REGULATION OF HSV-1 IMMEDIATE EARLY GENE EXPRESSION

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ABSTRACT

Herpes simplex Virus Type 1 expresses three different classes of genes, immediate early, early, and late, during a lytic infection. Immediate early genes are the first class of genes expressed and they are the only genes expressed independently of de novo viral protein synthesis. This unique characteristic is thought to be the result of the activation of immediate early genes by Vmw65, a protein brought into the cell as a component of the infecting virion. Vmw65 transactivates through the target sequence TAATGARAT (R= purine) which is present at least once in all immediate early transcription regulatory regions. By inserting minimal synthetic promoters, containing the TAATGARAT sequence, into the thymidine kinase locus of the herpes simplex virus type 1 genome I determined that transactivation by Vmw65 is not sufficient to confer on the linked sequences the complete immediate early pattern of gene expression. Furthermore through a transient expression assay I determined that the TAATGARAT sequence element, by itself, when linked to a TATA box is sufficient to act as a target for Vmw65 transactivation.

DEDICATION

My thesis is dedicated to my parents, Manfred and Rosemary Hupel, for without their constant love and support I would not have been able to complete this work.

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Table of Contents

List of Figures

1.1 Herpes Simplex Virus: General Introduction

Herpesviridae is a large family of viruses containing greater than 80 distinct herpesviruses which cause a wide variety of pathologies. With respect to biological properties such as host range, duration of reproductive cycle, characteristics of latent infection, and cytopathology, Herpesviridae can be classified into the three subfamilies: alphaherpesvirinae, betaherpesvirinae, and gammaherpesvirinae (Roizman, 1982). Alphaherpesviruses, exemplified by herpes simplex virus, usually infect the epithelial cells of skin, oral cavity, eyes, genital and respiratory tract, and cause mild primary lesions. After primary infection, alphaherpesviruses may persist latently in the neurons of ganglia innervating the primary infected area and periodically reactivate, resulting in re-occurring lesions of the respective epithelial cells. Alphaherpesviruses have a broad host range *in vitro* and a reproductive cycle of less than 18 hours. Betaherpesviruses, such as cytomegalovirus, have a narrow host range *in vivo* and cells frequently become enlarged upon infection. Latent infections may persist in secretory cells, kidneys and other tissues. *In vitro*, betaherpesviruses have a relatively long reproductive

cycle compared to alphaherpesviruses. Gammaherpesviruses usually infect and persist latently in cells of the lymphoid cell lineage as well as some epithelial and fibroblastic cells. Epstein-Barr virus is a representative of the gammaherpesviruses and is a causative agent of human infectious mononucleosis and is also associated with Burkitt's lymphoma and nasopharyngeal carcinoma (Honess, 1984).

Herpes simplex viruses (HSV), whose natural host is humans, can be further divided into type one (HSV-1) and type two (HSV-2) with HSV-1 being the focus of the research presented in this thesis. The HSV-1 infectious virion consists of a double stranded linear DNA core approximately 150 000 base pairs (bp) in length surrounded by an icosohedral capsid which is enveloped in a lipid bilayer. The viral tegument is the name given to the space located between the icosohedral capsid and the viral envelope (Honess, 1984).

The program of HSV transcription provides an excellent model to study differential eukaryotic gene expression. Upon infection HSV-1 usurps control over the host cell's transcription machinery and through interactions with viral encoded proteins and host cell proteins a regulated cascade of HSV gene expression ensues.

DNA sequence analysis of the entire HSV-1 genome has predicted that HSV encodes approximately 70 genes which can be divided into three classes (McGeoch et al., 1988). These classes are distinguished according to kinetics of expression in infected cells as well as the dependence on viral gene products and viral DNA replication prior to their expression (Honess and Roizman, 1974). Immediate early (IE) or alpha genes are the first genes to be expressed upon infection and are the only viral genes expressed in the absence of de novo viral protein synthesis (Watson et al., 1979). Expression of IE genes peaks at approximately 3 hours post infection and declines thereafter. IE genes encode proteins which modulate transcription and are required for expression of the other two classes of viral genes (see below, and reviewed in Everett, 1987a). The next class of viral genes expressed are the early (E) or beta genes. Early gene expression does not depend on viral DNA replication, peaks at approximately 6 hours post infection and subsequently declines. Early gene expression is however, strictly dependent on the presence of IE gene products. Many early genes encode proteins which are involved in viral DNA replication (Olivo et al., 1989). Late (L) or gamma genes are expressed maximally only after viral DNA replication and they are also dependent on the products

of IE genes for expression (Harris-Hamilton and Bachenheimer, 1985). Some late genes encode structural virion proteins.

The three classes of HSV genes have been defined on the basis of the expression patterns of a limited subset of all HSV genes. Therefore it is important to note that the gene classes are not strictly defined and a continuum of temporal classes probably exist. For example, L genes can be further divided into leaky late (gamma-1) and true late (gamma-2) genes. Leaky late genes are expressed before viral DNA replication but unlike E genes are expressed maximally after DNA replication. In contrast, expression of true late genes is not detected before viral DNA replication. Despite this continuum of temporal classes the IE gene class remains distinct since IE genes are the only genes expressed when de novo viral protein synthesis is inhibited (Watson et al., 1979).

All HSV genes are transcribed from individual promoters by the host cell RNA polymerase II (pol II) (Wagner, 1985). Furthermore, the three levels of HSV gene expression are regulated mainly at the level of transcription (Godowski and Knipe, 1986), however, some evidence exists for post translational control (Weinheimer and McKnight, 1987). Promoter transplant

experiments, in which promoters of one temporal class were substituted with promoters from different temporal classes, have revealed that transcription of HSV genes is regulated by promoter sequences (Post et al., 1981, Silver and Roizman, 1985, Homa et al., 1988). These experiments have prompted numerous studies attempting to identify specific cis acting sequences involved in temporal control of transcription. Such pursuits are important because HSV transcription is mediated by an interplay between host and viral functions. Any insights into mechanisms of HSV temporal control will lead to a better understanding of eukaryotic transcription. Before reviewing the information regarding HSV promoters and temporal regulation of transcription it is important to review the information on the cis acting DNA sequences found in eukaryotic transcription control regions and the trans acting protein factors which modulate transcription through these defined cis acting sequences.

1.2 Eukaryotic Transcription

Eukaryotic transcription compared to prokaryotic transcription appears to be much more complex. For example, prokaryotic transcription is carried out by a single RNA polymerase enzyme. This RNA polymerase is a

complex enzyme composed of 5 sub-units (McClure, 1985). In eukaryotic cells the situation is far more complex since three different proteins are used to catalyze the synthesis of RNA from a DNA template. These complex enzymes, RNA polymerase (pol) I, pol II, and pol III, each consist of 2 large subunits (>100 kDa) and 7-12 smaller subunits with a total molecular mass of 5-6 X 10^5. Each enzyme is responsible for the production of different types of RNA. For example RNA pol I synthesizes large ribosomal RNAs, pol III synthesises small RNAs such as tRNA and 5S ribosomal RNA, and pol II, the main focus of this thesis, synthesises RNAs which will be translated into proteins (Chambon, 1975).

Enormous efforts in molecular biology are targeted towards determining how genes, particularly pol II specific genes, are differentially regulated. Most of these efforts attempt to determine how RNA pol II recognises which genes are to be transcribed. Insights into site selection by pol II are crucial in determining the mechanisms that regulate differential gene expression. Advances in the field have determined that pol II transcription is mediated and regulated by specific cis-acting DNA sequences, promoters and enhancers, and the specific proteins, transcription factors, which interact with these sequences.

1.2.1 Promoters and Enhancers

Functional analysis of deletions and mutations engineered into a large number of pol II transcribed genes has revealed that pol II transcription is regulated by two broad classes of cis acting DNA sequences called promoters and enhancers (for a review see Maniatis et al., 1987). Promoters consist of short DNA sequences, promoter elements, which are generally located within 100 bp upstream of the transcription start site (for examples see Myers et al., 1986, McKnight and Kingsbury, 1982). DNA footprinting experiments have further delimited the boundaries of promoter elements and have demonstrated that promoter elements constitute binding sites for transcription factors (for an example see Jones et al., 1985).

Dissection of a number of different promoters has demonstrated a common pattern of organization (Maniatis et al., 1987, Dynan, 1989). Typically, promoters contain a sequence motif with a TA rich core sequence variously termed the TATA or Hogness box or the Goldberg-Hogness box generally located between 25 and 30 bp upstream of the mRNA cap site, the transcription initiation site. In some cases disruption of the TATA sequence results in

transcription starting at multiple sites suggesting that the TATA box is important in directing pol II to start transcribing at a defined distance from the TATA box itself. (Benoist and Chambon, 1981, Mathis and Chambon, 1981). This point is further confirmed since deletion of the cap site still results in transcription initiation starting at a defined point from the TATA box (Grosschedl and Birnsteil, 1980, Grosveld et al., 1981). In addition, mutational analysis has shown that the TATA box is a critical element in determining transcription efficiency both in vivo and in vitro (Grosveld et al., 1982, Corden et al., 1980, Grosveld et al., 1981). In some cases the TATA and cap site sequences, by themselves, can promote low level basal transcription in vitro (Sassone-Corsi et al., 1981, Sawadago and Roeder, 1985a,) which suggests that the TATA box and cap site constitute the minimal core promoter. Furthermore, since the TATA box is present in many genes in the context of different combinations of other upstream promoter elements the proteins which interact with the TATA box are considered general transcription factors required for pol II transcription.

In contrast to the prokaryotic RNA polymerase, purified pol II, by itself, is unable to accurately initiate transcription in vitro. However accurate initiation is observed upon the addition of whole cell

extracts (Weil et al., 1979). Furthermore, accurate transcription initiation can be reconstituted using partially purified fractions of a whole cell extract. Accurate initiation requires, in addition to pol II, four activities; TFIIA, TFIIB, TFIID, and TFIIE. Addition of these general transcription factors results in accurate transcription initiation from a minimal core promoter consisting of only a TATA box and cap site (Sawadago and Roeder, 1985b, Reinberg et al., 1987, Buratowski et al., 1989).

The most extensively studied general transcription factor is TFIID. In the absence of any other protein, purified TFIID is able to bind to the TATA box and this binding is the first step in the assembly of a functional initiation complex on the core promoter (Sawadago and Roeder, 1985a, Nakajima, et al., 1988, Buratowski et al., 1989). TFIID binding is subsequently stabilized by binding of TFIIA to the TATA-TFIID complex. Thereafter TFIIB, pol II, and TFIIE complete a functional initiation complex and transcription initiation ensues (Buratowski et al., 1989). Furthermore, TFIID binding prior to nucleosome assembly prevents nucleosome mediated repression of transcription (Workman and Roeder, 1987). Taken together, these experiments demonstrate the key role that TFIID plays in assembling functional

transcription complexes and further suggest that TFIID may be a target for other transcription regulatory events.

The promoters of some pol II transcribed genes such as the HMG CoA reductase gene and the lymphocyte specific terminal deoxynucleotidyltransferase (TdT) gene do not contain a TATA box (Reynolds et al., 1984, Smale and Baltimore, 1989). However, at least in the case of the TdT gene, transcription is efficiently initiated at a single nucleotide suggesting that in some cases a TATA box is not required. Analysis of sequences spanning the cap site of the TdT gene has identified a sequence, the "initiator", which, by itself, is able to efficiently and accurately initiate transcription. The initiator, therefore constitutes the simplest functional promoter. Addition of a heterologous TATA box to the initiator significantly increases the efficiency of transcription suggesting cooperative interaction between the initiator and the TATA box (Smale and Baltimore, 1989). Identification of the initiator element has resolved the question concerning the activation of at least one gene that does not possess a TATA box.

