuclease digestion, whereas  $\alpha 1$  transcripts should protect only ca. 120 nt. Infected MRC5 and HeLa cell RNAs gave rise to 212 and ca. 120 nt products which comigrated with those obtained with control reticulocyte RNA (Fig. 1B), indicating that both of the endogenous  $\alpha$ -globin genes are activated by HSV infection.

**The  $\beta$ - and  $\zeta$ -globin genes are not activated.** Previous studies have indicated that HSV IE proteins do not induce the endogenous  $\beta$ -globin gene in rabbit fibroblasts (25). To determine whether infection of human cells leads to a more global induction of globin genes, HeLa cells were infected with 10 PFU/cell with HSV-1 KOS Paa<sup>r</sup>5 in the presence or absence of aphidicolin to block viral DNA replication, and RNA extracted at 6 and 12 hours post infection was scored for  $\alpha$ -,  $\beta$ -, and  $\zeta$ - globin transcripts by primer extension (Fig. 2). As shown in Fig. 2 and further documented below, aphidicolin prevents the decline in  $\alpha$ -globin RNA levels that otherwise occurs at late times post-infection, resulting in enhanced signals at the 12 hour time point. Although  $\alpha$ -globin transcripts were induced, neither  $\beta$ - nor  $\zeta$ -globin

**Fig. 2. Primer extension analysis of  $\alpha$ -,  $\beta$ -, and  $\zeta$ -globin transcripts. HeLa cells were infected with 10 PFU of HSV-1 KOS strain PAA<sup>r5</sup> per cell in the presence and absence of 10  $\mu$ g of aphidicolin (aph) per ml. Total RNA extracted at 0, 6, or 12 h postinfection was analyzed by primer extension using 5'-labeled oligonucleotides complementary to  $\alpha$ -,  $\beta$ -, and  $\zeta$ -globin mRNAs, and the extension products were resolved on an 8% polyacrylamide sequencing gel. RNA extracted from human blood was used as positive control (C) for  $\alpha$ - and  $\beta$ -globin transcripts, while RNA from K562 cells was used as the positive control (C) for detection of  $\zeta$ -globin RNAs. M, size markers (see the legend to Fig. 1).**

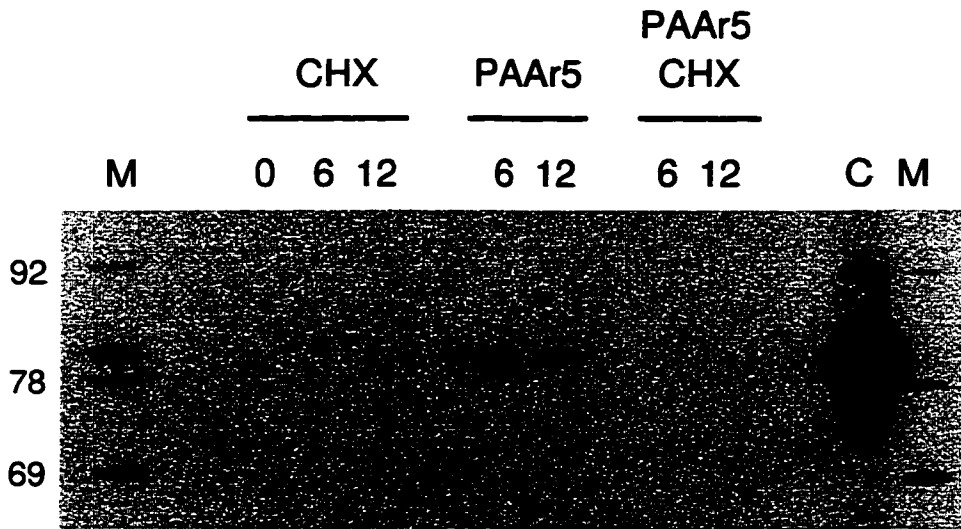
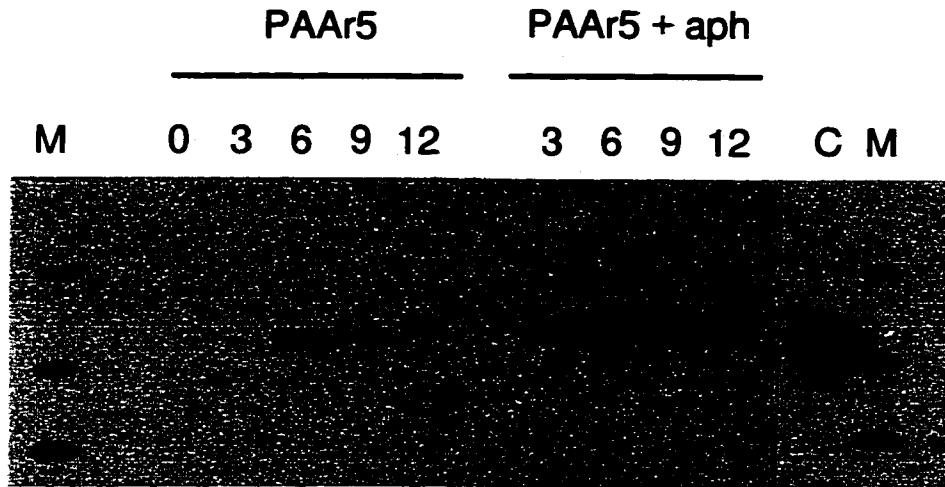


transcripts could be detected in the infected cell RNA samples. Inasmuch as the  $\beta$ - and  $\zeta$ - primers gave rise to strong signals in primer extension reactions with control RNA from human reticulocytes and K562 erythroleukemia cells (Fig. 2), we conclude that HSV-infection does not detectably activate the endogenous  $\beta$ - or  $\zeta$ -globin genes. These data confirm the previous observations made with the rabbit  $\beta$ -globin gene, and further demonstrate that HSV infection does not induce all members of the  $\alpha$ -globin gene family (as evidenced by the finding that the  $\zeta$ -globin gene is not expressed).

**Time course of  $\alpha$ -globin RNA accumulation.** We examined the time course and general characteristics of  $\alpha$ -globin transcript accumulation in order gain information about the temporal class of the HSV gene product(s) responsible for induction. Globin RNA was detected by 3 hours post infection, increased in abundance by 6 hours, then declined at later times (Fig. 3). Induction was not blocked by inhibiting viral DNA replication with aphidicolin (Fig. 3) or phosphonoacetic acid (see Fig. 5C); instead, these drugs prevented the decline of accumulated  $\alpha$ -globin mRNA at later times. In contrast, induction was blocked when viral protein synthesis was prevented with cyclohexamide (Fig. 4), a response that distinguishes the  $\alpha$ -globin gene from viral IE genes. In all of these respects, the expression pattern of the endogenous  $\alpha$ -globin gene resembled that of an HSV E gene. This in turn suggests that one or more IE polypeptides serve to activate  $\alpha$ -globin expression.

**Fig. 3. Time course of accumulation of  $\alpha$ -globin transcripts in infected cells. HeLa cells were infected with 10 PFU of HSV-1 KOS strain PAA<sup>r5</sup> per cell in the presence or absence of 10  $\mu$ g of aphidicolin (aph) per ml. Total RNA extracted at the indicated times (hours) postinfection was analyzed for  $\alpha$ -globin RNA by primer extension. Human blood RNA was used as the positive control (C). M, size markers (see the legend to Fig. 1).**

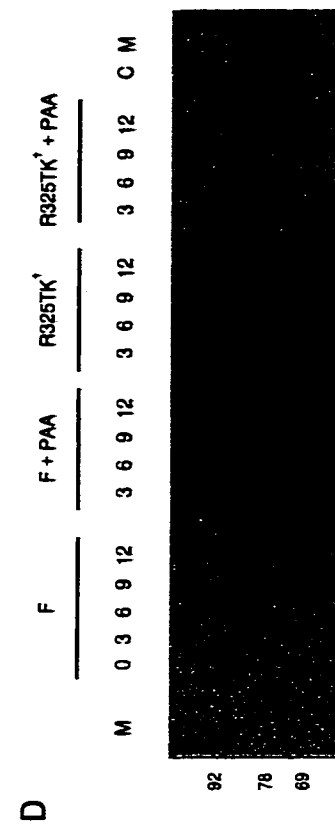
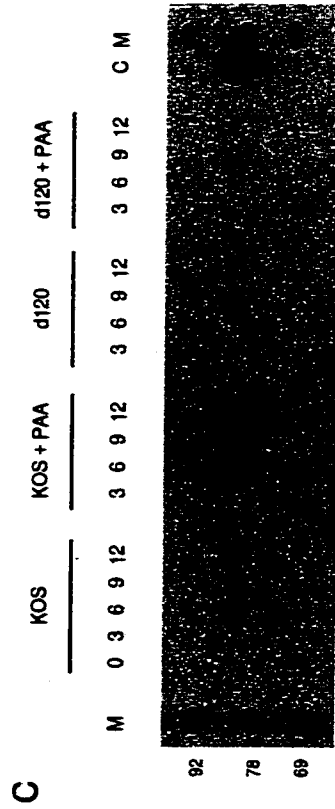
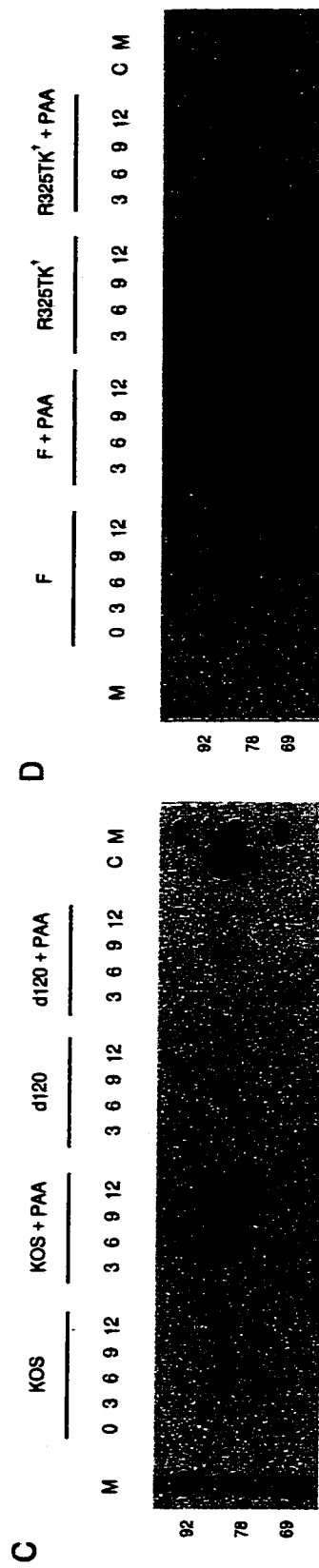
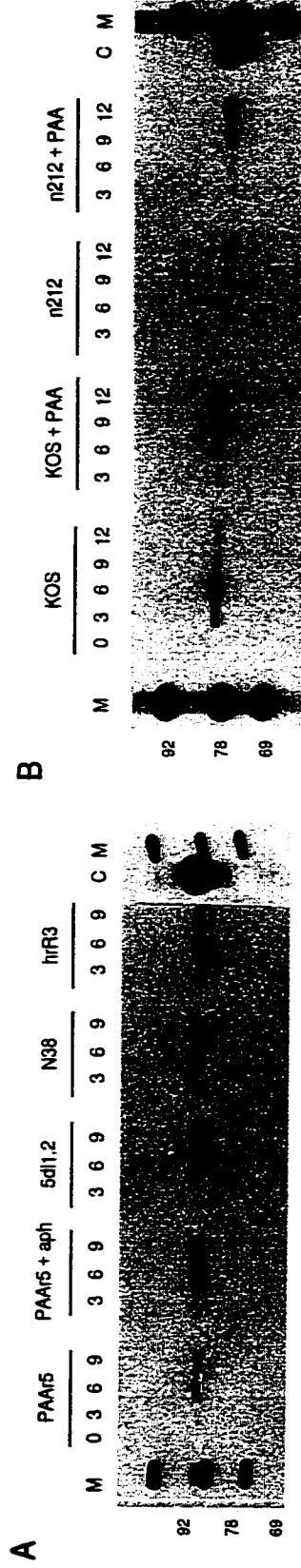
**Fig. 4. Effects of cycloheximide on induction of  $\alpha$ -globin transcripts. HeLa cells were infected with 10 PFU of HSV-1 KOS strain PAA<sup>r5</sup> per cell in the presence or absence of 100  $\mu$ g of cycloheximide (CHX) per ml, and total RNA harvested at the indicated times (hours) postinfection was analyzed for  $\alpha$ -globin RNA by primer extension. C, control; M, size markers (see the legend to Fig. 1).**



**At least two HSV immediate-early proteins are required for efficient accumulation of  $\alpha$ -globin mRNA in infected cells.** HSV encodes five IE proteins, and four of these (ICP0, ICP4, ICP22, and ICP27) have been shown to regulate gene expression in HSV-infected cells (reviewed in references 29 and 83). The E protein ICP6 is also induced by VP16 and expressed very early during infection (23). We used viral mutants bearing lesions in each of these six genes to examine their roles in activating  $\alpha$ -globin expression during infection of HeLa cells (Fig. 5). Accumulation of ICP0 mRNA was also monitored in each case as a control for infection (data not shown), except in the case of the ICP0 mutant *n212*, where ICP4 mRNA was examined. This analysis revealed that mutations that inactivate ICP27 (5dl1.2), ICP47 (N38), or ICP6 (hr3) did not impair induction of  $\alpha$ -globin RNA (Fig. 5A). Rather, these mutant strains displayed an extended period of induction, in that globin RNA levels did not decline during the later stages of infection. By contrast, viral mutants bearing lesions in the genes encoding ICP4 (d120), and ICP22 (R325TK<sup>+</sup>) displayed greatly reduced levels of globin RNA relative to the wild-type parental-strains (Fig. 5C and D). The ICP0 mutant *n212* showed a slightly impaired induction compared to the parental KOS strain (Fig. 5B); however, this may be a result of the decreased amount of ICP4 expressed compared to the parental-strain infected cells (data not shown). These results indicate that although two (and possibly three) IE proteins contribute to induction, none of these gene products is absolutely required

Fig. 5. Effects of mutations in various HSV IE genes on induction of  $\alpha$ -globin RNAs. HeLa cells were infected with 10 PFU of the indicated HSV strains per cell, and RNA extracted at the indicated times (hours) postinfection was analyzed for  $\alpha$ -globin transcripts by primer extension. Where indicated, aphidicolin (aph; 10  $\mu$ g/ml) or phosphonoacetic acid (PAA; 100  $\mu$ g/ml) was added to block viral DNA replication. Panels A to D represent independent experiments. The HSV strains used are described in Materials and Methods. C, control; M, size markers (see the legend to Fig. 1).



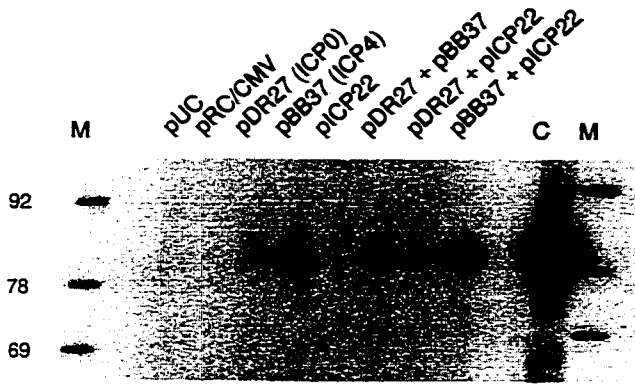


The interpretation of these results is further complicated by the fact that ICP0, ICP4 and ICP22 each modulate the expression of other HSV genes: ICP4 is required for activation of E and L gene expression (20, 100), ICP22 stimulates L gene expression in certain cell types (86), and ICP0 stimulates expression of HSV genes belonging to all three temporal classes (13, 71). Thus, it was possible that some of all of these IE proteins contribute to induction indirectly by stimulating the expression of one or more additional viral proteins which serve as the true inducer. To distinguish between direct and indirect effects, it was necessary to examine activity in the absence of other HSV gene products.

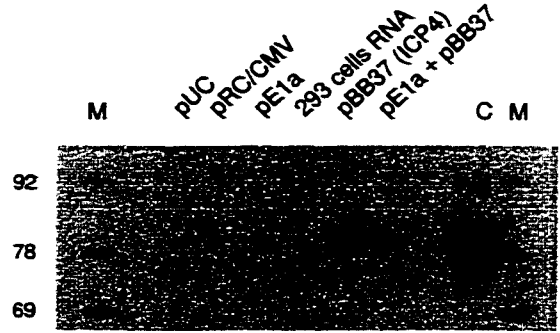
**ICP0 and ICP4 are each sufficient to trigger expression of the endogenous  $\alpha$ -globin gene in HeLa cells.** We used a transient transfection approach to determine the effects of ICP0, ICP4, and ICP22 on expression of the endogenous  $\alpha$ -globin gene in the absence of other HSV gene products. HeLa cells were transfected with expression vectors bearing these IE genes under the control of the HCMV IE promoter, and RNA samples harvested thirty-six hours later were scored for  $\alpha$ -globin RNA by primer extension (Fig. 6A). Cells transfected with the ICP0 (pDR27) and ICP4 (pBB37) expression vectors displayed easily detectable levels of  $\alpha$ -globin RNA, whereas the ICP22 vector (pICP22) did not show any activation effect (Fig. 6A). The levels of  $\alpha$ -globin expression were consistently higher in cells transfected with pBB37 (ICP4) than with pDR27 (ICP0); inasmuch as

Fig. 6. Induction of  $\alpha$ -globin transcripts in cells transiently expressing HSV IE polypeptides. (A) HeLa cells were transfected with control or expression vectors encoding ICP0, ICP4, ICP22 (pDR27, pBB37, and pICP22, respectively), separately or in combination, using the calcium phosphate transfection method. Total RNA extracted 36 h posttransfection was scored for  $\alpha$ -globin transcripts by primer extension. C, control; M, size markers (see the legend to Fig. 1). (B) HeLa cells were transfected with control or expression vectors encoding adenovirus type 5 E1a (pE1a) or HSV ICP4 (pBB37), separately or together. Total RNA was extracted and assayed for  $\alpha$ -globin RNA as for panel A. (C) HeLa cells were transfected with a plasmid bearing a modified  $\alpha$ 2-globin gene (pUC $\alpha$ 2-NsiI) in combination with vectors expressing ICP0, ICP4, ICP22, and E1a. Total RNA was extracted and assayed for  $\alpha$ -globin RNA by primer extension analysis. The plasmid-derived transcripts were 12 nt longer than the blood control RNA, giving rise to products approximately 92 nt long.

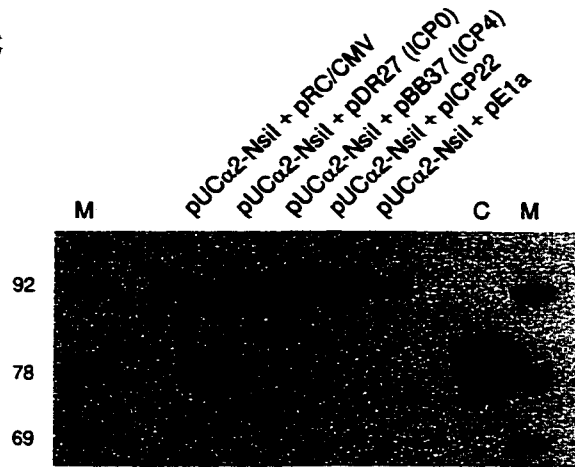
A



B



C



both plasmids bear the same vector backbone, this observation suggests that ICP4 may be a more potent activator of  $\alpha$ -globin expression than ICP0. These data demonstrate that ICP0 and ICP4 are each able to induce detectable expression of the previously silent endogenous  $\alpha$ -globin gene in the absence of other HSV gene products. ICP4 and ICP0 did not display obvious synergism when provided in combination since globin RNA levels were not significantly increased, and ICP22 had no detectable effect on the activity of either of these proteins (Fig. 6A). One possible explanation for the lack of activity of ICP22 in these assays is suggested by data indicating that another HSV protein (UL13) mediates post-translational modifications of ICP22 that may be required for activity (78, 79). Further studies are required to test this hypothesis.

#### **Adenovirus E1a does not activate the endogenous $\alpha$ -globin genes.**

Previous studies have shown that adenovirus E1a activates expression of co-transfected copies of the  $\beta$ -globin gene in a fashion similar to HSV IE proteins (38). Moreover, E1a has often been compared to HSV ICP4 because both proteins are required for efficient expression of viral and cellular genes located in their respective viral genomes during infection (8, 29, 88). Given these findings, we examined whether E1a can activate the endogenous  $\alpha$ -globin genes in non-erythroid cells.  $\alpha$ -globin transcripts could not be detected in 293 cells (36) which express E1a (Fig. 6B), although HSV infection strongly induced  $\alpha$ -globin expression in this cell type (data not shown). Moreover, HeLa cells

transfected with an E1a expression vector (pE1a) did not display  $\alpha$ -globin transcripts (Fig. 6B). Inasmuch as pE1a activated cotransfected copies of the adenovirus E1b and E4 promoters in HeLa cells (not shown), these results argue that E1a does not stimulate expression of endogenous  $\alpha$ -globin genes.

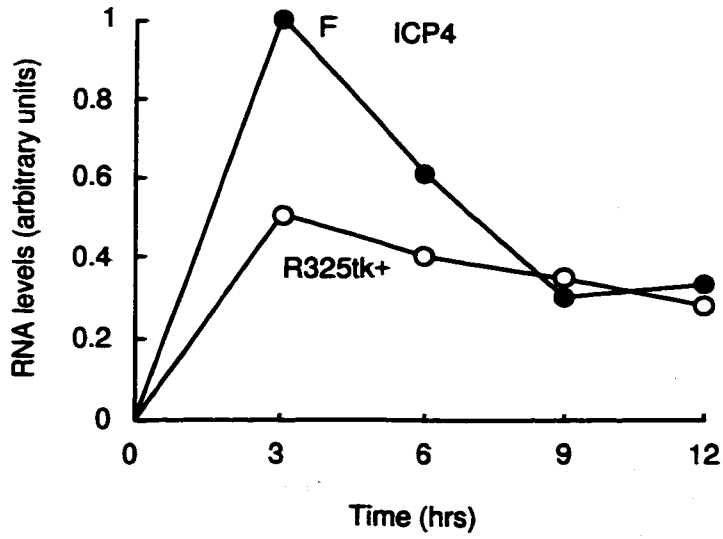
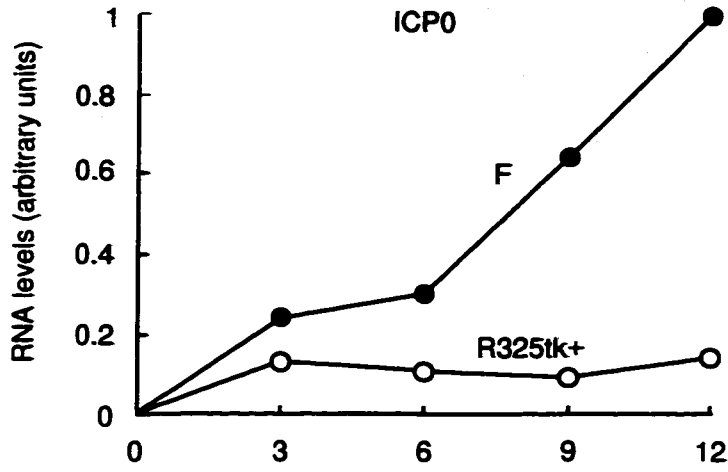
**Effects of ICP0, ICP4, ICP22, and E1a on transfected copies of the human  $\alpha$ 2-globin gene.** Although the endogenous chromosomal  $\alpha$ -globin gene is completely silent in non-erythroid cells, transfected copies are robustly expressed. Inasmuch as ICP0, ICP4, and E1a have been previously shown to enhance expression of newly introduced copies of a variety of viral and cellular genes, it was of interest to determine whether these viral proteins altered expression of a transfected  $\alpha$ -globin gene. To discriminate between transcripts of the transfected gene and those arising from the endogenous chromosomal gene, we added a 12 bp insert at the NcoI site of a cloned  $\alpha$ 2 gene (pUC $\alpha$ 2-NsiI). As a result, transcripts of this marked gene give rise to a primer extension product 12 nt longer than those of the endogenous gene. As expected, HeLa cells transfected with pUC $\alpha$ 2-NsiI expressed high levels of the modified  $\alpha$ 2 globin mRNA (Fig. 6C), illustrating the constitutive expression of transfected copies of this gene in this cell type. The ICP0 expression vector pDR27 had at best a small stimulatory effect, while the ICP4 vector consistently increased expression ca. 10 fold. A longer exposure of the gel confirmed that in both cases transcripts arising from the endogenous gene were induced

(data not shown). The ICP22 vector had no detectable effect, while pE1a consistently reduced expression of the transfected gene.

**Effects of an ICP22 mutation on accumulation of ICP4 and ICP0 transcripts during infection of HeLa cells.** The finding that ICP0 and ICP4 each induce expression of the endogenous  $\alpha$ -globin gene in transfected cells explains why neither protein is essential for detectable activation during HSV infection. However, these data do not explain why inactivating ICP22 function during infection reduces induction ca. 10-fold, while ICP22 has no effect in the transfection assay. Purves *et al* (78) have shown that ICP22 is required for the accumulation of ICP0 and several other viral mRNAs during infection of restrictive cell types. Inasmuch as the results presented above demonstrate that ICP0 and ICP4 are directly involved in  $\alpha$ -globin induction, we compared the accumulation of ICP0 and ICP4 RNAs during infection of HeLa cells with R325tk<sup>+</sup> and the parental F strain by primer extension (Fig. 7) The data indicated that the levels of ICP4 and ICP0 transcripts are reduced relative to wild-type levels in this cell type. With ICP4 RNA, the effect was relatively small (twofold reduction) and was evident only at the early time points. However, ICP0 RNA levels were reduced throughout infection, and the deficit relative to wild-type virus became progressively more pronounced as the infection progressed. These data raise the possibility that ICP22 plays an indirect role in  $\alpha$ -globin induction, by stimulating the expression of ICP0 and possibly ICP4.

Fig. 7. Accumulation of ICP0 and ICP4 transcripts during infection with R325TK<sup>+</sup>. HeLa cells were infected with 10 PFU of HSV-1 strain F or the ICP22-deficient mutant R325TK<sup>+</sup> per cell, and RNA samples extracted at the indicated times postinfection were analyzed for ICP0 (upper panel) and ICP4 (lower panel) transcripts by primer extension. Primer extension signals were then quantified by PhosphorImager analysis. For each transcript, values were normalized to the most intense signal observed during the time course.





## Discussion

The data presented in this paper demonstrate that correctly initiated transcripts derived from the endogenous human  $\alpha$ -1 and  $\alpha$ -2 globin genes accumulate during HSV infection of normal human fibroblasts and HeLa cells, and that the IE proteins ICP4 and ICP0 are each sufficient to trigger this effect. Inasmuch as Macleod *et al* could not detect transcription of the  $\alpha$ -globin gene in non-erythroid cells by using a nuclear run-on assay (65), these data imply that HSV IE proteins activate transcription of the previously silent endogenous gene. Previously silent endogenous globin genes are also activated when nonerythroid cells are fused with erythroid cells to form heterokaryons (3, 4). However, the HSV-mediated induction reported here is distinct from the erythroid cell-specific reprogramming of globin gene expression that occurs in heterokaryons, because the  $\alpha$ -globin gene is expressed whereas the  $\beta$ -globin gene is not.

The difference in responses of the endogenous  $\alpha$ - and  $\beta$ - globin genes to HSV infection is intriguing, as the  $\alpha$ - and  $\beta$ - globin promoters are both strongly activated by HSV IE polypeptides when they are newly introduced into cells as part of infecting recombinant HSV genomes (72, 88, 90). As reviewed in the Introduction, the  $\alpha$ - and  $\beta$ -globin gene clusters adopt very distinct chromatin structures in non-erythroid cells: the  $\alpha$ -globin locus is packaged in a relatively "open" early-replicating configuration (18, 98), while the  $\beta$ -cluster displays a

DNaseI resistant, late replicating, tightly "closed" conformation (32, 41). It seems likely that these differences in accessibility account, at least in part, for the differential responses of the endogenous  $\alpha$ - and  $\beta$ -globin promoters to HSV IE proteins. If so, then the large number of other tissue-specific genes that are assembled into late-replicating, closed chromatin in non-expressing cells may also be shielded from activation. However, this explanation probably cannot account for the inactivity of  $\zeta$ -globin gene, which is located in the  $\alpha$ -globin gene cluster and presumably packaged in a relatively open conformation. It will therefore be interesting to determine whether or not the  $\zeta$ -promoter is inherently susceptible to activation by HSV IE proteins, by assaying the activity of  $\zeta$ -globin genes delivered into cells as part of an infecting HSV genome.

The relatively open chromatin structure of the  $\alpha$ -globin gene may stem in part from its CpG-rich promoter region (10); CpG islands are in general hypomethylated and less condensed than bulk chromatin (94). (Although Antequera *et al* reported that the entire  $\alpha$ -globin gene and promoter are heavily methylated in HeLa cells [11, 2], we find that only the transcribed body of the gene is methylated in our subline [data not shown].) However, the possibility that all tissue-specific genes bearing CpG islands are activated by HSV infection appears to be excluded by our observation that hepatocyte-specific retinol binding protein mRNA does not accumulate during infection of HeLa cells (data not shown).

The complete silence of the endogenous  $\alpha$ -globin gene in non-erythroid cells contrasts strikingly with the constitutive activity of the  $\alpha$ -globin promoter borne on transfected templates (11, 16, 68). These data have led to the hypothesis that chromatin-dependent repression mechanisms act to prevent expression of the endogenous gene (11, 16). Our data indicate that HSV ICP0 and ICP4 either bypass or disrupt these silencing mechanisms. It is interesting to relate this activity to previous information about the functions of ICP0 and ICP4 in the HSV life cycle.

Little is known of the biochemical mechanism of action of ICP0; however, biological studies have demonstrated that it stimulates expression of all three temporal classes of HSV genes during lytic infection (12, 71, 85, 93). Moreover, it plays a pivotal role in triggering the onset of the viral lytic cycle, as evidenced by the finding that infecting HSV genomes often enter a transcriptionally quiescent state in the absence of ICP0 function (84, 93). A remarkable feature of this quiescent state is that otherwise constitutively active heterologous promoters embedded in the viral genome are silenced along with the HSV lytic promoters (53). Provision of ICP0 *in trans* breaks this quiescent state, leading to expression of the heterologous transgene and activation of the lytic cycle (44, 53). ICP0 also plays an essential role in reactivation of the HSV genome from *in vivo* latency in sensory neurons (60). Taken in combination, these data suggest that ICP0 acts to convert HSV genomes from an inactive state to an active

conformation. The observation that ICP0 induces expression of the resident  $\alpha$ -globin gene without stimulating expression of transfected copies of the same gene raises the possibility that an analogous transition is involved. If so, then further study of this system will provide a valuable model for understanding the mechanisms of HSV latency and reactivation.

ICP4 is an essential transcriptional activator that induces expression of HSV E and L genes and represses IE gene transcription (20, 100). It displays both sequence-specific and generalized DNA binding activity (24, 30, 31, 55, 69, 101), and mutations that inactivate DNA binding abolish the transcriptional regulatory properties of the protein (19, 21, 22, 73, 74, 87). Although appropriately positioned ICP4 binding sites target promoters for repression by ICP4 (57, 61, 81, 82), specific binding sites are not required for transactivation (42, 52, 89), which appears to be mediated through interactions with TBP, TF IIB, and TAF250 (15, 91). The transcriptional activation function of ICP4 almost certainly accounts for stimulation of transfected  $\alpha$ -globin templates. Perhaps ICP4 and components of the basal transcriptional apparatus can access the TATA region of the globin promoter despite the restrictive chromatin conformation that excludes upstream activator proteins. Inasmuch as ICP4 provides the functional equivalent of the activation signal delivered by Sp1 (50), this might prove sufficient to induce expression. Alternatively, ICP4 might actively disrupt the restrictive chromatin structure as a prelude to

activation. These possibilities could be distinguished by high-resolution *in vivo* footprinting of the gene before and after induction by ICP4. Irrespective of the precise mechanism involved, the ability of ICP4 to induce expression from a restrictive chromatin environment is likely to be significant during emergence of the HSV genome from latency.

We found that inactivating ICP22 function reduced induction during HSV infection ca. 10-fold. However, the ICP22 expression vector did not stimulate the  $\alpha$ -globin gene in transfected cells, nor did it show synergistic effects with ICP0 or ICP4 in cotransfection assays. The possibility that the defect observed during infection stems from other unsuspected mutations in the R325tk<sup>+</sup> isolate appears to be excluded by the finding that another independently isolated ICP22 mutant (75) displays the same phenotype (data not shown). One possible explanation is that ICP22 is required for efficient expression of ICP4 and/or ICP0 during infection of HeLa cells. Consistent with this view, we found that ICP4 and ICP0 mRNA levels were reduced relative to wild-type levels during infection with R325TK<sup>+</sup>. However, these data do not exclude the possibility that ICP22 also directly contributes to activation. If so, then the results of the transfection assays suggest that this effect requires the presence of one or more additional viral proteins. Supporting this latter view, Roizman *et al* have provided evidence that ICP22 is phosphorylated by the viral protein kinase encoded by gene UL13 during infection (79).

Furthermore, the activity of ICP22 may depend on this posttranslational modification, as evidenced by the finding that ICP22 and UL13 mutants display similar defects in viral gene expression (78). ICP22 is involved in HSV-induced changes to the phosphorylation status of the carboxy-terminal domain of the large subunit of the cellular RNA polymerase II (80). Inasmuch as such changes may alter the elongation properties of polymerase II (7), it may prove informative to explore the effects of ICP22 (with and without UL13) on the processivity of transcription of the endogenous  $\alpha$ -globin gene in infected cells.