In addition to the core TATA box and cap site, typical promoters contain various promoter elements generally found between 20 and 100 base pairs upstream of

the TATA box. Promoter elements are also recognition sites for sequence specific DNA binding proteins which modulate transcription. Well characterized promoter elements include the CCAAT box, the GC box, and the octamer sequence ATGCAAAT. The CCAAT box has been found in a variety of different gene promoters including the HSV thymidine kinase gene (tk), the murine sarcoma virus long terminal repeat, and the rabbit beta-globin gene, and is located between 60 and 80 base pairs upstream of the transcription initiation site (Graves et al., 1986, Myers et al., 1986). A variety of different proteins have been shown to bind to the CCAAT sequence motif (Chodosh et al., 1988a). Another example of a promoter element is the GC box, GGGCGG, which binds the cellular transcription factor Sp1 (Dynan and Tjian, 1983). Sp1 was originally identified as a factor which binds to viral promoter sequences but subsequently a variety of cellular genes including the mouse dyhydrofolate reductase gene and the human metallothionein gene were shown to also contain potential Sp1 binding sites (reviewed in Dynan and Tjian, 1985). Sp1 binding sites may be located proximal to the TATA box as in the adenovirus E1B promoter (Berk, 1986) or be located at larger distances from the TATA box as in the HSV IE gene promoters (Jones and Tjian, 1985). Purified Sp1 is able to activate

transcription in a reconstituted in vitro transcription reaction (Jones and Tjian, 1985). The octamer sequence motif is another type of promoter element found in many different gene promoters including; the histone H_2B gene promoter, immunoglobulin light and heavy chain gene promoters, and the HSV tk gene promoter (Parslow et al., 1984, Pruijn et al., 1987, Parslow et al., 1987). Both tissue specific and ubiquitous proteins are able to bind and activate transcription through interaction with the octamer sequence motif (see below).

Analysis of the structural organization of promoters has revealed that promoters may be a mosaic of different promoter elements. For example, the HSV tk gene promoter contains two GC boxes, a CCAAT box, and an octamer sequence all located with 100 base pairs from the TATA box (McKnight and Kingsbury, 1982, Parslow et al., 1987). Simpler promoters, for example the E1B promoter, consist of only one GC box immediately upstream of a TATA box (Berk, 1986). Some promoter elements, such as GC boxes, are able to function irrespective of their orientation relative to the TATA box (Dynan and Tjian, 1985). Therefore promoters may contain single promoter elements or contain combinations of repeated promoter elements. Correct spacing of promoter elements with respect to the TATA box may be necessary. In one case,

insertion of odd multiples of half turn DNA between promoter elements and the TATA box resulted in decreased transcription whereas insertion of even multiples of half turn DNA had no detrimental affect (Takahashi et al., 1986). This latter observation suggests that protein-protein interaction between transcription factors is important and this interaction requires stereospecific alignment.

A second broad class of cis acting DNA sequences, enhancers, are also involved in regulating transcription. Enhancers function to increase the density of RNA polymerase molecules on the corresponding gene (Weber and Schaffner, 1985, Triesman and Maniatis, 1985). Characteristics of enhancers include the ability to activate transcription from heterologous promoters, function in an orientation independent manner, and unlike promoters function irrespective of distance from the transcription initiation site (Maniatis et al., 1987). For example, the first identified enhancer, the SV40 72 base pair repeat, can stimulate transcription from the heterologous beta-globin promoter when present in either orientation both 1400 bp upstream and 3300 bp downstream of the mRNA cap site (Banerji et al., 1981). Subsequently, many other viral and cellular enhancers have been identified (reviewed in Maniatis et al., 1987).

Enhancers can display tissue specific activity, as is the case for the immunoglobulin heavy chain gene enhancer (Banerji et al., 1983), or display ubiquitous activity, such as the SV40 72 bp repeat enhancer, being active in a variety of cell types (Schirm et al., 1987). Some enhancers display transcription activating activity in response to external stimuli. Examples of such inducible enhancers include the heat shock gene enhancer and the enhancer present in the mouse mammary tumour virus long terminal repeat. These enhancers are induced upon heat shock and presence of steroid hormones respectively (Bienze and Pelham, 1986, Chandler et al., 1983).

The SV40 enhancer is the most extensively studied enhancer and therefore provides an excellent example to illustrate enhancer structure and function. Structural analysis has demonstrated that the SV40 enhancer consists of multiple sequence motifs, some of which display tissue specific activity, that act synergistically to generate enhancer activity (Herr and Clarke, 1986, Schirm et al., 1987). Fine structural analysis has demonstrated that a hierarchy of organization exists within the enhancer. The basic unit of an enhancer, called an enhancon, corresponds to a short 8-10 bp DNA sequence which, by itself, has no enhancer activity. Enhancons when duplicated or when present directly next to a different

enhancon form enhancer elements which are, by themselves, inactive. Duplication of enhancer elements results in enhancer activity (Ondek et al., 1988). Different classes of enhancons, however, exist which can display enhancer activity on their own (Fromental et al., 1988). Taken together these experiments illustrate that enhancer function is the result of the synergistic action of a repetitive array of short DNA sequences.

DNAase 1 footprinting has revealed that the individual sequence motifs, enhancons, constitute binding sites for cellular proteins (Wildeman et al., 1986, Davidson et al., 1986). Two lines of evidence indicate that these enhancer motif binding proteins function to activate transcription; (1) mutation of a tissue specific enhancer motif has no effect on enhancer activity in cells which do not posses the respective sequence specific binding protein (Xiao et al., 1987), and (2) mutation of a motif which disrupts protein binding also reduced transcription activation by the enhancer (Wildeman et al., 1986, Davidson et al., 1986). Together, these experiments demonstrate that transcription activation is mediated by sequence specific DNA binding proteins which bind to sequences within the enhancer.

The examination of both promoters and enhancers has demonstrated that both are similarly organized.

Indeed the differences between promoters and enhancers are slight. For example, certain elements, such as the octamer sequence, can function both as a promoter element and as an enhancer element (Parslow et al., 1987). Furthermore, the SV40 enhancer can functionally substitute for the beta-globin promoter (Treisman and Maniatis, 1985). In addition, certain promoter elements when duplicated can function as an enhancer (Bienze et al., 1986). Finally, in some cases promoters and enhancers act synergistically to activate transcription to high levels as well as regulate tissue specific gene expression. (Garcia et al., 1986, Parslow et al., 1987, Fischer and Maniatis, 1988). These studies indicate that promoters and enhancers activate transcription by similar mechanisms. Enhancers, however, generally lack motifs which determine the transcription initiation site.

1.2.2 Transcription Factors

Most of the regulatory proteins which bind to specific DNA sequences contain a limited number of common structural motifs. One type of motif involved in DNA binding is the helix-turn-helix motif. This motif is common to the large number of homeotic genes since it comprises part of the DNA sequence recognition homeobox

domain (reviewed in Johnson and McKnight, 1989). Another common sequence motif, the zinc finger, is also found in a variety of DNA binding proteins including the pol III transcription factor TFIIIA, the yeast transcription activating protein GAL4, transcription factor Sp1, and in the DNA binding domain of steroid hormone receptors (Kadonaga et al., 1987. and also reviewed in Evans and Hollenberg, 1988). A third common structural motif, the leucine zipper, was originally identified in the CCAAT box and enhancer binding protein (C/EBP), and is characterized by a heptad repeat of leucines which extend along one side of an amphipathic helix (Landschultz et al., 1988). The leucine zipper is also common to proteins such as fos, myc, jun, and the yeast transcription activator GCN4 (Johnson and McKnight, 1989). The zipper motif constitutes an oligomerization interface whereby protein homodimers and heterodimers form (Sellers and Struhl, 1989). Specifically, leucine zippers determine what types of protein complexes can form, which in turn determines the DNA binding specificity of the complex (Sellers and Struhl, 1989, Kouzardides and Ziff, 1989). Such a motif is therefore not required for DNA binding, per se, but is required for subunit association in the case of proteins such as C/EBP and jun which only bind to DNA as dimers or heterodimers (Johnson and McKnight,

1989, Halazonetis et al., 1988).

Elucidating the mechanism whereby transcription is activated by sequence specific binding proteins is difficult because some proteins can bind to remarkably different sequences and the same sequence can be bound by completely different proteins. An excellent example to illustrate both of these complexities involves the octamer sequence and its respective binding proteins.

The octamer sequence (ATGCAAAT consensus) is found in the enhancer and promoter regions of the immunoglobulin (Ig) heavy chain gene as well as in Ig light chain gene promoters. Expression of these genes is strictly confined to cells of the B-lymphocyte origin. By itself, the octamer sequence, when linked immediately upstream of a TATA box is sufficient for B-cell specific activation of the linked gene (Wirth et al., 1987). The octamer is also present as part of the SV40 72 base pair enhancer. In the context of the enhancer, the octamer displays B-cell specific activity (Schirm et al., 1987, Tanaka et al., 1988). The octamer sequence is also involved in the expression of genes which are ubiquitously expressed. For example, the cell cycle regulation of the histone H_2b gene requires an intact octamer (LaBella et al., 1988). Furthermore, the octamer sequence is required for efficient expression of snRNA

genes (Carbon et al., 1987). All snRNA genes, U1 to U6, contain sequences homologous to the octamer in their distal sequence elements (Sollner-Web, 1988). Interestingly, U2 snRNA transcribed by pol II and U6 snRNA transcribed by pol III both compete for binding of the same octamer binding protein (Carbon et al., 1987). That pol II and pol III require the same transcription factor is not surprising since both enzymes share — subunits and the largest subunits of each enzyme display sequence homology (Allison et al., 1985). Finally the octamer sequence is an essential component of the adenovirus type 4 origin of DNA replication (Pruijn et al., 1987).

Consistent with the presence of the octamer sequence in both lymphoid specific and ubiquitously expressed genes, Staud et al., (1986) described two species of nuclear proteins which specifically bound to the octamer sequence. One protein OCT-2 (variously named OTF-2 or NF-A2) is present in cells of lymphoid origin (Staud et al., 1986), and is able to activate B-cell specific genes dependent on the presence of an intact octamer (Scheidereit et al., 1987, Muller et al., 1988). Similarly, OCT-1 (variously named OTF-1, NF-A1, or OBP100) is expressed ubiquitously (Staud et al., 1986) and is able to activate <u>in vitro</u> transcription of the H_2b

gene (Fletcher et al., 1987). OCT-2 is therefore responsible for the expression of B-cell specific genes and OCT-1 mediates the activation of octamer containing ubiquitously expressed genes. Furthermore, O'Neil et al., (1988), demonstrated that OCT-1 is identical to the adenovirus DNA replication factor NF-III which recognizes the octamer sequence. Interestingly, both OCT-1 and 2 bind indistinguishably to the octamer sequence. Different proteins are therefore able to bind to the same DNA sequence. (Staud et al., 1986, Scheidereit et al., 1987). Another example of a DNA sequence recognized by different proteins is the CCAAT box (Johnson and McKnight et al., 1989). Three different HeLa cell proteins have been identified which specifically recognize the CCAAT box motif (Chodosh et al., 1988a).

Recently cDNAs encoding both OCT-1 (Sturm et al., 1988) and OCT-2 (Staudt et al., 1988, Clerc et al., 1988, Scheidereit et al., 1988, Muller et al., 1988) have been cloned. Sequence comparisons have indicated that both OCT-1 and 2 are highly homologous within a 150 amino acid region. This region, called the POU (pronounced POW) domain, can be subdivided into two domains, the homeobox domain because of the homology with Drosophila homeotic genes, and the POU specific domain (Herr et al., 1988). The homeobox of Drosophia genes contains a helix-turn-

helix motif required for DNA binding (reviewed in Levine and Hoey, 1988). Deletion analysis of OCT-1 has demonstrated that the homeobox specific domain is required for DNA binding which is further stabilized by the POU specific domain (Sturm and Herr, 1988). The high homology (87%) in the DNA binding domains (POU boxes) of both OCT-1 and 2 explains the identical DNA binding properties of the two proteins (Herr et al., 1988).

In addition to specific binding to the conserved octamer sequence, both OCT-1 and 2 can bind to remarkably degenerate octamer sequences. For example, OCT-2 is capable of binding to sequences recognized by Drosophila homeotic proteins and yeast mating type proteins, both of which contain a homeobox domain (Schiedereit et al., 1988, Ko et al., 1988). Moreover, OCT-2 can activate transcription through DNA sequences which are normally recognized by the Drosophila homeotic genes ultrabithorax and Abdominal-b (Thali et al., 1988). In addition, both OCT-1 and OCT-2 are able to bind to the Ig heptamer sequence which shares no sequence similarity to the octamer sequence (Poellinger et al., 1989, LeBowitz et al., 1989). Finally, OCT-1 can recognize the TAATGARAT (R=purine) motif of HSV IE genes (Baumruker et al., 1988). Taken together these observations demonstrate that both OCT-1 and 2 recognize sequences other than the

octamer sequence. Given the binding flexibility of homeotic genes (Desplan et al., 1988) and the observation that certain Drosphila homeotic genes bind the consensus octamer sequence (Thali et al., 1988), perhaps all homeotic genes have the potential of modulating transcription through the octamer motif.

1.2.3 Activation of Transcription

Numerous cis acting DNA sequences which regulate transcription and the trans acting proteins which interact with these sequences have been extensively characterised. The mechanism whereby sequence specific DNA binding proteins alter the activity of RNA pol II, however, still remains largely an enigma. Most information concerning the mechanisms of transcription activation has been obtained from studies on yeast.

The transcription regulatory regions of yeast genes are structurally very similar to those of higher eukaryotes. Typically, transcription is regulated by cis acting sequences located approximately 250 base pairs upstream of the transcription start site. These sequences, called upstream activating sequences (UAS) are functionally similar to higher eukaryotic enhancer sequences, except that UASs cannot activate transcription

when present downstream of the transcription initiation site. In addition, yeast genes contain a TATA homology which is present 60-120 base pairs upstream of the RNA start site (reviewed in Guarante, 1988). Both UASs and the TATA box are targets for sequence specific DNA binding proteins which modulate transcription of the linked gene (reviewed in Struhl, 1987). The study of GAL4, a DNA binding protein which activates transcription by binding to the UASs of the GAL1 and GAL10 genes, has provided insights into the mechanism of transcription activation by transcription factors.

Dissection of GAL4 has determined that, in the simplest case, transcription activators are bipartite, consisting of a DNA binding domain (discussed above) and a transcription activating domain (Keegan et al., 1986). In fact the DNA binding specificity of GAL4 can be altered without altering the activation function (Brent and Ptashne, 1985). The bipartite nature of GAL4 is similar to the structural organization of steroid hormone receptors. In response to hormone binding, hormone receptors activate transcription by binding to specific DNA sequences called hormone responsive elements located upstream of the mRNA cap site. The DNA binding domain and transcription activating domain of some steroid hormone receptors can also be separated (reviewed in Beato et

al., 1989).

Deletion studies have revealed that GAL4 contains two activation domains either of which can activate transcription when fused to a DNA binding domain. Both of these activation domains contain a large number of acidic amino acids and therefore posses a net negative charge (Ma and Ptashne, 1987a). Similarly, the activation domain of another yeast transcription activating protein GCN4 also contains a net negative charge due to a large number of negatively charged amino acids (Hope and Struhl, 1986). Random E. coli genomic fragments can substitute for the GAL4 activation domains provided that they encode an oligopeptide with a net negative charge (Ma and Ptashne, 1987b). This latter experiment suggests that there is no strict structural requirement for activation domains beyond carrying a net negative charge. Some evidence, however, exists which suggests that the activation domain must form an amphipathic helix (Giniger and Ptashne, 1987). The above studies indicate that transcription activation in yeast is mediated by interactions between the acidic domain of transcription activators and other proteins involved in transcription such as the TATA box binding proteins or RNA pol II itself.

Numerous studies indicate that the mechanism of

transcription activation is highly conserved across all eukaryotes. For example, GAL4 is able to specifically activate transcription in mammalian cells containing a GAL4 UAS. Activation is dependent on the presence of the acidic activation domain (Kakidani and Ptashne, 1988, Webster et al., 1988). GAL4 derivatives can also activate transcription in Drosophila larvae as well as in plant cells (reviewed in Ptashne, 1988). Furthermore, the yeast TATA box binding factor, TFIID, can functionally substitute for mammalian TFIID in an in vitro transcription reaction (Buratowski et al., 1988, Cavallini et al., 1988). Other mammalian transcription factors including AP1 and a CCAAT box binding protein also have functional homologues in yeast (Harshman et al., 1988, Chososh et al., 1988b). A fusion protein consisting of the mammalian fos protein linked to the GAL4 DNA binding domain can activate transcription from genes containing the GAL4 UAS in yeast (Lech et al., 1988). Finally, the human oestrogen receptor can induce transcription, in yeast, from genes containing the oestrogen responsive element (Metzger et al., 1988). Taken together, these experiments strongly suggest that the mechanism of transcription activation is evolutionarily conserved among eukaryotic cells.

The target for acidic activation domains and for

transcription factors which bind to upstream promoter and enhancer sequences is not known. GAL4 is able to activate transcription from a minimal promoter consisting only of a GAL4 UAS linked immediately upstream of a TATA box (Webster et al., 1988). The possible targets for the GAL4 activation domain therefore include the TATA box binding proteins or pol II and its associated proteins. Recently, Horikoshi et al., (1988a), demonstrated that GAL4 binding qualitatively alters the TFIID footprint over the TATA box region, therefore suggesting that TFIID is the target for the activation domain. Similarly, the binding of ATF, a transcription factor involved in activation of the adenovirus E4 promoter, also alters TFIID binding to the E4 TATA box (Horikoshi et al., 1988b). The alteration of TFIID binding by ATF facilitates the assembly of the other general transcription factors and pol II into a complete pre-initiation complex (Hai et al., 1988). Furthermore, USF, a factor required for maximal expression of the adenovirus major late promoter, may also cooperatively interact with TFIID (Sawadago and Roeder, 1985a, Van Dyke et al., 1988). TFIID may also be the target of viral transcriptional regulatory proteins such as adenovirus EIA (Wu et al., 1987, Leong et al., 1988), the pseudorabies virus IE protein (Abmyer et al, 1988, Workman et al., 1988), and the HSV ICP4 protein

(Everett, 1988).

In summary, transcription by RNA pol II is regulated by specific proteins called transcription factors which recognise specific DNA sequences. The binding of transcription factors to their cognate sequences in promoters and enhancers facilitates the formation of initiation complexes by general transcription factors and RNA pol II. Once a complete initiation complex is formed transcription initiation ensues. Both protein-protein and protein-DNA interactions are important in the assembly of the initiation complex.

1.3 HSV Promoters and Temporal Regulation

As previously mentioned, promoter replacement experiments have demonstrated that the kinetics of expression of HSV genes are determined by the promoter sequences of these genes (Post et al., 1981, Silver and Roizman, 1985, Homa et al., 1988). Extensive analysis of promoters from the three kinetic classes has revealed a correlation between promoter complexity and kinetic class. HSV IE genes contain the most complex promoter regions, E genes contain simpler promoters, and L genes are driven by the least complex promoters (reviewed in

Everett, 1987a).

1.3.1 Immediate Early Genes:

Upon infection five IE genes, encoding the proteins ICP (infected cell polypeptide) 0 (Vmw110), ICP4 (Vmw175), ICP27 (Vmw63), ICP22 (Vmw68), and ICP47 (Vmw12), are coordinately induced in the absence of de novo viral protein synthesis (Everett, 1987a). The coordinate induction of IE genes is brought about by a protein brought into the cell as part of the infecting virion (Campbell et al., 1984). This protein, Vmw65, is assembled into the tegument component of the virion and is present at a copy number of 400-600 molecules per virus particle (Heine et al., 1974).

Sequence analysis and functional studies of IE promoters have identified conserved functional sequences present in all five IE regulatory regions. These regions include a proximal sequence element located within 100 bp upstream of the transcription start site. The proximal promoter element contains a TATA homology found approximately 25-30 bp upstream of the transcription start site, as well as, GA and GC rich stretches (Mackem and Roizman, 1982a, Whitton et al., 1983, Cordingly et al., 1983). These sequences constitute binding sites for

cellular transcription factors. For example, some GC boxes present in the proximal element bind the cellular transcription factor Sp1, and this interaction is essential for *in vitro* transcription of IE genes (Jones and Tjian, 1985). The proximal promoter element is sufficient to confer constitutive expression in the absence of transinduction by Vmw65 (Mackem and Roizman, 1982b).

In addition to the proximal promoter element, IE gene promoters contain a far upstream element located greater than 100 bp upstream of the transcription start site (Mackem and Roizman, 1982b). This far upstream element contains GA and GC rich sequences similar to those in the proximal element but in addition contains the sequence TAATGARAT (R=purine). The TAATGARAT motif, present at least once in all IE far upstream regions, is usually found greater than 100 bp and as far as 600 bp upstream of the IE cap site (Mackem and Roizman, 1982a, Smith et al., 1989). The far upstream region is analogous to a transcription enhancer, even in the absence of Vmw65 transactivation, since it can function in either orientation and at distances greater than 1000 bp away from the cap site to enhance transcription (Lang et al., 1984, Preston and Tannahill, 1984). However conflicting evidence exists as to whether the far upstream region can

function downstream of a promoter to enhance transcription (Lang et al., 1984, Preston and Tannahill, 1984). Even though the far upstream region can enhance transcription in the absence of any viral transinduction, deletion analysis and promoter reconstruction experiments have identified the TAATGARAT motif, present in the far upstream region, as being strictly required for Vmw65 transactivation (Cordingly et al., 1983, Preston et al., 1984, Gaffney et al., 1985, Bzik and Preston., 1986, O'Hare and Goding, 1988).

In summary, IE genes contain a rather complex transcription regulatory region consisting of a proximal promoter element as well as a far upstream enhancer like element. Both can operate to increase transcription in the absence of transactivation by viral functions, however, the far upstream region can further be induced to enhance transcription since the TAATGARAT motifs, in this region, are targets for Vmw65 transactivation.