We suspect that further studies of the mechanisms by which HSV IE proteins stimulate expression of the endogenous  $\alpha$ -globin gene will reveal novel activities of these regulators and shed light on mammalian gene silencing mechanisms.

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**Chapter 4: Analysis of the methylation and chromatin structure of the endogenous  $\alpha$ -globin gene**

## Preface

The experiments described in this chapter address the tissue-specific regulation of the human  $\alpha$ -globin gene. In particular, I present data showing that the endogenous  $\alpha$ -globin gene in HeLa cells can be activated by treating cells with a combination of DMSO and cycloheximide or anisomycin. Also, I examined several possible mechanisms that may silence expression of this gene in non-erythroid cells and proposed that the deposition of a nucleosome at the TATA box and transcription start site of the  $\alpha$ -globin gene is involved in the repression of this gene in non-erythroid cells.

Although this project mainly focused on the  $\alpha$ -globin gene, it is important to note that transcription of members of the  $\alpha$ - and  $\beta$ -globin clusters are regulated not only individually by proximal *cis*-acting elements, but also collectively, as gene families, by distal enhancers, and by factors that alter chromatin structure and methylation status of the globin loci. In this introduction, I have provided a brief overview of our current understanding of the developmental and tissue-specific expression of the globin genes, and highlighted some key areas of ongoing research. Put into perspective, our study is just one piece of a complex regulatory puzzle that governs expression of the globin genes in erythroid and non-erythroid cells.



## Introduction

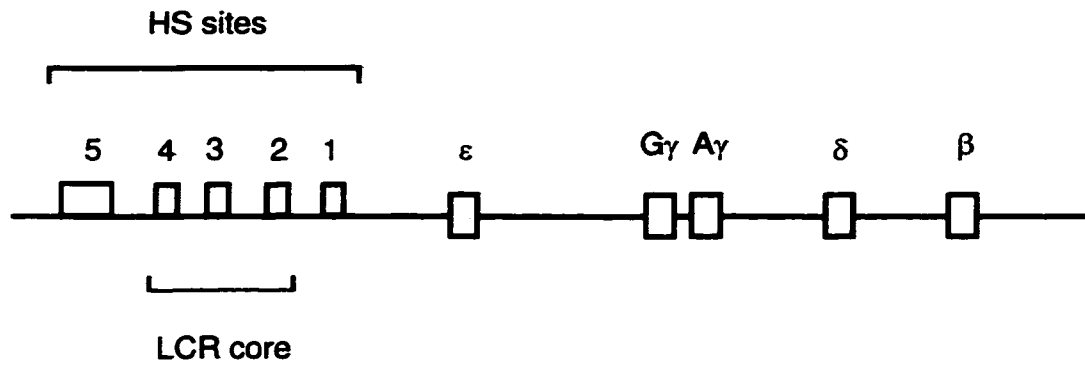
### 4.1 Organisation and expression of the $\alpha$ - and $\beta$ -globin genes

The  $\alpha$ -like and  $\beta$ -like globin genes are two multigene families that are tissue-specific and developmentally regulated. They are only expressed in erythroid tissues and their gene products together form the heterotetrameric haemoglobin molecules (e.g.,  $\alpha_2\beta_2$ ). In humans, it is believed that the haematopoietic stem cells migrate to different organs during development and hence the site of globin synthesis switches accordingly as well. Erythropoiesis initially occurs in the primitive erythroblasts of the yolk sac, but switches to the fetal liver after approximately 7 - 10 weeks of gestation. Soon after birth, a second switch takes place and the bone marrow becomes the adult site of haematopoiesis for the rest of the organism's life span (reviewed in Higgs, 1990; Wood, 1993; Grosveld, et al., 1993).

Coordinated with the changes in the site of erythropoiesis, different members of the globin gene families are expressed at different developmental stages. In mammals, the arrangement of the genes in the globin loci mirrors their developmental order of expression (reviewed in Orkin, 1995; Higgs, 1990). For example, within the human  $\beta$ -globin cluster on chromosome 11, the embryonic  $\epsilon$ -globin gene is located upstream of the fetal  $\gamma$ -globin genes ( $G\gamma$  and  $A\gamma$ ),

**Fig. 1. Schematic drawing of the  $\beta$ - (A) and  $\alpha$ -globin loci (B). The  $\beta$ -globin cluster is comprised of  $\epsilon$ , G $\gamma$ , A $\gamma$ ,  $\delta$  and  $\beta$ -globin genes, and the five DNase hypersensitive sites (HS) are located 6 to 20 kb upstream of the  $\epsilon$ -globin gene. The  $\alpha$ -globin cluster is comprised of  $\zeta$ 2,  $\alpha$ 2 and  $\alpha$ 1 genes and the HS -40 is located 40 kb upstream of the  $\zeta$ 2-gene.**

A

 $\beta$ -globin locus on human chromosome 11

B

 $\alpha$ -globin locus on human chromosome 16

which in turn are 5' to the adult  $\delta$ - and  $\beta$ -globin genes (Fig. 1A). Expression of the  $\gamma$ -globin genes is restricted to fetal liver cells, and is repressed soon after birth and replaced by the expression of  $\delta$ - and  $\beta$ -globin genes in the adult bone marrow. Analogously, within the human  $\alpha$ -globin locus ( $\zeta 2$ - $\alpha 2$ - $\alpha 1$ , Fig. 1B) on chromosome 16, the  $\zeta 2$  gene is expressed at the embryonic stage whereas the  $\alpha 2$  and  $\alpha 1$  genes are expressed in both fetal and adult life. The  $\alpha 2$  and  $\alpha 1$  genes are almost identical in sequence and encode the same protein product (Orkin and Goff, 1981). While both of these genes are expressed throughout fetal and adult stages, their relative level of expression changes during development (Liebhaber, et al., 1986; Albitar, et al., 1992). Initially, the two  $\alpha$ -globin genes are expressed in equal amounts in the fetus, but by adulthood, the level of  $\alpha 2$  expression is approximately 3 times higher than  $\alpha 1$  as measured by both mRNA and protein levels. The exact cause of this change is not known, but is believed to be mediated at the transcription level, and is related to the positioning of the two genes relative to the upstream enhancer element HS -40 (this element will be discussed in more details later).

Although the two globin families are organised as two separate gene clusters on different chromosomes, their expression is tightly coordinated to provide equal amounts of  $\alpha$ - and  $\beta$ -globin chains for assembly into haemoglobin molecules. The mechanism responsible for the coordinated regulation is not known; however, the ratio of  $\alpha$ - to  $\beta$ -globin chains in normal individuals is maintained as  $1 \pm 0.05$ .

Mutations or deletions of globin DNA sequences can result in imbalanced expression of  $\alpha$ - or  $\beta$ -globin chains in affected individuals which in turn leads to serious health problems (Higgs, 1993; Schrier, 1994). Research into the molecular causes of these genetic diseases has led to significant advances in our understanding of the control of individual globin genes as well as the molecular mechanisms that determine their tissue- and developmental stage-specific expression. However, the overall regulation of these genes is highly complex and the current knowledge of the mechanisms involved is far from complete.

#### **4.2 Developmental regulation of globin genes**

By far the most intensely studied, and hence best understood, area of globin gene regulation is the developmental switching of the  $\beta$ -globin genes. This perhaps reflects the clinical relevance of this area of research since there is a higher incidence of patients suffering from severe  $\beta$ -thalassemia (a condition caused by the loss of  $\beta$ -globin expression) than those suffering from  $\alpha$ -thalassemia. As the  $\alpha_2$  and  $\alpha_1$  genes are functionally equivalent, there are a total of four copies of the adult  $\alpha$ -globin gene per cell. Individuals with mutations that inactivate one to three copies of this gene can still survive with the remaining  $\alpha$ -globin gene(s), but suffer from mild to chronic anaemia depending on the number of functional genes left (Weatherall, 1997; Weatherall, 1997). Since the  $\alpha$ -globin gene is essential throughout fetal

development and adulthood,  $\alpha$ -globin-null fetuses do not normally develop and most often die *in utero*. As a result, it is rare to find patients that do not express any  $\alpha$ -globin at all. In contrast, the  $\beta$ -globin genes are only required in the adult stage of development and  $\beta$ -globin-null fetuses develop relatively normally, but suffer from severe anemia and related growth defects when expression of the fetal  $\gamma$ -globin gene is switched off at birth (Weatherall, 1997). Incidentally, patients with homozygous defects in their  $\beta$ -globin genes suffer from severe anemia (Cooley's anemia) not because of the lack of functional hemoglobin molecules, but due to the destruction of red blood cells caused by the precipitation of uncoupled  $\alpha$ -globin chains (Schrier, 1994). Consequently, much more effort is focused on the study of the developmental switching of  $\beta$ -globin genes and the possible reactivation of the fetal  $\gamma$ -globin genes in adult tissues to complex with the excess  $\alpha$ -globin chains.

#### **4.3 Identification of the LCR and HS -40 enhancer elements**

The use of transgenic mouse models is ideal for studying the developmental switching of globin genes since this process cannot be duplicated in tissue-culture cells. However, it should be noted that mice do not have a fetal-specific globin gene, and the human fetal-specific  $\gamma$ -globin gene is expressed in the embryonic stage of development in transgenic mice. Early studies introducing human globin transgenes into the mouse genomes showed that, for example,

$\gamma$ - and  $\beta$ -globin transgenes were correctly expressed in the mouse erythroid tissues; however, their expression was low compared to the endogenous mouse  $\beta$ -globin genes, and was variable depending on the site of integration (Chada, et al., 1986; Kollias, et al., 1986; Magram, et al., 1985; Townes, et al., 1985). In contrast, studies using mouse erythroid cell somatic hybrids containing the whole human chromosome 11 showed that the human  $\beta$ -globin gene in these cells was expressed at high levels equivalent to that seen for the mouse endogenous  $\beta$ -globin gene (Chao, et al., 1983; Zavoday, et al., 1983; Papayannopoulou, et al., 1985). Therefore, crucial regulatory elements present on the intact chromosome must be missing in the constructs used to generate the transgenic animals. Together with the observation that some  $\beta$ -thalassemic patients have intact  $\beta$ -globin genes but have large deletions in the sequences upstream of the  $\beta$ -globin locus (Van der Ploeg, et al., 1980; Driscoll, et al., 1989), researchers eventually identified the Locus Control Region (LCR) element upstream of the  $\beta$ -globin gene cluster. When linked to the human globin genes and introduced into transgenic mice, the LCR not only directed high level expression of the globin transgenes, but also allowed them to be expressed in a copy number-dependent and integration site-independent manner (Grosveld, et al., 1987).

The LCR is defined as a series of developmentally-stable, erythroid-specific DNase I hypersensitive (HS) sites (HS1 to HS5) located 6 to 20 kb upstream of the  $\epsilon$ -globin gene (Fig. 1A) (Tuan, et al.,

1985; Forrester, et al., 1986; Forrester, et al., 1987; Grosveld, et al., 1987). Analysis of individual HS sites showed that their formation is dependent on the binding of ubiquitous (such as Sp1) and erythroid-specific (GATA-1 and NF-E2) transcription factors to their core sequences (reviewed in Grosveld, et al., 1993). Of the five sites, HS2, HS3, and HS4 form the functional core responsible for the LCR activity (Forrester, et al., 1989; Ryan, et al., 1989; Fraser, et al., 1990; Collis, et al., 1990). Each of these three HS sites can augment transcription of the linked globin genes in erythroid cells; however, only HS2 functions as a *bona fide* enhancer element (Talbot, et al., 1990; Ney, et al., 1990; Ney, et al., 1990). The LCR is also required for establishing the open chromatin conformation of the  $\beta$ -globin locus in erythroid cells and for the early replication timing of this region in S phase of the cell cycle. Naturally occurring deletions of the LCR seen in  $\beta$ -thalassemic patients showed that in its absence, the  $\beta$ -globin locus and its surrounding region (~200 kb in total) was folded into condensed chromatin in erythroid cells, and replicated at late times during S phase (Forrester, et al., 1990). This property likely allows the LCR to define the chromatin structure of its surrounding region when introduced into transgenic mice and insulate the linked transgenes from any restrictive chromatin effects at the site of integration.

A LCR-like element was also identified upstream of the  $\alpha$ -globin locus. Initial transgenic studies showed that mice containing the human  $\zeta$ - or  $\alpha$ -globin transgene alone did not express the human



globin genes in any tissue (Palmiter and Brinster, 1986); however, when the LCR was linked to the  $\alpha$ -globin gene, this transgene was expressed at high-levels and in an erythroid-specific manner (Hanscombe, et al., 1989; Ryan, et al., 1989). A search for DNase I hypersensitive sites upstream of the  $\alpha$ -globin locus found a number of erythroid-specific DNase I HS sites, but only one of these sites, HS -40 (hypersensitive site minus 40), located 40 kb upstream of the  $\zeta$ -globin gene, had enhancer activity that could drive high level expression of linked globin genes in erythroid cells (Higgs, et al., 1990; Jarman, et al., 1991; Sharpe, et al., 1992). Analysis of this HS site showed that it resembles HS2 of the LCR both structurally (containing binding sites for ubiquitous and erythroid-specific transcription factors) and functionally (able to act as powerful erythroid-specific enhancer) (Jarman, et al., 1991; Strauss, et al., 1992). Transgenic mice carrying human  $\zeta$ - or  $\alpha$ -globin transgenes linked to the HS -40 expressed the human globin genes at high levels in the appropriate tissue and developmental stages. However, even with the HS -40, expression of the  $\alpha$ -globin transgene was not proportional to the number of copies present, and its high level expression declined as the animal aged (Higgs, et al., 1990; Sharpe, et al., 1992). From these experiments, it is clear that the LCR and the  $\alpha$ -HS -40 are not functionally equivalent. Unlike the  $\beta$ -globin locus which is organised in a condensed chromatin conformation in non-expressing cells, the  $\alpha$ -globin locus already lies in a region of open chromatin in all cell types (Vyas, et al., 1992); therefore, HS -40 may not have a chromatin opening mechanism

analogous to the LCR. It is not known why the expression of the  $\alpha$ -globin transgene is repressed over time even in the presence of HS -40. Other distal regulatory elements may be required for continual expression of the  $\alpha$ -globin genes throughout development; however, extensive searches for such additional elements have not been fruitful thus far (Sharpe, et al., 1993).

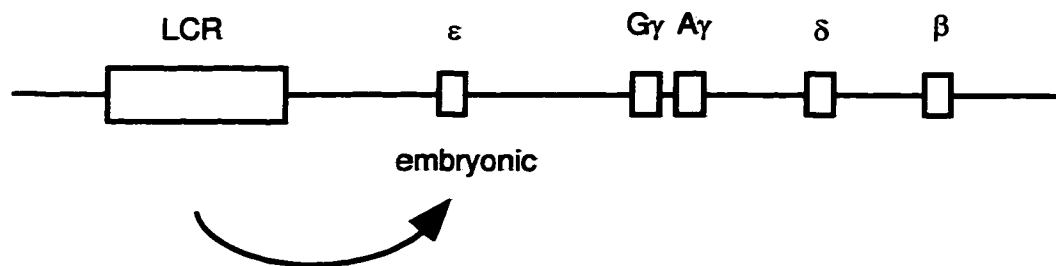
#### **4.4 Developmental switching of the $\beta$ -globin genes is mediated by the competition of individual globin promoters for the LCR**

The current theory suggests that the LCR performs a two-step process important for the expression of the  $\beta$ -globin genes in erythroid cells: it is first required to decondense the chromatin surrounding the  $\beta$ -globin locus, and once an open chromatin domain has been established, the LCR then acts as powerful enhancer element driving high level expression of the downstream globin genes. Transfection analysis showed that all members of the  $\beta$ -globin locus are activated by the LCR, and they may also compete for its enhancement effects (reviewed in Grosveld, et al., 1993). As the genes within the  $\beta$ -globin cluster are arranged in their order of expression during development, it has been suggested that competition among these genes for the LCR mediates the developmental switching of the  $\beta$ -globin genes (Orkin, 1990; Epner, et al., 1992; Grosveld, et al., 1993). In this model, the relative positioning of the globin genes as well as developmental stage-specific factors determine which globin gene is preferentially activated

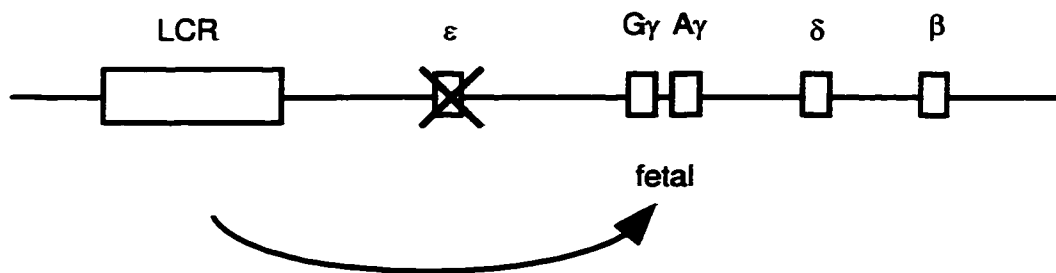
by the LCR. For example, during embryonic development, the most proximal  $\epsilon$ -globin gene is the first globin gene activated by the LCR. Sequential silencing of the  $\epsilon$ - and  $\gamma$ -globin promoters during development would then allow corresponding expression of the downstream  $\gamma$ - and  $\beta$ -globin genes (Fig. 2). This hypothesis is supported by the results of a number of studies. First, studies examining how the order of globin genes relative to the LCR affected their expression showed that, in general, the gene closest to the LCR was expressed at higher levels than the distal gene (Hanscombe, et al., 1991; Peterson and Stamatoyannopoulos, 1993). These findings are consistent with the idea that the arrangement of the globin genes within the  $\beta$ -globin cluster is important for determining the order of expression. Second, Kim *et al.* used homologous recombination to introduce a hygromycin B resistance ( $\text{hygro}^R$ ) gene driven by the Friend virus LTR between the first two HS sites of the LCR in a cell line normally expressing the adult  $\beta$ -globin gene. They found that expression of the inserted  $\text{hygro}^R$  gene was activated upon cellular differentiation, and this in turn silenced expression of the downstream  $\beta$ -globin gene. Therefore, the more proximal  $\text{hygro}^R$  gene can out-compete the distal  $\beta$ -globin gene for activation by the LCR (Kim, et al., 1992). Third, when the human  $\beta$ -globin transgene linked to the LCR was introduced to embryonic stem cells to generate transgenic mice, these mice inappropriately expressed the human  $\beta$ -globin transgene at all developmental stages (Behringer, et al., 1990; Enver, et al., 1990). Proper developmental regulation was established when the  $\gamma$ -globin

Fig. 2. Schematic drawing illustrating the competition model which directs the developmental switching of the  $\beta$ -globin genes. In the embryonic yolk sac, the  $\beta$ -globin LCR activates expression of the closest  $\epsilon$ -globin gene. During fetal development, the  $\epsilon$ -globin promoter is silenced and the LCR activates expression of the fetal stage-specific  $\gamma$ -globin genes. Finally, in adulthood, the  $\gamma$ -globin genes are also repressed and the LCR directs expression of the adult  $\delta$ - and  $\beta$ -globin genes.

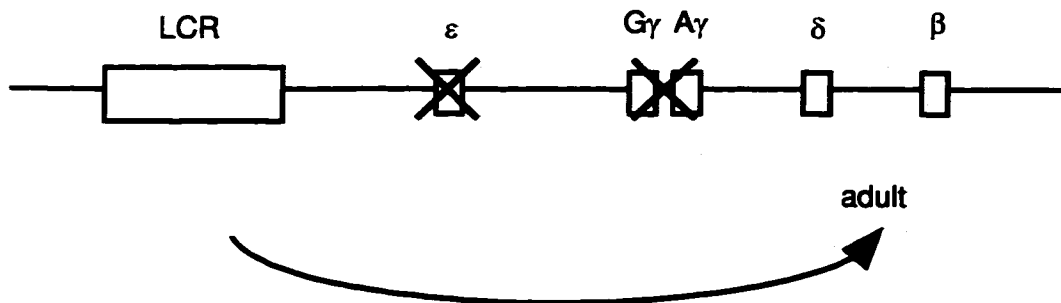
embryonic yolk sac



fetal liver



adult bone marrow



gene was placed between the LCR and the  $\beta$ -globin gene, suggesting that developmental regulation of the  $\beta$ -globin gene was mediated, at least in part, by a competition between the two globin promoters for the LCR. Fourth, analysis of fetal liver cells from transgenic mice carrying the human  $\beta$ -globin locus showed that developmental switching occurs at this site and a significant number of these cells express both  $\gamma$ - and  $\beta$ -globin within the same cell. By using *in situ* hybridisation to detect  $\gamma$ - or  $\beta$ -globin intron-containing primary transcripts as a measure of current transcription (intron-containing transcripts are quickly spliced and therefore short-lived), Wijgerde *et al.* elegantly showed that the  $\gamma$ - and  $\beta$ - globin genes were never co-transcribed at the same time (Wijgerde, et al., 1995). Instead, transcription of these two genes flip-flopped within the same cell to accumulate both  $\gamma$ - and  $\beta$ -globin mRNA. This finding argues that the HS sites within LCR functions as a single unit and activates transcription of the globin genes one at a time. Furthermore, competition of the  $\gamma$ - or  $\beta$ -globin promoters for the interaction with the LCR can then determine which globin gene is dominantly expressed.

According to the competition model, expression of globin genes is also determined by developmental stage-specific factors that direct interaction of the preferred globin promoter with the LCR element. By analysing expression of transfected globin promoters, Jane *et al.* identified elements within the human  $\gamma$ -globin gene that bind to fetal stage-specific factors and allow the  $\gamma$ -globin promoter to preferentially

interact with the linked HS2 enhancer element (Jane, et al., 1992; Amrolia, et al., 1995). In their studies, the authors made constructs containing the HS2 linked to two tandemly placed reporter genes (CAT and luciferase) driven by the  $\beta$ - and  $\gamma$ -globin promoters respectively. When these constructs were transfected into K562 cells (a human erythroleukemic cell line that normally expresses  $\epsilon$ - and  $\gamma$ - but not the adult  $\beta$ -globin genes) and assayed for the relative expression of CAT and luciferase, the authors found that the  $\gamma$ -globin promoter was preferentially expressed over the  $\beta$ -globin promoter which in turn down-regulated expression of the adjacent  $\beta$ -globin promoter. This effect was shown to be mediated by the competition of the two promoters for the linked HS2 enhancer since it was dependent on the presence of a functional HS2 enhancer element. Deletion analysis further identified two separate elements, one at around position -50 relative to the transcription start site, and the other in the 5' untranslated region of the  $\gamma$ -globin gene, which allowed preferential expression of the  $\gamma$ -globin promoter in the K562 cells. These two elements, called stage selector elements (SSE), were found to bind nuclear factors, named stage specific proteins (SSP) (Jane, et al., 1995), and directed enhancer activity of HS2 to the  $\gamma$ - instead of the  $\beta$ -globin promoter. Insertion of the -50 $\gamma$  element into the  $\beta$ -globin promoter allowed expression of this modified gene in fetal stage-specific K562 cells, suggesting that it was a genuine developmental stage-specific element (Jane, et al., 1992).

#### 4.5 Analysis of the individual globin promoters

Although the competition between  $\gamma$ - and  $\beta$ -globin promoters for activation by the LCR element likely determines the adult stage-specific expression of the  $\beta$ -globin gene, this mechanism is not universally employed by all globin genes. For example, transgenic mice carrying the individual  $\epsilon$ - or  $\gamma$ -globin transgenes linked to the LCR showed developmental stage-specific expression, suggesting that they were autonomously regulated by the flanking sequences within the transgene constructs (Raich, et al., 1990; Dillon and Grosveld, 1991). *In vitro* mapping studies have shown that each globin promoter contains numerous nuclear factor binding sites (reviewed in Grosveld, et al., 1993), and transfection assays have identified multiple regulatory elements that either positively or negatively regulate transcription of these globin genes. For example, analysis of the  $\epsilon$ -globin promoter by transfection assay showed that a negative regulatory element was present in the promoter sequences between -392 to -177 relative to the transcription start site (Cao, et al., 1989). Deletion of this sequence resulted in 3 fold and 10 fold higher expression of the transfected reporter genes in K562 (erythroid) and HeLa (non-erythroid) cell lines. Conversely, addition of this sequence to a heterologous TK promoter reduced the CAT reporter gene transcribed from this promoter in transfected cells by 4 to 10 fold. Transgenic mice carrying a construct containing the LCR cassette linked to the  $\epsilon$ -globin transgene with a deletion in the silencer element



showed extended expression of the  $\epsilon$ -globin gene into the fetal and adult stages (Raich, et al., 1990). However, expression of this gene at these stages was 45 to 60 fold lower than in the embryonic yolk sac. Therefore, in spite of some leaky expression of the  $\epsilon$ -globin gene in later developmental stages, the identified silencer element was not entirely responsible for the down-regulation of this gene during development.

Analysis of the  $\gamma$ -globin promoter by transfection and *in vitro* binding assays showed that a number of ubiquitous and erythroid-specific factors bind to its 5' flanking promoter sequences (Anagnou, et al., 1986; Gumucio, et al., 1991; Jane, et al., 1992). Studies using transgenic mice with constructs containing the LCR linked to truncated versions of the  $\gamma$ -globin promoter showed that several positive and negative elements were present in this promoter sequences (Perez-Stable and Costantini, 1990; Stamatoyannopoulos, et al., 1993). These elements were important for the expression of the human  $\gamma$ -globin transgene in fetal liver as well as for its down-regulation in adult tissue. For example, Stamatoyannopoulos *et al.* showed that transgenic mice carrying the human  $\gamma$ -globin gene with 141 bp of the 5' flanking promoter sequence linked to the LCR showed low levels of  $\gamma$ -globin gene expression at the fetal stage which was down-regulated in the adult stage. Mice carrying an additional 60 bp of the promoter sequence (201 bp of the promoter in all) showed higher level of  $\gamma$ -globin expression at the fetal stage cells and even higher level

of expression in the adult stage, suggesting that a positive element was present between -210 and -141 of the  $\gamma$ -globin promoter which enhanced expression of the  $\gamma$ -globin transgene. Mice containing  $\gamma$ -globin transgenes with 382 bp of the promoter gave similar expression profiles as in mice with 201 bp of the  $\gamma$ -globin promoter. However, mice with 730 bp of the  $\gamma$ -globin promoter regained the proper down-regulation of the  $\gamma$ -globin transgene expression in adult tissues, suggesting that an element between -730 and -378 of the  $\gamma$ -globin promoter also mediated the fetal-specific expression of the  $\gamma$ -globin gene.

Although the previous study showed that the 5' flanking sequences were sufficient to mediate developmental stage-specific expression of this gene, complete silencing of the  $\gamma$ -globin gene in adult tissues also depended on expression of the downstream  $\beta$ -globin gene. Several studies have shown that the erythroid Krüppel-like factor (EKLF) is an erythroid-specific transcription factor critical for the expression of the  $\beta$ -globin gene in adult erythroid cells. When one copy of the mouse EKLF gene was knocked out in transgenic mice harbouring the entire human  $\beta$ -globin locus on a yeast artificial chromosome, the expression of human  $\beta$ -globin gene was reduced whereas expression of the *cis*-linked human  $\gamma$ -globin gene was correspondingly increased (Perkins, et al., 1995; Nuez, et al., 1995). These observations reported by two independent studies suggested that EKLF was required for the  $\gamma$  to  $\beta$ - globin developmental switching

process. However, it should be noted that in human, silencing of the  $\gamma$ -globin gene in adult tissue is not dependent on the expression of the downstream  $\beta$ -globin gene since  $\beta$ -thalassemic patients with homozygous defects in both  $\beta$ -globin genes do not normally have extended expression of the  $\gamma$ -globin genes in adulthood. Therefore, EKLF may have a separate effects on the silencing of the  $\gamma$ -globin gene and the activation of the  $\beta$ -globin gene in adult tissues.

#### **4.6 Reactivation of the $\gamma$ -globin genes in adult tissues**

Although the fetal  $\gamma$ -globin genes in  $\beta$ -globin null patients are not normally expressed in adult life, there are some cases when these patients have co-inherited mutations in their  $\gamma$ -globin genes which sustained expression of the fetal  $\gamma$ -globin gene into their adult lives. This condition, known as hereditary persistence of fetal haemoglobin (HPFH), can ameliorate the severity of the  $\beta$ -thalassemia symptoms since the  $\gamma$ -globin chain can complex with the excess  $\alpha$ -globin chains to reduce the damaging effects caused by the precipitation of  $\alpha$ -globin molecules. HPFH is often associated with deletions or point mutations in the  $\gamma$ -globin promoter (reviewed in Wood, 1993). There are at least 10 point mutations that are commonly associated with HPFH located from -114 to -202 in the  $\gamma$ -globin promoter. Although several of these mutations clustered around -200, there is no clear correlation between these mutations and the putative negative regulatory elements identified so far. The mutations around -200 did affect binding of the

ubiquitous transcription factor Sp1 to that region and suggested that Sp1 binding was involved in the silencing of the  $\gamma$ -globin gene in adult tissue. In one interesting case, it was found that the point mutation at -202 (C  $\rightarrow$ G) created a novel binding site for the nuclear stage-specific protein (SSP) that bound to the -50 $\gamma$  SSE (Jane, et al., 1993). As mentioned earlier, Jane *et al.* defined two stage-specific elements (SSE) within the  $\gamma$ -globin promoter which appeared to be important for the interaction between the HS2 element with the  $\gamma$ -globin promoter as well as the silencing of the linked  $\beta$ -globin gene. They also found that methylation of the -50 $\gamma$  SSE increased the affinity of Sp1 for this site and the binding of Sp1 is believed to interfere with the binding of SSP to this element. During development, the  $\gamma$ -globin gene is first methylated at the embryonic stage, then unmethylated at the fetal development stage, and methylated again at the adult stage (Van der Ploeg and Flavell, 1980; Mavilio, et al., 1983). It is believed that methylation of the  $\gamma$ -globin gene is part of the mechanism that maintains its silence in adult tissue, perhaps by blocking the interaction between the -50 $\gamma$  SSE with the SSP. Interestingly, methylation of the novel HPFH -202 (C  $\rightarrow$ G) SSE site did not affect binding of SSP to this site. The persistent binding of SSP to the methylated HPFH -202 SSE may therefore sustain the  $\gamma$ -globin gene expression into the adult stage of development.

The observation that HPFH could lessen the severity of the  $\gamma$ -thalassemia condition has prompted great interest in the clinical research community to study the mechanisms responsible for adult

stage-silencing of the  $\gamma$ -globin gene and to develop ways to reactivate expression of this gene in  $\beta$ -globin null patients (reviewed in Olivieri, 1996). Since methylation of the  $\gamma$ -globin gene has been shown to correlate with the transition of fetal to adult stages of development, it has been suggested that this modification may cause the silencing of the  $\gamma$ -globin gene. However, careful analysis of the timing of  $\gamma$ -globin expression and its methylation during human development suggested that the  $\gamma$ - to  $\beta$ -globin switch occurs prior to methylation of this gene (Enver, et al., 1988), therefore methylation is not likely the cause of repression, but may play a role in the maintenance of its silence in adult tissues. Nevertheless, several clinical studies have tried to reactivate the  $\gamma$ -globin genes in  $\beta$ -thalassemic patients by treating them with chemical agents, such as 5-azacytidine, which cause general demethylation. Short term treatment with this compound did increase the expression of  $\gamma$ -globin gene in some patients; however, its effectiveness was variable and transient, and its usefulness was further limited by the toxicity of the demethylation compounds. Another feature that correlates with the silencing of the  $\gamma$ -globin gene is the change in the chromatin structure of this gene (Groudine, et al., 1983). Mapping of DNase I hypersensitive sites showed that some of these sites around the  $\gamma$ -globin gene were lost in adult tissues. Interestingly, a number of studies have found that treatment of adult erythroid cells with sodium butyrate could reactivate the  $\gamma$ -globin genes (Olivieri, 1996). It is believed that butyrate inhibits the activity of cellular deacetylases and causes hyperacetylation of nucleosome-

associated histones. Acetylation of the nucleosomal histones is currently thought to be important in transcriptional activation by loosening the interaction of histone tails with the DNA, and creating a chromatin environment more accessible to transcription factors (reviewed in Brownell and Allis, 1996; Grunstein, 1997). The reactivation of the  $\gamma$ -globin genes by sodium butyrate suggests that chromatin structure is involved in maintaining the silence of the  $\gamma$ -globin gene in adult tissue. Clinical trials treating  $\beta$ -thalassemic patients with butyrate compounds to reactivate the fetal  $\gamma$ -globin genes showed some success among the patients, and its full effectiveness remains to be determined.

#### **4.7 EKLF and the $\beta$ -globin promoter**

As mentioned earlier, developmental regulation of the  $\beta$ -globin gene is mediated by its competition with the linked  $\gamma$ -globin gene for the LCR enhancer activity. During the fetal- to adult- stage switching process, the activation of  $\beta$ -globin gene expression is likely due to the combination of repression of  $\gamma$ -globin gene as well as promotion of  $\beta$ -globin expression in adult tissues. Transfection studies showed that expression of the  $\beta$ -globin gene was greatly dependent on EKLF since mutations in their binding sites greatly reduced expression of this gene in adult tissue (Miller and Bieker, 1993; Feng, et al., 1994). Also, naturally occurring mutations in the EKLF binding sites of the  $\beta$ -globin promoter have been correlated to cases of  $\beta$ -thalassemia

(Kulozik, et al., 1991). Recently, Donze *et al.* demonstrated that EKLF was expressed at a 3 fold higher level in adult erythroid tissue than in fetal tissue, bound to the  $\beta$ -globin promoter 8 fold more efficiently than to the  $\gamma$ -globin promoter, and transactivated the  $\beta$ -globin promoter > 300 fold better than the  $\gamma$ -globin promoter (Donze, et al., 1995). These studies together strongly suggest that EKLF acts as an adult-stage specific transcription factor that promotes  $\beta$ -globin expression in adult erythroid tissues.