1.3.2 Early Genes:

The second class of HSV genes expressed upon infection are the E genes. Early gene expression is maximal approximately 6 hours post infection and levels of E gene mRNA declines thereafter. Expression of early

genes is not dependent on viral DNA replication (Harris-Hamilton and Bachenheimer, 1985). Some early genes encode essential functions for viral DNA replication such as the viral DNA polymerase and various DNA binding proteins (Olivo et al., 1989). Early gene expression is strictly dependent on activation by IE gene products, particularly ICP4 (Preston, 1979, Watson and Clements, 1980, Dixon and Schaffer, 1980, Deluca et al., 1985, Persson et al., 1985). No E genes are expressed when infection is carried out in conditions which preclude the synthesis of IE proteins such as in the presence of protein synthesis inhibitors.

Most of our knowledge regarding promoter complexity and regulation of E gene expression is derived from extensive analysis of the HSV thymidine kinase (tk) gene promoter. Deletion analysis as well as linker scanning mutations spanning the tk promoter has delimited a region extending to position -110, with respect to the start of transcription, as being essential for efficient tk transcription. These studies have identified various defined sequence elements including a TATA box homology at position -25, two GC boxes at position -60 and -105, a CCAAT sequence at -80 (McKnight et al., 1981, McKnight and Kingsbury, 1982). In addition, an octamer sequence exists at position -138 (Parslow et al., 1987). The

octamer sequence at this location is however not important during virus infection of cultured cells (Boni and Coen, 1989). At least two different cellular proteins specifically interact with these sequences including the transcription factor Sp1 (Jones et al., 1985). Early gene promoters consist of binding sites for various cellular transcription factors and therefore resemble the proximal promoter region of IE gene promoters.

The above studies have identified promoter domains required for the constitutive expression of tk in the absence of any viral regulators. During a productive viral infection E gene expression requires IE gene products, therefore, it is important to identify essential sequences which may act as targets to mediate this transinduction. Sequence analysis of E gene promoters has not identified a consensus sequence element analogous to the TAATGARAT motif of IE promoters which may serve as a target to facilitate the coordinate induction of E genes by IE polypeptides. Interestingly, the promoter domains required for transactivation of a plasmid born copy of tk by superinfecting virus in a transient transfection assay are the same as those required for constitutive tk activity in the absence of superinfecting virus (Eisenberg et al., 1985). In

addition, the sequences required for expression of tk when resident in the viral genome during infection, are also the same as those essential for constitutive activity of plasmid borne copies of tk (Coen et al., 1986). Taken together, these studies show that the promoter domains of tk required under conditions of trans induction, correspond to those important for recognition by cellular transcription factors. This observation suggests that IE proteins activate transcription of tk by modulating the activity of cellular transcription factors.

The results of functional analysis of the HSV glycoprotein D gene (gD) promoter, another E gene, are in agreement with the conclusions obtained from functional studies of the tk gene regarding regulation of E gene expression. All promoter domains necessary for full regulated expression of the gD gene map within 83 bp of the transcription start site (Everett, 1983). This region contains sequences potentially recognized by cellular transcription factors including a TATA box and GC rich regions. Furthermore, the sequences required for cis activation of the gD promoter by the SV40 enhancer correspond to the sequences necessary for trans activation by viral IE gene products, suggesting that IE proteins function by modulating the activity of cellular

transcription factors (Everett, 1984a).

The functional studies of the tk and gD promoters demonstrate that there are no virus specific sequences responsible for the activation of E gene activation by IE polypeptides. This point is confirmed by experiments demonstrating that cellular genes are regulated as bonafide viral genes when they are inserted in the viral genome. The rabbit beta-globin gene is efficiently expressed from it's own promoter and regulated as a viral gene when inserted into an E gene, tk, or a late gene, glycoprotein C (gC). (Smiley et al., 1987, Panning and Smiley, 1989). To test the possibility that there are fortuitous sequences homologous to virus specific sequences present in the beta-globin promoter, Panning and Smiley (1989) demonstrated that the human alpha-globin gene, which contains no sequence similarity to the promoter regions of beta-globin, is also regulated as an E gene when resident in the viral genome at the tk locus. Furthermore, like true viral E genes, expression of viral transduced beta-globin and alpha-globin is dependent on IE proteins, namely ICP4 (Duncan and Smiley, manuscript in preparation). These studies clearly demonstrate that E genes do not contain any virus specific target sequences required for IE transactivation. Temporal regulation can be achieved by cellular DNA sequence elements suggesting

that IE proteins function by re-directing the activity of cellular transcription factors.

The studies of the tk and gD promoters as well as experiments on virally transduced cellular genes have demonstrated that E gene promoters are contained within 150 bp of the transcription start site and consist of sequence elements which serve as recognition sites for cellular transcription factors. Furthermore, it appears that there are no virus specific sequences required for efficient activation of E gene promoters by IE polypeptides.

1.3.3 Late Genes:

Late genes, the last class of HSV genes expressed upon infection, can be divided into two subclasses which differ in their dependence on viral DNA replication for maximum expression. Leaky late genes are significantly expressed prior to viral DNA replication but are expressed maximally after replication. True late genes, in contrast, are more strictly dependent on viral replication for maximal expression (Wagner, 1985).

Most of the information regarding L gene expression comes from studies on the true late genes US11 and gC. In contrast to E genes, a number of experiments

have demonstrated that sequences upstream of the TATA box are not required for efficient regulated expression of L genes. Firstly, mutant viruses, in which regions of the gC gene promoter and untranslated leader have been deleted have demonstrated that sequences between -34 and +124 are required for efficient expression of gC. Contained within this region is the TATA homology (Homa et al., 1986, Shapira et al., 1987). More extensive deletion analysis has demonstrated that the minimal functional gC promoter corresponds to a 15 base pair sequence which spans the TATA box. Furthermore, this 15 base pair promoter is sufficient to serve as a target for transactivation by ICP4 and ICP0 in transient transfection assays (Homa et al., 1988). Secondly, deletion analysis of the US11 promoter has shown that all DNA sequence elements necessary for fully efficient regulated expression of US11 are within 31 base pairs of the RNA cap site. Contained within this region is a TATA homology (Johnson and Everett, 1986a,b). Thirdly, the TATA box alone of the adenovirus type 2 major late promoter produces a transcript with leaky late kinetics when it is inserted into the HSV-1 viral genome (Keith and Smiley, manuscript in preparation). Taken together, these experiments demonstrate the importance of the TATA box and demonstrate that no sequences upstream of the

TATA box are needed for L gene expression.

Other experiments have demonstrated that, in addition to the TATA box, sequences around the cap site and untranslated leader are required for maximum expression as well as correct temporal regulation of some late genes. For example, the US11 TATA box is alone sufficient to drive transcription, however the sequence of the US11 cap site from -11 to +39 is required for maximum expression. The US11 cap site region increases the dependence of the Ad 2 MLP TATA box promoter on DNA replication when linked downstream of the TATA box (Kibler and Smiley, manuscript in preparation). In addition sequences downstream of the gamma-42 true late gene TATA box are also required for proper temporal regulation of this gene (Mavromara-Nazos and Roizman, 1989). These experiments demonstrate the requirement for sequences downstream of the TATA box for efficient temporal regulation of L gene expression. The role of these downstream sequences is not known.

A number of observations have determined that in addition to promoter sequence the genomic environment of HSV genes is also important in temporal class regulation. For example, a wild type tk gene is constitutively expressed in the absence of any viral functions when introduced transiently or stably into cultured cells

(Leiden et al., 1976). In contrast tk delivered into the cell via the viral genome during an infection is not expressed in the absence of IE proteins (Preston, 1979). Similarly, the endogenous mouse beta-globin gene in Friend erythroleukemia cells is repressed upon HSV infection. A viral transduced globin gene, however, is efficiently expressed and regulated as a bonafide viral gene (Smiley et al., 1987). Furthermore genes driven by true late promoters behave indistinguishably from genes driven by early promoters upon viral superinfection when integrated into the cellular genome. The same late promoter is subject to true late control when it is present on the viral genome (Silver and Roizman, 1985). These observations indicate that the genomic context of the gene affects its temporal regulation.

One possibility to explain the genomic position effect is that the HSV genome is associated with specific compartments within the nucleus and this compartmentalization is key to temporal regulation. Evidence for this possibility comes from studies which show that the HSV genome is present in "replication compartments". These replication compartments form at late times post infection and contain ICP4 and ICP8 (the major DNA binding protein) which are required for transcription and replication respectively (Knipe et al.,

1987). Both functions are required for late gene expression, therefore late genes may not be expressed until the viral genome becomes assembled into the "replication compartments"

1.4 Immediate Early Proteins

Upon infection by HSV the genes encoding the five IE proteins ICP4, ICP0, ICP27, ICP22, and ICP47 are the first to be expressed. These genes form a distinct kinetic class since their expression does not require any de novo viral protein synthesis. Expression of IE gene products is required for the proper expression of the E and the L genes (Honess and Roizman, 1975). Information regarding the function of the individual IE proteins in regards to HSV gene regulation is derived from two types of experiments: the genetic analysis of viruses containing mutations in the various IE genes, and transient transfection studies whereby IE genes are transfected into cells independently or in the presence of a very limited subset of other viral functions.

1.4.1 ICP4:

The major regulatory protein of HSV, ICP4,is a

large phosphoprotein which is strictly essential for virus replication. ICP4 has multiple phosphorylation states and therefore migrates as a diffuse band of approximately 175,000 daltons on sodium dodecyl sulfate (SDS) polyacrylamide gels. (Courtney and Benyesh-Melnick, 1974, Wilcox, et al., 1980). Furthermore, ICP4 is a complex molecule consisting of multiple functional domains (DeLuca et al., 1984, DeLuca and Schaffer, 1988, Paterson and Everett, 1988). ICP4 localizes to the nucleus of infected cells and becomes sequestered into discrete sub-nuclear compartments (Courtney and Benyesh-Melnick, 1974, Knipe et al., 1987). When in the nucleus ICP4 proteins may associate and function as homodimers (Metzler and Wilcox, 1985, Shepard and DeLuca, 1989).

Mutant viruses containing a variety of temperature sensitive lesions or deletions in the gene encoding ICP4 all essentially exhibit the following phenotypic characteristics: (i) over production of IE mRNA, (ii) under representation or absence of E and L mRNA, and (iii) absence of viral DNA replication (DeLuca et al., 1985). These observations suggest that ICP4 functions to down regulate IE genes as well as activate E and L gene expression. Furthermore, ICP4 is required continuously for E and L gene expression at the level of transcription (Preston, 1979, Dixon and Schaffer, 1980,

Watson and Clements, 1980, DeLuca et al., 1985). Taken together, these experiments demonstrate that ICP4 carries out essential viral functions and is required for the transition from IE to E and L phases of the replication cycle.

A number of other studies have confirmed and extended the results obtained with ICP4 mutant viruses with respect to ICP4 function. For example, genes driven by E and L promoters, either integrated into the cellular genome or transiently expressed, are activated by viral superinfection in an ICP4 dependent fashion (Dennis and Smiley, 1984, El Kareh et al., 1984, Mosca et al., 1985, O'Hare and Hayward, 1984). In addition, transient expression assays have demonstrated that genes driven by E and L promoters are activated when co-transfected with ICP4 alone (Everett, 1984b, Gelman and Silverstein, 1985, O'Hare and Hayward, 1985a, Quinlan and Knipe, 1985, Mavromara-Nazos et al., 1986, Everett, 1986). ICP4 has also been reported to down regulate expression of reporter genes driven by the ICP4 promoter itself thereby displaying an autoregulatory function (O'Hare and Hayward, 1985b, 1987). Finally, partially purified ICP4 added to an uninfected cell extract stimulates in vitro transcription from E and L promoters but represses transcription from IE gene promoters (Pizer et al.,

1986). Collectively, these experiments have demonstrated that ICP4, by itself, is able to both activate gene expression from HSV promoters as well as down regulate expression from its own promoter and these experimental observations agree strongly with the phenotypic characteristics of the ICP4 mutant viruses.

The mechanism whereby ICP4 activates transcription from some promoters and inhibits transcription from others is not understood. A large number of reports have demonstrated that ICP4 can bind to specific DNA sequences present in the vicinity of the promoters of all three temporal classes of genes. This observation has led to the model that ICP4 functions through interaction with these sequences. ICP4 can form specific DNA protein complexes with a number of HSV genes including: the early genes gD (Faber and Wilcox, 1986, Tedder et al., 1989) and perhaps tk (Kristie and Roizman 1986a), the IE genes ICP4 (Kristie and Roizman 1986a,b, Muller 1987, Faber and Wilcox, 1988, Roberts et al., 1988), ICP0 (Kristie and Roizman 1986a,b, Kattar-Cooley and Wilcox, 1989, Roberts et al., 1988), ICP27 (Kristie and Roizman, 1986a), and the L genes ICP42 and ICP25 (or Vmw65) (Michael et al., 1988). Although a consensus ICP4 binding sequence which includes the core nucleotides ATCGTC has been suggested (Faber and Wilcox, 1986), ICP4

can also bind to unrelated sequences such as those found in the untranslated leader of L genes as well as sequences far upstream of the gD gene (Micheal et al., 1988, Tedder et al., 1989).

Evidence that ICP4 binding sites play a role in gene activation by ICP4 is mainly derived from in vitro transcription reactions. Addition of partially purified ICP4 to uninfected cell extracts can increase expression from the gD promoter (Beard et al., 1986). Furthermore, all three ICP4 binding sites within the gD promoter contribute to ICP4 mediated transactivation and addition of multiple copies of synthetic oligonucleotides upstream of the gD promoter enhance the response to ICP4 (Tedder et al., 1989, Tedder et al., 1988). In addition, the transactivation function of ICP4 is dependent on the integrity of the DNA binding domain (Paterson and Everett, 1988, DeLuca and Schaffer, 1988, Shepard and DeLuca, 1989). Taken together, these experiments provide evidence that binding of ICP4 to specific DNA sequences plays a role in activating transcription from certain promoters.

Additional evidence exists which indicates that binding of ICP4 to its respective binding site is not required for activation of gene expression. For example, only 83 base pairs upstream of the gD cap site are

required for proper expression in transient assays. This region does not include any HSV specific regulatory signals including the ICP4 binding site located approximately 100 base pairs away from the transcription initiation site (Everett 1983, 1984). Removal of the putative ICP4 binding site within the tk gene has no effect on tk expression in viruses bearing such mutant tk genes (Coen et al., 1986). In transient expression systems ICP4 is also able to activate transcription from a variety of non HSV genes, which presumably do not contain any ICP4 binding sites. These genes include the HIV LTR (Albrecht et al., 1989), Human Papilloma Virus (HPV) 16 early region (McCusker and Bacchetti, 1988), the SV40 early region (Everett, 1988) adenovirus E2a (Tremblay et al., 1985, Spessot et al., 1989) and the rabbit beta-globin promoter (Everett, 1984b). HSV transduced cellular genes such as rabbit beta-globin and human alpha-globin are regulated as true viral genes in an ICP4 dependent fashion (Smiley et al., 1987, Panning and Smiley, 1989, Duncan and Smiley, in preparation). From these different studies it is clear that ICP4 does not activate transcription by binding to specific DNA sequences located in the vicinity of gene promoters. It is conceivable, however, that activation by ICP4 requires specific DNA binding but the ICP4 binding sites can be

located at a distance from the promoter. Therefore, heterologous genes and gD, in transient assays, may be activated by binding of ICP4 to the reported ICP4 binding sites in the pBR322 vector (Faber and Wilcox, 1986). Furthermore, cellular genes integrated in the viral genome and HSV tk genes lacking an ICP4 binding site may still be regulated by ICP4 through distantly located sites also within the viral genome.

The only clear role of ICP4 binding to DNA is in ICP4 autoregulation. ICP4 has been shown to bind to sequences around the transcription initiation site of the ICP4 promoter (Muller, 1987, Faber and Wilcox, 1988). Binding of ICP4 to its cap site is strictly required to repress ICP4 transcription (Roberts et al., 1988). Recently, DiDonato and Muller (1989) demonstrated that binding of ICP4 to the ICP4 gene promoter changes the DNA geometry of the TATA box region. This conformational change may interfere with the binding of TFIID to the TATA box thereby inhibiting transcription initiation. The mechanism whereby ICP4 down regulates expression from the other IE genes is not known.

In summary, ICP4 provides an essential function required for HSV replication. ICP4 is required for the expression of E and L genes as well as the down regulation of IE gene expression. The role of ICP4

binding sites in the control of HSV gene expression is not clear and an understanding of the significance of these sites as well as the nature of interactions between ICP4 and host cell proteins is essential for an overall understanding of HSV gene regulation.

1.4.2 ICP0:

The IE gene ICP0 encodes a 110 000 dalton nuclear phosphoprotein which binds DNA in crude cell extracts (Pereira et al., 1977, Hay and Hay , 1980, Ackermann et al., 1984). Analysis of mutant viruses containing deletions in both copies of the ICP0 gene has demonstrated that ICP0 carries out an important viral function but is not strictly essential for viral replication in tissue culture. Viruses with a defective ICP0 gene display a profile of viral polypeptide synthesis and DNA replication similar to wild type virus. The burst size, however, is 10 to 1000 fold reduced. Moreover, the plaque size was reduced and the burst contained an extremely high particle to plaque forming unit (pfu) ratio when compared to wild type. Viral replication was impaired at low multiplicities of infection (moi) and this was overcome at a high moi (Stow and Stow, 1986, Sacks and Schaffer, 1987). These

experiments demonstrate that ICP0 carries out an important viral function that is not essential at higher multiplicities of infection in tissue culture.

Structure function studies have revealed that like ICP4, ICP0 is a complex protein which contains multiple essential domains (Everett, 1987b, Cai and Schaffer, 1989). Transient transfection studies have demonstrated that ICP0, by itself, is able to activate expression from HSV promoters. Specifically, ICP0 can transactivate promoters from all three kinetic classes of genes (Gelman and Silverstein, 1985, O'Hare and Hayward 1985a,b, 1987, Quinlan and Knipe , 1985, Everett, 1986). In addition, ICP0 and ICP4 can act synergistically to activate promoters to a greater extent than each gene individually (Everett, 1984b, 1986, O'Hare and Hayward, 1985a, Gelman and Silverstein, 1985, Quinlan and Knipe, 1985). ICP0 is also able to activate expression of heterologous genes such as the SV40 early region (Everett, 1988, HPV upstream regulatory region (McCusker and Bacchetti, 1988), and Adenovirus E3 gene (O'Hare et al., 1986). Paradoxically, ICP0 can activate transcription, by itself, in transient expression assays but is unable to activate expression from E and L genes during an infection with an ICP4 deletion mutant (DeLuca et al., 1985). The high particle to pfu ration observed

with ICP0 deletion viruses is probably the result of impaired E gene expression (Cai and Schaffer, 1989).

Although various studies have demonstrated that ICP0 encodes a transactivation function, the role of ICP0 during a viral infection is not clear. ICP0 is essential in the reactivation of HSV into the lytic cycle from the latent state (Harris et al., 1989). The observation that ICP0 can activate transcription from the ICP4 promoter (O'Hare and Hayward, 1985b, 1987) suggests that ICP0 initiates the lytic cycle by activating ICP4.

1.4.3 ICP27:

The gene for ICP27 encodes a 63 000 MW phosphorylated nuclear protein (Ackermann et al., 1984). Studies of viruses with temperature sensitive mutations mapping to the ICP27 gene have shown that ICP27 encodes an essential function for viral replication. (Sacks et al., 1985). Temperature sensitive ICP27 mutants display the following phenotypic characteristics: (i) over production of certain IE proteins, (ii) over expression of some E proteins as well as reduced expression of other E proteins, and (iii) greatly impaired ability to express L gene functions (Sacks et al., 1985). In addition, ICP27 null mutant viruses are DNA replication deficient

(McCarthy et al., 1989). Taken together, the analysis of various ICP27 mutant viruses has shown that ICP27 appears to possess both stimulatory as well as inhibitory activity and these activities are involved in regulating the cascade of HSV gene expression.

Results of transient transfection experiments are in agreement with the analysis of viral mutants. ICP27, by itself, is unable to affect the basal level of promoter activity of isolated viral genes of all three kinetic classes except gB (Gelman and Silverstein, 1985, O'Hare and Hayward, 1985a, Everett, 1986, Sekulovich et al., 1988, Rice and Knipe, 1988). However, ICP27 is able to repress the activation of IE and some E genes by ICP4 and ICP0 (Sekulovich et al., 1988) and enhance the ICP4 and ICP0 dependent activation of some E and L genes (Sekulovich et al., 1988, Everett, 1986). Recently, Rice colleagues have shown that the stimulatory and inhibitor activities of ICP27 map to different functional domains of the ICP27 polypeptide and both activities can be mutationally separated (Rice et al., 1989). Some overlap in the transactivation and repression domains however exists (Hardwicke et al., 1989). Collectively, these experiments show that ICP27, directly or indirectly, can facilitate both up and down regulation of HSV genes and therefore plays an essential role in regulating HSV gene

expression.

1.4.4 ICP22 and ICP47:

The genes encoding ICP22 and ICP47 encode polypeptides with molecular weights of 68 000 and 12 000 respectively. Analysis of viruses containing truncated versions of the ICP22 gene has demonstrated that this gene is required for viral replication in some cell types including Rat-1 and human embryonic lung cells (HEL), but is not required in HEp-2 and Vero cells. The growth defect of ICP22 mutant viruses can be attributed to the lack of late viral gene expression in non permissive cells (Sears et al., 1985). However, since the ICP22 mutants still synthesize the amino terminal 30 % of the protein it is not known if ICP22 is strictly essential for viral replication in all cell types (Post and Roizman, 1981). Perhaps in some cell types the amino terminus of ICP22 suffices for proper late gene expression and other cell types require larger portions of the ICP22 polypeptide. Construction of a true ICP22 null mutant is required to determine if this polypeptide carries out an essential viral function.

Viruses deleted for the entire ICP47 coding sequences have been constructed. These viruses are

replication competent in all cell types tested therefore ICP47 is dispensable for growth in tissue culture (Longnecker and Roizman, 1986, Mavromara-Nazos et al., 1986).

1.5 Vmw65 and Immediate Early Gene Activation

The coordinate induction of IE gene transcription is activated positively by the virion structural protein Vmw65. As previously mentioned, a variety of experiments including promoter transplant in combination with deletion analysis and promoter reconstruction experiments have demonstrated that the TAATGARAT motif is required for responsiveness to Vmw65 transinduction (Post et al. 1981, Batterson and Roizman, 1983, Cordingly et al., 1983, Kristie and Roizman, 1984, Preston et al., 1984, Gaffney et al., 1985, Bzik and Preston, 1986). The TAATGARAT is found at least once in all IE far upstream regions (Mackem and Roizman, 1982a).