#### **4.8 Developmental switching of the $\alpha$ -globin genes**

In contrast to the switching of the  $\beta$ -globin genes, the switch from the  $\zeta$ - to  $\alpha$ -globin during development is not well studied. Transgenic mice with constructs containing the LCR linked to individual  $\zeta$ - or  $\alpha$ -globin genes showed that these human transgenes were expressed at the correct developmental stages, and therefore, they were autonomously regulated by their 5' flanking sequences (Spangler, et al., 1990; Sabath, et al., 1993; Albitar, et al., 1991). Early transfection studies examining the promoter elements of the  $\zeta$ -globin gene suggested that a negative regulatory element was located between -167 to -148 of the  $\zeta$ -globin promoter (Lamb, et al., 1989). However, subsequent studies using constructs that contained the HS -40 element did not replicate this result. Pondel *et al.* observed that transgenic mice carrying constructs with the HS -40 element linked to the first 67 bp of the  $\zeta$ -globin promoter sequence showed erythroid-specific

expression of the lacZ reporter gene (Pondel, et al., 1996). The authors also reported that the lacZ expression dropped 40 to 100 fold from 10.5 days to 16.5 days post-fertilisation, suggesting that this short  $\zeta$ -globin promoter fragment was sufficient to direct tissue- and embryonic stage-specific expression of the reporter gene. To date, there has not been any developmental stage-specific silencing elements identified for either  $\zeta$ - or  $\alpha$ -globin genes. Unlike the developmental switching program of the  $\beta$ -globin locus which involved competition among the  $\beta$ -like globin genes, the  $\zeta$ - and  $\alpha$ -globin promoters may just be independently regulated by developmental stage-specific transcription factors.

#### **4.9 Tissue-specific regulation of the globin genes**

In addition to the developmental regulation, expression of globin genes is also subjected to tissue-specific regulation. Little is known about this process, but it is believed that the lack of  $\beta$ -globin expression in non-erythroid cells is mostly due to the inaccessible chromatin structure of the  $\beta$ -globin locus in these cells. In addition, transient transfection studies showed that none of the  $\beta$ -like globin gene promoters function well in non-erythroid cells. For example, the transfected  $\beta$ -globin gene is not transcribed in non-erythroid cells unless an enhancer element such as the SV40 enhancer is present on the plasmid construct (Humphries, et al., 1976; Triesman, et al., 1983; Green, et al., 1983). Promoter mapping analysis showed that



transcription of these  $\beta$ -like globin genes in erythroid cells was dependent on erythroid-specific transcription factors (e.g., GATA-1, NF -E2), and therefore, the lack of these factors would also limit their transcription in non-erythroid cells. In one transgenic mouse study, Perez-Stable found that a  $\gamma$ -globin promoter-driven SV40 T-antigen transgene was expressed at low levels in the mouse non-erythroid tissues (Perez-Stable, 1994); however, the transgene construct did not contain the LCR, and no attempt was made to define the sequences required for the tissue specific expression of the transgene. This study may be an exception since most transgenic mouse studies found that the globin transgenes are expressed only in the appropriate tissues. As yet, no elements have been identified within the globin promoters that determine tissue-specificity.

In contrast to the  $\beta$ -globin locus, the  $\alpha$ -globin locus lies in a region of open chromatin in both erythroid and non-erythroid cells. This locus replicates early in all cell types and a number of non tissue-specific genes have been found in its surrounding region. Therefore, unlike the  $\beta$ -globin locus, the tissue-specificity of the  $\alpha$ -globin locus is not regulated by cell type-dependent chromatin condensation. Transient transfection analysis showed that the  $\zeta$ -globin promoter does not function well in non-erythroid cells (Proudfoot, et al., 1984). This promoter contains binding sites for the erythroid specific transcription factor GATA-1 and *in vitro* mutagenesis studies showed that these binding sites are important for  $\zeta$ -globin expression in erythroid cells

(Watt, et al., 1990; Sabath, et al., 1995). These studies therefore argue that the tissue-specific expression of the  $\zeta$ -globin gene is determined by its dependence on erythroid-specific factors. In contrast, expression of the  $\alpha$ -globin gene does not depend on the presence of any erythroid-specific factors. This is illustrated by at least three examples: First, the  $\alpha$ -globin promoter is unlike the other globin promoters in that it does not contain any binding sites for erythroid-specific transcription factors. Second, the human  $\alpha$ -globin gene when transiently transfected into non-erythroid cells, such as HeLa cells, are readily expressed, indicating that ubiquitous transcription factors are sufficient to activate transcription of this gene (Humphries, et al., 1976; Mellon, et al., 1981; Triesman, et al., 1983). Third, the stably transfected human  $\alpha$ -globin gene in mouse erythroleukemia (MEL) cells is constitutively expressed regardless of the differentiation status of the cells (Charnay, et al., 1984). In contrast, the mouse endogenous globin genes as well as the stably transfected human  $\beta$ -globin gene are only expressed after the MEL cells are induced to differentiate. In spite of the promiscuous nature of the  $\alpha$ -globin gene in transfection assays, this gene is normally only expressed in differentiated erythroid cells; therefore, a repression mechanism must prevent the constitutive expression of this gene in non-erythroid and undifferentiated erythroid cells.

Our general understanding of the regulated expression of the human  $\alpha$ -globin gene is, thus far, rather limited. Since the  $\alpha$ -globin

gene lies in a region of open chromatin in both expressing and non-expressing cells and is surrounded by other constitutively expressed genes, the repression mechanism likely acts locally at the region of the  $\alpha$ -globin promoter and gene. In this study, we investigated the silencing of this gene by examining three mechanisms that are often associated with transcription repression: the presence of a soluble repressor, methylation of the promoter sequences, and nucleosome-mediated interference. Our data suggest that the silencing of this gene is not mediated by the first two mechanisms, but may be mediated at the local chromatin level. Further studies dissecting the chromatin architecture of the  $\alpha$ -globin gene may yield more information regarding the tissue-specific regulation of the  $\alpha$ -globin gene.

## **Materials and Methods**

**Cell culture and viruses.** HeLa, HepG2, and Vero cells were grown as monolayers in  $\alpha$ -MEM supplemented with 10%, 10%, and 5% fetal bovine serum respectively. HSV Paa<sup>r</sup>5 strain was propagated on Vero cells and used to infect HeLa cells at an MOI of 10. For all infections, aphidicolin was added at 10  $\mu$ g/ml to prevent viral DNA replication.

**Treatment of cells with DMSO and cycloheximide or anisomycin.** Subconfluent layers of HeLa cells were treated with a final concentration of 1% DMSO and either 100  $\mu$ g/ml cycloheximide or 10  $\mu$ g/ml anisomycin. Total RNA was harvested from these cells after various times of exposure to the drugs and was then used for primer extension analysis.

**Primer extension analysis.** Total RNA from HeLa or HepG2 cells was isolated with Trizol (Gibco BRL). 10  $\mu$ g of total RNA was used per sample for primer extension analysis as previously described (Cheung, et al., 1997). 25mer oligonucleotides were used as primers for reverse transcription of the  $\alpha$ -globin RNA, or the retinol binding protein RNA.

**Transient transfection assays.** Increasing amounts of pUC $\alpha$ 2 (30 - 60 $\mu$ g) or pUC $\alpha$ 2Nsi (5 - 60 $\mu$ g) were transfected into HeLa cells using the calcium phosphate method. Carrier pUC DNA was added to make up

a total amount of 60 $\mu$ g of DNA used per transfection sample. HeLa cells were exposed to the DNA/calcium phosphate precipitate for 16 hr and were allowed to recover for another 24 hr after removal of the precipitate before total RNA was harvested with Trizol (Gibco BRL).

**S1 nuclease protection assay.** 10 $\mu$ g of total RNA from pUC $\alpha$ 2 transfected cells were used in this assay for detection of  $\alpha$ 2 and  $\alpha$ 1 globin RNA. The protocol for S1 nuclease protection assay was as described in (Cheung, et al., 1997).

**Preparation of genomic DNA.** This technique was adapted from the published protocol by Laird *et al.* (Laird, et al., 1991). HeLa cells were grown to subconfluency on 150mm tissue culture plates. At 6 hours post infection, mock-infected or HSV-infected cells were washed once with ice-cold PBS. 2 ml of lysis buffer (100mM Tris-Cl, pH 8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl, 100 $\mu$ g/ml proteinase K) was then added to each plate of cells. Cell lysates were collected into 15ml Corning conical tubes, and incubated overnight at 48 $^{\circ}$ C. Equal volume of isopropanol was added to the cell lysate and mixed gently by inversion to precipitate the genomic DNA. The precipitated DNA pellet was removed with a pipette tip, washed once in 70% EtOH, and air-dried briefly before being resuspended in the appropriate volume of TE.

**Genomic Southern blot analysis.** 50 - 100 $\mu$ g of total HeLa genomic DNA was first cleaved with the restriction enzyme Pst I since two Pst I sites conveniently flank the human  $\alpha$ -globin gene of interest. The Pst I restricted genomic DNA was precipitated with EtOH, pelleted, and resuspended in TE at a concentration of approximately 1 $\mu$ g/ $\mu$ l. 10 $\mu$ g of Pst I cut DNA was then further cleaved with 50 units of either Hpa II or Msp I. To control for complete cleavage of the genomic DNA, one-tenth volume of this restriction enzyme reaction was removed and 1 $\mu$ g of pUC 18 DNA was added during incubation at 37°C. If all the Hpa II or Msp I sites on the pUC 18 plasmid were cut, this would imply that sufficient enzyme was added to cleave the genomic DNA. Restricted or mock-restricted genomic DNA was loaded onto a 1.5% agarose gel and separated by electrophoresis at 15V/cm. The DNA in the agarose gel was then treated with 0.1M HCl, denatured in NaOH/NaCl, renatured in Tris/NaCl, transferred onto uncharged nylon membrane (Nytran) by capillary blotting or pressure blotting. The DNA was cross-linked to the membrane by UV-crosslinking, hybridized to a  $^{32}$ P-labelled probe containing the  $\alpha$ -globin fragment, exposed to X-ray films, and detected by autoradiography.

**Preparation of HeLa nuclei.** HeLa cells were grown on 150mm tissue-culture plates and were either mock-infected or infected with Paa<sup>F5</sup> strain HSV. At 6 hpi, cells were washed once with ice-cold PBS, collected by scraping in PBS, and pelleted by centrifugation. Approximately  $2 \times 10^7$  cells were resuspended in 1 ml of buffer I

(0.32M sucrose, 3mM CaCl<sub>2</sub>, 2 mM MgAcetate, 0.1mM EDTA, 0.1% TritonX-100, 1mM DTT, 10mM Tris, pH8), and homogenized with a dounce homogenizer. 2 ml of buffer II (1.85M sucrose, 5mM MgAcetate, 0.1mM EDTA, 1mM DTT, 10mM Tris, pH8) was added and mixed with this crude extract, and the released nuclei were pelleted over a 1.8 ml buffer II cushion by ultracentrifugation at 37,000 rpm in a SW50.1 rotor. The pelleted nuclei were resuspended in 200µl nuclei storage buffer (75mM KCl, 0.25mM MgCl<sub>2</sub>, 1mM EDTA, 0.5mM EGTA, 0.15mM spermine, 0.5M spermidine, 20mM HEPES, pH7.9, 10mM β-mercaptoethanol, 0.23M sucrose) supplemented with 0.1mM PMSF and 5µg/ml final concentration each of pepstatin A, leupeptin, and chymostatin. Resuspended nuclei were then stored at -70°C until needed.

**Analysis of nuclear DNA by restriction enzyme digestion, micrococcal nuclease, or DNase treatment.** For digestion with Msp I, HeLa cell nuclei were washed and resuspended in a buffer containing 100mM NaCl, 50mM Tris, pH8, 5mM MgCl<sub>2</sub>, 0.1mM EGTA, and 1mM β-mercaptoethanol. A small aliquot of the resuspended nuclei was counted with a haemocytometer to estimate the total number of nuclei present, and approximately  $1 \times 10^6$  nuclei in a volume of 100µl were used per sample. The aliquoted nuclei were cut with 50 units of Msp I for 1 h at 37°C.

For micrococcal nuclease treatment, HeLa nuclei were washed and resuspended in a buffer containing 15mM Tris, pH8, 50mM NaCl,

1.4mM CaCl<sub>2</sub>, 0.2mM EGTA, 0.2mM EDTA and 5mM β-mercapatoethanol. Again, approximately 1 x 10<sup>6</sup> nuclei were aliquoted into 100μl samples and parallel samples were treated with increasing amount of micrococcal nuclease (0 to 4 units) for 15 minutes at 37°C.

For DNase I treatment, HeLa nuclei were washed and resuspended in a buffer containing 60mM KCl, 0.1 mM EGTA, 5% glycerol, 15mM Tris, pH7.5, 0.5mM DTT. MgCl<sub>2</sub> at a final concentration of 5mM was added to samples just prior to addition of DNase I, and samples of 1 x 10<sup>6</sup> nuclei were treated with DNase I (0 to 15 units) for 15 minutes at 37°C.

After treatment by restriction enzyme, micrococcal nuclease, or DNase I, HeLa nuclei were lysed by adding equal volume of lysis buffer (2% SDS, 20 mM EDTA, and 100μg/ml proteinase K) and incubated at 37°C for 1 h. The samples were then phenol-chloroform extracted and the genomic DNA was precipitated with ethanol, pelleted, and resuspended in TE. The DNA samples were further cut with Pst I and separated on 1.5% agarose gel and analysed by Southern blot as described above.

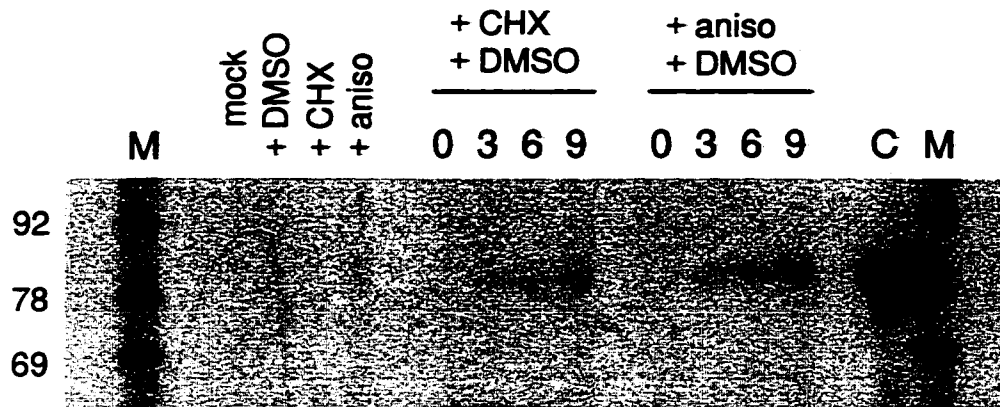


## Results

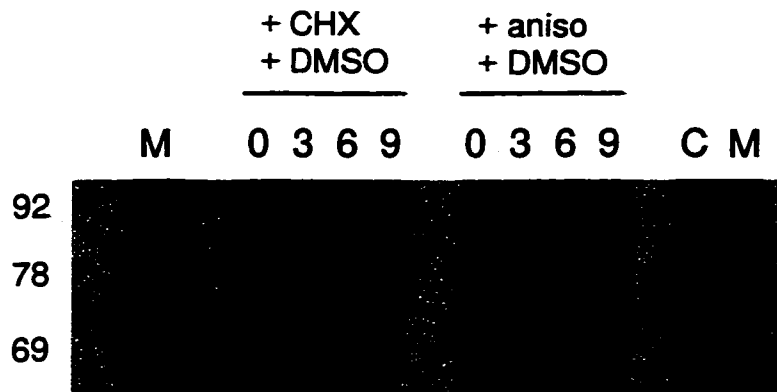
**Activation of the endogenous  $\alpha$ -globin gene in HeLa cells by cycloheximide/anisomycin and DMSO.** One of the main questions addressed in this chapter is how is the cellular  $\alpha$ -globin gene silenced in non-erythroid cells. In our previous study, we found that the endogenous  $\alpha$ -globin gene in non-erythroid cells can be activated by herpes simplex virus (HSV) infection. This suggested that the chromosomal copy of this gene is not irreversibly repressed in non-expressing cells. This conclusion was again illustrated by our finding that the endogenous  $\alpha$ -globin gene in HeLa cells could also be activated simply by treating cells with cycloheximide or anisomycin in the presence of DMSO. In this experiment, dishes of HeLa cells were treated with 100  $\mu\text{g/ml}$  of cycloheximide (CHX) or 10  $\mu\text{g/ml}$  of anisomycin (aniso) in the presence of 1% DMSO. At different time points, total RNA was extracted from the drug-treated cells and was assayed for  $\alpha$ -globin RNA by primer extension analysis. As shown in Fig. 3A, no globin RNA was detected in mock-treated cells whereas low levels of  $\alpha$ -globin RNA was detected within 3 hours of DMSO plus protein synthesis inhibitor treatment, and the amount of  $\alpha$ -globin RNA continued to accumulate from 3 to 9 hours of drug exposure. The levels of  $\alpha$ -globin RNA in drug-treated cells were low, but can clearly be seen in Fig. 3B which represented a longer and darker exposure of another trial of the experiment. Since this gene is normally transcriptionally silent in HeLa cells, the detection of  $\alpha$ -globin RNA

**Fig. 3. Primer extension analysis for  $\alpha$ -globin RNA in drug-treated HeLa cells. (A) HeLa cells were mock-treated or treated with 1% DMSO, 100  $\mu$ g/ml of cycloheximide (CHX), 10  $\mu$ g/ml of anisomycin (aniso), DMSO plus CHX, or DMSO plus aniso. Total RNA was harvested at the indicated time points (hours post treatment), and 10  $\mu$ g per sample of total RNA was used for primer extension analysis with a 5'  $^{32}$ P-labelled 25mer oligonucleotide primer complementary to the human  $\alpha$ -globin RNA. (B) Long exposure of the primer extension analysis from another trial of the experiment. RNA extracted from human blood was used as a positive control (C), and HpaII digested pBR322 DNA was used as size markers (M).**

A



B



indicated that transcription of this gene was activated by the drugs. Cells treated with cycloheximide, anisomycin, or DMSO alone did not accumulate  $\alpha$ -globin RNA (Fig. 3A), indicating that activation of this repressed gene require both DMSO and protein synthesis inhibitors. In repeated trials (data not shown), there were consistently higher amounts of accumulated  $\alpha$ -globin RNA in cells treated with anisomycin and DMSO than those treated with cycloheximide and DMSO. Although it is not clear how these compounds together affect the regulation of  $\alpha$ -globin expression, our finding that expression of this gene occurred in the presence of protein synthesis inhibitors suggested that the activation process does not require *de novo* synthesis of new regulatory factors.

**The DMSO and anisomycin treatment only activated the endogenous  $\alpha$ -globin gene.** In addition to  $\alpha$ -globin genes, other globin genes are also regulated in a tissue-specific manner. To determine whether globin genes in general are activated by our drug treatment, HeLa cells were treated with 10  $\mu$ g/ml of anisomycin in 1% DMSO and total RNA was harvested at various times post treatment and was assayed for the presence of  $\alpha$ -,  $\beta$ -, and  $\zeta$ -globin RNA by primer extension. As shown in Fig. 4, anisomycin plus DMSO-treated cells expressed  $\alpha$ -globin RNA, but neither  $\zeta$ - or  $\beta$ -globin genes were activated. A similar result was seen with the HSV-induced expression of  $\alpha$ -globin gene. Together these results illustrate that the silencing mechanism of the  $\alpha$ -globin gene differs from the mechanisms that regulate tissue-

**Fig. 4. Examination for the presence of  $\alpha$ -,  $\beta$ -, and  $\zeta$ -globin RNA in HeLa cells treated with DMSO and anisomycin. Parallel dishes of HeLa cells were treated with 1% DMSO and 10  $\mu\text{g/ml}$  of aniso, and RNA harvested at 0, 3, 6, 9, and 12 hours post treatment was examined by primer extension analysis using  $^{32}\text{P}$ -labelled oligonucleotides complementary to  $\alpha$ -,  $\beta$ -, and  $\zeta$ -globin RNA. RNA extracted from human blood was used as the positive controls for  $\alpha$ - and  $\beta$ -globin and RNA from K562 cells was used as the positive control for  $\zeta$ -globin RNA (C).**

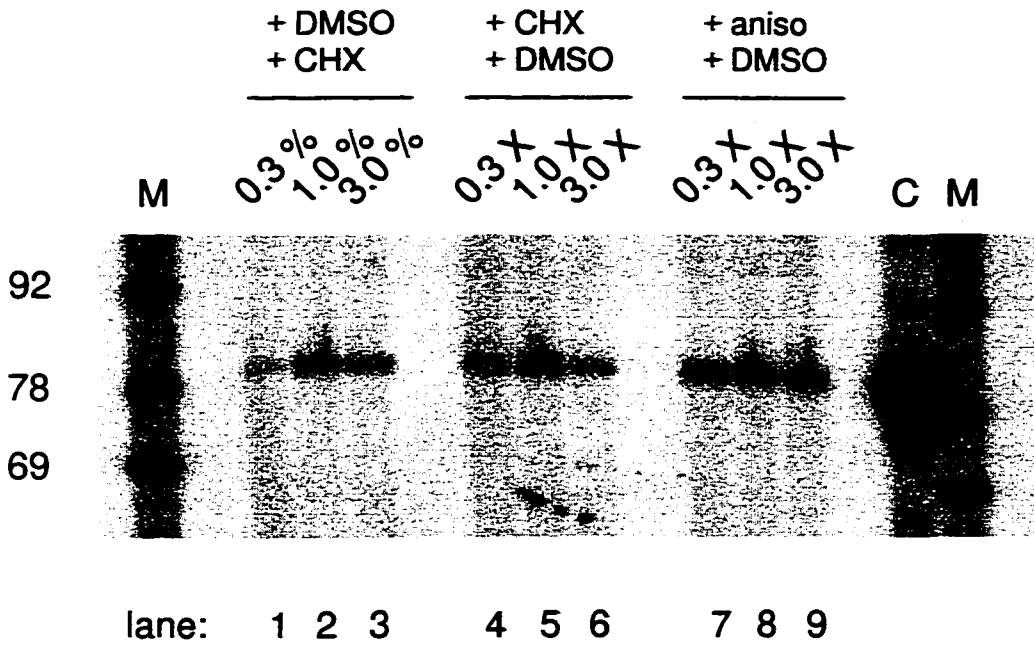


specificity of the  $\beta$ - and  $\zeta$ -globin genes, and suggest that regulation of the  $\alpha$ -globin gene may be easily disrupted.

**Optimization of the drug-induced activation of the  $\alpha$ -globin gene.** To determine the optimal concentration of DMSO and protein synthesis inhibitors for inducing expression of the endogenous  $\alpha$ -globin gene, HeLa cells were treated with combinations of various amounts of DMSO and cycloheximide or anisomycin for 6 hours and total RNA was harvested and assayed for  $\alpha$ -globin RNA by primer extension analysis. The results are shown in Fig. 5: in lanes 1 to 3, a constant amount of cycloheximide (100  $\mu$ g/ml) was used in each sample in combination with 0.3, 1 or 3% of DMSO. For lanes 4 to 6, 1% of DMSO was added to each sample in combination with 30, 100 or 300  $\mu$ g/ml of cycloheximide (0.3X, 1X, and 3X), and for lanes 7 to 9, 1% of DMSO was added to each sample in combination with 3, 10 or 30  $\mu$ g/ml of anisomycin (0.3X, 1X, and 3X). By comparing the amount of accumulated  $\alpha$ -globin RNA in these samples, the optimal conditions for induction of the  $\alpha$ -globin gene were determined as 1% DMSO with 100  $\mu$ g/ml of cycloheximide or 1% DMSO with 10  $\mu$ g/ml of anisomycin. Lowering or elevating the amount of protein synthesis inhibitors used compared to the optimal concentration still activated the transcription of the  $\alpha$ -globin gene; however, a lower amount of transcripts accumulated under such conditions.

Fig. 5. Determination of the optimal concentrations of DMSO, CHX and aniso for inducing expression of  $\alpha$ -globin RNA. HeLa cells were treated with various concentrations and combinations of DMSO, CHX and aniso for 6 hours, and total RNA was extracted and 10  $\mu$ g of each sample was examined for  $\alpha$ -globin RNA by primer extension analysis. For lanes 1 to 3, cells were treated with 100  $\mu$ g/ml of CHX in combination with 0.3, 1.0, or 3.0% DMSO. For lanes 4 to 6, cells were treated with 1.0% DMSO in combination with 33, 100 or 300  $\mu$ g/ml of CHX. For lanes 7 to 9, cells were treated with 1% DMSO in combination with 3.3, 10 or 30  $\mu$ g/ml of aniso.

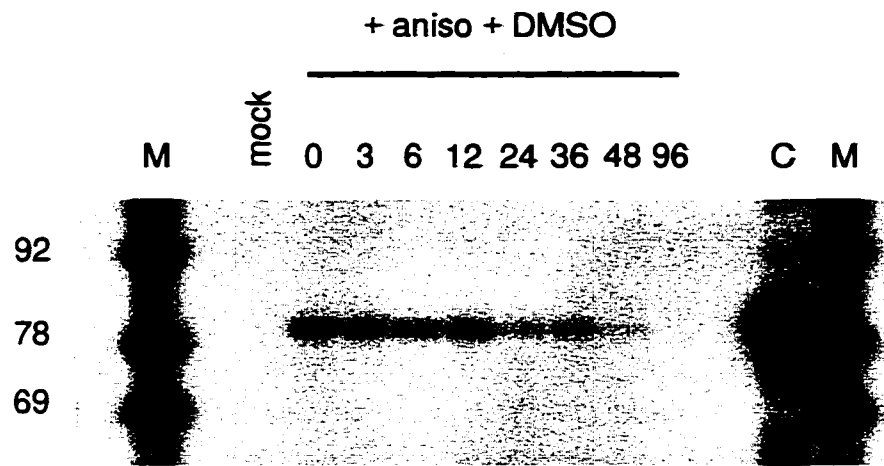




**Removal of the DMSO and the protein synthesis inhibitors resulted in recovery of the repression mechanism.** To determine whether the drug treatment causes a permanent change in the expression status of the endogenous  $\alpha$ -globin gene, parallel dishes of HeLa cells were treated with 1% DMSO and 10  $\mu\text{g/ml}$  of anisomycin for 6 hrs, at which time the cells were extensively washed, and allowed to recover in fresh medium. Total RNA was harvested from these cells at various times post drug-removal and was assayed for  $\alpha$ -globin RNA by primer extension analysis. As shown in Fig. 6, the level of  $\alpha$ -globin RNA steadily declined after removal of the anisomycin and DMSO, suggesting that transcription of the  $\alpha$ -globin gene did not continue after removal of the drugs. Therefore, the repression mechanism was not permanently disrupted by the anisomycin plus DMSO treatment.

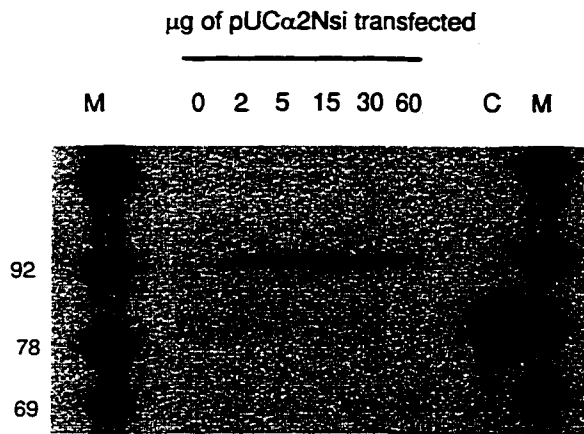
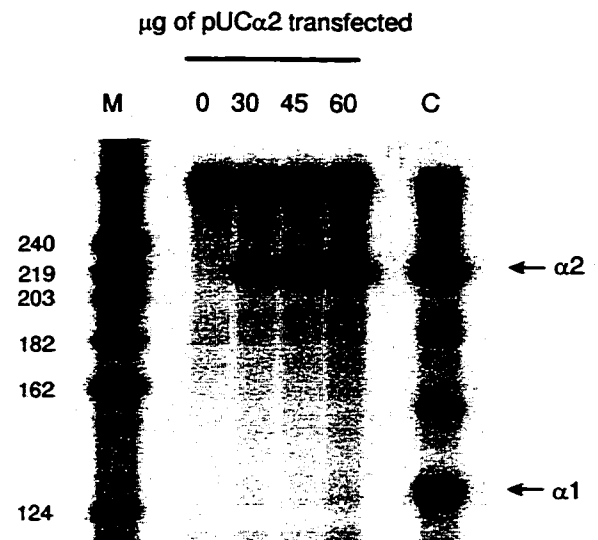
**There is no evidence for the presence of a labile repressor.** The activation of gene expression by cycloheximide and anisomycin has been described for a number of genes (see discussion), and several possible mechanisms are involved. In some cases, the induction effect is due to the inhibition of expression of a labile repressor molecule which normally regulates expression of the induced gene. To test whether a labile repressor normally binds to the  $\alpha$ -globin gene, we transfected plasmids containing the  $\alpha$ -globin gene into HeLa cells and determined whether they could de-repress the endogenous  $\alpha$ -globin gene by competing away the putative repressor. Increasing amounts of pGEM4Z $\alpha$ Nsi were transfected into HeLa cells and total RNA

Fig. 6. Examination of the levels of  $\alpha$ -globin RNA in HeLa cells at different times post removal of DMSO and aniso. HeLa cells were treated with 1% DMSO and 10  $\mu$ g/ml of aniso for 6 hours and then allowed to recover in fresh medium for the various times indicated (hours post drug removal). Total RNA from these cells were then harvested and 10  $\mu$ g of each sample was examined for  $\alpha$ -globin RNA by primer extension analysis.



harvested from these cells at 36 hrs post transfection was assayed for  $\alpha$ -globin RNA by primer extension analysis. The pGEM4Z $\alpha$ Nsi plasmid contained a tagged version of the human  $\alpha 2$  gene which allowed us to distinguish between the transcripts derived from this gene and the endogenous  $\alpha$ -globin gene by a difference of 12 nt in the RNA length. Primer extension analysis of total RNA harvested from transfected cells showed abundant accumulation of the tagged version of  $\alpha$ -globin RNA, but no endogenous  $\alpha$ -globin transcripts were detected in the same samples (Fig. 7A). We also transfected increasing amounts of the pUC $\alpha 2$  construct which contained a 4 kb fragment containing the human  $\alpha 2$  gene with its 5' and 3' flanking sequences, and then used S1 nuclease protection analysis to assay for the presence of  $\alpha 2$  and  $\alpha 1$  transcripts in transfected cells. Since  $\alpha 2$  and  $\alpha 1$  genes share the same promoter and coding sequences, and differ only in their 3' untranslated region, detection of  $\alpha 1$  transcripts in cells transfected with the  $\alpha 2$  gene would indicate activation of the endogenous  $\alpha$ -globin gene. As shown in Fig. 7B, although we detected abundant  $\alpha 2$  transcripts, presumably derived from the transfected plasmid, we could not detect any  $\alpha 1$  transcripts that would indicate expression of the endogenous  $\alpha 1$  gene. The results from both transfection studies indicated that the endogenous  $\alpha$ -globin gene could not be de-repressed by excess copies of  $\alpha$ -globin sequences. This suggested that either the silencing mechanism is not mediated by a limiting amount of soluble repressor molecules, or the transfected  $\alpha$ -globin sequences cannot titrate away the repressor molecules from the

Fig. 7. Transfection of excess copies of  $\alpha$ -globin gene into HeLa cells. Various amounts (0 to 60  $\mu\text{g}$ ) of pUC $\alpha$ 2Nsi (A) or pUC $\alpha$ 2 (B) were transfected into HeLa cells using the calcium phosphate method and total RNA was harvested from the transfected cells at 36 hours post transfection. (A) 10  $\mu\text{g}$  per sample of total RNA from pUC $\alpha$ 2Nsi transfected cells was examined by primer extension analysis using 5' end  $^{32}\text{P}$ -labelled primers complementary to  $\alpha$ -globin RNA; (B) 10  $\mu\text{g}$  per sample of total RNA from pUC $\alpha$ 2 transfected cells was examined by S1 nuclease protection assays using 3' end  $^{32}\text{P}$ -labelled ss DNA probes complementary to the 3' end of the  $\alpha$ 2 gene.

**A****B**

endogenous genes. So far, we do not have any evidence supporting the existence of an  $\alpha$ -globin specific repressor that regulates its tissue-specific expression.

**HSV infection does not activate the CpG island-containing, liver cell-specific, retinol binding protein gene.** The effects of cycloheximide/anisomycin and DMSO on the expression of the endogenous  $\alpha$ -globin gene could be due to several other causes (as enumerated in the discussion section of this chapter); however, there is no obvious explanation that could be unequivocally tested. To continue our examination of how the  $\alpha$ -globin gene is silenced in non-erythroid cells, we further investigated our previous finding that HSV infection could activate expression of this normally silent gene in HeLa cells. One feature of the  $\alpha$ -globin gene, as opposed to members of the  $\beta$ -globin gene family, is its high G+C content and the presence of a CpG island within its promoter and coding sequences. CpG islands are GC rich sequences most often associated with actively-transcribed house-keeping genes, but are also found in a number of tissue-specific genes such as the  $\alpha$ -globin gene (Antequera, et al., 1990; Antequera and Bird, 1993)). To test if this unique feature is linked to its inducibility by HSV infection, we tested whether another CpG island-containing tissue-specific gene, the liver cell-specific retinol binding protein (RBP) gene, could also be activated by HSV infection. HeLa cells were infected with HSV at various MOIs and total RNA was harvested at 6 hpi. The RNA was assayed for the presence of  $\alpha$ -globin as well as for retinol binding



protein RNA by primer extension analysis using primers specific for these two genes respectively. As shown in Fig. 8A and B,  $\alpha$ -globin, but not RBP RNA was detected in the HSV-infected HeLa cells. These results suggest that although both of these tissue-specific genes contain CpG islands, they do not respond to activation by HSV the same way.

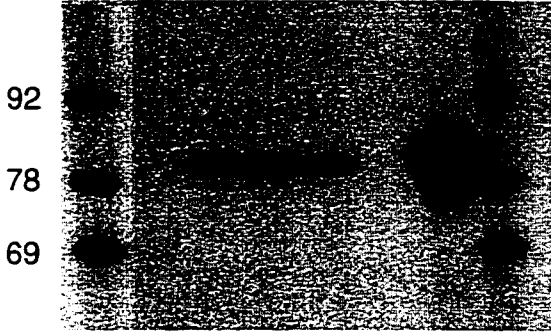
**The  $\alpha$ -globin promoter is not methylated in our HeLa cell line, and HSV infection does not alter the methylation status of the endogenous  $\alpha$ -globin gene.** Although CpG islands are generally hypomethylated in all primary cell lines, Antequera *et al.* have shown that the CpG islands of inactive genes are often heavily methylated in long-term passaged cell lines (Antequera, et al., 1990). For example, the authors found that all except one of the 20 CCGG sites within the 1.5 kb PstI to PstI fragment containing the  $\alpha$ -globin promoter and gene were methylated in their HeLa cell line. Since methylation is generally thought to be associated with repression of gene expression, we were interested to determine whether the  $\alpha$ -globin gene of our HeLa cell line was also heavily methylated and whether HSV infection altered its methylation status. Following the methods described by Antequera *et al.*, we extracted genomic DNA from mock-infected or HSV-infected HeLa cells, and digested these samples with the restriction enzymes HpaII or MspI. Both of these enzymes recognise and cleave at the CCGG sequence; however, HpaII does not cut this sequence if the cytosine residues are methylated, whereas MspI is not sensitive to the

**Fig. 8. Primer extension analysis for  $\alpha$ -globin and retinol binding protein (RBP) RNA. HeLa cells were infected with HSV at an MOI of 0, 3, 10, or 30, and total RNA was harvested from these cells at 6 hpi. 10  $\mu$ g per sample of total RNA was examined by primer extension using  $^{32}$ P-labelled oligonucleotides complementary to  $\alpha$ -globin (A) or RPB (B) RNA. RNA from human blood and HepG2 cells were used as positive controls for  $\alpha$ -globin and RBP respectively (C).**

**A**

+ HSV

M 0 3 10 30 C M



$\alpha$ -globin

**B**

+ HSV

M 0 3 10 30 C

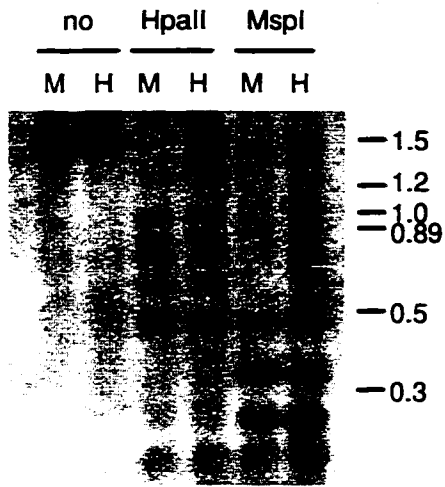


retinol binding protein

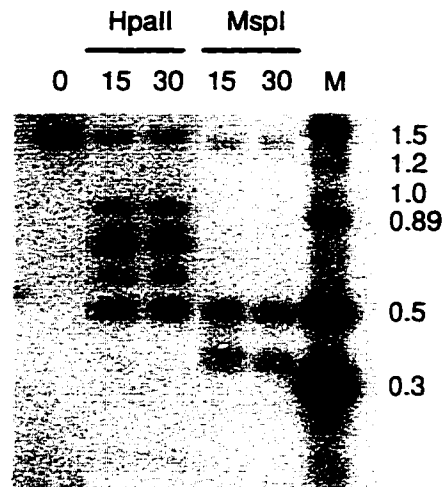
methylation status of this site. A comparison of the digestion pattern of these two enzymes therefore illustrates which of the CCGG sites are methylated. As a control for complete HpaII and MspI digestions, one  $\mu\text{g}$  of pUC18 DNA was added to a small aliquot of the genomic DNA/restriction enzyme reaction to test whether sufficient enzyme was present in the sample to also digest the pUC18 control DNA (data not shown). The HpaII- or MspI- cleaved genomic DNA was further digested with PstI (two PstI sites flank the human  $\alpha$ -globin gene as illustrated in Fig. 9C), and then separated on an agarose gel for Southern blot analysis. As shown in Fig. 9A and B, the HpaII- and MspI-digestion patterns at the  $\alpha$ -globin locus were not identical, indicating that some of the 20 CCGG sites within the  $\alpha 2$  PstI fragment were methylated and not cleaved by HpaII. However, this HpaII digestion pattern was different than that seen in Antequera's report, indicating that our HeLa cell line is differently methylated compared to their cells. The genomic DNA harvested from mock-infected (M) or HSV-infected (H) samples showed identical HpaII or MspI digestion patterns (Fig. 9A), and therefore demonstrated that HSV infection did not alter the methylation status of this locus. The Southern blot shown in Fig. 9A was probed with a  $^{32}\text{P}$ -labelled 1.5 kb PstI  $\alpha 2$  fragment, and the MspI digested DNA showed the expected bands generated from digestion of all MspI sites within this DNA region. These include the 468, 327, 162 bp fragments, and smaller fragments that ran at the bottom and off the gel. Based on the known MspI sites within the 1.5 kb fragment, we could determine that the 468 bp fragment was derived

Fig. 9. Examination of the methylation status of the endogenous  $\alpha$ -globin gene in HeLa cells. (A) HeLa cells were infected (H) or mock-infected (M) with HSV at an MOI of 10, and total genomic DNA was harvested at 6 h.p.i. 10  $\mu$ g per sample of genomic DNA was mock-digested or digested with 30 U of the restriction enzymes HpaII or MspI, and then digested with Pst I. Restriction enzyme digested genomic DNA samples were examined by Southern blot analysis using the  $^{32}$ P-labelled 1.5 kb PstI to Pst I  $\alpha$ 2 fragment as probe. (B) Total genomic DNA from uninfected HeLa cells was harvested and 10  $\mu$ g per sample of the genomic DNA was first digested with 15 or 30 U of Hpa II or Msp I, and then with Pst I. Digested samples were examined by Southern blot analysis using the  $^{32}$ P-labelled 300 bp BstEII to PstI fragment corresponding to the 3' end of the  $\alpha$ 2 gene as probe. (C) Schematic drawing of the  $\alpha$ 2 globin gene and the methylation status of the Hpa II / Msp I sites along the  $\alpha$ -globin locus. P = Pst I sites; B = BstE II sites; close circles represent methylated sites; semi-open circles represent differentially methylated sites; and open circles represent unmethylated sites.

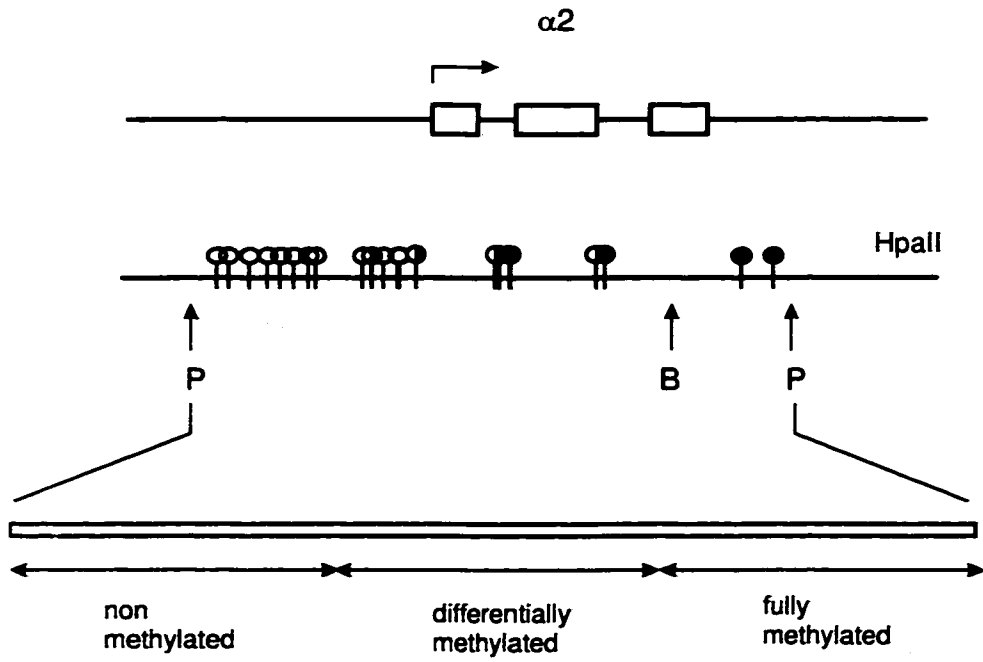
**A**



**B**



**C**



from the  $\alpha 1$  gene, whereas the 327 bp was derived from the  $\alpha 2$  gene. These two  $\alpha$ -globin genes have identical DNA sequences except for a few differences within the last 130 bp of their 3' ends. For example, the  $\alpha 2$  gene has two additional HpaII sites within its 3' end compared to the  $\alpha 1$  gene (Fig. 9C), and hence the  $\alpha 1$  gene gave rise to the 468 bp fragment upon MspI cleavage, whereas this region of the  $\alpha 2$  gene was further cleaved to generate 327, 70, and 58 bp fragments. The 468 and 327 bp bands were also detected in the MspI digested genomic DNA when a similar blot was hybridised with a  $^{32}\text{P}$ -labelled 300 bp 3' end probe (Fig. 9B), confirming that these two bands were derived from the 3' end of the  $\alpha 2$  and  $\alpha 1$  genes. In contrast to the MspI digestion pattern, HpaII digested DNA showed a series of bands ranging from approximately 450 to 1000 bp when hybridised with either the full length or 3' end-specific probes (Fig. 9A and B). Since these fragments were detected by the 3' end probe, they must represent 3' co-terminal DNA fragments. These fragments did not arise from partial digestion of the genomic DNA since the pUC18 DNA was fully digested in control samples that tested for presence of sufficient HpaII enzymes (data not shown). Instead, they were likely generated as a result of variations in the extent of methylation within the HeLa cell population; ie. the HeLa cell population was made up of several sub-groups that had different extents of methylation at the  $\alpha$ -globin locus. It is important to note that this 300 bp 3' end probe overlaps the two extra HpaII sites at 3' end of the  $\alpha 2$  gene (Fig. 9C) which makes interpretation of the data potentially more difficult. However, it

appears that these two HpaII sites of the  $\alpha 2$  gene were methylated since, the 3' end probe only detected the 468 bp band but not the 327 bp band which should have been present if the last two HpaII sites of the  $\alpha 2$  gene were unmethylated and cleaved. Assuming that these two sites were methylated in the  $\alpha 2$  locus and that HpaII digestion of the  $\alpha 2$  and  $\alpha 1$  genes generated the same bands, our data showed that the sizes of these bands in fact correlated very well with the known HpaII sites within the  $\alpha 2$  and  $\alpha 1$  coding region, and suggested that these 3' co-terminal fragments corresponded to DNA fragments with successive increases in the number of methylated HpaII sites at the 3' end of the  $\alpha$ -globin gene. Since this series of 3' co-terminal bands only extended up to approximately 1000bp in length, this suggested that 500 bp region at the 5' end of the 1.5 kb PstI to PstI region was either fully methylated or completely unmethylated. We believe that this region, which contains the  $\alpha$ -globin promoter, is unmethylated since if this 5' end region were completely methylated, one would predict to see significantly different or additional bands (representing intact fragments that correspond to the methylated 5' end of the  $\alpha$ -globin locus) detected by the full length probe compared to the fragments detected by the 3' end probe (Fig. 9A and B). Since this was not the case (in fact it appears that almost the same bands were detected in both blots), we concluded that the promoter region of the  $\alpha$ -globin gene was not methylated. The collective data from this experiment suggested that our HeLa cells were made up of subpopulation of cells that differ in their extents of methylation at the  $\alpha$ -globin locus. Consequently,



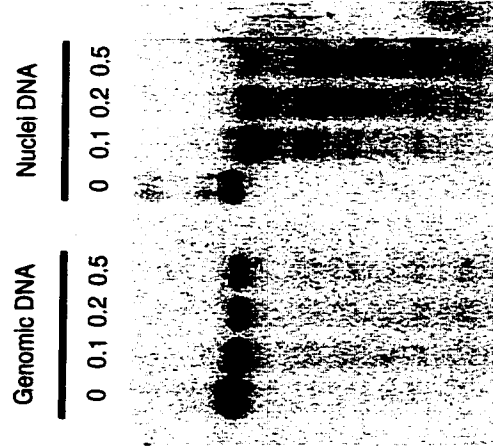
digestion of total genomic DNA with HpaII enzyme generated a series of 3' co-terminal bands. The sizes of these bands suggested that the differentially methylated HpaII sites were located in middle 500 bp region of the PstI to PstI  $\alpha$ -globin fragment, and the length of each fragment correspond to the first unmethylated HpaII site counting from the 3' end of the  $\alpha$ -globin locus of each subpopulation of HeLa cells. In contrast to the observation by Antequera *et al.*, these data suggest that the promoter region of the  $\alpha$ -globin gene in our HeLa cell population is not methylated. Other laboratories have also found that the  $\alpha$ -globin promoter was methylation-free in HeLa cells (Rein, et al., 1995). These variable findings likely reflect HeLa sub-lines that have been generated over many years of HeLa cell passage. Insofar as methylation is in general inhibitory to gene expression, our finding that the promoter region of our HeLa cells is not methylated is consistent with the observations that this gene can be activated in these cells, and that activation upon HSV infection does not alter the methylation status of this gene.

**Positioned nucleosomes were found in the coding region, but not in the promoter region of the  $\alpha$ -globin gene.** Many attempts by different laboratories to replicate the tissue-specific expression of the endogenous  $\alpha$ -globin gene with transfected  $\alpha$ -globin constructs have ended in failure and this has prompted researchers to conclude that the silencing of the endogenous  $\alpha$ -globin gene is mediated at the chromatin level. To examine the chromatin structure of the

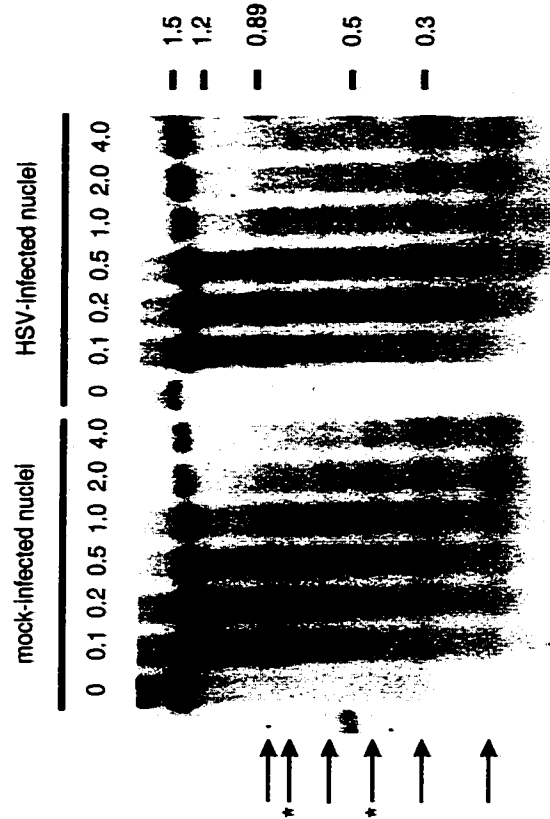
endogenous human  $\alpha$ -globin gene, we tested for the presence of positioned nucleosomes at chromosomal region surrounding the  $\alpha$ -globin gene. Intact nuclei were harvested from HeLa cells and parallel samples were treated with increasing amounts of micrococcal nuclease (MNase) which preferentially cleaved at the inter-nucleosomal linker DNA. As a control, naked genomic DNA was also digested with MNase to show that the enzyme did not show any sequence specific digestion of naked DNA. After MNase treatment, the intact nuclei were lysed and genomic DNA was harvested. This DNA was further cleaved by PstI and the  $\alpha$ -globin sequences were examined by genomic Southern analysis by probing with  $^{32}\text{P}$ -labelled 300 bp  $\alpha$ -globin 3' end fragment. As shown in Fig. 10A, MNase digested control naked genomic DNA at random sites and resulted in an undetectable smear of  $\alpha$ -globin sequences. In contrast, nuclei DNA digested with MNase showed a distinct 200bp ladder when hybridised with the  $\alpha$ -globin probe, indicating that the endogenous  $\alpha$ -globin gene did contain positioned and phased nucleosomes (Fig. 10A and B). By mapping the MNase sensitive sites, we found that 5 nucleosomes were positioned in the coding region, but not in the promoter region of the  $\alpha$ -globin gene (Fig. 10C). Interestingly, the MNase digestion pattern showed that the first nucleosome was positioned over the TATA element and the transcription start site of this gene. Moreover, while the average distance between the bands that mark the MNase hypersensitive sites was approximately 200 bp in the coding region of the gene, the distance between the sites flanking the transcription

**Fig. 10. Micrococcal nuclease (MNase) digestion of genomic DNA or intact nuclei. (A) 10  $\mu$ g per sample of genomic DNA or intact nuclei from uninfected HeLa cells were treated with increasing amounts (0 to 0.5 U) of MNase. The intact nuclei were then lysed and total genomic DNA samples were digested with PstI and examined by Southern blot analysis using the  $^{32}$ P-labelled 1.5 kb Pst I to Pst I  $\alpha$ 2 fragment as probe. (B) HeLa cells were infected or mock-infected with HSV at an MOI of 10 and intact nuclei were harvested from the infected cells at 6 h.p.i. Parallel samples of nuclei were treated with increasing amounts (0 to 4.0 U) of MNase and the genomic DNA was then harvested and examined by Southern blot analysis using the  $^{32}$ P-labelled 1.5 kb Pst I to Pst I  $\alpha$ 2 fragment as probe. (C) Schematic drawing showing the MNase hypersensitive sites (i.e. the boundaries of the positioned nucleosomes) superimposed onto a diagrammatic representation of the  $\alpha$ -globin transcript.**

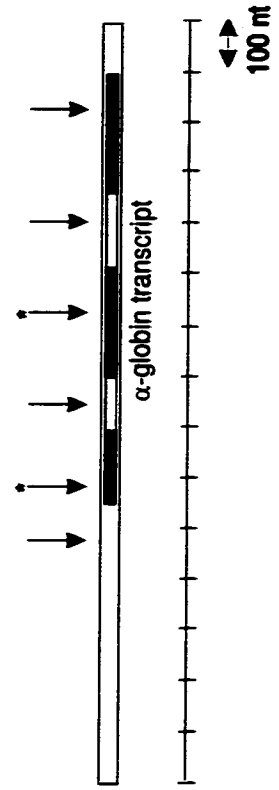
**A**



**B**



**C**

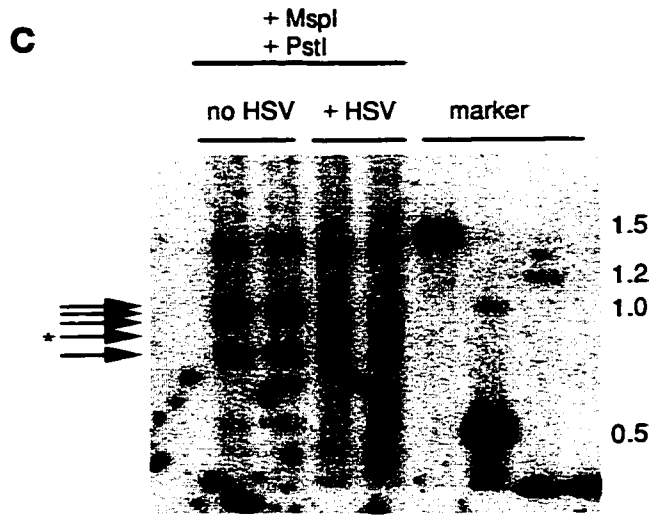
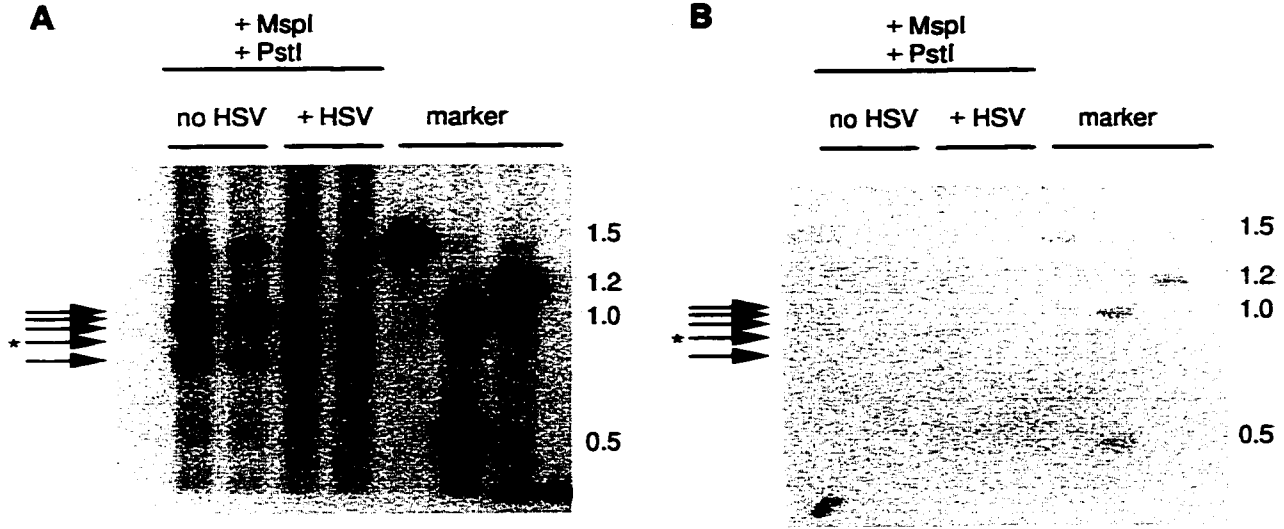


start site was consistently closer to approximately 150bp, suggesting that the DNA may be tightly wound around this positioned nucleosome. The positioning of this nucleosome over the TATA box and transcription start site of this gene may occlude these elements from the transcription initiation complex and prevent its transcription. Examination of the positioned nucleosomes in the  $\alpha$ -globin region after HSV-infection (Fig. 10B) showed subtle changes in the MNase digestion pattern. For example, the internucleosomal MNase hypersensitive sites marked by \* in Fig. 10B and C were consistently less distinct after HSV infection, suggesting that some of the nucleosomes may be less rigidly positioned upon activation. These results together suggest that activation of the  $\alpha$ -globin gene by HSV does not involve eviction of specific nucleosomes, but subtle changes to the chromatin may be sufficient to allow interaction of the TATA element and transcription start site with the transcription initiation machinery. Alternatively, the lack of any detectable changes in the nucleosome positioning at the  $\alpha$ -globin promoter upon activation may indicate that only a subset of the HeLa cells is activated upon HSV-infection. To distinguish between these two possibilities, one can use *in situ* hybridisation to determine the proportion of HSV-infected cells that express  $\alpha$ -globin RNA.

**Activation of the  $\alpha$ -globin gene by HSV altered the accessibility of the nucleosome-bound DNA sequences to restriction enzyme digestion.** To determine whether the nucleosome-bound  $\alpha$ -globin gene was

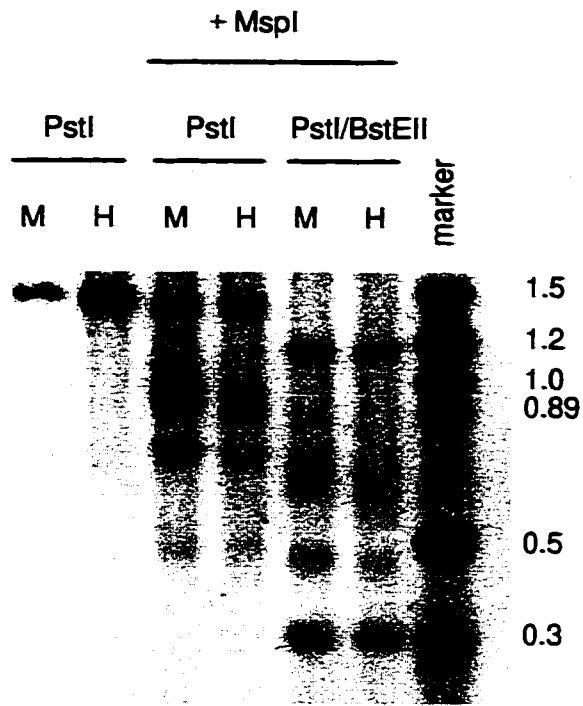
accessible by nuclear factors, we added the restriction enzyme MspI to intact nuclei to assay which of its recognition sites within the chromatin-bound  $\alpha$ -globin gene were accessible and cleaved by this enzyme. Nuclei harvested from mock-infected (M) and HSV-infected (H) HeLa cells at 6 hpi were first digested with 50 units of MspI. Genomic DNA was then extracted, cut with PstI, and examined by genomic Southern blot analysis as described before. When the DNA extracted from the MspI-digested nuclei was probed with a  $^{32}\text{P}$ -labelled 1.5 kb PstI fragment (Fig. 11A), the digestion pattern obtained was different from that seen with MspI digested naked genomic DNA (Fig. 9A), indicating that some of the MspI sites were not accessible when folded into chromatin. By stripping and reprobing this blot (Fig. 11B and C), we found that the same bands were detected by a 300 bp 3' end probe (the BstEII to PstI fragment of the  $\alpha 2$  gene). This result suggested that the MspI sites at the 3' end of this gene were not accessible to restriction enzyme digestion when assembled into chromatin, a finding which is consistent with our previous observation that nucleosomes were positioned along the transcription unit of the  $\alpha$ -globin gene. Insofar as these bands were detected by the 3' end probe, this suggested that they were 3' co-terminal fragments that were likely generated as a result of minor variations in the MspI accessible sites within the HeLa cell population (similar to the variation in the methylation status illustrated in Fig. 9). That these bands were 3' co-terminal was confirmed by digesting genomic DNA harvested from MspI digested nuclei with PstI and BstEII which

Fig. 11. Digestion of intact nuclei with Msp I. HeLa cells were mock-infected or infected with HSV at an MOI of 10, and intact nuclei were harvested from the infected cells at 6 h.p.i. Duplicate samples of intact nuclei were digested with 50 U of Msp I, and the genomic DNA harvested from the digested nuclei was further digested with Pst I and then examined by Southern blot analysis. (A) The blot was hybridised with the <sup>32</sup>P-labelled 1.5 kb Pst I to Pst I  $\alpha$ 2 fragment probe. (B) The same blot used in (A) was stripped and exposed to film. (C) The stripped blot in (B) was rehybridised with the <sup>32</sup>P-labelled 300 bp BstEII to Pst I fragment corresponding to the 3' end of the  $\alpha$ 2 gene. The arrow with the \* indicates the extra band that is present in HSV-infected nuclei. (D) Intact nuclei from mock-infected (M) or HSV-infected (H) were digested with Msp I and the genomic DNA harvested from these nuclei were further digested with Pst I or Pst I and BstEII, and then examined by Southern blot analysis using the <sup>32</sup>P-labelled 1.5 kb  $\alpha$ 2 fragment as probe. (E) Schematic diagram representing the 3' co-terminal DNA fragments, and indicating the MspI accessible and inaccessible regions of the  $\alpha$ -globin locus.

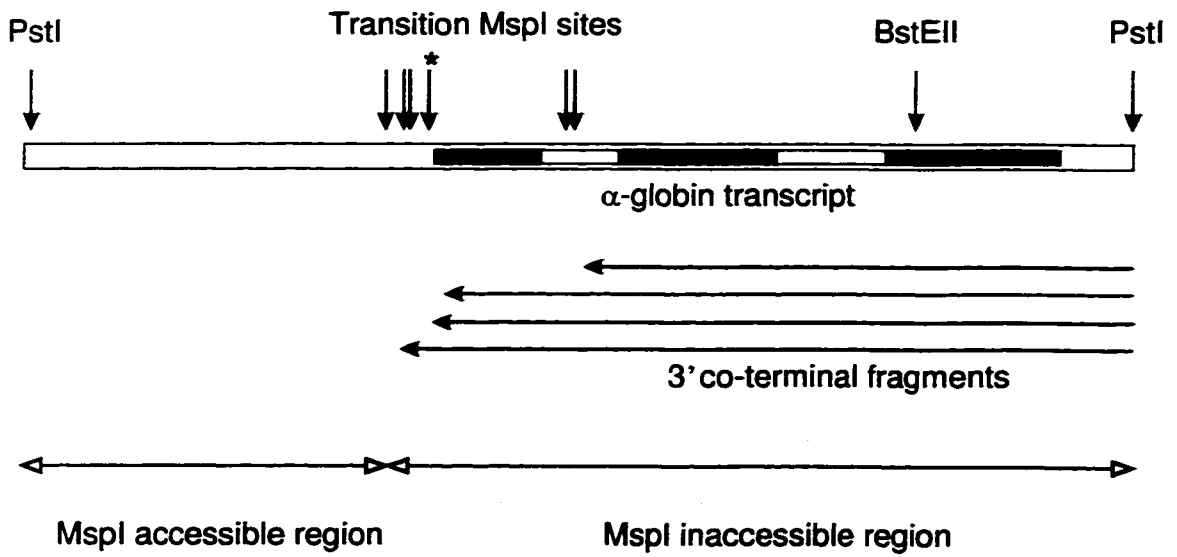




**D**



**E**

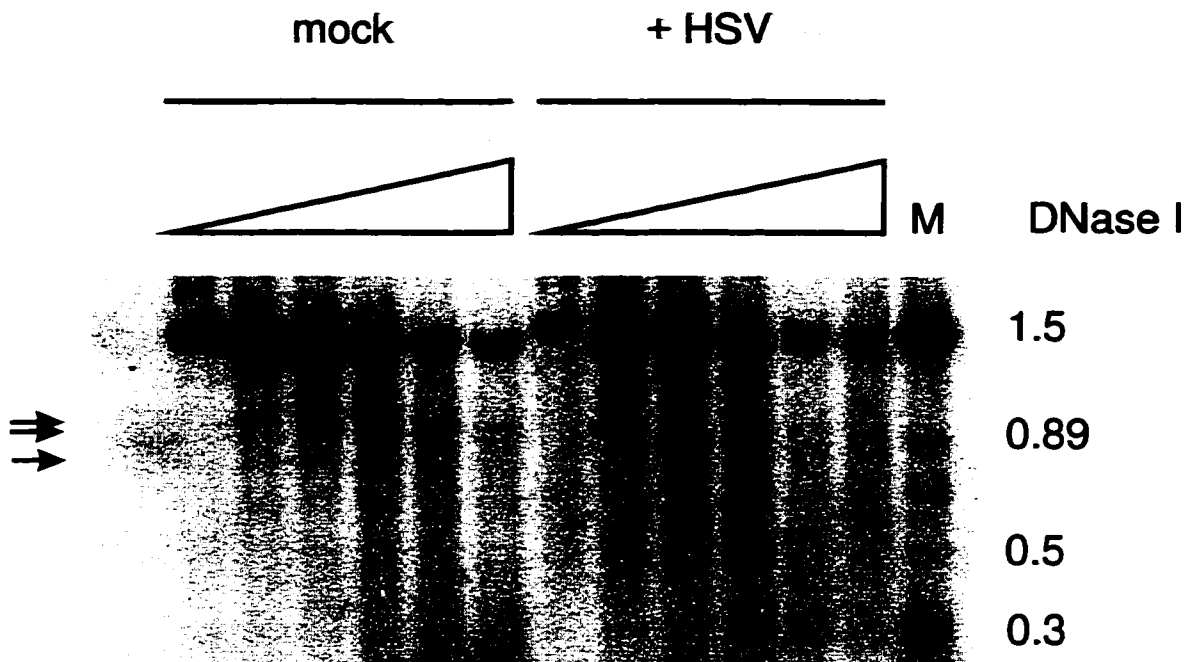


generated a different set of fragments that were each 300 bp shorter than the corresponding fragments generated from PstI digestion alone (Fig. 11D). By correlating the sizes of these 3' co-terminal fragments with the known MspI sites, we determined that the MspI sites within the transcription unit of the  $\alpha$ -globin gene were inaccessible (Fig. 11E). Since no additional bands were detected by the full length probe compared to the 3' end probe (Fig 11A and C), this suggested that the MspI sites at the promoter region were accessible and cleaved into fragments that were too small to be detected. These results suggested that the nucleosome bound region of the  $\alpha$ -globin locus was inaccessible by MspI enzymes, whereas the  $\alpha$ -globin promoter region which was free of positioned nucleosomes was accessible and cleaved by MspI. Also, the presence of the 3' co-terminal fragments suggested that the transition points between the accessible and inaccessible regions may vary among the HeLa cells within the population. Interestingly, when HSV-infected nuclei were examined and compared to the mock-infected nuclei, there was a subtle but distinct change in the accessibility of one or more of the MspI sites at the transition site. Upon activation, there appeared to be a new accessible MspI site (indicated by \* in Fig. 11A, C and E) which mapped to immediately 5' of the  $\alpha$ -globin transcription start site. This finding suggested that a change in accessibility may be required for the activation of this gene, and is consistent with our hypothesis that a nucleosome normally occludes accessibility of this region of the  $\alpha$ -globin gene and silences the expression of this gene in non-erythroid

cells. In order to further confirm this hypothesis, one will need to develop experimental techniques that yield results with better resolution to better examine the changes in accessible sites upon HSV infection.