Attempts to demonstrate specific binding of Vmw65 to TAATGARAT have failed, indeed Vmw65 does not bind to DNA at all (Marsden, 1987). Instead, Vmw65 mediates transactivation of IE genes by associating with cellular proteins which in turn bind to the TAATGARAT element (McKnight et al., 1987, O'Hare and Goding, 1988, Preston

et al., 1988). These cellular proteins include the octamer binding protein OCT-1 and at least one other unidentified cellular protein (Gerster and Roeder, 1988, apRHYS et al., 1989, O'Hare et al., 1988, Stern et al., 1989). Some TAATGARAT motifs contain an overlapping octamer sequence but OCT-1 is able to recognize and bind to TAATGARAT sequences which do not contain an overlapping octamer sequence (Baumruker et al., 1988).

Mutational analysis of Vmw65 has revealed 2 separate regions which are both necessary for transactivation of IE gene transcription. The amino terminal 411 amino acids are required for binding to host cell proteins OCT-1 and perhaps others, while the carboxyl terminal domain is required for stimulating transcription. The transactivation domain, specified by the carboxyl terminal amino acids 411 to 490, contains a high proportion of acidic amino acids residues and therefore contains a net negative charge (Triezenberg et al., 1988a, Ace et al., 1988). When this acidic tail of Vmw65 is fused to the DNA binding domain of the yeast transcription activating protein GAL4 the resultant fusion protein is a potent transactivator of promoters containing the GAL4 binding site (Sadowski et al, 1988). Vmw65 therefore belongs to the class of transcription factors which activate transcription via an acidic tail,

similar to GAL4 and GCN4. Presumably the acid tail of Vmw65 interacts with other proteins involved with transcription regulation (reviewed in Ptashne, 1988). The nature of these proteins and their interaction with Vmw65 is not known.

1.6 Project Rational

The experiments described in this thesis were designed to specifically address two main questions:

1) Is the TAATGARAT element, by itself, when present immediately upstream of a TATA box sufficient to serve as a target for Vmw65 transactivation ?

The TAATGARAT motif was identified as a target for Vmw65 by sequence deletion experiments and by promoter reconstruction experiments, as previously mentioned (Cordingly et al., 1983, Kristie and Roizman, 1984, Preston, 1984, Gaffney et al., 1985, Bzik and Preston, 1986). In both of these types of experiments various IE gene upstream regions were fused to reporter genes already containing a functional promoter which was previously unresponsive to Vmw65. Even though these experiments identified the TAATGARAT element as a cis

acting target for Vmw65 transactivation, they did not determine whether TAATGARAT was the only motif required. The previous experiments did not limit the possible protein-protein interactions with Vmw65 since other cis acting sequence elements present in the promoter of the reporter gene may have bound transacting proteins which provided essential contacts with Vmw65. Indeed, deletion studies of IE promoter regions have demonstrated that sequences flanking the TAATGARAT motif affect the ability of Vmw65 to activate transcription (Preston et al., 1984, Bzik and Preston, 1986, Triezenberg et al., 1988b).

An answer to this first question is necessary to gain an understanding of the mechanism by which Vmw65 activates transcription. Such an understanding may in turn lead to insights into how all transcription factors with acidic domains activate transcription.

2) Is the TAATGARAT element ,by itself, and subsequent transactivation by Vmw65 responsible for directing IE gene kinetics ?

Even though a correlation exists between promoter complexity and kinetic class little is known about the exact sequence requirements essential for temporal regulation of each class of HSV genes. The presence of

the TAATGARAT motif in all five IE genes suggests that transactivation of IE genes by Vmw65 may be sufficient for high level of expression during the earliest stages of viral infection before any transcription occurs from the E or L viral genes. This suggestion cannot however be supported by the experiments which initially identified the TAATGARAT motif as a Vmw65 target for two reasons. Firstly, as mentioned above, in all of the deletion analysis or promoter reconstruction experiments other sequence elements where present in addition to TAATGARAT. Secondly, most of the experiments which identified the TAATGARAT motif involved transient transfection experiments whereby a reporter gene driven by a promoter containing a TAATGARAT motif was a target for either a co-transfected Vmw65 or Vmw65 supplied by viral infection (Cordingly et al., 1983, Kristie and Roizman, 1984, Preston et al., 1984, Gaffney et al., 1985, Bzik and preston, 1986). In these types of experiments the TAATGARAT containing target gene was not in the context of the viral genome and therefore the role that TAATGARAT plays in mediating IE temporal regulation could not be determined. An understanding of the sequence elements which direct RNA pol II to transcribe HSV genes at certain times during infection is necessary for elucidating the mechanisms of differential gene

regulation of HSV genes.

1.7 Experimental Design

Answers to both of the above questions require the construction of minimal promoters containing only well defined sequence elements. To this end I have constructed synthetic promoters built by linking tandem repeats or single copies of an oligonucleotide containing the sequence of the TAATGARAT motif found between position -147 and -164 of the ICP0 gene immediately upstream of another synthetic oligonucleotide containing the TATA sequence of the adenovirus type 2 major late promoter. To determine if the synthetic promoters could serve as targets for Vmw65 transactivation a rabbit beta-globin reporter gene was linked to one of the synthetic promoters and co-transfected into cells with a plasmid harboring the Vmw65 gene. To determine the role of Vmw65 transactivation in mediating IE gene kinetics various synthetic promoters were introduced into the viral genome. The transcripts produced by these synthetic promoters were then assayed for temporal appearance during infection with the recombinant viruses.

MATERIALS AND METHODS

2.1 Growth of Bacterial Cells

Bacterial cells (E. coli strain HB101) were grown either in suspension culture or on solid Luria Broth (LB) agar surfaces. Suspension cultures were grown with agitation at 37°C in LB or M9 media. Unless otherwise indicated all percent concentrations are weight/volume. LB consists of: 1% Bactotryptone (Difco), 0.5% Yeast extract (Difco), 125 mM NaCl, 10 mM Tris HCl pH 7.6, and 0.4% glucose. M9 media consists of: 40 mM Na_2HPO_4, 22 mM KH_2PO_4, 12.5 mM NaCl, 19 mM NH_4Cl, 0.1% Casamino Acids (Difco), 1mM $MgSO_4$, 0.0001% Thiamine, 0.1 mM $CaCl_2$ and 0.2% glucose. Where appropriate antibiotic selection was performed by adding 20 ug/ml ampicillin (Sigma). Bacterial colonies were grown at 37°C on solid LB agar which consists of LB supplemented with 1.5% bacto-agar (Difco) and selection was carried out by addition of ampicillin when required.

2.2 Isolation of Plasmid DNA

2.2.1 Large Scale Isolation of Plasmid DNA

20 ml of a saturated overnight (o/n) culture of HB101 was used to inoculate 1 litre (L) of LB or M9 containing ampicillin and this culture was incubated for 12-16 hours. Alternatively, the large culture was allowed to grow until the OD_{600} was 0.7-0.8 at which time plasmid amplification was carried out by the addition of 170 mg of chloramphenicol and then allowed to incubate for a further 12-16 hours.

Saturated cultures were centrifuged for 20 minutes (min) at 6 000 RPM and resuspended in 20 ml of glucose buffer (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA) containing 5 mg/ml lysozyme (Boeringer Manheim) and incubated at room temperature for 5 min. 40 ml of freshly made alkaline SDS (0.2 M NaOH, 1% SDS) was then added with subsequent incubation on ice for 10 minutes (min.) whereupon 30 ml of 5 M KAc pH 4.8 was added. This mixture was then incubated for a further 15 min on ice.

The resulting mixture was then centrifuged at 20 OOO RPM for 20 min at 4^{o}C using an SW28 Beckman rotor. DNA in the supernatant was precipitated with 2.5 volumes of isopropanol and pelleted by centrifugation at 10 000 RPM for 15 min at 4^{o}C. DNA was resuspended in 5-8 ml TE (10 mM Tris pH 7.5, 1mM EDTA) and an equal volume of 5M

LiCl was added with subsequent incubation on ice for 15-30 min. The RNA precipitate was pelleted by centrifugation at 10 000 RPM for 15 min at 4°C and the DNA in the supernatant was precipitated with 2.5 volumes of 95% ethanol and pelleted by centrifugation at 10 000 RPM for 15 min at 4°C. The DNA was resuspended in 0.5 ml TE (as above) placed in an eppendorf tube and treated with 40 ug/ml RNase A for 15 min at 37°C. One volume of a polyethylene glycol (PEG) solution (13% PEG, 1.6 M NaCl) was then added with subsequent incubation on ice for 30 min. The resulting DNA precipitate was then pelleted by centrifugation at 13 000 RPM in an eppendorf centifuge and resuspended in 0.5 ml of distilled water.

An equal volume of phenol/chlorophorm (1:1 v/v, the phenol was prepared as in Maniatis et al. 1982, the chlorophorm is a solution consisting of 24 parts chlorophorm to 1 part isoamyl alcohol) was then added, vortexed briefly, centrifuged at 13 000 RPM for 5 min. and the aqueous layer removed. The aqueous layer was extracted with phenol/chlorophorm until no precipitate was seen at the aqueous interphase. DNA in the aqueous phase was precipitated by adding NaCl to a final concentration of 0.15 M and 2.5 volumes of 95% ethanol followed by centrifugation at 13 000 RPM for 15 min. The pellet was resuspended in 0.5 ml of 0.3 M sodium acetate,

and the DNA was re-precipitated with 2.5 volumes of ethanol folowed by centrifugation at 13 000 RPM for 15 min. The DNA was then washed first with 70% ethanol and then with 95% ethanol and dried using a Speed-Vac lyophilizer and redissolved in 1 ml of distilled water.

In all other protocols 'washing' the DNA indicates that the DNA was first washed with 70% ethanol and then washed with 95% ethanol. Furthermore 'drying' indicates that the DNA was dried in a Speed-Vac lyophilizer.

2.2.2 Small Scale Isolation of Plasmid DNA

The procedure used is a modification of the procedure devised by Birnboim and Doly, 1979. 1 ml of a confluent overnight culture of E.coli was centrifuged for 30 seconds at 13 000 RPM . The bacterial pellet was resuspended in 100 ul of glucose buffer containing 5 mg/ml lysozyme (Boeringer Manheim) and incubated at room temperature for 5 min. 200 ul of alkaline SDS was then added with subsequent incubation on ice for 5 min. whereupon 150 ul of 3 M sodium acetate pH 4.8 was added. The mixture was then incubated for another 15 min. on ice followed by centrifugation in an eppendorf centrifuge for 5 min. The supernatant was extracted once with

phenol/chlorophorm (as above). DNA was precipitated by adding 2.5 volumes of ethanol, pelleted by centrifugation, redissolved in 200 ul of 0.3 M sodium acetate, and re-precipitated by adding 2.5 volumes of ethanol. The DNA was washed, dried, and resuspended in 50 ul distilled water and treated with 40 ug/ml RNase A for 15 min. at 37^{o}C.

In cases when DNA isolated from this small scale plasmid isolation procedure was used as a template for double stranded DNA sequencing (see below) 3 M sodium acetate was added to the resuspended DNA after RNase A treatment to a final concentration of 0.3 M in a volume of 0.2 ml. The DNA was then reprecipitated with ethanol, washed, dried, and resuspended in 30 ul of distilled water.