**DNase I hypersensitive sites are present around the transcription start site of the endogenous  $\alpha$ -globin gene in HeLa cells.** Another assay that is commonly used to determine chromatin structure is the identification of DNase I hypersensitive sites (HS). These sites generally indicate DNA sequences within the chromatin that are exposed and accessible for binding by nuclear factors. In this experiment, HeLa cells were mock-infected or infected with HSV and intact nuclei were harvested from these cells at 6 hpi. Parallel samples of isolated nuclei were treated with increasing amounts of DNase I and then lysed by proteinase K and SDS. The harvested genomic DNA was cleaved by PstI, fractionated on an agarose gel, and examined by Southern blot analysis with the  $^{32}\text{P}$ -labelled 300 bp 3' end probe. In the mock-infected samples, weak DNase I HS sites were found within the  $\alpha$ -globin gene with the dominant HS sites located around the transcription start site (Fig. 12). In the HSV-infected samples, DNase I HS sites were seen at similar locations although the intensities of the bands may be weaker than in the mock-infected samples. At this level of resolution it is difficult to conclude whether HSV infection altered the formation of DNase I hypersensitive sites;

Fig. 12. DNase I digestion of intact nuclei. HeLa cells were mock-infected or HSV-infected at an MOI of 10, and intact nuclei were harvested from infected cells at 6 h.p.i. Parallel samples of nuclei were treated with increasing amounts (0 to 16 U) of DNase I and genomic DNA extracted from these nuclei was digested with Pst I. The digested genomic DNA was then examined by Southern blot analysis using the  $^{32}\text{P}$ -labelled 300 bp BstEII to Pst I fragment corresponding to the 3' end of the  $\alpha 2$  gene.



however, it is clear that HS sites are present within the  $\alpha$ -globin gene, particularly around the transcription start site. These results again suggest that the region around the transcription start site of the endogenous  $\alpha$ -globin gene may adopt unusual chromatin structures which may be involved in its silencing in non-erythroid cells.

## Discussion

It is widely accepted that the endogenous human  $\alpha$ -globin gene is regulated by a repression mechanism that silences its expression in non-erythroid cells (Brickner, et al., 1991; Rein, et al., 1995). However, our understanding of this process has so far been limited, in part, due to the lack of an experimental model that faithfully reproduces the erythroid-specific expression of this gene in cell culture. For example, transiently transfected copies of the  $\alpha$ -globin gene are promiscuously transcribed in all cell types, and to date, no tissue-specific regulatory element has been found. In our previous study, we found that the endogenous  $\alpha$ -globin gene in non-erythroid cells is activated upon HSV infection (Cheung, et al., 1997). This observation shows that the repression mechanism of this gene can be disrupted, and suggests that analysis of this phenomenon may be an alternative approach to elucidate the tissue-specific regulation of the  $\alpha$ -globin gene.

While studying the activation of the endogenous  $\alpha$ -globin gene by HSV, we found that cycloheximide plus dimethyl sulfoxide (DMSO) can also induce expression of this gene in non-erythroid cells. This discovery was made purely by serendipity when we first noticed that HeLa cells treated with cycloheximide and aphidicolin accumulated  $\alpha$ -globin RNA even without HSV infection (data not shown). Further analyses showed that it was not aphidicolin, but the DMSO that it was dissolved in, that in combination with cycloheximide activated

expression of the  $\alpha$ -globin gene. The drug-treated cells accumulated only low levels of globin RNA, and this effect was specific to the  $\alpha$ -globin gene since neither  $\zeta$ -, nor  $\beta$ -globin genes were activated. We also showed that anisomycin can substitute for cycloheximide for the induction effect, suggesting that inhibition of protein synthesis may be a contributing factor. It is not clear whether DMSO and cycloheximide/anisomycin independently affect different aspects of the silencing mechanism for the  $\alpha$ -globin gene, or whether they synergize to activate its expression. Nevertheless, since expression of the  $\alpha$ -globin gene occurred in the presence of protein synthesis inhibitors, the induction process must not require *de novo* synthesis of new transcription factors. This observation, together with the fact that the  $\alpha$ -globin gene is normally under repression, suggests that the drug-induced expression may be a result of derepression rather than activation of this gene.

DMSO, cycloheximide, and anisomycin have each been shown to independently affect gene expression. DMSO is a free radical scavenger that is often used to induce differentiation of myeloid or erythroid-leukemic cell lines such as HL60 and MEL cells (Perussia, et al., 1981; Friend, et al., 1971). The mechanism leading to differentiation is not known; however, this process correlates with many cellular changes including alteration of proto-oncogene expression (Ramsay, et al., 1986), activation of globin genes (Profous-Juchelka, et al., 1983) and changes in inositol metabolite levels



(Faletto, et al., 1985) and cellular ion concentrations (Lannigan and Knauf, 1985). DMSO has been reported to enhance transcription of various genes. In some cases, such as the apolipoprotein A-I, induction of transcription was mediated directly through an "antioxidant response element" present in the promoter sequences (Tam, et al., 1997). However, for the  $\alpha$ -globin gene, such an element has not been identified. In addition to its direct activation of specific genes, DMSO also affects the general chromatin structure of the genome. For example, biochemical analysis showed that DMSO can alter the thermostability of chromatin and transform it to a more extended and relaxed state (Lapeyre and Bekhor, 1974). Also, MEL cells induced by DMSO have increased levels of phosphorylation and acetylation of histones (Neumann, et al., 1978; Leiter, et al., 1984). These post-translational modifications of histones affect the interactions between DNA and nucleosomes and are often associated with activation of transcription. Therefore, these effects of DMSO on chromatin may facilitate disruption of the silencing mechanism that regulates the  $\alpha$ -globin gene.

Similar to the effects of DMSO, cycloheximide and anisomycin have also been shown to enhance expression of a number of genes. This effect is most often associated with the phenomenon known as "superinduction" which results in the over-accumulation of cellular immediate early (IE) genes when protein synthesis inhibitors are added to mitogen activated cells (Cochran, et al., 1983; Lau and

Nathans, 1987). The best studied example of this effect is the activation of the *c-fos* gene: addition of cycloheximide or anisomycin to mitogen activated cells increases the stability of the otherwise highly labile *c-fos* mRNA, enhances its transcription rate, and prolongs the duration of *c-fos* transcription (reviewed in Edwards and Mahadevan, 1992). These effects enhance expression of the cellular IE genes that are designed to be rapidly activated and transiently expressed; however, they are unlikely to contribute to the activation of the  $\alpha$ -globin gene whose expression is usually regulated in an all or none manner.

Cycloheximide also activates expression of other inducible genes such as IL-2, IL-6, and the glycoprotein hormone  $\alpha$ -subunit gene (Zubiaga, et al., 1991; Faggioli, et al., 1997; Cox, et al., 1990). For these cases, an often proposed induction mechanism suggests that protein synthesis inhibitors prevent the expression of a labile repressor and thus lead to derepression of these genes. For example, such a putative repressor has been identified for the glycoprotein hormone  $\alpha$ -subunit gene and accumulation of this repressor quickly declined upon cycloheximide treatment (Cox, et al., 1990). In the case of  $\alpha$ -globin, the possibility that a tissue-specific repressor regulates its expression has been raised but not directly tested. For example, using *in vivo* footprinting analysis, Rein *et al.* demonstrated that nuclear factor I (NFI) was bound to the endogenous  $\alpha$ -globin promoter in HeLa cells but not in erythroid K562 cells, and suggested that it may act as a repressor to prevent expression of this gene in non-erythroid cells

(Rein, et al., 1995). In this study, we attempted to substantiate the existence of a tissue-specific repressor for the  $\alpha$ -globin gene. We reasoned that a repressor molecule that binds to the  $\alpha$ -globin sequences may be titrated away from the endogenous  $\alpha$ -globin gene by excess copies of this gene introduced into the cells. Our results however showed that transfected copies of the  $\alpha$ -globin gene could not activate expression of the endogenous  $\alpha$ -globin genes. Interestingly, a recent study showed that transfection of plasmids containing the human  $\epsilon$ -,  $\gamma$ -, or  $\beta$ -globin gene into non-erythroid HeLa cells induced transcription of the previously silent intergenic regions of the chromosomal  $\beta$ -globin locus (Ashe, et al., 1997). This phenomenon, known as "transinduction", only activated transcription of the intergenic sequences, but not the  $\epsilon$ -,  $\gamma$ -, or  $\beta$ -globin genes themselves. For transinduction to occur, the transfected globin genes must be actively transcribed and required the linkage of a SV40 enhancer to the globin genes; however, their transcription could also be driven by non-globin promoters and still induced transcription of the endogenous locus. *In situ* hybridisation experiments showed that the transfected plasmids localised close to the chromosomal  $\beta$ -globin locus. These observations led the authors to speculate that transcription of the globin genes on the plasmids could recruit transcription factors to the region and activate the endogenous  $\beta$ -globin locus, or alternatively, the transfected genes could relocate the chromosomal locus into a region of the nucleus that could support active transcription. Although the mechanism mediating this effect is not known, the activation of

sequences within the chromosomal  $\beta$ -globin locus raises questions about the hypothesis that the  $\beta$ -globin cluster in non-erythroid cells is folded into condensed chromatin and is not accessible to transcription factors. Perhaps the nuclear factors recruited by the transfected globin genes could affect the accessibility of the condensed endogenous  $\beta$ -globin locus. The authors also showed that transfection of the  $\alpha$ -globin gene into HeLa cells did not activate transcription of the  $\beta$ -globin locus; however, they did not examine the transcription of the  $\alpha$ -globin locus in these transfected cells. In our case, transfection of actively transcribing  $\alpha$ -globin genes into HeLa cells did not activate transcription of the endogenous  $\alpha$ -globin genes. In light of this transinduction effect on the  $\beta$ -globin locus, it would be interesting to determine whether plasmids containing  $\alpha$ -globin sequences colocalise with the endogenous  $\alpha$ -globin locus and whether they can activate transcription of the intergenic sequences of the  $\alpha$ -globin locus.

Cycloheximide and anisomycin are unique among protein synthesis inhibitors in that they can also stimulate the same nuclear signaling responses as growth factors and lead to rapid phosphorylation of chromatin associated proteins (Mahadevan, et al., 1991). For example, both compounds can stimulate phosphorylation of histone H3 and anisomycin can prolong phosphorylation of the nucleosome binding HMG-14 protein in mitogen activated cells (Mahadevan, et al., 1991; Barratt, et al., 1994). These effects are not related to protein synthesis inhibition since they can be induced at

drug concentrations lower than required for inhibition of protein synthesis and also since other translation inhibitors do not possess the same abilities. Although our data showed that lowering the concentration of cycloheximide or anisomycin reduced the accumulation of  $\alpha$ -globin RNA, we did not conclusively determine whether induction of the  $\alpha$ -globin gene was dependent on inhibition of protein synthesis. Further studies using other translation inhibitors such as puromycin which do not lead to phosphorylation of chromatin proteins may give a more definitive answer. It is nevertheless intriguing that both DMSO and cycloheximide/anisomycin affect general chromatin structure and histone modifications. Combined with our data on the chromatin organisation of the endogenous  $\alpha$ -globin gene, it is tempting to speculate that the regulation of this gene is mediated through histone structure and nucleosome formation.

The role of chromatin structure on regulation of  $\alpha$ -globin expression has been demonstrated by studies on a medical condition known as ATR-X syndrome. This genetic disorder results in X-linked mental retardation and  $\alpha$ -thalassemia in affected individuals (Higgs, 1993). Although these patients lack  $\alpha$ -globin expression, the associated genetic defect lies not in the  $\alpha$ -globin locus, but in the X-chromosome. A recent study identified its causative defect in the X-linked XH2 gene which when mutated, down-regulated expression of the structurally intact  $\alpha$ -globin gene (Gibbons, et al., 1995). XH2 encodes a protein belonging to the SNF2 family of putative helicases which in turn

belongs to the SWI/SNF family of proteins. These proteins are mammalian transcription activators that remodel restrictive chromatin structures to facilitate transcription initiation (reviewed in Peterson, 1996; Tsukiyama and Wu, 1997). The dependence of  $\alpha$ -globin gene expression by XH2 suggests that this gene is regulated, at least in part, by its chromatin structure. It would be of interest to determine whether expression of XH2 is tissue-specific, and whether it is also essential for the transcription of the  $\alpha$ -globin gene in non-erythroid cells.

Other studies using transient and stable transfection assays also implicated that chromatin plays an important role in the regulation of  $\alpha$ -globin gene. Pondel *et al.* found that deletions of the  $\alpha$ -globin 5' flanking G+C rich region which contained six Sp1 binding sites had no effect on the expression of the reporter gene when these constructs were transiently transfected into erythroid cells, but showed a 90% reduction in promoter activity compared to constructs retaining the deleted regions when stably integrated into the cellular genome (Pondel, et al., 1995). Since this effect was only seen upon integration, this suggested that the deleted sequences played an important role in facilitating transcription of the  $\alpha$ -globin promoter in the chromatin context. The authors further demonstrated that full promoter activity of the integrated constructs could be restored when six tandem Sp1 binding sites were added to the deletion mutants, and proposed that binding of Sp1 could alter the chromatin structure of the

$\alpha$ -globin promoter. Using a different series of reporter constructs that contained the rabbit  $\alpha$ -globin promoter and coding region as well as varying lengths of the 5' flanking sequences, Shewchuk *et al.* found a similar effect in that constructs lacking extended 5' flanking regions showed very low transcription activity compared to those containing the G+C rich regions (Shewchuk and Hardison, 1997). Again, this difference was only evident in stably transfected, but not transiently transfected cells. However, in this case, the authors showed that deletions of the Sp1 sites within the 5' flanking sequences did not affect transcription activity, and suggested that Sp1 binding was not critical to the expression of the stably integrated gene. Instead, they demonstrated that the G+C rich 5' flanking sequences of the non-functional  $\theta$ -globin gene could substitute for the  $\alpha$ -globin 5' flanking regions, and proposed that it was the G+C rich characteristic of these regions that was important in the expression of the  $\alpha$ -globin promoter in the chromatin context. They also reported that CpG island DNA had a lower affinity for nucleosome formation *in vitro*, and implied that the presence of the CpG island established a nucleosome-free region at the  $\alpha$ -globin promoter to facilitate its transcription.

The presence of a CpG island within the  $\alpha$ -globin gene no doubt has a great influence on its chromosomal structure and expression. As demonstrated by Tazi *et al.*, CpG islands adopt an "active" chromatin structure and differ from bulk chromatin in at least three ways: these regions contain low levels of linker histone H1, the

associated H3 and H4 histones are highly acetylated, and nucleosome-free regions are present (Tazi and Bird, 1990). Also, the CpG dinucleotides within the islands are generally hypomethylated and this has been demonstrated for the  $\alpha$ -globin gene in both expressing and non-expressing cells. Antequera *et al.*, however, showed that the CpG islands of the  $\alpha$ -globin and other tissue-specific genes become methylated in long-term cultured non-expressing cell lines (Antequera, et al., 1990). Using HpaII and MspI digestion to assay for the methylation status of CCGG sequences within a 1.5 kb PstI - PstI fragment containing the  $\alpha$ -globin gene, they found that all except one of the HpaII sites within this region were methylated in HeLa cells. In contrast to their results, we found that the 5' flanking region of the  $\alpha$ -globin gene of our HeLa cell line was free of methylation, and its coding region was differentially methylated. We believe that this difference was due to variations in the methylation extent of the  $\alpha$ -globin CpG island among the numerous sublines of long-term cultured HeLa cells. Even in Antequera's study, they observed variable degrees of methylation, ranging from moderate to high, of the  $\alpha$ -globin CpG island in different non-erythroid cell lines. By genomic sequencing, Rein *et al.* found that the promoter region of the  $\alpha$ -globin gene in their HeLa cell line was unmethylated as well (Rein, et al., 1995). Insofar as methylation is often associated with repression of gene expression, the finding that the  $\alpha$ -globin promoter of our HeLa cells is unmethylated is consistent with its transcriptional competence in HSV-infected cells. Incidentally, we recently found an independent



HeLa cell line that did not display activation of the endogenous  $\alpha$ -globin gene upon HSV infection (Kim Ellison, personal communication). It would be of interest to test whether the inducibility of the  $\alpha$ -globin gene correlates with the methylation status of its promoter sequences.

In this study, we assayed whether another CpG island-containing tissue-specific gene could be activated by HSV infection. We found that the liver-specific retinol binding protein (RBP) gene remained silent in HSV-infected HeLa cells even though the  $\alpha$ -globin gene was activated. However, it is possible that this gene is heavily methylated in our HeLa cells and thus cannot be activated. Antequera found that the RBP gene was fully methylated in all cell lines tested even in the cells where they found variable methylation of the  $\alpha$ -globin gene (Antequera, et al., 1990). Determination of the RBP gene methylation status in our HeLa cells will have to be done to further define whether the CpG island feature is associated with the activation by HSV.

Our analysis of the chromatin structure of the endogenous  $\alpha$ -globin gene showed that an array of phased nucleosomes was positioned in the coding region of this gene in our HeLa cell line. However, no positioned nucleosomes was observed in the 5' flanking promoter region, suggesting that either this region contained randomly placed nucleosomes, or it may be free of nucleosomes. It is likely that at least part of this region is nucleosome-free. This region

of DNA contains the highest density of CpG dinucleotides within the  $\alpha$ -globin CpG island, and CpG islands often contain nucleosome-free regions. In addition, several DNase I hypersensitive sites were found at around the transcription start site (Fig. 10), and similarly, DNase I hypersensitive sites are believed to be nucleosome-free or disrupted regions of chromatin. We also found that this region of the  $\alpha$ -globin gene was free of methylation and these data together suggested that the 5' flanking region of the endogenous  $\alpha$ -globin gene was relatively unobstructed. Consistent with our finding, Rein *et al.* demonstrated by *in vivo* footprinting analysis that a variety of factors were bound to the  $\alpha$ -globin promoter in HeLa cells, thus indicating that this part of the  $\alpha$ -globin gene was accessible to nuclear factors in non-erythroid cells (Rein, et al., 1995).

If transcription factors were bound to the endogenous  $\alpha$ -globin promoter even in non-erythroid HeLa cells, then how is transcription of this apparently primed gene suppressed in non-erythroid cells? From their genomic footprinting analysis of the  $\alpha$ -globin promoter in HeLa cells, Rein *et al.* found several dimethyl sulfate (DMS) hypersensitive sites located immediately 5' upstream of the TATA element which suggested that the DNA conformation of that region was distorted. As suggested by the authors, this DMS hypersensitivity could be due to the presence of a positioned nucleosome which could cause the DNA to bend or kink sharply. Our data were completely consistent with their hypothesis as we found the first of the array of

phased nucleosomes was positioned over the TATA element and transcription start site. Insofar as a nucleosome positioned over the TATA box or transcription start site has been shown to be sterically incompatible with the assembly of transcription initiation complex in *in vitro* transcription assays (Lorch, et al., 1992), a similar *in vivo* arrangement at the  $\alpha$ -globin gene would likely obstruct transcription of this gene as well. Positioned nucleosomes are also found in the chicken and mouse  $\beta$ -globin genes (McGhee, et al., 1981; Benezra, et al., 1986). In the latter case, an array of positioned nucleosomes was found to extend from -3000 to +1500 relative to the transcription start site of the mouse  $\beta$ -globin gene in non-erythroid cells. This same region was covered by an array of positioned nucleosomes in erythroid cells, with the exception of an approximately 500 bp region of the promoter that was nucleosome-free, suggesting that expression of the endogenous  $\beta$ -globin gene required an unobstructed and accessible promoter. A recent study using low-copy number mini-chromosomes containing the  $\beta$ -LCR linked to the  $\epsilon$ -globin gene found that an array of positioned nucleosomes covered the mini-chromosome  $\epsilon$ -globin gene from position -800 to +600 in both erythroid and non-erythroid cells (Gong, et al., 1996). Activation of this  $\epsilon$ -globin gene in erythroid cells required displacement of one positioned nucleosome covering a GATA-1 binding site and the TATA element of this gene. Furthermore, this remodeling of the  $\epsilon$ -globin promoter was dependent on binding of the erythroid-specific transcription factor NF-E2 to the HS2 of the  $\beta$ -LCR. The consensus of these findings indicates that positioned nucleosomes

are not compatible with active transcription and suggests that transcription of the chromatin bound  $\alpha$ -globin gene likely require alterations in the deposition of nucleosomes.

For a number of eucaryotic promoters that are regulated by positioned nucleosomes, such as the yeast Pho 5 gene and the MMTV promoter, their induction involves eviction of specific nucleosomes to allow access of the underlying sequences by transcription factors (Svaren and Horz, 1996). We found that the activation of the  $\alpha$ -globin gene in HeLa cells did not involve complete removal of positioned nucleosomes; however, activation did lead to subtle changes in the chromatin structure. It is possible that only a small fraction of the HeLa cells responded to the activation of the  $\alpha$ -globin gene and the chromatin structure of the non-responsive cells may mask activation-related removal of specific nucleosomes. This possibility could be clarified by determining what fraction of the HSV-infected HeLa cells expressed the  $\alpha$ -globin RNA by *in situ* hybridisation techniques. Nevertheless, we did find distinct changes in the accessibility of the MspI sites around the transcription start site in association with transcription activation, indicating that the positioning of the nucleosome over the TATA element and transcription start site was shifted upon activation. In particular, an additional MspI site close to the transcription start site became accessible to MspI digestion upon transcriptional activation of the  $\alpha$ -globin gene, suggesting that the transcription start site may become accessible to nuclear factors upon

activation. Our data so far have been obtained by detection of genomic Southern blots with indirect labeling methods. In order to map the exact changes in the accessible MspI sites, one will have to develop higher resolution protocols such as by adding a primer extension step after digestion of nuclei DNA by MspI. Such finer analysis of the data could identify the underlying DNA elements whose accessibility may correlate with transcription activation, and this could strengthen our hypothesis that the TATA element and transcription start site may be occluded from the transcription initiation complex to silence expression of this gene in non-erythroid cells.

The transcription of nucleosome bound DNA does not always require eviction of nucleosomes from the promoter region, but can also result from alterations of the nucleosome-DNA contacts. Current studies of transcription regulation mediated by chromatin show that there are two general mechanisms that modify chromatin in preparation for transcription. One mechanism involves ATP-dependent chromatin remodeling complexes that facilitate binding of transcription factors to nucleosome bound DNA elements (Peterson, 1996; Tsukiyama and Wu, 1997), and a second mechanism involves enzymatic alterations of the nucleosomal histones (Brownell and Allis, 1996; Grunstein, 1997). Recent advances in chromatin research have identified eucaryotic chromatin remodeling factors in yeast, drosophila, and mammals. The best studied example of these factors is the yeast SWI/SNF complex which is found to associate with the

RNA polymerase II holoenzyme complex (Wilson, et al., 1996). SWI/SNF is required for the expression of a variety of yeast genes and biochemical studies showed that the yeast and human homologues can disrupt histone-DNA contacts. This reaction does not lead to complete displacement of the nucleosome, but does result in a >30-fold increase in the affinity of transcription factors to the nucleosomal binding site (Cote, et al., 1994). As mentioned earlier, the X-linked XH2 gene encodes a protein belonging to this family of proteins and is essential for transcription of the  $\alpha$ -globin gene in erythroid-cells. Elucidation of how this protein affects transcription of the  $\alpha$ -globin gene would be invaluable to the understanding of how chromatin structure can regulate expression of this gene.

In addition to chromatin remodeling complexes, enzymatic modifications of nucleosomal histones also plays an important role in the transcription process. In particular, histone acetylation has long been associated with transcription competence (active genes have acetylated histones and conversely inactive genes have underacetylated histones) (Allfrey, et al., 1964). It is believed that acetylation of the lysine residues of histone H3 and H4 would neutralise their positive charge and loosen their association with the negatively charged DNA (Wolffe, 1994). Recent advances found that a number of transcription coactivators, such as GCN5, and TAF<sub>II</sub>250, possess nuclear histone acetyl-transferase (HAT) activities, thus strengthening the regulatory link between histone acetylation and

transcription activation (reviewed in Wolffe and Pruss, 1996). It is most interesting that DMSO and cycloheximide/anisomycin have all been shown to affect histone acetylation. Moreover, HSV ICP4, which we have previously shown to be sufficient to activate expression of the endogenous  $\alpha$ -globin gene, is known to physically associate with TAF<sub>II</sub>250 (Carrozza and DeLuca, 1996), and this nuclear factor has been shown to possess HAT activity (Mizzen, et al., 1996). Taking all our data into account, they provide corroborative evidence that transcription activation of the endogenous  $\alpha$ -globin gene in non-erythroid cells may involve modification of the histones that make up the nucleosomes positioned at the  $\alpha$ -globin gene. Although our hypothesis is only based on circumstantial evidence, it merits further experimental testing especially with the development of antibodies specific for acetylated forms of histone H3 (C. D. Allis, personal communication) which makes it possible to assay the acetylation status of the nucleosomes associated with the  $\alpha$ -globin promoter. A link between histone acetylation and transcriptional activation of the  $\alpha$ -globin gene would strengthen our hypothesis that a positioned nucleosome at the TATA element and transcription start site of this gene can repress expression of this gene in non-erythroid cells.

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**Chapter 5: The effects of herpes simplex virus (HSV) immediate-early proteins ICP4, ICP22, and ICP27 on the expression of the activated endogenous  $\alpha$ -globin gene in HSV-infected nonerythroid cells**

Manuscript to be submitted to Journal of Virology.

### **Abstract**

We previously reported that the silent endogenous human  $\alpha$ -globin gene is activated upon herpes simplex virus (HSV) infection of non-erythroid cells. We report here that this process involves transcriptional and post-transcriptional controls mediated by several HSV immediate-early regulatory proteins. Viral mutants bearing null mutations in the genes encoding ICP4 and ICP22 each displayed a ca. 8-10 fold reduction in the levels of accumulated globin RNA. Nuclear run-on analysis confirmed that activation involves increased levels of globin gene transcription, and showed that ICP4 is required for much of this increase. In contrast, cells infected with an ICP22 null mutant displayed wild-type levels of globin transcription, suggesting that ICP22 acts post-transcriptionally to enhance the accumulation of globin transcripts. ICP22 has previously been shown to be phosphorylated by the viral kinase U<sub>L</sub>13, and genetic evidence indicates that at least some aspects of ICP22 function depend on U<sub>L</sub>13. However, we found that cells infected with a mutant lacking U<sub>L</sub>13 accumulated the same levels of  $\alpha$ -globin RNA as those infected with wild type HSV, indicating that U<sub>L</sub>13 is not required for the regulatory contribution of ICP22 in this system. Northern blot analysis revealed that cells infected with wild-type HSV accumulated both spliced and unspliced globin RNAs. ICP27 was required for the accumulation of unspliced RNA, yet deletion of the ICP27 gene did not increase either the levels or rate of accumulation of fully spliced  $\alpha$ -globin mRNA.

Inasmuch as ICP27 had no effect on  $\alpha$ -globin gene transcription, these data suggest that ICP27 stimulates the accumulation of globin pre-mRNA without greatly inhibiting splicing of globin transcripts. This conclusion contrasts with those reached in previous studies by other investigators, where it was suggested that ICP27 globally inhibits RNA splicing and thereby prevents expression of cellular genes.

## Introduction

Herpes simplex virus (HSV) is a large enveloped DNA virus that infects a wide variety of mammalian cells in culture (reviewed in reference Roizman and Sears, 1996). Upon infection, the viral genome is transported to the cell nucleus where viral gene expression occurs. HSV genes bear a significant resemblance to eucaryotic genes: their promoters are recognized and transcribed by cellular RNA polymerase II, and the viral transcripts are 5' capped, 3' polyadenylated, and are translated by the host protein synthesis machinery. The HSV genome contains more than 80 genes which are broadly grouped into three classes, immediate-early (IE), early (E), or late (L), based on their temporal order of expression. These three classes of genes are sequentially expressed in a cascade fashion during lytic infection (Hones and Roizman, 1974). The expression of IE genes is induced upon binding of the transactivation complex composed of virion-associated VP16 and cellular Oct1 factors to the TAATGARATTC elements present in the promoters of IE genes (Gerster and Roeder, 1988; O'Hare, et al., 1988; Preston, et al., 1988). With the exception of ICP47, which has an immune-evasion function (Hill, et al., 1995; York, et al., 1994), the other IE gene products (ICP0, ICP4, ICP22, and ICP27) are regulatory proteins that activate expression of the viral E and L genes (reviewed in Roizman and Sears, 1996 and Ward and Roizman, 1994). E genes are transcribed following the expression of IE gene products and in general encode for proteins involved in viral

DNA synthesis, whereas L genes are transcribed after initiation of viral DNA replication and encode for virion structural proteins. The IE transactivators play a critical role in the HSV lytic life cycle. Honess and Roizman demonstrated that incorporation of amino acid analogues into newly synthesized IE polypeptides rendered them non-functional and prevented expression of other viral genes (Honess and Roizman, 1975). Also, a number of studies have shown that viruses containing mutations in ICP4, ICP22, and ICP27 have severe defects in E or L gene expression (DeLuca, et al., 1985; Smith and Schaffer, 1987; Sacks, et al., 1985; McCarthy, et al., 1989; Sears, et al., 1985). Therefore, these IE proteins are key regulatory factors that drive the progression of the HSV gene expression cascade.

In coordination with the execution of the viral gene expression programme, HSV strongly inhibits expression of endogenous cellular genes. The shutoff of host genes presumably facilitates viral gene expression by minimizing competition for the cellular transcription and translation machineries. This process is initially triggered by a virion component brought in by the infecting viral particles, then later bolstered by one or more newly synthesized viral proteins (Fenwick and Clark, 1982; Fenwick, 1984). Early studies demonstrated that the early, or virion-directed, shutoff of host protein synthesis is caused by disaggregation of polyribosomes and degradation of mRNA (Sydiskis and Roizman, 1966; Nishioka and Silverstein, 1978). Read and Frenkel isolated a viral mutant, named *vhs1* (virion host shutoff), which lacks

this activity (Read and Frenkel, 1983), and subsequent studies mapped this mutation to the U<sub>L</sub>41 gene of the HSV genome (Kwong, et al., 1988). This gene encodes the tegument associated vhs protein which has been demonstrated to be responsible for early host shutoff activity (Fenwick and Everett, 1990; Read, et al., 1993. Smibert, et al., 1992). The vhs protein appears to affect the turnover of cellular and viral mRNA (Kwong and Frenkel, 1987; Oroskar and Read, 1989; Schek and Bachenheimer, 1985); however, its precise activity is still not fully understood. In contrast to the vhs-directed activity, the viral protein(s) responsible for the delayed shutoff function is not known. This activity requires *de novo* protein synthesis and therefore suggests that the delayed host shutoff is mediated by one of the newly synthesized viral proteins (Nishioka and Silverstein, 1978; Fenwick, 1984; Spencer, et al., 1997). Based on the observation that viral mutants lacking ICP27 were defective in the second-stage shutoff activity (Sacks, et al., 1985), it has been hypothesized that ICP27 may be responsible for this inhibitory activity. However, proof for the direct involvement of ICP27 in this process has not yet been provided.

The different fates of cellular and viral genes during viral infection indicate that HSV must be able to differentiate between the two gene populations; however, the basis for that distinction remains unclear. Early studies in our laboratory showed that expression of the endogenous  $\beta$ -globin gene in mouse erythroleukemia (MEL) cells is repressed upon HSV infection: pre-existing  $\beta$ -globin RNA is rapidly

degraded, and transcription of this gene is greatly inhibited in HSV-infected cells (Smibert and Smiley, 1990). In contrast, expression of a rabbit  $\beta$ -globin gene present in the genome of the infecting recombinant HSV is activated and regulated by viral proteins. These results demonstrate that the differential regulation of the highly homologous mouse and rabbit  $\beta$ -globin genes during HSV infection is not based on their DNA sequences, but is dictated at least in part by their genomic context. Experiments using a recombinant virus containing the human  $\alpha$ -globin gene showed that, when placed in the viral genome, this cellular gene is also activated and regulated as a viral gene (Panning and Smiley, 1989). These findings therefore suggest that HSV transactivators do not differentiate between cellular and viral promoters and can effectively activate transcription of both. We have recently reported that the previously silent endogenous  $\alpha$ -globin gene is activated in HSV-infected non-erythroid cells (Cheung, et al., 1997, Chap. 3 of this thesis), and found that maximal expression of this cellular gene depends on the presence of ICP0, ICP4 and ICP22 during infection. Furthermore, transient expression assays showed that ICP0 or ICP4 is sufficient to induce expression of the endogenous  $\alpha$ -globin gene in HeLa cells, demonstrating that viral transactivators can access and activate cellular genes within the host genome. Here we further examine the effects of HSV regulatory proteins on the expression of the endogenous  $\alpha$ -globin gene, and report our findings about the levels of action of ICP4, ICP22, and ICP27.

ICP4 is a 175 kD phosphonuclear protein essential for the expression of HSV E and L genes during infection (DeLuca, et al., 1985; Everett, 1984; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985). It also has a repressor function which down regulates its own expression and that of other IE genes (DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985). Both transactivation and repression activities involve binding of ICP4 to DNA sequences, and mutations in the DNA binding domain of ICP4 abolish its ability to regulate gene expression (DeLuca and Schaffer, 1988; Paterson and Everett, 1988; Paterson and Everett, 1988; Shepard, et al., 1989). Activation of gene expression by ICP4 does not require sequence binding sites in the target genes (Gu and DeLuca, 1994; Imbalzano, et al., 1990; Smiley, et al., 1992), but repression by ICP4 involves specific positioning of ICP4 at the target promoters through consensus binding motifs (Leopardi, et al., 1995; Michael and Roizman, 1993; Roberts, et al., 1988). Although the precise modes of action for this viral regulatory protein are not known, genetic evidence showed that transactivation by ICP4 requires a functional TATA element (Gu and DeLuca, 1994; Smiley, et al., 1992). Also, biochemical studies indicated that ICP4 physically interacts with cellular transcription factors such as TATA-binding protein, TFIIB, and TAF250 (Smith, et al., 1993; Carrozza and DeLuca, 1996), suggesting that the function of ICP4 is likely mediated through its interaction with the basal transcription machinery.



ICP22 is perhaps the least studied member of the IE protein family. It is a 68 kD nuclear protein that is extensively modified post-translationally by phosphorylation and nucleotidylation (Wilcox, et al., 1980; Blaho, et al., 1993). Electrophoretic analysis of HSV-infected cell extracts using denaturing polyacrylamide gels showed that 5 distinct forms of ICP22 molecules, which differ in their extents of phosphorylation, are present in infected cells (Ackermann, et al., 1985). At present, it is not known how these differentially phosphorylated forms of ICP22 correlate with the function of this viral protein. Deletion of the U<sub>g</sub>1 gene, which encodes the ICP22 protein, results in HSV mutants that display severe growth defects in specific cell types (Sears, et al., 1985). While growth of this viral mutant is unaffected in Vero and HEp-2 cell lines, its plating efficiency is greatly reduced on rodent cell lines and human cell strains such as Rat-1 and HEL (human embryonic lung) cells. The poor yield of this mutant virus on the restrictive cell cultures correlates with, and is likely a result of, defects in the expression of ICP0 and a subset of viral late genes (Purves, et al., 1993; Sears, et al., 1985). These data demonstrate that ICP22 regulates expression of specific viral genes, and that a cell type-specific host protein may substitute for its function during infection of permissive cells.

Cells infected with a U<sub>L</sub>13-defective mutant do not contain the various phosphorylated forms of ICP22 (Purves and Roizman, 1992), providing genetic evidence that ICP22 is phosphorylated by the U<sub>L</sub>13

kinase. Moreover, U<sub>L</sub>13-negative mutants display a growth phenotype identical to ICP22-defective mutants (Purves, et al., 1993), suggesting that the function of ICP22 may depend on U<sub>L</sub>13. Examination of the role of ICP22 in viral gene expression indicated that it may affect the stability of ICP0 mRNA (see Discussion section of this paper, Carter and Roizman, 1996). In addition, nuclear run-on analysis of viral gene transcription showed that defects in late gene expression in ICP22<sup>-</sup> mutant-infected cells stem at least in part from reduced level of transcription (Rice, et al., 1995). The functional link between ICP22 and the transcription process is further illustrated by the finding that ICP22 is required for the HSV-mediated alteration of the phosphorylation status of the host RNA polymerase II (Rice, et al., 1995). In normal cells, the carboxy-terminal domain (CTD) of the largest subunit of RNA pol II exists in two forms: the hypophosphorylated II<sub>o</sub> form associated with the transcription initiation complex, and the hyperphosphorylated II<sub>a</sub> form associated with elongating polymerase (Lu, et al., 1991; Cadena and Dahmus, 1987; Payne, et al., 1989). Rice *et al.* initially showed that upon HSV infection, the II<sub>a</sub> form of the RNA polymerase is no longer detected, and is replaced by a form of RNA polymerase containing an intermediate level of phosphorylation which is designated as II<sub>i</sub> (Rice, et al., 1994). The authors further showed that ICP22 is required for the generation of the II<sub>i</sub> form of RNA polymerase by demonstrating that cells infected with an ICP22 defective mutant lack both II<sub>a</sub> and II<sub>i</sub> forms of RNA polymerase (Rice, et al., 1995). Although it is not clear

how ICP22 induces accumulation of the III<sub>i</sub> form of the polymerase, this effect suggests that ICP22 may globally affect the transcription process in HSV-infected cells.

ICP27 is a 68 kD nuclear phosphoprotein which, similar to ICP4, is essential for viral propagation (Sacks, et al., 1985). Cells infected with viral mutants lacking ICP27 display defects in both viral DNA replication and late gene expression (Sacks, et al., 1985; McCarthy, et al., 1989; Rice and Knipe, 1990). ICP27 is a multifunctional protein which can positively or negatively regulate gene expression at the post-transcriptional level (Sandri-Goldin and Mendoza, 1992). Early transfection assays showed that ICP27 alone has little effect on co-transfected reporter genes driven by HSV promoters. However, when ICP0 and ICP4 expression clones are added to activate expression of the reporter genes, ICP27 can then further activate or repress expression of the reporter genes, suggesting that ICP27 can act in synergy with ICP4 and ICP0 to alter reporter gene expression (Everett, 1986; Rice and Knipe, 1988; Sekulovich, et al., 1988; McMahan and Schaffer, 1990). Using this transfection scheme, Sandri-Goldin and Mendoza discovered that ICP27 enhanced expression of genes that have weak 3' end polyadenylation signals, but repressed expression of reporter genes that contained intron sequences (Sandri-Goldin and Mendoza, 1992). Subsequent studies showed that ICP27's induction effect on gene expression stems from its ability to promote polyadenylation at weak 3'

polyadenylation sites and to stabilize the 3' ends of normally labile mRNA (Brown, et al., 1995; McGregor, et al., 1996). This function may be of particular importance in the transition of early to late gene expression since HSV late genes appear to have inherently less efficient 3' end processing signals (McGregor, et al., 1996). In addition, a recent study showed that ICP27 is required for the expression of several early genes that encode the components necessary for viral DNA replication (Uprichard and Knipe, 1996). This finding provides a direct explanation for the lack of viral DNA replication observed in cells infected with ICP27-defective mutants.

Independent of its effect on mRNA 3' end processing, ICP27 also affects the cellular splicing process. A link between ICP27 and the host cell splicing machinery has been demonstrated by biochemical data and supported by results from a variety of functional assays. For example, Sandri-Goldin *et al.* showed that ICP27 co-immunoprecipitated with splicing factors that react to the anti-Sm antiserum and appeared to affect the phosphorylation status of some of these factors (Sandri-Goldin and Hibbard, 1996). Also, ICP27 was found to be necessary and sufficient to induce redistribution of the snRNPs in HSV-infected cell nuclei, and colocalized with the redistributed snRNPs (Phelan, et al., 1993). Sandri-Goldin and Mendoza demonstrated that when ICP4 and ICP0 expression clones were co-transfected with reporter genes, the additional presence of ICP27 significantly lowered accumulation of spliced reporter mRNA

in transfected cells (Sandri-Goldin and Mendoza, 1992). Furthermore, Hardy and Sandri-Goldin showed that *in vitro* splicing reactions were inhibited in nuclear extracts from wild type HSV-infected cells but not in those harvested from cells infected with an ICP27 defective mutant (Hardy and Sandri-Goldin, 1994). These findings together suggest that ICP27 inhibits host cell splicing and prevents accumulation of spliced mRNA. Hardwicke and Sandri-Goldin also showed that the steady-state levels of three cellular transcripts were significantly lower in cells infected with wild type HSV compared to those infected with ICP27-defective mutants (Hardwicke and Sandri-Goldin, 1994). Since only four HSV genes contain intron sequences whereas most cellular genes have multiple introns, these observations prompted Sandri-Goldin and co-workers to propose that ICP27 selectively represses host gene expression by inhibiting cellular splicing activity, and that this viral protein is responsible for the delayed host shutoff activity seen in HSV-infected cells.

In this study, we examined the effects of ICP4, ICP22, and ICP27 on the expression of the endogenous  $\alpha$ -globin gene during HSV infection. Our data indicate that ICP4 induces transcription of this cellular gene whereas ICP22 regulates accumulation of its RNA at the post-transcriptional level. Furthermore, we directly tested whether ICP27 inhibits cellular gene expression by examining its effects on  $\alpha$ -globin gene expression and splicing of its RNA. The results show that ICP27 induces accumulation of unspliced  $\alpha$ -globin RNA; however,

wild type HSV-infected cells accumulates the same levels of spliced  $\alpha$ -globin mRNA as compared to cells infected with the ICP27-defective mutants. Therefore, these findings indicate that ICP27 induces accumulation of unspliced  $\alpha$ -globin RNA, but it neither inhibits splicing of the  $\alpha$ -globin RNA, nor suppresses expression of this intron-containing cellular gene.

## Materials and Methods

**Cells and viruses.** HeLa and Vero cells were grown in  $\alpha$ -minimal essential medium ( $\alpha$ -MEM) containing 10% and 5% fetal bovine serum respectively. The following HSV-1 strains were propagated on Vero cells: wild type KOS and F; R325tk<sup>+</sup> (Post and Roizman, 1981) and del22Z (Poffenberger, et al., 1993), two different ICP22 mutants; R7356, an U<sub>L</sub>13 deletion mutant (Purves and Roizman, 1992); and N38 (Umene, 1986), a mutant lacking ICP47. The ICP4 null mutant d120 (DeLuca, et al., 1985), was grown on complementing E5 cells, and the ICP27 deletion mutant, 5dl1.2 (McCarthy, et al., 1989), was propagated on complementing V27 cells. The ICP0 amber mutant n212 (Cai and Schaffer, 1989), was grown and titrated on U2OS cells.

**Nuclear run-on transcription assay.** The nuclear run-on assay was adapted from the protocol described by Spencer *et al.* (Spencer, et al., 1990). In brief, HeLa cells were mock infected or infected with wild-type or mutant HSV strains at an MOI of 10 in the presence of 300  $\mu$ g/ml of phosphonoacetic acid (PAA). At 6 hours post infection, cells were lysed in a hypotonic buffer, and nuclei were harvested by centrifugation and resuspended in nuclear storage buffer (50 mM Tris pH 8.0, 5 mM MgCl<sub>2</sub>, 40% glycerol, and 0.5 mM DTT). Approximately  $2 \times 10^7$  nuclei were used per sample for the run-on transcription assay and transcription was performed in a buffer containing <sup>32</sup>P-UTP and 150 mM KCl. Following *in vitro* transcription, nuclei were treated

with DNase I and then lysed in proteinase K/SDS solution. Labeled RNA products were precipitated by isopropanol and unincorporated  $^{32}\text{P}$ -UTP was removed by passage through sephadex G-50 spin-columns. Purified transcripts were hybridized to Nytran Plus (S & S) filters bearing single-stranded bacteriophage M13 DNA probes specific for  $\alpha$ -globin, HSV TK, and the cellular  $\gamma$ -actin gene. Hybridized filters were washed and treated with RNase A to remove nonhybridized portions of the labeled RNA. Washed filters were exposed to X-ray films or to phosphorimager screens for densitometry analysis.

The single-stranded DNA probes were derived from M13mp18 constructs containing different DNA fragments corresponding to the genes and regions of interest. Three different probes corresponding to the human  $\alpha$ -globin gene were used (see Fig. 2A). The full length probe corresponded to the PstI fragment of the  $\alpha 2$  globin gene which encompassed the entire  $\alpha$ -globin transcript. The 5' proximal probe was complementary to +1 to +156 of the  $\alpha$ -globin transcript, and the 3' proximal probe was complementary to +357 to +493 of the  $\alpha$ -globin transcript. Three corresponding antisense probes were also constructed and used as negative controls. The single-stranded M13 probes for HSV TK and  $\gamma$ -actin were described in (Smibert and Smiley, 1990) and (Spencer, et al., 1997) respectively.

**Primer extension analysis.** RNA from mock infected or HSV infected HeLa cells was isolated with Trizol (Gibco BRL). 10  $\mu\text{g}$  of total RNA was used per sample for primer extension analysis as previously



described (Cheung, et al., 1997). A 25mer oligonucleotide corresponding to +55 to +80 of the  $\alpha$ -globin transcript was used as primer for cDNA synthesis.

**Northern blot analysis.** 10  $\mu$ g of total RNA was used per sample and denatured RNA samples were separated on formaldehyde/agarose (6%/1.5%) gels by electrophoresis at 1.5V/cm. The formaldehyde/agarose gels were denatured, renatured, and blotted onto Nytran Plus membranes. The RNA was UV cross-linked to the membrane filters with a Stratalinker (Stratagene) and the filters were then hybridized with  $^{32}$ P-labeled probes corresponding to the entire or different parts of the  $\alpha$ -globin transcript.

Full length probe: PstI fragment of  $\alpha 2$  globin gene.

exon 1 probe: 5' AGGCGGCCTTGACGTTGGTCTTGTC 3'

intron 1 probe: 5'GAGGGTGGCCTGTGGGTCCGGGCGGGCGAG 3'

**S1 nuclease protection assay.** Strand separation, isolation of single-stranded DNA, and nuclease treatment were performed as described previously (Cheung, et al., 1997). In brief, 10  $\mu$ g of total RNA per sample was hybridized with excess  $^{32}$ P-labeled single-stranded probe and then treated with S1 nuclease. Protected products were resolved on 8% polyacrylamide sequencing gels and exposed to film or phosphorimager screen for densitometry analysis. The single-stranded probe was generated from a construct containing 224 bp of

the exon 1-intron 1 junction of the  $\alpha$ -globin gene. This region was amplified by PCR with the following primer pair:

5' GCGGGATCCGCGGCCCCGGCACTCTTC 3' and

5' GCGGGATCCGGGGCCAGGACGGTTGA 3', and was cloned into the BamHI site of pUC18.

**RNase H cleavage.** 10  $\mu$ g of total RNA per sample was hybridized to 300 ng of oligo(dT) (Pharmacia) plus or minus 300 ng of oligonucleotides complementary to  $\alpha$ -globin exon 2 or exon 3 sequences in a buffer containing 20 mM Tris pH 7.5, 10 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 50 mM NaCl, 1 mM DTT, and 30  $\mu$ g/ml of BSA at 60°C. RNase H (Promega) was added to a final concentration of 0.025 units/ $\mu$ l and the samples were incubated at 30°C for 1h. The cleavage products were precipitated with ice-cold ethanol and analysed by Northern blot. The sequences for the exon 2 and exon 3-specific oligonucleotides are as follows:

exon 2: 5' GCCCGTGGCTCAGGTCGAAGT 3'

exon 3: 5' CCGAGGCTCCAGCTTAACGGTATTT 3'

**Transient transfection assay.** Transient transfections were done using the calcium phosphate method and was previously described in (Cheung, et al., 1997). 20  $\mu$ g of each plasmid was used per transfection and pUC18 was used as carrier DNA such that each transfection sample contained 60  $\mu$ g of total DNA. pBB37 is an HSV ICP4 expression clone obtained from Peter O'Hare. pC27 is the ICP27

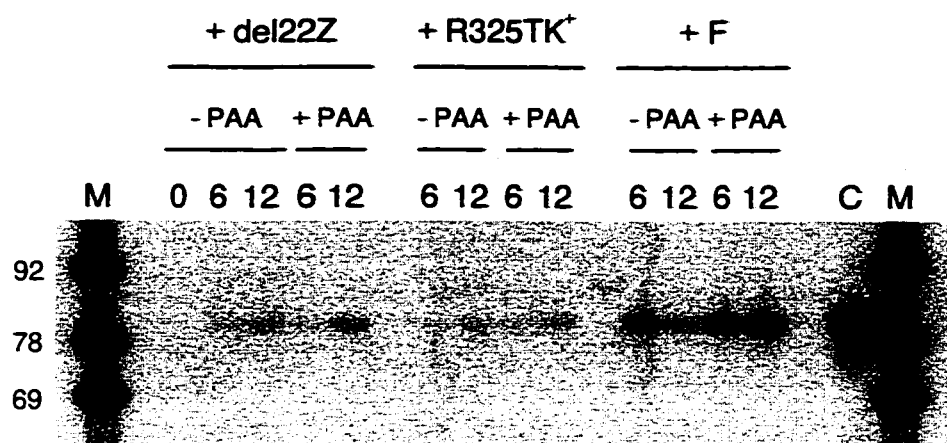
expression clone and pUHD10-3 was the parental vector for pC27. Both these plasmids were obtained from Stephen Rice. Total RNA was harvested from transfected cells at 36 h post transfection and was treated with RNase free DNase I (Promega) to remove any contaminating plasmid DNA. 10  $\mu$ g of the DNA-free RNA was then used in Northern blot analysis.

## Results

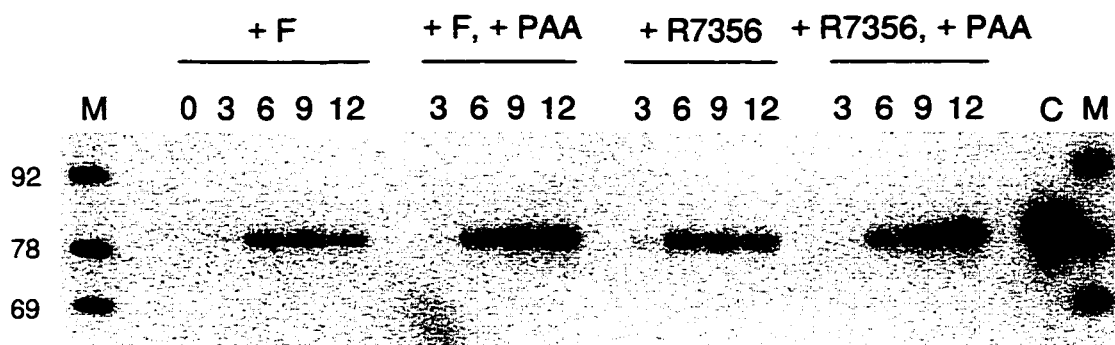
**Accumulation of  $\alpha$ -globin RNA in HSV infected cells does not require UL13.** Our previous report showed that accumulation of the  $\alpha$ -globin RNA in HSV-infected cells was dependent on ICP0, ICP4, and ICP22 in that cells infected with mutants lacking any one of these genes contained significantly less globin RNA compared to wild type HSV-infected cells (Cheung, et al., 1997). Furthermore, we found that transient expression of either ICP0 or ICP4 was sufficient to induce accumulation of  $\alpha$ -globin RNA in transfected cells, indicating that these viral proteins play direct roles in the activation of this gene during infection. In contrast, the role of ICP22 in the expression of the  $\alpha$ -globin gene during infection was not clear since expression of ICP22 alone had no effect on the endogenous  $\alpha$ -globin gene of the transfected cells. One possible explanation for this result was that the effect of ICP22 may depend on other HSV proteins. For example, Purves *et al.* have shown that ICP22 is phosphorylated by the viral kinase UL13 during infection, and suggested that this post-translational modification is required for the function of ICP22. To test whether UL13 is required for the accumulation of  $\alpha$ -globin RNA during infection, total RNA was harvested from HeLa cells infected with wild type F strain, ICP22<sup>-</sup> or UL13<sup>-</sup> mutants, and the steady-state amount of  $\alpha$ -globin RNA was assayed by primer extension analysis. Phosphonoacetic acid (PAA) was added to some of the infected cell samples since this was previously shown to prevent the decline of the

Fig. 1. Primer extension analysis for  $\alpha$ -globin RNA in HSV-infected HeLa cells. HeLa cells were mock-infected or infected with wild type HSV F strain, mutants lacking ICP22 (del22Z or R325TK<sup>+</sup>), or a mutant lacking U<sub>L</sub>13 (R7356) in the presence or absence of phosphonoacetic acid (PAA). Total RNA was harvested at various times post infection and 10  $\mu$ g of each sample was used in primer extension analysis. (A) Comparison of the steady-state level of  $\alpha$ -globin RNA in del22Z, R325TK<sup>+</sup>, or F strain HSV-infected cells. (B) Comparison of the steady-state level of  $\alpha$ -globin RNA in F or R7356 strain HSV-infected cells. In both figures, C represents RNA from the blood RNA positive control and M represents HpaII digested pBR322 DNA size markers.

A



B



accumulation of  $\alpha$ -globin transcripts at late times during infection (Chap. 3). As shown in Fig. 1, cells infected with the ICP22<sup>-</sup> strains (del22Z and R325TK<sup>+</sup>) accumulated approximately 8 to 10 fold less  $\alpha$ -globin RNA compared to the F strain-infected cells. In contrast, R7356 (UL13<sup>-</sup>) and F strain infected cells showed almost identical levels of  $\alpha$ -globin RNA, indicating that UL13 was not required for accumulation of  $\alpha$ -globin RNA in infected cells. Insofar as our results demonstrated that ICP22<sup>-</sup> and UL13<sup>-</sup> mutants had different effects on the accumulation of  $\alpha$ -globin RNA, these data implied that not all functions of ICP22 depend on UL13.

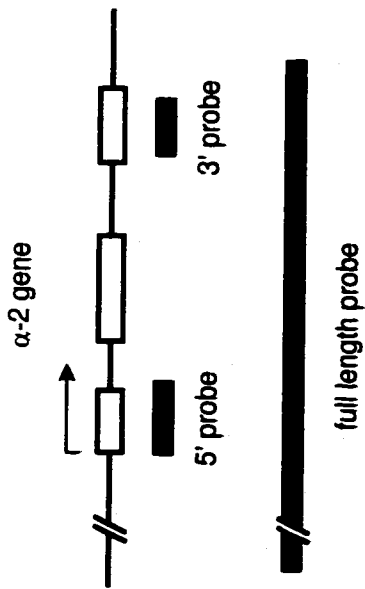
**ICP22 affects accumulation of  $\alpha$ -globin RNA at the post-transcriptional level.** In our original study, the activation of the  $\alpha$ -globin gene was inferred from the HSV-induced accumulation of  $\alpha$ -globin RNA in the infected cells. We now used nuclear run-on analysis to determine the transcription activity of the  $\alpha$ -globin gene before and after infection. Cells were either mock-infected, infected with wild-type HSV strains (KOS or F), or with mutants lacking ICP4 (d120), ICP22 (del22Z), or ICP27 (5dl1.2). Infections were carried out in the presence of PAA to minimize any differences in the progression of the viral lytic cycle among the different mutants. At 6 hours post infection (hpi), nuclei were harvested and used for nuclear run-on assay. The level of on-going transcription was determined by the amount of <sup>32</sup>P-labeled transcripts made during run-on transcription as measured by hybridization to single-stranded DNA probes complementary to the

RNA of interest. In all, six different  $\alpha$ -globin probes (3 sense and 3 antisense) were used. DNA sequences encompassing the entire  $\alpha$ -globin transcription unit, or just the 5' or 3' regions of the transcript (see Fig. 2A) were cloned into M13 vectors in both sense and antisense orientations, and single-stranded phage DNA generated from these recombinants was then used for detection of the  $\alpha$ -globin run-on transcripts. In this experiment the sense probes were designed to hybridize to the  $\alpha$ -globin RNA whereas the antisense probes were used as negative controls. In addition, an HSV TK/M13 SS DNA probe was used to assay for TK transcription as a control for HSV infection, and the M13 SS DNA probe containing the sequences of the 3' end of human  $\gamma$ -actin was used to monitor general cellular transcription. Lastly, M13 SS DNA was also used as a control for non-specific hybridisation of RNA transcripts to the vector M13 sequences. In the mock infected nuclei, run-on transcription was detected for the control  $\gamma$ -actin gene, but not for the  $\alpha$ -globin gene (Fig. 2B), confirming that the  $\alpha$ -globin gene is indeed transcriptionally silent in HeLa cells. In contrast, abundant  $\alpha$ -globin run-on transcripts were detected in the HSV-infected nuclei, indicating that activation of this previously silent gene by HSV is mediated at the transcription level. For all the HSV strains tested, with the exception of d120, we consistently detected more run-on transcription at the 5' end of the  $\alpha$ -globin gene than at the 3' end. Densitometric analysis indicated that there were approximately 4 times more run-on transcripts that hybridized to the 5' probe than the 3' probe even after correction for the differences in the

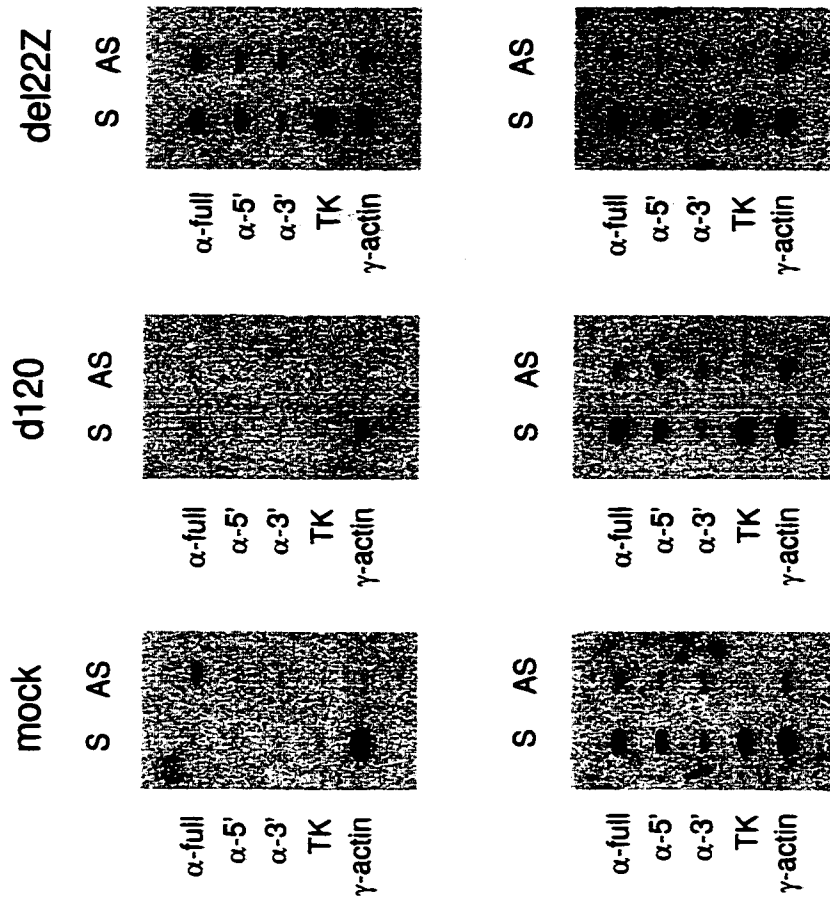


Fig. 2. Nuclear run-on analysis of  $\alpha$ -globin gene in mock-infected or HSV-infected HeLa cells. (A) Schematic diagram showing the  $\alpha$ -globin gene with the exon regions represented by open rectangles and the intron regions represented by the lines between the exons. The filled rectangles indicate regions of the gene used as probes for nuclear run-on analysis. (B) HeLa cells were mock-infected or infected with wild type and mutant HSV strains in the presence of PAA. At 6 hpi, nuclei were harvested and used for nuclear run-on analysis. The  $^{32}\text{P}$ -UTP labeled run-on transcripts were hybridized to filters containing SSDNA probes that detects sense (S) and antisense (AS) RNA corresponding to the regions of the  $\alpha$ -globin gene shown in (A), as wells to the HSV TK gene (TK), and the human  $\gamma$ -actin gene. Hybridized signal was quantified by phosphorimager analysis. The ICP4<sup>-</sup> (d120) mutant, and the ICP27<sup>-</sup> (5dl1.2) mutant were derived from the wild type KOS strain; whereas the ICP22<sup>-</sup> (del22Z) mutant was derived from the wild type F strain.

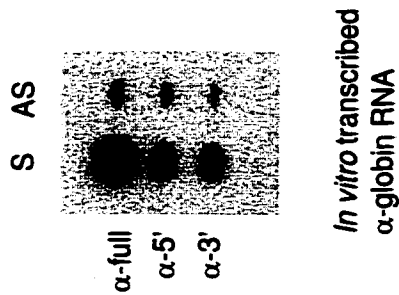
**A**



**B**



**C**



F

5d11.2

KOS

length of complementarity of the probes and the uracil content of the transcripts. This was not due to differences in the annealing efficiency of the two probes since *in vitro* transcribed  $\alpha$ -globin RNA hybridised equally well to both probes (Fig. 2C). Instead, this result suggested that a significant portion of the RNA polymerase engaged at the 5' end of the gene failed to reach the end of the gene. Comparing the  $\alpha$ -globin transcription signal in cells infected with HSV wild type and mutant strains by densitometry, we observed similar levels of transcription for the cells infected with wild-type KOS or the ICP27<sup>-</sup>5dl1.2 strains. This was in agreement with our previous finding that ICP27 did not greatly affect the overall accumulation of  $\alpha$ -globin RNA in the infected cells. Our previous study also showed that deletion of either ICP4 or ICP22 resulted in severe reduction of the accumulation of  $\alpha$ -globin RNA. Densitometric analysis showed that nuclei from d120-infected cells had almost no transcription of the  $\alpha$ -globin gene at 6 hpi (approximately a 10 fold decrease compared to the wild type KOS strain); whereas nuclei from the del22Z infected cells showed near wild type level of transcription of the  $\alpha$ -globin gene (approximately 75% of the F strain level in this particular experiment). Consistent with the known function of ICP4 as a transcription activator, our data indicated that it was required for the transcription of the  $\alpha$ -globin gene. In contrast, deletion of ICP22 did not drastically alter the transcription level of this gene, suggesting that ICP22 may affect the accumulation of  $\alpha$ -globin RNA at a post-transcriptional level. In the multiple trials of this experiment using the ICP22<sup>-</sup> mutant, we consistently observed

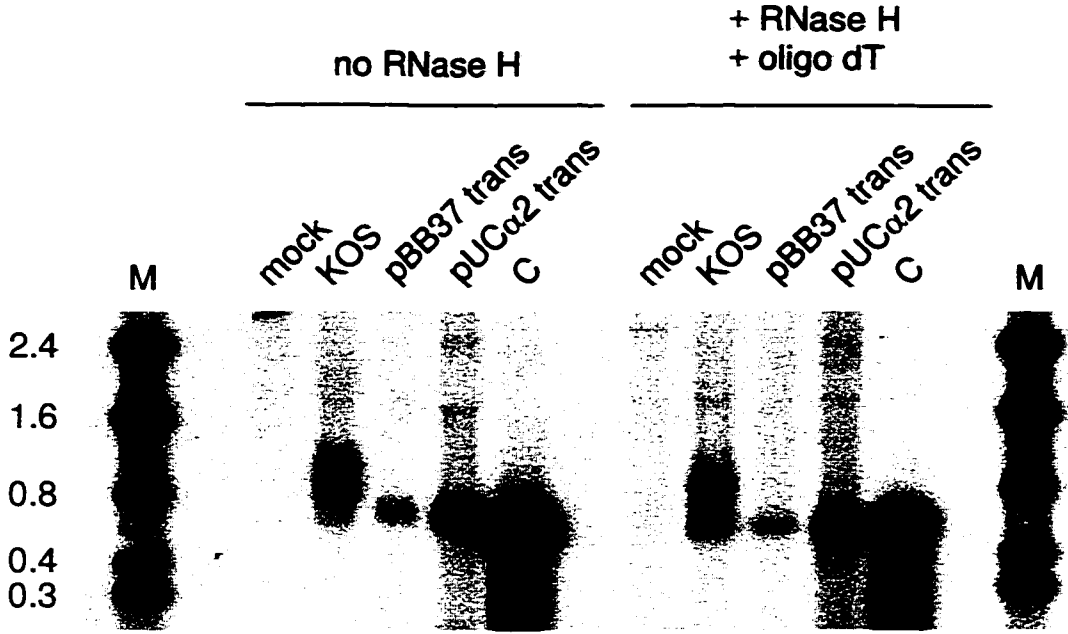
a slightly lower level of transcription activity of the  $\alpha$ -globin gene compared to the wild type infected cells (average of 70%). These results therefore do not exclude the possibility that ICP22 also directly or indirectly affects the transcription rate of this gene. In addition, infection by the mutant lacking ICP22, as compared to other infected samples, resulted in a higher level of anti-sense transcription as detected by the anti-sense probes. This was consistently observed in several trials; however, we do not know how this effect is related to the function of ICP22.