2.3 Generation of Recombinant Plasmids

Most of the cloning procedures followed are detailed in Maniatis et al. 1982. Similarly, digestions using restriction endonucleases were performed according to the conditions outlined in Maniatis et al. 1982. Restriction enzymes and other enzymes used for cloning were obtained mainly from Bethesda Research Laboratories (BRL) or Pharmacia.

When the insert to be cloned consisted of double stranded oligonucleotides tandemly repeated, the insert was prepared as follows: 2 ug of each complementary oligonucleotide was 5' end labelled with gamma-^{32}P ATP (New England Nuclear) according to the procedure outlined in Maniatis et al. (1982) for 5' end labelling linkers. The oligonucleotides were then annealed by mixing 0.5 ug of each 5' end labelled oligonucleotide in 9 ul of 1 X ligase buffer (50 mM Tris HCl pH 7.6 , 10 mM $MgCl_2$, 1mM ATP, 1mM dithiothreitol) and incubating this solution at 37^{o}C for 1/2 hour. The annealed oligonucleotides were then ligated together by adding 1 unit of T4 DNA ligase to the above solution and allowing the reaction to incubate at 4^{o}C for 4 hours. When the insert to be cloned was a single copy of a double stranded oligonucleotide the complementary single stranded oligonucleotides were annealed in 1 X ligase buffer as above. Oligonucleotides were purchased from the Central Facility of the Institute for Molecular Biology and Biotechnology of McMaster University.

2.4 Gel Electrophoresis

Throughout this work 2 types of gel matrices, agarose and polyacrylamide, were employed to separate DNA

molecules. Agarose gels were prepared by melting agarose (Sigma) 0.7-1% in TBE (0.089 M Tris, 0.089 M Boric acid, 0.002 M EDTA, pH 8.3) and casting gels into either horizontal or vertical gel molds. Vertical polyacrylamide gels were also prepared with TBE and final polyacrylamide concentrations ranged from 5-10%. Acrylamide stock solutions contained 40 parts acrylamide (Bio-Rad) and 1 part bis-acrylamide (Bio-Rad). Polymerization was catalyzed with 50-200 ul TEMED (N,N,N',N'-tetramethylethylene diamine) (Bio-Rad). Both types of gels were run with a TBE buffer system using a Pharmacia constant voltage power supply. Visualization of the DNA was performed by soaking the gel in TBE containing 0.5 ug/ml ethidium bromide followed by illumination by an ultra violet light source. Alternatively the gel was exposed to Kodak XAR-5, fast, X-ray film for autoradiography in cases when the DNA molecules were labelled with a radioactive isotope.

Isolation of DNA fragments from preparative polyacrylamide gels involved excising the appropriate band from the gel and placing it in an eppendorf centrifuge tube. The gel fragment was then mashed with a teflon plunger with subsequent incubation in gel elution buffer (0.5 M sodium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS) at 37^{o}C o/n. The following day the

polyacrylamide was pelleted by centrifugation and the DNA in the supernatant was precipitated with ethanol.

2.5 Bacterial Transformation

2.5.1 Making Competent Cells

E. coli strain HB101 were made competent for DNA transformation using the calcium chloride/ rubidium chloride procedure modified from Maniatis et al. 1982. 5 ml of a saturated culture of HB101 was used to inoculate 50 ml of LB. This culture was then alowed to grow untill the OD_{600} reached 0.6-0.7 (approximately 5 X 10^7 cells/ml). The bacteria were pelleted by centrifugation at 2700 RPM for 10 min. at 4°C in an IEC table-top centrifuge and subsequently resuspended in 20 ml MOPS I (morpholinopropane sulfonic acid) (50 mM MOPS pH 7.0, 10 mM RbCl). Bacterial cells were recovered by centrifugation at 2700 RPM in the IEC centrifuge (as above) and were resuspended in 20 ml of MOPS II (115 mM MOPS pH 6.5, 80 mM $CaCl_2$, 11.5 mM RbCl). Bacterial cells were again pelleted as before and resuspended in 2 ml MOPS II and stored at 4°C.

2.5.2 Transformation of Bacterial Cells

DNA (10 ul) was added to 100 ul of competent cells with subsequent incubation on ice for 30 min. The cells were then heat shocked at 42°C for 1 min. One ml of LB was then added to the transformed cells. Aliquots of up to 100 ul of transformed bacteria were plated on solid LB plates containing ampicillin and incubated o/n at 37°C to allow for colonies to grow.

2.6 Colony Hybridizations

Bacterial colonies were grown on solid LB agar surfaces until they reached a size of 2-3 mm in diameter. Circular disks of nitrocellulose (0.45 um pore size) (Schleicher and Schuell) were then placed on top of the colonies and allowed to make contact with all of the colonies. The disks were then carefully removed ensuring that most of the bacterial colony remained attached to the nitrocellulose filter. The nitrocellulose disks were then peeled off of the LB agar plate and placed on 4 layers of Watman 3MM paper soaked in 0.5 M NaOH for 1 hour, with the colonies on the upper surface. Subsequently, the disks were soaked, twice, in 1 M Tris pH 7.5 for 10 min, then soaked in a solution of 1 M Tris pH 7.2, 1.5 M NaCl for 10 min., and finally soaked in 20

X SSC for 10 min. The disks were then dried at room temperature and baked for 2-3 hours at 80°C.

The baked nitrocellulose disks were then soaked in 6 X SSC, sealed in a plastic bag (4-6 disks/bag) and 20 ml of pre-hybridization solution (6 X SSC, 0.4% bovine serum albumin, 0.4% Ficoll, 0.4% polyvinylpyrolidone and 250 ug/ml denatured salmon testes DNA) was added. Pre-hybridization was allowed to proceed for 2-4 hours at room temperature with agitation provided by a rotating plate. The pre-hybridization solution was then replaced by 20 ml hybridization solution consisting of 6 X SSC, 10% dextran sulfate, 125 ug/ml denatured salmon testes DNA, 10 mM Tris-HCl pH 7.5, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrolidone and 1 X 10^6 cpm/ml of 5' end labelled oligonucleotide. Oligonucleotides were 5' end labelled with gamma-p^{32} ATP according to the protocol of Maxam and Gilbert, 1980. Hybridization proceeded o/n at room temperature using a rotating plate. The following day the hybridization solution was removed and the disks were washed according to the conditions specific to the different oligonucleotide probes used. These conditions are summarized as follows; AB 228: 6 X SSC for 15 min. at room temperature followed by 0.1 X SSC for 3 min. at room temperature followed by 0.1 X SSC for 3 min. at 36°C, AB

38 and AB 16: 3, 5 min. washes with 2 X SSC containing 0.1% SDS at 60°C. The disks were then allowed to air dry and were exposed to Kodak XAR-5 film in a cassette containing intensifier screens.

2.7 DNA Sequencing

All sequences were generated with the chain-termination method using denatured double stranded DNA as a template (Zhang, et al. 1988).

2.7.1 Preparation of the Template

DNA was denatured by adding 10 ug of large scale LiCl prepared DNA, or 10 ul of small scale isolated DNA (see above) to a solution of 250 mM NaOH, 8 mM EDTA in a total volume of 20 ul and left to stand at room temperature for 5 min. This solution was then quickly neutralized by adding 2 ul of 2M ammonium acetate (pH 4.6), and the DNA was precipitated by adding 3 volumes of cold ethanol. The DNA was washed, dried, and resuspended in 16 ul distilled water.

2.7.2 Annealing Template and Primer

The annealing reaction was performed by mixing 7 ul of denatured template (prepared as above), 1 ul of oligonucleotide primer (0.25 OD/ml) and 2 ul 5 X sequenase buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl). This mixture was then incubated in a 65^{o}C water bath for 5 min. and allowed to cool slowly to room temperature for 30 min.

2.7.3 Labelling Reaction

To the 10 ul mixture containing the annealed template and primer (above) the following was added: 1 ul 0.1 M dithiothreitol, 2 ul of labelling mix (7.5 uM dGTP, 7.5 uM dATP, 7.5 uM dTTP) diluted 1:5, 1 ul alpha-^{32}P dCTP (specific activity 3000 Ci/mmol) (Dupont) and 2 ul of sequenase enzyme (United States Biochemical) diluted 1:8 in ice cold TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA). This mixture was then incubated at room temperature for 5 min.

2.7.4 Termination Reactions and the Sequencing Gel

All of the termination mixes contained 80 uM dGTP, 80 uM dATP, 80 uM dTTP, and 80 uM dCTP, in addition the G,A,T, and C termination mixes contained 5 uM ddGTP,

5 uM ddATP, 5 uM ddTTP, and 5 uM ddCTP repectively. Prior to the labelling reaction 4 eppendorf tudes were set up, each containing 2.5 ul of one of the termination mixes. After the labelling reaction 3.5 ul of the labelling reaction mixture was added to each eppendorf containing the termination mixes. These mixtures were then incubated for 10 min. at 37^{o}C and the reactions were stopped by adding 4 ul sequencing dye (90% formamide (v/v), 0.02% (w/v) bromophenol blue, 0.02% xylene cyanol, 0.089 M Tris, 0.089 M Boric acid, 0.002 M EDTA). The reaction mixtures were then incubated at 80-90^{o}C for 2 min. and placed immediately on ice. Three ul of each reaction was run on a pre-warmed 8% polyacrylamide sequencing gel containing 7M urea. The gel was then dried and exposed to Kodak XAR-5 or Kodak XRP-1, slow, x-ray film in a screenless cassette.

2.8 Mammalian Cell Culture

Vero cells (African green monkey kidney cells) and mouse fibroblasts (LTA cells) were grown as monolayer cultures in Corning tissue culture flasks or plates. Both types of cells were grown at 37^{o}C in a humidified atmosphere enriched with 5% (v/v) CO_2. Vero cells were maintained in alpha-modified minimal essential medium

(alpha-MEM) (Gibco) supplemented with 5% (v/v) fetal bovine serum (Gibco), 1% (v/v) L-glutamine (Gibco), 1% (v/v) penicillin-streptomycin (Gibco), and 2% $NaHCO_3$. LTA cells were maintained in the same medium containing the same supplements except 10% fetal bovine serum was added.

2.9 Growth and Titration of Virus

Monolayers of Vero cells (approximately 2 X 10^7 cells per 150 cm^2 plate) were infected at an MOI of approximately 0.05 in a volume of 10 ml of serum free alpha-MEM supplemented with 1% L-glutamine, 1% penicillin-streptomycin, and 2% $NaHCO_3$. Two hours later 20 ml of alpha-MEM containing 5% FBS was added to the infected plates. When confluent cytopathic effect (cpe) was evident (2-3 days post infection) the cells were harvested by scraping the plates and resuspended in phosphate buffered saline (PBS) (0.137 M NaCl,2.68 mM KCl, 0.88 mM KH_2PO_4, 6.41 mM Na_2HPO_4, pH 7.3). The cells were recovered by centrifugation at 3000 RPM at 4^oC in a table top centrifuge and subsequently resuspended in alpha-MEM containing 5% FBS. Virus was recovered by disruptiong the cells by sonication and cell debris was removed by centrifugation. The supernatant (viral stock) was then stored frozen at -70^oC.