#### **HSV induced accumulation of multiple species of $\alpha$ -globin RNA.**

Previous analysis of the HSV-induced  $\alpha$ -globin RNA showed that the majority of these transcripts were initiated at the  $\alpha$ -globin promoter, and terminated at the globin poly (A) addition site. To determine whether post-transcriptional processing of these transcripts was affected by HSV gene products, we used Northern blot analysis to examine the  $\alpha$ -globin RNA from HSV KOS strain-infected cells. As a comparison, we examined RNA from cells transfected with a plasmid containing the human  $\alpha 2$  gene (pUC $\alpha 2$ ), or transfected with an ICP4 expression clone (pBB37) since our previous study showed that transfected copies of the human  $\alpha 2$  globin gene are readily transcribed in HeLa cells, and transfection of the ICP4 expression clone could induce expression of the endogenous  $\alpha$ -globin gene. Total RNA was harvested from the KOS strain-infected cells at 6 hours post infection, or from the transfected cells 24 hours after removal of the calcium

phosphate precipitate, and these RNA samples were examined by Northern blot analysis. As shown in Figure 3, several species of  $\alpha$ -globin RNA were detected in the HSV infected cells whereas only a single  $\alpha$ -globin species was detected in either of the transfected samples. The majority of the  $\alpha$ -globin RNA in the blood control RNA was also of a single species although it was much over-loaded on this gel and was not as evident at the exposure shown. We note that the  $\alpha$ -globin RNA from different sources did not share the same mobility through the agarose gel, suggesting that they were of different sizes. To analyse this further, we treated the harvested RNA with RNase H in conjunction with oligo dT to remove the poly (A) tails of the transcripts. Upon treatment, the HSV induced  $\alpha$ -globin RNA was resolved into 3 distinct species (Fig. 3, + RNase H), and the smallest  $\alpha$ -globin RNA from the KOS-infected cells now comigrated with the  $\alpha$ -globin RNA from the transfected cells as well as with the positive control blood RNA. This fastest migrating RNA species likely represents spliced globin transcripts since its estimated size corresponds well with the predicted size (576 nt) of the processed  $\alpha$ -globin mRNA. Estimation of the sizes of the two larger  $\alpha$ -globin RNA species showed that they correspond to the predicted sizes of  $\alpha$ -globin transcripts containing one (688 or 718 nt) or two (835 nt) introns, and suggested that they may represent  $\alpha$ -globin transcripts that are partially or not processed. These results not only indicate that the various  $\alpha$ -globin transcripts are polyadenylated, but also show that the

**Fig. 3. Northern blot analysis for  $\alpha$ -globin RNA. Total RNA was harvested from mock-infected, KOS-infected HeLa cells or from HeLa cells transfected with the ICP4 expression clone (pBB37) or with a plasmid containing the  $\alpha 2$  gene (pUC $\alpha 2$ ). RNA from each sample, or from the blood control sample (C) was either mock-treated or treated with RNaseH in the presence of oligo dT and separated on an 1.5% agarose gel. The  $\alpha$ -globin transcripts were detected by hybridizing to a  $^{32}\text{P}$ -labeled  $\alpha$ -globin probe. M represents *in vitro* transcribed RNA size markers.**



apparent variations in the length of  $\alpha$ -globin RNA in infected and transfected cells is due to the differences in their poly (A) tail lengths.

This difference in the poly (A) tail length could be a result of viral induced alteration in the polyadenylation process; however, it is more likely due to the difference in average age post synthesis of the  $\alpha$ -globin transcripts. As noted earlier, the RNA from the infected cells were harvested at 6 hours post infection whereas the transfected cells were harvested at 24 hours post removal of the calcium phosphate precipitate. Examination of the  $\alpha$ -globin transcripts harvested from transfected cells at various time points between 2 to 24 hours post precipitate-removal showed a rapid and progressive shortening of the poly (A) length over the 24 hour period (data not shown). Therefore, the difference between the time of harvest for the infected and transfected cells is the likely cause for the apparent difference in the poly (A) lengths of  $\alpha$ -globin transcripts.

**The HSV mutant lacking ICP27 only accumulated the fastest migrating species of  $\alpha$ -globin RNA.** To determine if the presence of the multiple  $\alpha$ -globin RNA species seen in the HSV-infected cells correlates with expression of specific HSV proteins, we used Northern blot analysis to examine the RNA from cells infected with wild type HSV strains or with mutants lacking specific immediate-early genes. HSV infections were carried out in the presence of PAA and total RNA was harvested at 6 hpi for Northern blot analysis. Multiple  $\alpha$ -globin

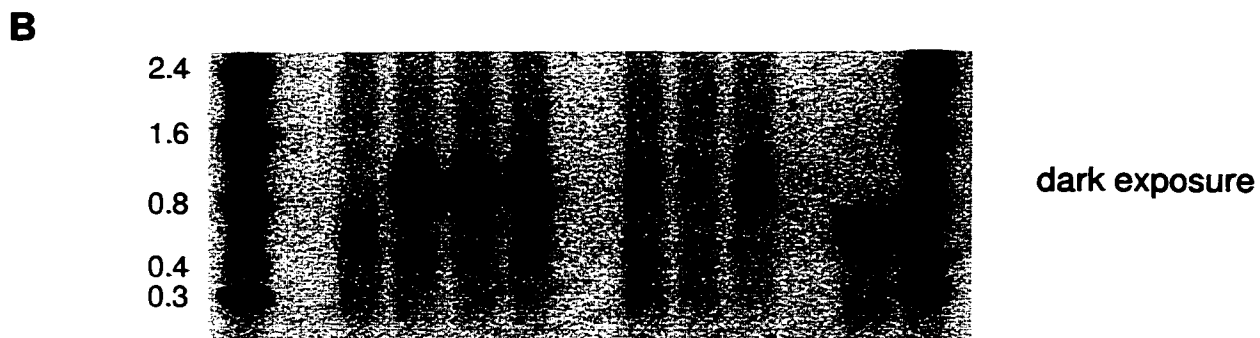
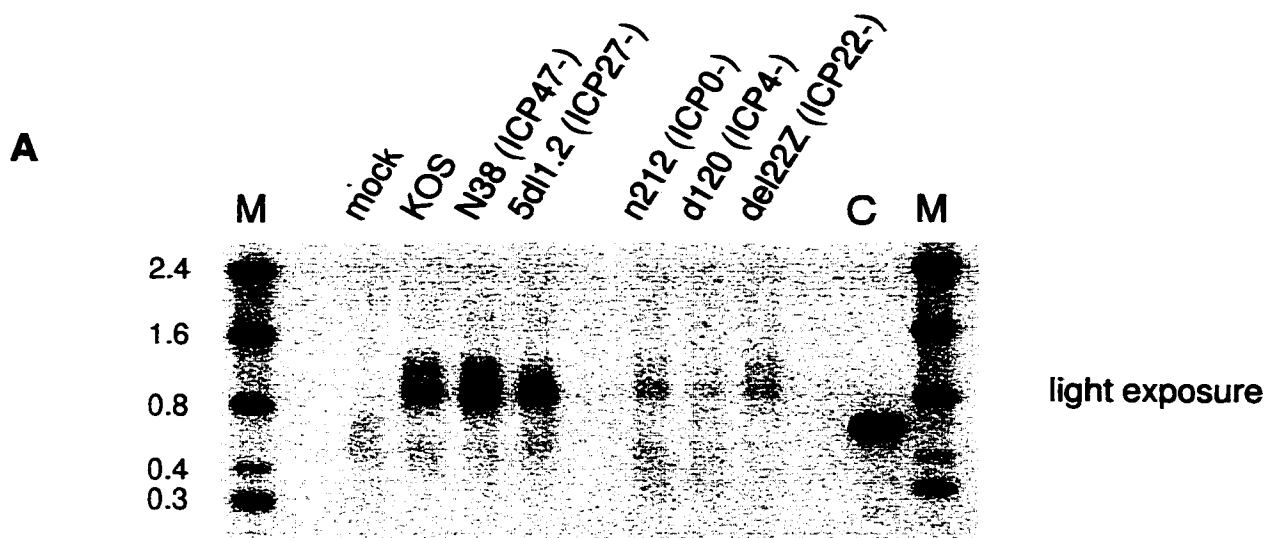


species were observed in cells infected with KOS, F, and the ICP47<sup>-</sup>N38 strains (Fig. 4A). The same RNA species were also seen in cells infected with mutants lacking ICP0, ICP4, and ICP22 even though much less globin RNA was found in these cells (Fig. 4B). In contrast, cells infected with the ICP27<sup>-</sup> 5dl1.2 strain only accumulated the fastest migrating form of globin RNA, suggesting that the presence of the two larger  $\alpha$ -globin RNA species was dependent on the expression of ICP27. This effect was better seen when the  $\alpha$ -globin RNA was treated with RNase H in the presence of oligo dT to remove the poly (A) sequences (Fig. 5B). The smallest species of  $\alpha$ -globin RNA in the KOS infected cells comigrated with the RNA in the 5dl1.2 infected cells as well as the blood control RNA, suggesting that they represent *bona fide* spliced  $\alpha$ -globin mRNA. As noted earlier, the lengths of the two larger RNA species correspond to the predicted sizes of  $\alpha$ -globin transcripts containing one or two introns. To ascertain whether these RNAs contain  $\alpha$ -globin intron sequences, we tested whether they hybridize to intron-specific probes by Northern blot analysis. As shown in Fig. 5A, only the slower migrating species hybridised to the intron-specific probe, whereas the exon-specific probe hybridized to all  $\alpha$ -globin bands. Together, these results confirmed that the two larger  $\alpha$ -globin species are intron-containing pre-mRNAs and that the fastest migrating species is fully processed  $\alpha$ -globin mRNA.

#### **ICP27 did not inhibit accumulation of fully spliced $\alpha$ -globin mRNA.**

Several reports have suggested that ICP27 inhibits the cellular

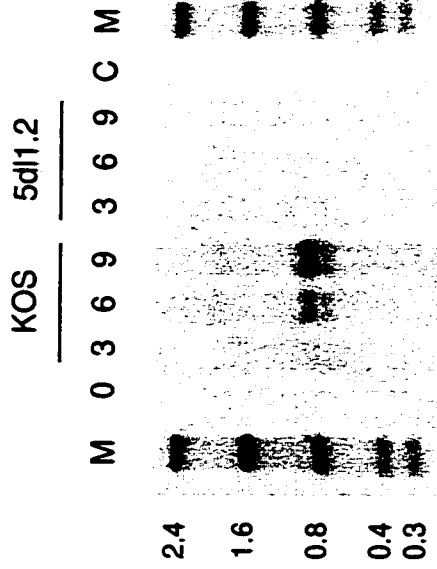
**Fig. 4. Northern blot analysis for  $\alpha$ -globin RNA from HeLa cells infected with various HSV strains. HeLa cells were mock-infected or infected with various strains of HSV in the presence of PAA. At 6 hpi, total RNA was harvested and used in Northern blot analysis to detect the  $\alpha$ -globin transcripts. Lane C represents the blood RNA positive control and lane M represents the RNA marker lane. (A) and (B) represent the same blot at two different exposures.**



**Fig. 5. Time course analysis of the accumulation of spliced and unspliced  $\alpha$ -globin RNA. HeLa cells were mock-infected or infected with the KOS or 5dl1.2 strains of HSV in the presence of PAA. At the indicated times, total RNA was harvested from the infected cells and was treated with RNase H in the presence of oligo dT to remove the poly (A) tails. The treated RNA was then resolved on 1.5% agarose gel, transferred to Nytran Plus filters and hybridised with  $^{32}\text{P}$ -labeled intron-1 specific (A) or exon-1 specific (B) probes. The resolved bands were quantified by phosphorimager analysis, categorized as processed or unprocessed, and presented as graphs in (C) and (D). For both graphs, the relative amounts of  $\alpha$ -globin RNA were normalized to the maximum amount of  $\alpha$ -globin RNA detected.**

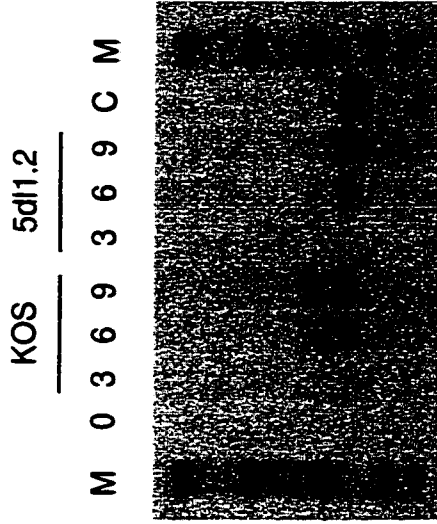
**A**

intron-1 specific probe



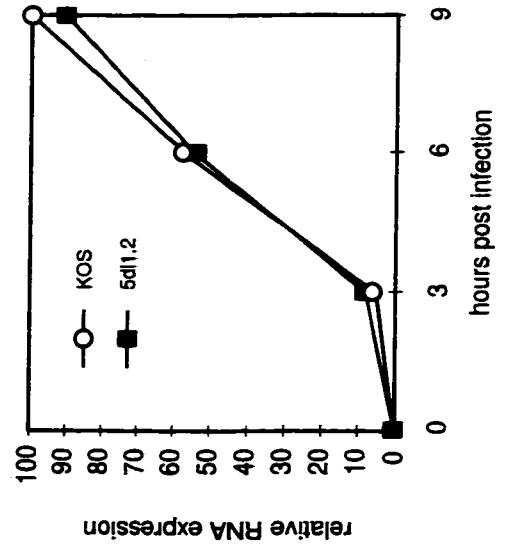
**B**

exon 1 probe



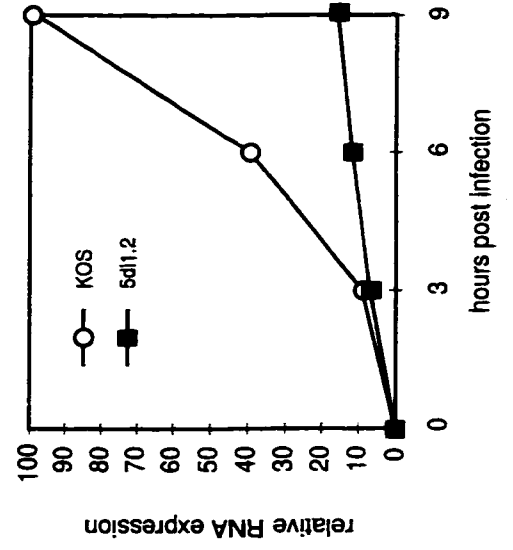
**C**

processed mRNA



**D**

unprocessed RNA



splicing machinery and prevents accumulation of spliced RNA (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994). To further examine the accumulation of spliced and unspliced  $\alpha$ -globin RNA in infected cells over time, total RNA from cells infected with KOS or 5dl1.2 strains was harvested at various time points post infection and analysed by Northern blot analysis. The amounts of spliced and unspliced  $\alpha$ -globin RNA from the different time points were quantified and their relative abundance was plotted on graphs in Fig. 5C and 5D. In this analysis, both intron-containing species were grouped as unprocessed RNA, whereas the  $\alpha$ -globin mRNA was defined as processed RNA. Consistent with our earlier observation, we found that there was a seven-fold difference in the accumulation of unspliced  $\alpha$ -globin RNA between the cells infected with the two HSV strains (Fig. 5D). However, the accumulation of fully spliced  $\alpha$ -globin RNA in the two infected cell populations was almost identical (Fig. 5C). Moreover, the steady increase in the amount of  $\alpha$ -globin transcripts over time suggested that transcription of this cellular gene was not inhibited over the course of infection. Indeed, our nuclear run-on results showed that the  $\alpha$ -globin gene in cells infected by wild type HSV or the ICP27<sup>-</sup> mutant displayed the same rate of transcription. Therefore, even though other studies have shown that ICP27 can inhibit cellular splicing and suggested that ICP27 functions to prevent expression of intron-containing cellular genes, our data did not support this hypothesis and suggested that ICP27's inhibitory effect may not be universal for all cellular genes.

**The continual accumulation of spliced and unspliced  $\alpha$ -globin RNA was seen by S1 nuclease protection assay.** The preceding data suggested that ICP27 was required for the accumulation of unspliced  $\alpha$ -globin RNA. As an independent test of this interpretation, we examined the  $\alpha$ -globin transcripts by S1 nuclease protection assay. In this assay, a single-stranded DNA probe corresponding to the exon 1 / intron 1 junction was hybridised to total RNA from infected cells (see Fig. 6A). Upon S1 nuclease treatment, intron-containing transcripts would generate a 177 nt protected fragment whereas fully spliced globin mRNA would result in a 98 nt protected fragment. The results shown in Fig. 6B confirmed that 5dl1.2-infected cells accumulated the same levels of fully spliced  $\alpha$ -globin mRNA as cells infected with wild type HSV. However, unlike the Northern blot analysis, this method detected a significant amount of intron-containing RNA in both infected cell populations. Densitometric analysis showed that there was a three-fold difference in the amount of intron-containing RNA in the KOS-infected cells compared to the 5dl1.2-infected cells. Although both RNA detection methods showed that equal amounts of spliced  $\alpha$ -globin mRNA were present in the KOS- and 5dl1.2- infected cells, they seemed to detect different amounts of unspliced  $\alpha$ -globin RNA present in the 5dl1.2-infected cells.

**Truncated unspliced  $\alpha$ -globin transcripts were present in both 5dl1.2 and KOS infected cells.** To explain the apparent discrepancy seen in the preceding sections, we note the nuclear run-on results showed that

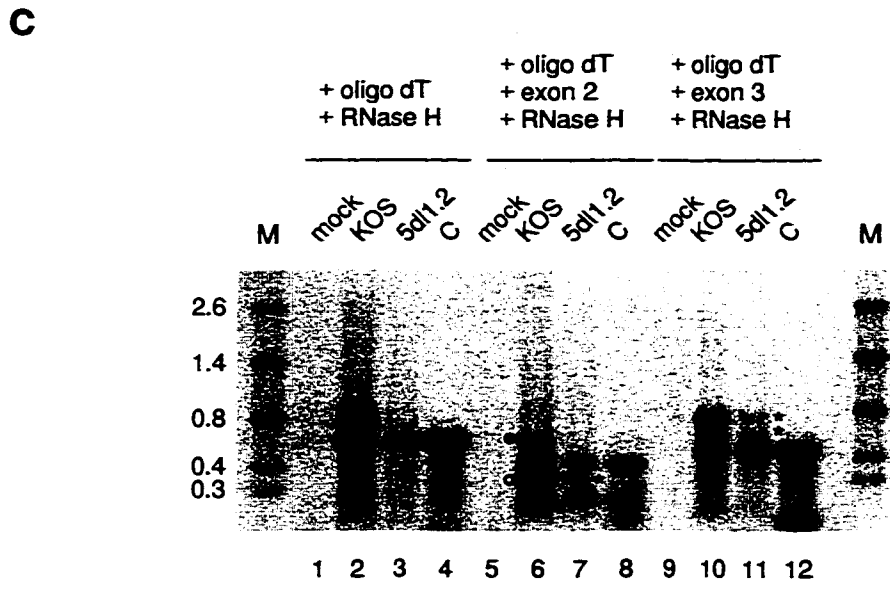
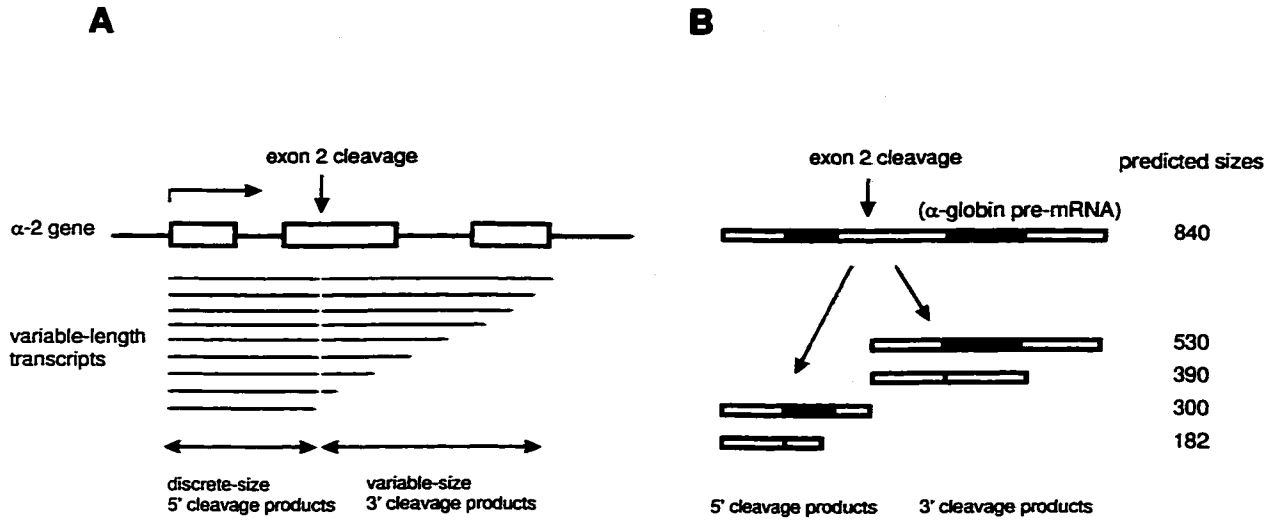
**Fig. 6. S1 nuclease protection assay for spliced and unspliced  $\alpha$ -globin RNA. (A) Schematic diagram showing exon 1, intron 1 and exon 2 regions of the  $\alpha$ -globin pre-mRNA. The complementary  $^{32}\text{P}$ -labeled SSDNA and the RNA protected fragments are shown as black lines. The predicted sizes for the protected fragments are also indicated on the right side. (B) HeLa cells were mock-infected or infected with the KOS or 5dl1.2 HSV strains in the presence of PAA. At the indicated times, total RNA was harvested from the infected cells and hybridised to the 3' labeled SSDNA probe. After S1 nuclease treatment, the samples were separated on an 8% sequencing gel and exposed to film for autoradiography.**





there were approximately 4-fold more engaged RNA polymerases present at the 5' end compared to the 3' end of the  $\alpha$ -globin gene in HSV-infected HeLa cells. This observation implies that the majority of engaged RNA polymerases fail to reach the 3' end of this gene. If this is true, then the transcribing polymerase may disengage anywhere along the  $\alpha$ -globin gene and could generate a series of truncated transcripts that are 5' co-terminal but have variable 3' ends (see Fig. 7A for illustration). These truncated transcripts would not be detected by Northern blot analysis since they would be spread out as a smear upon agarose gel electrophoresis, and the signal intensities of the labelled probes hybridising to these transcripts would be below the detection limit of this assay. In contrast, these variable-length transcripts could still be detected by S1 nuclease protection assay since the 3' labelled SSDNA will anneal to all transcripts containing RNA sequences complementary to the probe and generate protected fragments of discrete lengths upon S1 nuclease treatment. To test this hypothesis, we cleaved the  $\alpha$ -globin RNA from infected cells at an internal site and examined the molar ratios of the resulting 5' and 3' cleavage products. Cleavage of variable-length transcripts would generate discrete-sized 5' cleavage products that could then be detected by Northern blot analysis, but the 3' cleavage products would still vary in length and would not be detected (Fig. 7A). HeLa cells were mock-infected, or infected with KOS or 5dl1.2 HSV strains in the presence of PAA. At 6 h post infection, total RNA was harvested and 10  $\mu$ g samples were incubated with RNase H in the presence of oligo dT (to

**FIG. 7. Northern blot analysis for  $\alpha$ -globin RNA after RNase H cleavage at exon 2 or 3. (A) Schematic diagram showing the  $\alpha$ -globin gene with the exon regions indicated by open rectangles. Variable-length transcripts are illustrated as nested-lines and the predicted consequence after exon 2 cleavage is shown. (B) Schematic diagram showing the  $\alpha$ -globin pre-mRNA. The diagram also shows the four cleavage products from unspliced and spliced  $\alpha$ -globin mRNA after exon 2 cleavage. (C) Total RNA harvested from mock-infected, KOS or 5d1.2 infected cells were treated with RNase H in the presence of oligo dT and oligos complementary to exon2 and/or exon 3. The samples were then separated on an 1.5% agarose gel for Northern blot analysis. RNA bands identified by \* represent the 5' end products resulting from cleavage of the variable-length transcripts. The bands identified by the open and closed circles represent the 5' and 3' cleavage products of the intron-containing  $\alpha$ -globin RNA after cleavage at exon 2.**



remove any poly (A) sequences) and oligonucleotides complementary to exon 2 or exon 3 sequences. The cleavage products were analysed by Northern blot using a  $^{32}\text{P}$ -labeled probe that spans the entire  $\alpha$ -globin gene. Cleavage of full-length intron-containing  $\alpha$ -globin RNA at exon 2 should generate 300 and 530 nt products (schematically illustrated in Fig. 7B) and cleavage of full-length spliced  $\alpha$ -globin mRNA should generate 182 and 390 nt products. As shown in Fig. 7C, these 4 cleavage products were seen in internally cleaved KOS-infected cell RNA sample (lane 6), confirming that these cells contained full length unspliced and spliced globin RNA. Densitometric analysis of the 182 and 390 nt products showed that they were of equal abundance, suggesting that the majority of the spliced  $\alpha$ -globin mRNA in KOS-infected cells were full length transcripts. In contrast, there were approximately 3 fold more of the 300 nt 5' cleavage product compared to the 530 nt 3' product (comparing the two bands marked by open and closed circles in Fig. 7C, lane 6). Therefore, in addition to the full length unspliced  $\alpha$ -globin RNA, a greater portion of the intron-containing transcripts were of variable length. Similar treatment of RNA harvested from 5dl1.2-infected cells (Fig. 7C, lane 7) showed that there were also approximately equal amounts of the 182 and 390 nt bands. In addition, a 300 nt band (marked by \*) was clearly evident in this sample, indicating that unspliced  $\alpha$ -globin transcripts were indeed present in cells infected with the ICP27-defective mutant. Moreover, since the corresponding 530 nt 3' cleavage product was not detected in this sample, this suggested that only truncated intron-

containing RNA, but not full length unspliced  $\alpha$ -globin RNA, was present in 5dl1.2-infected cells. Entirely analogous results were seen in the infected-cell samples treated with RNase H in the presence of oligo dT and oligonucleotides complementary to exon 3 (Fig. 7C, lanes 9 to 12). In addition to the full length spliced  $\alpha$ -globin RNA, a small amount of cleavage products generated from unspliced  $\alpha$ -globin transcripts was found in the 5dl1.2-infected samples (those marked by \* in Fig. 7C, lane 11). The fact that these unspliced RNAs are only detected after internal cleavage supports our hypothesis that they are of variable length, and these results demonstrate that full length intron-containing transcripts are not present in the absence of ICP27. Although we can detect their presence, at present we do not know whether these partial transcripts seen in KOS and 5dl1.2-infected cells are generated by aborted transcription or from degradation of pre-existing  $\alpha$ -globin RNA.

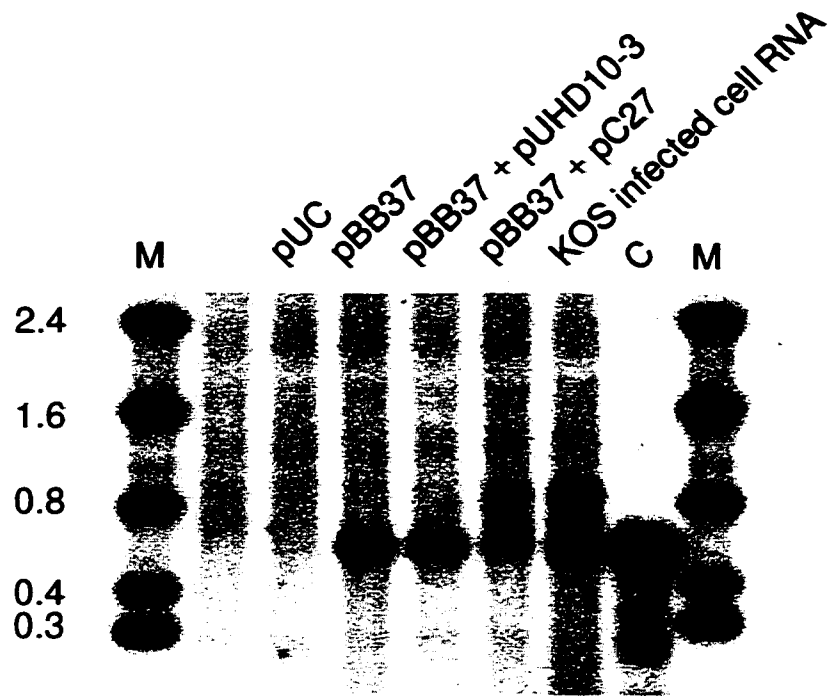
**Expression of ICP27 induced accumulation of unspliced  $\alpha$ -globin RNA.** The data presented so far showed a strong correlation between the accumulation of unspliced pre-mRNA with the presence of ICP27 during infection. To determine if ICP27 is directly responsible for this effect, we used a transient transfection approach to determine if ICP27 is sufficient to alter the splicing of  $\alpha$ -globin RNA. As demonstrated in the previous study (Cheung, et al., 1997), transfection of an ICP4 expression clone into HeLa cells induced expression of the endogenous

$\alpha$ -globin gene. We cotransfected the ICP4 expression clone with either the control vector plasmid (pUHD10-3) or with the expression clone for ICP27 (pC27) and examined the  $\alpha$ -globin transcripts by Northern blot analysis. As shown in Fig. 8, expression of ICP4 alone induced expression of the otherwise silent endogenous  $\alpha$ -globin gene.

Cotransfection of the ICP4 clone with pUHD10-3 vector did not alter the quality nor the quantity of the  $\alpha$ -globin RNA, whereas co-transfection of the ICP4 clone with pC27 induced accumulation of the same unspliced  $\alpha$ -globin species as seen in KOS infected cells. Thus, ICP27 is sufficient (in combination with ICP4) to induce accumulation of unspliced globin RNA in the absence of other HSV gene products. We observed a 40% reduction in the total  $\alpha$ -globin signal (spliced plus unspliced RNA) in the presence of ICP27, an effect which was not observed during infection with wild type versus ICP27-deficient HSV. Whether this reduction stems from previously described intron-dependent "repression" function of ICP27 remains to be determined.

**Fig. 8. Northern blot analysis for  $\alpha$ -globin RNA in transfected or KOS-infected HeLa cells. HeLa cells were transfected with the ICP4 expression clone (PBB37) in conjunction with pUC, the control vector (pUHD10-3) or the ICP27 expression clone (pC27) and total RNA was harvested at 36 hours post transcription. Total RNA from the transfected samples as well as from the KOS-infected cell sample was harvested and treated with RNase H and oligo dT. The RNA samples were then separated on an 1.5% agarose gel for Northern blot analysis for  $\alpha$ -globin RNA.**





## Discussion

The data presented in this paper show that expression of the endogenous  $\alpha$ -globin gene in HSV-infected cells is regulated by several viral proteins which affect the transcription initiation of this gene, as well as the processing and stability of its RNA. Nuclear run-on analysis showed that  $\alpha$ -globin gene is transcriptionally silent in non-erythroid HeLa cells, but can be activated upon HSV-1 infection. Efficient transcription of this gene requires the presence of ICP4 as indicated by our finding that cells infected with a mutant lacking the ICP4 gene have a ca. 10 fold lower transcription activity compared to those infected with its wild type parental strain. This observation is consistent with the known transcriptional activation properties of ICP4 and shows that the reduced accumulation of  $\alpha$ -globin RNA in cells infected with an ICP4 defective mutant stem at least in part from reduced levels of transcription.

ICP22 is required for maximal accumulation of  $\alpha$ -globin RNA in HSV-infected cells (Cheung, et al., 1997). However, the finding that cells infected with this mutant or with its parental strain both show similar levels of run-on transcription at the  $\alpha$ -globin locus (Fig. 2B) suggests that, in contrast to ICP4, the effect of ICP22 is mediated at the post-transcriptional level. This conclusion is reminiscent of recent observations by Carter and Roizman. These authors found that ICP0 intron 1 RNA, generated from the splicing of ICP0 pre-mRNA, is

surprisingly stable and accumulates in HSV-infected rabbit skin and Vero cells (Carter and Roizman, 1996). When these cells were infected with an ICP22 defective mutant, very low levels of ICP0 mRNA were found even though the ICP0 intron 1 RNA accumulated to the same level as seen in wild-type HSV-infected cells. Insofar as processing of ICP0 pre-mRNA should generate equal amounts of intron 1 RNA and mRNA products, the molar ratio imbalance between these two RNA populations in cells infected with the ICP22 defective mutant implied that transcription of the ICP0 gene was not affected by this mutation, but the stability and accumulation of ICP0 mRNA were dependent on ICP22. Similar to this situation, ICP22 may regulate the stability and accumulation of  $\alpha$ -globin RNA in HSV-infected cells. Our nuclear run-on data show that the transcription rate of the  $\alpha$ -globin gene in cells infected with an ICP22 defective mutant is consistently slightly lower (average about 70%) than that seen in parental strain-infected cells. Therefore, ICP22 may also indirectly affect transcription of this gene, perhaps by altering the expression of ICP4 and ICP0 during HSV infection as described in our previous study.

The precise function of ICP22 during HSV infection is currently not known. HSV-infected cells contain multiple forms of this molecule that are phosphorylated to different extents, and Purves and Roizman have demonstrated that phosphorylation of ICP22 was mediated by another viral protein, U<sub>L</sub>13 (Purves and Roizman, 1992). This viral kinase may regulate the function of ICP22 as demonstrated by studies

that showed that mutants lacking either of these proteins have almost identical cell type-specific defects in ICP0 and viral late gene expression (Purves, et al., 1993). Our studies demonstrate that, in contrast to the requirement of ICP22,  $\alpha$ -globin RNA accumulates to wild-type levels even in the absence of U<sub>L</sub>13. Therefore, this implies that not all functions of ICP22 depend on its post-translational modification by U<sub>L</sub>13. Insofar as ICP22 is an immediate-early gene expressed early during HSV infection whereas U<sub>L</sub>13 is a late gene, ICP22 may have two separate functions prior to and after the synthesis of U<sub>L</sub>13. Indeed, Purves *et al.* have demonstrated that phosphorylation of ICP22 by U<sub>L</sub>13 does not occur till late times post infection (Purves, et al., 1993). The effects of ICP22 on the accumulation of  $\alpha$ -globin RNA may reflect a function mediated by the unphosphorylated form of ICP22. Consistent with this hypothesis, Prod'hon *et al.* showed that transient expression of ICP22 down regulated expression of the co-transfected CAT reporter gene driven by an ICP4 promoter, and this activity was ablated by the additional expression of U<sub>L</sub>13 (Prod'hon, et al., 1996). Therefore, ICP22 may have a separate regulatory function in the absence of U<sub>L</sub>13.

In addition to the effects of ICP22 on the stability of ICP0 mRNA, it has been suggested to affect gene expression at the transcription level. Nuclear run-on analysis showed that the defect in viral late gene expression in cells infected with ICP22-negative mutants was mediated at the transcription level (Rice, et al., 1995). Furthermore,

Rice *et al.* demonstrated that ICP22 was required during HSV infection for the alteration in phosphorylation of the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II (RNAP II) (Rice, et al., 1995). Phosphorylation of the RNAP II CTD is normally associated with the transcription elongation process and at present it is not known how the HSV-induced alteration of the polymerase phosphorylation status would affect gene expression in general. Using the  $\alpha$ -globin gene as a reporter, we found that ICP22 does not have any effect on the transcription elongation of this gene during HSV infection. However, to our surprise, there appears to be an inherent polarity in the transcription of this gene even in wild type HSV-infected cells. We consistently find approximately 4-fold more engaged polymerases at the 5' end than at the 3' end of this gene in cells infected with the HSV strains tested with the exception of the mutant lacking ICP4 which only shows barely detectable levels of transcription of this gene. This difference in polymerase density is not due to a difference in the hybridization efficiency of the run-on transcripts to the 5' and 3' end probes, but instead, suggests that a significant amount of the initiated polymerase complexes are terminated before they reach the end of the gene. This gradient may be a common feature of the transcription of  $\alpha$ -globin gene in a non-erythroid setting, or alternatively, it may be induced by HSV infection, perhaps due to a competition for elongation factors between cellular and viral genes. Nuclear run-on analysis of the transcription of  $\alpha$ -globin genes introduced into HeLa cells either by transfection or

through infection with a recombinant HSV containing a copy of this gene will help to distinguish between these possibilities.

In this report, we also examined the effects of ICP27 on the expression of the  $\alpha$ -globin gene. In particular, we tested whether ICP27 inhibits splicing of the  $\alpha$ -globin gene and whether it represses expression of this cellular gene. Early studies have shown that host protein synthesis is rapidly shut off upon HSV infection (reviewed in Fenwick, 1984). This viral-induced inhibition of host gene expression occurs in two distinct stages, early and delayed, and results in the disaggregation of cytoplasmic polyribosomes, accelerated turnover of mRNA, and transcriptional shutoff of cellular genes. The early host shutoff is perhaps the better understood part of this process: genetic evidence has shown that it is mediated by the tegument protein vhs which induces rapid turnover of cellular and viral mRNAs (Nishioka and Silverstein, 1978; Read and Frenkel, 1983; Kwong, et al., 1988). In contrast, little is known about the mechanisms or viral proteins responsible for the delayed host shutoff activity. Genetic studies showed that HSV mutants lacking ICP27 are defective in the delayed shutoff activity (Sacks, et al., 1985), suggesting that this viral protein may be directly or indirectly involved in the repression of host gene expression. Functional studies of ICP27 suggested that it inhibits cellular splicing and represses expression of intron-containing genes. As a result, researchers have hypothesized that ICP27 may shut off host gene expression by inhibiting expression of intron-containing

genes (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994). Although this hypothesis is widely accepted, the direct effects of ICP27 on the accumulation of cellular transcripts during infection have not been examined. This is in part due to complications arising from pre-existing cellular transcripts and the vhs-induced mRNA turnover activity. Therefore, in this study, we took advantage of the fact that the endogenous  $\alpha$ -globin gene is expressed only after HSV-infection and used it to examine the effects of ICP27 on the expression of this intron-containing gene. A comparison of the  $\alpha$ -globin RNA present in wild type HSV and ICP27 defective mutant-infected cells shows a striking difference between the two RNA samples in that accumulation of full length unspliced transcripts occurs only in the presence of ICP27. This implies that ICP27, either directly or indirectly, alters the splicing machinery to induce accumulation of unspliced RNA during HSV infection. Although the presence of unspliced  $\alpha$ -globin transcripts suggests that the splicing machinery may be inhibited by the presence of ICP27, the data from both Northern blot and S1 nuclease protection assays show that fully spliced  $\alpha$ -globin mRNA continues to accumulate in an identical fashion in cells infected with wild type or ICP27 defective HSV strains. Therefore, our data indicate that ICP27-induced accumulation of unspliced transcripts does not interfere with the normal output of the splicing machinery. If this conclusion is correct, then the implication is that ICP27 rescues a population of unspliced transcripts which would otherwise be degraded.

Our conclusion that ICP27 does not inhibit accumulation of spliced globin mRNA has one caveat: the  $\alpha$ -globin gene is activated by viral transactivators ICP0 and ICP4, and therefore, the interaction of this gene with these viral regulators may preclude inhibition by ICP27. However, it is relevant to point out that the co-transfection studies which first demonstrated the repression of intron-containing reporter genes by ICP27 were in fact done in the presence of ICP4 and ICP0 transactivators (Sandri-Goldin and Mendoza, 1992). Early transfection studies found that ICP27 alone had little effect on the expression of reporter genes driven by HSV promoters; however, when ICP0 and ICP4 expression clones were added to activate expression of reporter genes, the additional expression of ICP27 could then enhance or repress expression of the reporter genes (Everett, 1986; Rice and Knipe, 1988; Sekulovich, et al., 1988; McMahan and Schaffer, 1990). Using this transfection scheme, Sandri-Goldin and Mendoza demonstrated that ICP27, in general, activated expression of reporter genes with weak 3' poly (A) addition sites, but repressed expression of reporter genes that contained intron sequences (Sandri-Goldin and Mendoza, 1992). These data therefore indicate that ICP27 can function synergistically with ICP0 and ICP4 to affect gene expression.

An examination of the current literature on ICP27 shows that many functions have been assigned to this viral regulator. For example, ICP27 is clearly required for viral DNA replication and late



gene expression during HSV infection (McCarthy, et al., 1989), and it has also been shown to influence mRNA 3' end processing (McGregor, et al., 1996), inhibit cellular splicing (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994), and alter the distribution of splicing factors in the nuclei of infected cells (Phelan, et al., 1993). In spite of the wealth of data regarding the various functions of ICP27, it is at times difficult to ascertain which effects are directly or indirectly attributable to this viral regulator protein. The effects of ICP27 on host cell splicing has been well addressed by a series of studies from Sandri-Goldin's laboratory. As mentioned earlier, Sandri-Goldin and Mendoza were the first to demonstrate the repressor function of ICP27 was directed at co-transfected intron-containing genes (Sandri-Goldin and Mendoza, 1992). In a follow-up study, Hardwicke and Sandri-Goldin found that the steady-state levels of  $\beta$ -tubulin, actin, and GADPH transcripts declined much more rapidly in cells infected with wild type HSV compared to those infected with an ICP27 defective mutant (Hardwicke and Sandri-Goldin, 1994). From this observation, the authors concluded that the rapid decline of these cellular transcripts was due to the presence of ICP27 in wild type HSV-infected cells which prevented accumulation of intron-containing transcripts. In another study, Hardy and Sandri-Goldin demonstrated that nuclear extracts harvested from wild type HSV-infected cells did not support splicing of reporter transcripts whereas nuclear extracts from cells infected with the ICP27 defective mutant were capable of supporting such *in vitro* splicing reactions (Hardy and Sandri-Goldin,

1994). The authors thus concluded that ICP27 inhibited the splicing capacity of the infected nuclear extracts. Although these studies provided strong correlative evidence that ICP27 was involved in the inhibition of splicing, possibly resulting in the shut off of cellular gene expression, demonstration of a direct involvement of ICP27 in the observed phenotypes has, in some cases, been lacking. For example, it has not been tested whether addition of purified ICP27 to nuclear extracts was sufficient to inhibit *in vitro* splicing reactions.

The use of ICP27 defective mutants is a convenient way to assay ICP27's function during infection; however, it should be noted that ICP27 mutants in general have additional defects in viral DNA replication as well as in late gene expression. These secondary differences between the wild type and mutant HSV strains may contribute to apparent effects of ICP27 on splicing. Although our examination of ICP27's effect on the accumulation of  $\alpha$ -globin transcripts also relies on a comparison between wild type and ICP27 defective mutant strains, we have attempted to minimize the secondary differences between the two HSV strains by adding PAA to block viral DNA replication and prevent late gene expression in HSV-infected cells. The use of PAA is particularly significant since we have previously shown that in the absence of PAA, the steady-state level of  $\alpha$ -globin RNA in HSV-infected cells peaked at approximately 6 hours post infection, but rapidly declined from 6 to 12 hours post infection (Cheung, et al., 1997). Given the extraordinary stability of  $\alpha$ -

globin transcripts in general, this finding suggests that a viral DNA replication-dependent activity can induce rapid turnover of the accumulated transcripts. Such an activity may also lead to the decline in the steady-state levels of  $\beta$ -tubulin, actin, and GAPDH transcripts in wild type HSV-infected cells seen by Hardwicke and Sandri-Goldin (Hardwicke and Sandri-Goldin, 1994), and complicates their original interpretation that the decline in the accumulation of these cellular transcripts is due to the inhibition of cellular gene expression by ICP27.

In this study, we performed transfection assays to examine the direct effects of ICP27 on expression of the endogenous  $\alpha$ -globin gene. We utilised the endogenous  $\alpha$ -globin gene as a reporter by inducing its expression in HeLa cells with the transfected ICP4 expression clone and found that the added expression of ICP27 reduced the overall amount of  $\alpha$ -globin transcripts by 40%. In addition, ICP27 induced accumulation of partially spliced and unspliced  $\alpha$ -globin transcripts identical to those seen in the KOS-infected cells. These observations strongly suggest that ICP27 is, at least in part, responsible for the accumulation of unspliced  $\alpha$ -globin RNA during wild type HSV infection. Since ICP4 was used to activate expression of the endogenous  $\alpha$ -globin gene, our data do not exclude the possibility that ICP4 is also involved in this process. This possibility is supported by the fact that ICP4 interacts both physically and functionally with ICP27 (Panagiotidis, et al., 1997; Sekulovich, et al., 1988). However, we

note that cells infected with the ICP4<sup>-</sup> mutant also accumulates unspliced  $\alpha$ -globin transcripts (Fig. 4); therefore, ICP4 is not required for this effect during HSV-infection.

Hardwicke and Sandri-Goldin also used a variety of transient transfection assays to study the effects of ICP27 on the expression of intron-containing reporter genes (Hardwicke and Sandri-Goldin, 1994). In contrast to our finding that the presence of ICP27 mildly inhibited expression of the reporter  $\alpha$ -globin gene, these authors found that ICP27 greatly inhibited expression of co-transfected intron-containing genes. In one experiment, cotransfection of an ICP27 expression clone with the pSV2-CAT intron-containing reporter construct reduced the accumulation of CAT RNA by 6 fold. Furthermore, the authors did not detect any unspliced CAT transcripts in the ICP27-expressing cells. The apparent discrepancies between these results and ours could be due to several differences in our transfection assays. First, these studies used different ICP27 expression plasmids: the pC27 clone we used was designed to be co-transfected with a tet-operator expression plasmid to enhance expression of ICP27 whereas Hardwicke's expression clone was driven by the CMV promoter. Although the pC27 plasmid readily expresses ICP27 in the absence of induction by tetracycline (Stephen Rice, personal communication), it is not clear how the expression level of this plasmid compares to the expression level of the clone used by Hardwicke and Sandri-Goldin. Second, we have chosen an

endogenous cellular gene as a reporter compared to the transfected CAT reporter used by Hardwicke and Sandri-Goldin. The differences in copy number and surrounding chromatin context between these reporter genes may result in differences in the transcription and processing of their RNA. Third, the CAT reporter plasmid used by Hardwicke and Sandri-Goldin contained added heterologous intron sequences which may respond differently to the effects of ICP27 compared to reporter genes that contained natural intron sequences. In a different series of experiments, Hardwicke and Sandri-Goldin used another intron-containing reporter plasmid, pTK-CAT-5'S-SVLPA CAT, and transfected it in conjunction with ICP4 and ICP0 expression plasmids into cells in the presence or absence of the ICP27 expression clone. They found that the presence of ICP27 reduced the amount of spliced ICP0 mRNA in transfected cells and concomitantly enhanced accumulation of unspliced ICP0 pre-mRNA. However, examination of the CAT transcripts in the transfected cells showed that accumulation of the spliced CAT RNA was reduced, but unspliced CAT transcripts were not detected in these transfected cells. Even though the CAT reporter and the ICP0 genes were present together in the same cells, these two intron-containing genes responded differently to the effects of ICP27. Therefore, the use of CAT genes with heterologous intron sequences as a reporter for ICP27's effects may lead to different results compared to reporter genes containing natural intron sequences.

Although the co-transfection assay results obtained from Sandri-Goldin's laboratory suggested that ICP27 strongly inhibits accumulation of spliced transcripts, expression of intron-containing genes does occur in HSV-infected cells. Hardy and Sandri-Goldin compared the accumulation of ICP0 RNA in wild type HSV- and ICP27-defective mutant-infected cells and found that the presence of ICP27 correlated with the accumulation of unspliced ICP0 RNA (Hardy and Sandri-Goldin, 1994). Nevertheless, spliced ICP0 mRNA continued to accumulate over the course of wild type HSV infection, suggesting that splicing and expression of this intron-containing viral gene was not affected by presence of ICP27. This accumulation profile is very similar to that seen for the  $\alpha$ -globin transcripts in PAA-treated, wild type HSV-infected cells. Therefore, these results suggest that while ICP27 displays an inhibitory effect on splicing in co-transfection assays, this effect may be significantly tempered during infection.

In this study, we came across two unexpected results: the apparent polarity in the transcription of the  $\alpha$ -globin gene in HSV infected cells, and the presence of nested-unspliced transcripts that were of variable lengths in both KOS and 5dl1.2-infected cells. It is possible that these two observations are linked since truncated transcription could produce 5' co-terminal truncated transcripts. Our data did not address whether the population of variable-length transcripts arose from incomplete transcription, partial degradation of the unspliced RNA, or perhaps a combination of both since

incomplete transcripts would likely be unstable. Although ICP27 was not involved in the generation of these variable-length transcripts, it may affect their stability or accumulation. One interpretation of our results suggests that ICP27 may not affect accumulation of fully spliced  $\alpha$ -globin RNA in HSV-infected cells, but may enhance accumulation of full-length unspliced RNA in HSV-infected cells. Given that the  $\alpha$ -globin gene in KOS and 5dl1.2 infected cells had similar transcription activity as shown by the nuclear run-on assay (Fig. 2), and that roughly equivalent amounts of processed mRNA was found in both infected cell samples as shown in the Northern blot and S1 nuclease protection assays (Fig. 5 and 6), the additional accumulation of unspliced RNA could be due to stabilisation of a population of unprocessed transcripts that are otherwise degraded. Intron-containing transcripts are normally recognised by splicing factors and assembled into spliceosomes. The protein-bound pre-mRNA is either productively spliced into mRNA, or is degraded by as yet not well understood discard pathways (Burgess and Guthrie, 1993). ICP27 may dissociate the newly synthesized RNA from the splicing and degradation pathways and lead to stable accumulation of unspliced RNA. A similar function was reported for the Rev protein of human immunodeficiency virus (HIV). HIV uses both spliced and unspliced transcripts to express different viral proteins and Rev induces accumulation of intron-containing transcripts for expression of late viral proteins. Rev functions not only by increasing export of unspliced RNA from the nucleus to the cytoplasm, but also by

increasing the stability of unspliced RNA in the nucleus (Emerman, et al., 1989; Febler, et al., 1989; Malim, et al., 1989; Schwartz, et al., 1992; Malim and Cullen, 1993).

It is possible that HSV, like HIV, utilizes unspliced viral transcripts for synthesis of viral gene products as well. Although only four HSV genes are commonly thought to bear introns, proteins translated from unspliced viral transcripts have been identified. For example, HSV glycoprotein C is a late gene product translated from an intron-containing transcript (Frink, et al., 1983). Also, truncated forms of ICP0 that were translated from ICP0 transcripts containing intron 2 sequences have been found (Everett, et al., 1993). Studies by Phelan *et al.* (Phelan, et al., 1996) and Hardy and Sandri-Goldin (Hardy and Sandri-Goldin, 1994) have shown that unspliced ICP0 and UL15 transcripts were present in both the nucleus and cytoplasm of HSV-infected cells, indicating that unspliced transcripts were present in the cytoplasm and accessible by the translation machinery. Preliminary data from our laboratory also showed that unspliced  $\alpha$ -globin RNA was found in both nuclear and cytoplasmic fractions of infected HeLa cells. This suggested that the unspliced  $\alpha$ -globin RNA was not preferentially retained in the nucleus of the infected cells, and argued against the hypothesis that ICP27 repressed expression of intron-containing genes by retaining unspliced transcripts in the nucleus. In contrast to this interpretation, Phelan *et al.* suggested unspliced viral transcripts were retained in the infected-cell nucleus



(Phelan, et al., 1996). Using *in situ* hybridization, the authors showed that significant amounts of unspliced ICP0 transcripts were present as clumps in the nucleus, and UL15 unspliced transcripts were present in both the nucleus and cytoplasm of HSV-infected cells at late times post infection. Furthermore, accumulation of the unspliced viral transcripts was not seen in cells infected with the ICP27 defective mutant, and suggested to the authors that ICP27 was responsible for retaining unspliced viral transcripts in the infected-cell nucleus. At present, the fate of the unspliced RNA in HSV-infected cells has not been unambiguously determined. Diaz *et al.* showed that the HSV late gene product US11 could functionally substitute for the HTLV Rex protein in promoting export of unspliced RNA into the cytoplasm of infected cells, and suggested that US11 may perform a similar function during HSV infection (Diaz, et al., 1996). As noted by Phelan *et al.*, this function would antagonize the hypothesized retention of unprocessed transcripts mediated by ICP27, and it would be difficult to reconcile the purpose of such contradictory functions. In addition, three recent papers demonstrated that ICP27 molecules physically shuttled between the nucleus and cytoplasm of infected cells (Phelan and Clements, 1997; Sandri-Goldin, 1998; Soliman, et al., 1997). This shuttling occurred only in the presence of active transcription of HSV late genes, suggesting that it is involved in RNA export rather than RNA retention. Based on these recent findings and our own conclusions, we believe that an alternative interpretation could account for the results seen by Phelan *et al.*. We suggest that ICP27 is

responsible for the production or accumulation of unspliced RNA in infected-cells, but has no effect on their cellular distribution. In cells infected with the ICP27 defective mutant, unspliced transcripts do not accumulate and therefore are not detected in the infected-cell nuclei. In addition, US11 may function to promote export of unspliced RNA at late times post infection. If so, then unspliced ICP0 RNA transcribed at early times prior to expression of US11 may accumulate as clumps in the infected nuclei whereas UL15 unspliced RNA transcribed around the time of US11 expression may be more efficiently exported into the cytoplasm and therefore is present in both nuclei and cytoplasm of infected cells. This hypothesis is consistent with the current available data; however, further experiments will be needed to test its validity.

The results obtained in this study raise the possibility that ICP27 may not inhibit the cellular splicing machinery *in vivo*. In earlier studies, ICP27's inhibitory effects on splicing were defined as a reduction in the accumulation of spliced mRNAs. By that definition, our data suggest that ICP27 did not inhibit splicing of the  $\alpha$ -globin transcripts since we did not observe any reduction in the accumulation of spliced  $\alpha$ -globin RNA in HSV-infected cells. Indeed, the presence of fully spliced HSV UL15 at late times post infection would attest to the activity of the cellular splicing machinery present throughout the course of infection. Our infection and transfection results show that ICP27 does affect the cellular splicing machinery by inducing

accumulation of unspliced transcripts during HSV infection; however, this effect does not interfere with the normal splicing process. Also, preliminary data from our laboratory showed that mutations in any part of the ICP27 molecule abolish the accumulation of unspliced  $\alpha$ -globin RNA during HSV infection. This mutation sensitivity is different from the C-terminal directed repressor activity reported by Sandri-Goldin's laboratory (Hardwicke, et al., 1989), suggesting that these two processes may be independent from one another. At present, the role of ICP27 in the shutoff of host gene expression is still not understood. As demonstrated by Spencer *et al.* (Spencer, et al., 1997), the shut off of cellular genes during HSV infection is mediated at least in part at the transcription level, and their nuclear run-on data suggested that transcriptional shutoff of some of the examined cellular genes depended on the presence of ICP27. It is not clear whether this apparent requirement of ICP27 is related to its effect on host cell splicing. Perhaps further analysis of the function of ICP27 will clarify its involvement in the host shutoff activities induced by HSV.

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## **Chapter 6: Thesis Discussion**

## Discussion

The control of herpes simplex virus gene expression has often been used as a model for studying regulation of eucaryotic gene expression. HSV promoters bear significant resemblance to their cellular counterparts, and are transcribed by host RNA polymerase II (reviewed in Roizman and Sears, 1996 and Wagner, et al., 1995). However, in spite of their similarities, the majority of cellular genes are selectively inhibited during the HSV lytic cycle, while viral genes are preferentially transcribed. The method by which HSV distinguishes between cellular and viral genes is not known, but earlier studies indicated that it is not based on DNA sequence (Smibert and Smiley, 1990). One peculiar exception to this general inactivation, as noted by our laboratory, is the activation of the endogenous  $\alpha$ -globin gene in HSV-infected cells. This observation suggests that in spite of the general repression of cellular gene expression, some endogenous genes are subjected to viral transactivation. Moreover, since this gene is normally transcriptionally silent in non-erythroid cells, the expression of the  $\alpha$ -globin gene implies that HSV infection can disrupt the tissue-specific regulation of this gene.

In this thesis I have further characterised this unusual finding. A search for the viral proteins responsible for this effect showed that HSV IE proteins ICP0 and ICP4 are each sufficient to activate the  $\alpha$ -globin gene in HeLa cells. In addition, characterisation of the

methylation status and chromatin structure of the endogenous  $\alpha$ -globin gene demonstrated that HSV infection does not alter its methylation, but appears to affect the nucleosome positioning at the  $\alpha$ -globin gene locus. Finally, analysis of the expression of  $\alpha$ -globin RNA in HSV-infected cells showed that ICP22 is required for maximal accumulation of  $\alpha$ -globin RNA in HSV-infected cells, possibly by affecting the stability of its RNA, and ICP27 affects the processing of globin RNA by inducing accumulation of unspliced transcripts.

These findings clearly established that HSV proteins activate and regulate expression of the endogenous  $\alpha$ -globin gene during infection, and raise the question regarding the biological significance of this effect. It is unlikely that this activation is intentional, since  $\alpha$ -globin expression does not apparently promote HSV infection, but rather, the  $\alpha$ -globin gene may be a fortuitous target of HSV-directed mechanisms that activate viral genes maintained under repression. For example, a significant parallel exists between the activation of  $\alpha$ -globin expression and the reactivation of HSV genome from latency. Similar to the endogenous  $\alpha$ -globin gene, the latent viral genome is maintained in a quiescent and repressed state (Wagner and Bloom, 1997). Furthermore, ICP0 is involved in activating gene expression in both situations. This study showed that ICP0 is sufficient to induce expression of the silenced  $\alpha$ -globin gene in non-erythroid cells. In addition, results from transfection studies showed that ICP0 does not significantly stimulate expression of plasmid-borne copies of  $\alpha$ -globin

genes, and therefore ICP0 may not enhance transcription of the  $\alpha$ -globin promoter, but its activation of the endogenous  $\alpha$ -globin gene may be mediated by relieving repression of its silenced promoter. ICP0 also plays a significant role in reactivation of HSV genomes from latency. Experiments using *in vivo* models showed that latent genomes lacking ICP0 fail to reactivate from explanted mouse trigeminal ganglia (Leib, et al., 1989). Moreover, *in vitro* studies demonstrated that provision of ICP0 *in trans* is necessary and sufficient to reactivate HSV latent genomes and induce them to enter the lytic replication cycles (Harris, et al., 1989). At present, little is known about the silencing of viral genes during latency or the events leading to reactivation. Insofar as ICP0 appears to relieve repression of both  $\alpha$ -globin gene and latent HSV genome, this may reflect a similar silencing mechanism used in both processes. Our data suggested that chromatin structure plays a role in inhibiting expression of the  $\alpha$ -globin gene in non-erythroid cells. Indeed, latent viral genomes are bound by nucleosomes (Deshmane and Fraser, 1989), and the assembly of chromatin may also restrict transcription of the latent-state viral genome. If so, ICP0 may generally counteract any chromatin-mediated restrictions on transcription, a property which could explain its promiscuous nature as a transcription activator.

The other viral protein which can activate expression of the silenced  $\alpha$ -globin gene is ICP4. It is a potent transactivator which is essential for the transcription of HSV E and L genes during infection

(DeLuca and Schaffer, 1985; DeLuca, et al., 1985). Nuclear run-on analysis showed that ICP4 is also required for the transcription of the cellular  $\alpha$ -globin gene in HSV-infected cells, and co-transfection studies indicated that it enhances transcription from the  $\alpha$ -globin promoter by 10 fold. These data suggested that ICP4 induces expression of the endogenous  $\alpha$ -globin gene through activation of its promoter. Since transcription of the  $\alpha$ -globin gene is negatively regulated in non-erythroid cells, ICP4 must also be able to bypass this regulatory mechanism. ICP4 may normally function to transactivate viral genes that are negatively regulated. Several lines of evidence suggest that genes residing in the HSV genome are subjected to repression. For example, the HSV TK gene is an E gene that is expressed only upon induction by ICP4 during infection (Imbalzano, et al., 1990). However, when it is placed in the context of the cellular genome or when transfected into uninfected cells, it is readily expressed in the absence of ICP4 (Roizman and Sears, 1996; Leiden, et al., 1976; Kit, et al., 1978). Similarly, the human  $\alpha$ -globin gene is robustly transcribed when transfected as a plasmid into tissue culture cells, but its expression becomes dependent on ICP4 when recombined into the HSV genome (Smiley and Duncan, 1992). This general repression effect mediated by the HSV genome likely ensures tight regulation of the viral gene expression cascade by keeping the E and L viral genes silent until they are activated by ICP4.

The mechanism which silences expression of the  $\alpha$ -globin gene in non-erythroid cells has remained a mystery despite significant progress in the understanding of globin gene regulation in erythroid tissues. One perplexing observation is that while expression of the endogenous  $\alpha$ -globin gene is tightly regulated in a tissue-specific manner, transfected copies of this gene are robustly transcribed in non-erythroid cells (Humphries, et al., 1976; Mellon, et al., 1981; Triesman, et al., 1983). As yet, no tissue-specific regulatory element has been identified within the  $\alpha$ -globin gene and flanking sequences, and this has led researchers to suggest that repression of this gene may be mediated by the chromatin structure of the  $\alpha$ -globin locus (Charnay, et al., 1984; Brickner, et al., 1991). The finding that HSV can alleviate repression of this cellular gene prompted us to examine HSV-induced changes in chromatin structure of the  $\alpha$ -globin gene as a novel approach to investigate its tissue-specific silencing mechanism. The data reported in Chapter 4 suggested that regulation of  $\alpha$ -globin expression may not involve soluble repressor molecules, nor is it mediated by methylation of the promoter. Instead, this process may be regulated by nucleosomes positioned along the endogenous  $\alpha$ -globin gene. In particular, our data showed that one nucleosome is positioned over the TATA box and transcription start site of the  $\alpha$ -globin gene, and that accessibility of the underlying sequences is altered upon activation by HSV infection. Insofar as a similar arrangement of nucleosomes in *in vitro* assembled templates is incompatible with active transcription (Lorch, et al., 1992), it is highly

probable that such an arrangement *in vivo* would also be inhibitory to transcription initiation.

The possibility that nucleosome positioning regulates the tissue-specific expression of the  $\alpha$ -globin gene is an attractive hypothesis which deserves further investigation. For example, fine mapping of the DNA sequences bound by the positioned nucleosomes would provide a better idea which sites are occluded or accessible upon nucleosome assembly. Also, one important question that needs to be addressed is whether the change in chromatin structure observed accompanying transcription activation is a prerequisite step or a consequence of on-going transcription. To answer this question, one can determine whether ICP0 or ICP4 alters the accessibility of the  $\alpha$ -globin TATA box in the absence of on-going transcription. Such an experiment may not be easily done since it would require expression of ICP4 while general transcription is inhibited. One possible approach is to generate HeLa cell lines that express temperature sensitive ICP4 molecules that are non-functional at non-permissive temperatures. Coordination of the addition of transcription inhibitors, such as  $\alpha$ -amanitin, with a shift to permissive temperature, could deliver functional ICP4 and inhibit transcription of the  $\alpha$ -globin gene at the same time. Finally, generation of cell lines that inducibly express ICP4 or ICP0 would be useful for further investigating how each of these activators affects the chromatin structure of the  $\alpha$ -globin gene,

and for examining their effects on the expression of other cellular genes.

While studying how HSV infection activates expression of the endogenous  $\alpha$ -globin gene, we serendipitously found that this gene can also be activated by treatment of cells with DMSO in conjunction with cycloheximide or anisomycin. It is not known how this combination of drugs affect the tissue-specific regulation of the  $\alpha$ -globin gene, but several suggestions were discussed in Chapter 4. Insofar as activation of this gene occurs in the presence of protein synthesis inhibitors, this implies that induction does not require synthesis of new transcription factors. Instead, these drugs may activate expression of this gene by de-repressing its tissue-specific regulation. It would be informative to analyse the chromatin structure of the  $\alpha$ -globin gene before and after activation by DMSO plus cycloheximide/anisomycin, and determine whether this induction also involves changes in the nucleosome positioning at the  $\alpha$ -globin transcription start site.

In the final part of this thesis, I used the endogenous  $\alpha$ -globin gene as a reporter to examine the effects of ICP22 and ICP27 on the expression of this cellular gene. The data showed that ICP22 is required for maximal expression of the  $\alpha$ -globin gene during infection, possibly by affecting the accumulation of globin transcripts at the post-transcriptional level. However, ICP22 alone cannot induce expression of the endogenous  $\alpha$ -globin gene, nor does it affect the accumulation of



$\alpha$ -globin transcripts expressed from plasmid copies of the  $\alpha$ -globin gene. These findings indicate that ICP22 may only affect  $\alpha$ -globin gene expression in the context of viral infection, or perhaps its function requires the activities of other viral factors. Purves *et al.* demonstrated that ICP22 is phosphorylated by U<sub>L</sub>13 (Purves and Roizman, 1992; Purves, et al., 1993); however, our data indicated that ICP22's effect on the accumulation of  $\alpha$ -globin RNA is not dependent on its modification by U<sub>L</sub>13. It would be of interest to determine whether phosphorylation of ICP22 is essential for its function, and whether other viral proteins also regulate the phosphorylation level of ICP22 in the absence of U<sub>L</sub>13. At present, the precise function of ICP22 during HSV infection is not known. Mutants lacking ICP22 have limited expression of HSV L genes in certain cell types, suggesting that a cell-type specific activity may complement the function of ICP22 (Sears, et al., 1985). In addition, ICP22 has been demonstrated to be involved in the alteration of the phosphorylation status of the RNA polymerase (Rice, et al., 1995). The consequence of this effect on general transcription has not been clearly determined, and further studies of how ICP22 affects  $\alpha$ -globin gene expression may help elucidate the function of this enigmatic viral protein.

One of the unexpected findings of this thesis is that ICP27 induces accumulation of unspliced  $\alpha$ -globin RNA, but does not affect the overall levels of spliced  $\alpha$ -globin mRNA in HSV-infected cell. This observation disagrees with the currently accepted view that ICP27

inhibits RNA splicing (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994). A number of different studies have shown a link between ICP27 and the cellular splicing machinery (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994; Phelan, et al., 1993; Sandri-Goldin, et al., 1995). For example, this and other studies have documented a direct correlation between the presence of ICP27 and the accumulation of unspliced transcripts in HSV-infected cells. However, in contrast to the interpretation that these unspliced transcripts are by-products resulting from an inhibition of splicing, our data suggested that ICP27 actively promotes expression of unspliced transcripts independent of the efficiency of cellular splicing. A better analysis of ICP27's direct effects on the cellular splicing machinery will be required to distinguish between these two interpretations. ICP27 has also been suggested to be responsible for the delayed shutoff of host gene expression during HSV infection by virtue of its putative ability to inhibit splicing (Hardwicke and Sandri-Goldin, 1994). Again, the data presented in this thesis do not support this hypothesis since expression of the intron-containing cellular  $\alpha$ -globin gene is unaffected by the presence or absence of ICP27 in HSV-infected cells. Further examination of the effects of ICP27 on the expression of other cellular genes (there are a few cellular genes that remain active during HSV infection) may provide a better consensus on ICP27's effect on cellular gene expression.

The investigation of activation of the endogenous  $\alpha$ -globin gene by HSV transactivators has provided new information regarding the effects of viral proteins on cellular gene expression as well as tissue-specific regulation of the endogenous  $\alpha$ -globin gene. Nevertheless, one key question that remains unanswered is how this gene differs from the other cellular genes that are transcriptionally shut off upon HSV infection. One unique feature of the  $\alpha$ -globin gene is its tissue-specific regulation, and perhaps viral host shutoff mechanisms only target cellular genes that are transcriptionally active. It is becoming increasingly clear that intranuclear organisation is an important part of gene regulation and inactive cellular genes are probably localised to different nuclear domains compared to active genes (reviewed in Davie, 1997; and Jackson, 1997). It would be interesting to use *in situ* hybridisation techniques to examine the nuclear localisation of the endogenous  $\alpha$ -globin genes before and after HSV infection, and test whether they co-localise with viral transcription and replication compartments during infection. Such an examination will provide valuable insights into how HSV infection affects the host cell's intranuclear architecture and how these changes affect the expression of cellular genes.

## **Chapter 7: Thesis References**

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