For titration of viral stocks serial dilutions were made ranging from 10^{-2} to 10^{-7} in serum free alpha-MEM. These dilutions were used to infect confluent monolayers of Vero cells growing in 24 well Nunc dishes. Two hours latter the dishes were overlaid with alpha-MEM containing 5% FBS serum and 0.05% human immune serum (Connaught) and plaques were counted 3 days post infection.

2.10 Construction of Recombinant Viruses

Recombinant viruses were constructed according to the method of Smiley, 1980. Infectious viral DNA and linearized plasmid DNA, harbouring the herpes simplex virus tk gene containing the desired mutation, were co-transfected into Vero cells with subsequent isolation of tk deficient viruses (see below).

2.10.1 Preparation of Infectious Viral DNA

Ten plates of confluent Vero cells were infected with virus at an MOI of 10. When confluent cpe was evident the cells were harvested, as described above, and the cell pellet was resuspended in 10 ml 0.2 M EDTA pH 8.0. SDS and proteinase K (Boeringer Manheim) were added

to final concentrations of 0.5% and 100 ug/ml respectively followed by incubation o/n at 37°C. The following day the mixture was extracted 4 times with phenol and transferred to 15.9 mm diameter standard cellulose dialysis tubing. Dialysis was against 0.1 X SSC with multiple changes over 3-4 days. Viral DNA was collected from the dialysis tubing in a sterile laminar flow hood and stored in a sterile 50 ml Corning tube at 4°C. Various volumes of the viral DNA, ranging from 1 to 100 ul, were transfected into Vero cells (as described below) to titre for infectivity.

2.10.2 Transfection of Cells

This procedure is a modification of the method devised by Graham and Van der Eb, 1973. A $CaCl_2$ precipitate of viral and plasmid DNA (when appropriate) was prepared by mixing 0.250 ml of HEPES buffered saline (42 mM HEPES, 27 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 11 mM dextrose, pH 7.1), 20-60 ul infectious viral DNA, 1 ug linearized plasmid (when appropriate), 10 ug denatured salmon testes DNA, and H_2O to a final volume of 0.475 ml. 25 ul of 2.5 M $CaCl_2$ was then added and the mixture was aggitated by shaking the eppendorf tube briefly but vigorously. The transfection cocktail was then allowed to

stand at room temperature for 30 min. Subsequently the transfection cocktail was added to 50% confluent monolayers of Vero cells growing in 60 cm^2 plates containing 5 ml of alpha-MEM supplemented with 5% FBS. The plates were then incubated at 37°C for 4-6 hours before being subjected to a glycerol shock.

Glycerol shocking was performed by aspirating the medium and transfection cocktail from the transfected plates and adding 1 ml of serum free alpha-MEM containing 15% glycerol. This medium was left on the cells for 1 min whereupon the medium was aspirated and the cells were washed twice with 5 ml of serum free alpha-MEM. Five ml of alpha-MEM containing 5% FBS was then added to the cells with subsequent incubation at 37°C until confluent cpe was evident (5-7 days post transfection). Virus obtained from transfection was harvested and titred as described above.

2.10.3 Isolation of tk^- Virus

Thymidine kinase deficient mutants were isolated on the basis of their resistance to the base analogue 5-bromo-2'-deoxycytidine (BrdC). BrdC is efficiently incorporated into the DNA by wild type tk (tk+) viruses resulting in the "poisoning" of tk+ viruses.

Confluent monolayers of Vero cells growing in 100 cm^2 dishes were infected with 2 X 10^4 plaque forming units (pfu) of virus obtained from co-transfections using 3 ml serum free alpha-MEM. Two hours later 7 ml of alpha-MEM containing 5% FBS, 100 ug/ml 5-bromo-2'-deoxycytidine (Sigma), and 0.05% (v/v) human immune serum (Connaught) was added with subsequent incubation until plaques were visible (2-3 days). The medium was aspirated, and using a sterile wooden stick virus infected cells, from individual plaques, were transferred into one well of Nunc 24 well dishes in which confluent monolayer cultures of Vero cells were growing. Infection took place in the presence of 100 ug/ml Budc to further select for tk deficient viruses. Virus was harvested for rapid extraction of viral DNA from those wells which displayed confluent cpe.

2.11 Rapid Extraction of Viral DNA

At confluent cpe cells were harvested from Nunc 24 well dishes by scraping each well and placing 1/2 of the volume in an eppendorf tube. The remaining 1/2 volume was frozen down and stored at -70^{o}C. Cells were pelleted by centrifugation in an eppendorf centrifuge and resuspended in 0.4 ml pronase buffer (10 mM Tris-HCl pH

7.8, 10 mM EDTA, 0.5% SDS). Pronase was added to a final concentration of 1 mg/ml with subsequent incubation at 37°C for 3 hours. The mixture was then extracted twice with phenol/chloroform and the DNA was precipitated with 2.5 volumes of ethanol, washed, dried and resuspended in 30 ul H_2O.

2.12 Nick Translation

DNA was labelled by mixing 1 ug of plasmid DNA with the following reagents in a final volume of 45 ul: 55 mM Tris-HCl pH 7.4, 5.5 mM $MgCl_2$, 5.5 mM 2-mercaptoethanol, 0.17 mM each of dATP, dTTP, dGTP, and 100 uCi alpha-^{32}P labelled dCTP (specific activity 3000 Ci/mmol, New England Nuclear). 250 pg of activated DNase (see below) was then added in a volume of 2.5 ul and the reaction was allowed to proceed for 1 min. at room temperature. 20 units of E.coli DNA polymerase I (BRL) was then added with subsequent incubation at 14°C for 2 hours. The reaction was stopped by adding EDTA and SDS to final concentrations 20 mM and 0.1% respectively in a total volume of 200 ul. This mixture was then extracted once with phenol/chloroform and placed in a 15 ml glass Corex centrifuge tube. DNA was precipitated by adding 100 ug E.coli carrier tRNA (Boeringer Manheim) and 7.5 ml ice

cold 10% trichloroacetic acid with subsequent incubation on ice for 15 min. DNA was pelleted by centrifugation at 12 000 rpm for 15 min. at 4°C. The pellet was washed twice with 10 ml of nick translation wash solution (70% (v/v) ethanol, 10 mM Tris-HCl pH 8.0, 1mM EDTA) and resuspended in 1 ml TE (100 mM Tris-HCl pH 8.0, 1 mM EDTA). The nick translated DNA was denatured by boiling for 15 min. before being used as a probe.

DNase 1 was activated by adding 90 ul activation buffer (10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 150 mM NaCl, and 1 mg/ml of boiled bovine serum albumin pH 7.4) to 10 ul DNase (1 mg/ml in 0.1 M HCl) with subsequent incubation on ice for 2 hours. Before use the activated DNase was diluted 1000 fold in activation buffer.

2.13 Southern Blots

The protocol followed is a modification of the technique developed by Southern, 1975. After running the agarose gel, the gel was soaked in the following solutions: 0.25 M HCl for 30 min with fresh HCl added after 15 min., 0.5 M NaOH for 60 min., 1.5 M NaCl/ 1 M Tris-HCl pH 7.5 for 30 min, and 20 X SSC for 30 min. The transfer apparatus was assembled as follows: 4 pieces of Watman 3 MM paper presoaked in 20 X SSC were placed on

top of a larger piece of saran wrap. The gel was then placed on top of the presoaked 3 MM paper and 1 piece of nitrocellulose was then placed on top of the gel. Two more pieces of 3 MM paper presoaked in 20 X SSC were then placed over the 3 MM paper. The saran wrap was then rolled around the edges of the apparatus to provide a reservoir for the 20 X SSC. Finally, a stack of paper towels were placed on top of the apparatus and weighted down. After allowing the gel to blot o/n the nitrocellulose was soaked for 5 min in 2 X SSC and baked at 80^{o}C for 2 hours.

The baked nitrocellulose was then soaked in 2 X SSC and sealed in a plastic bag. 20 ml of prehybridization solution (2 X SSC, 0.4% bovine serum albumin, 0.4% Ficoll, 0.4% polyvinylpyrolidine, 250 ug/ml denatured salmon testes DNA) was then added and the blot was allowed to prehybridize for 3-4 hours. The prehybridization solution was then replaced with hybridization solution (2 X SSC, 10 mM Tris-HCl pH 7.5, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpryrolidone, 10% dextran sulfate, and 1 X 10^{6} cpm/ml denatured nick translated probe) and the blot was allowed to hybridize o/n at 60^{o}C. The next day the blot was washed 3 times with wash solution 1 (2 X SSC, 0.1% SDS) at 67^{o}C followed by three washes with wash solution

2 (0.1 X SSC, 0.1% SDS) at 54°C. Subsequently, the blot was air dried and exposed to Kodak XAR-5 x-ray film in a cassete containing intensifier screens.

2.14 Extraction of Cytoplasmic RNA

Cytoplasmic RNA was extracted by NP40 lysis of cells as described in Berk and Sharp, 1977. All solutions used in this procedure were rendered RNase free by treatment with 0.1% diethylpyrocarbonate (DEPC) followed by autoclaving.

Vero or LTA cells were collected by scraping the cell monolayers and suspending the cells in PBS. The cells were then placed in a 15 ml Corning tube and pelleted by centrifugation in a table top centrifuge at 2700 rpm at 4°C. Following centrifugation the cell pellet was then resuspended in 1 ml isotonic buffer (0.15 M NaCl, 10 mM Tris-HCl pH 7.8, 1.5 mM $MgCl_2$) such that the cell concentration was 1-3 X 10^7 cells/ml and transferred to a 15 ml Falcon tube. One ml of isotonic buffer containing 2% NP40 was then added with subsequent incubation on ice for 5 min. This mixture was the vortexed for 5 seconds and cell nuclei were pelleted by centrifugation at 5000 rpm for 5 min. at 4°C using a pre-cooled Beckman JA20 rotor and pre-cooled Beckman J2-21

centrifuge. The supernatant was then transferred to a new Falcon tube and 2 ml of urea/SDS (7M urea, 0.35 M NaCl, 10 mM Tris-HCl pH 7.8, 10 mM EDTA, 1% SDS) was added with subsequent incubation at room temperature for 5 min. The mixture was then extracted three times with phenol/chloroform and the aqueous phase was transferred to a 15 ml glass corex tube which was made RNase free by baking at 250°C for 4 hours. RNA was precipitated by adding 3 volumes of ice cold 95% ethanol and the RNA was pelleted by centrifugation at 12 000 RPM for 30 min. at 4°C. Subsequently the RNA pellet was washed 4 times with RNA wash solution (2.5:1 solution of 95% ethanol: 0.4 M NaAc pH 5.2) followed by 1 wash with 95% ethanol. After the washes the RNA was dried using a Speed-Vac lyophilizer (without heat), and was resuspended in 0.6 ml DEPC treated H_2O. RNA concentration was determined by UV absorbance spectroscopy at 260 nm. The RNA was stored frozen at -70°C.

2.15 Gel Purification of Oligonucleotides

Oligonucleotides were 5' end labelled with gamma-^{32}P ATP according to the method of Maxam and Gilbert, 1980. Labelled oligonucleotides were then run on an 8% sequencing gel containing 7M urea. The gel was then

exposed to Kodak XAR-5 x-ray film for 2 min. The fragment of the gel containing the oligonucleotides was then cut from the gel and allowed to incubate in 0.5 ml elution buffer (0.5 M NaAc, 10 mM $MgCl_2$, 1mM EDTA, 0.1% SDS), containing 15 ug E . coli carrier tRNA, overnight at 37°C. The following day the polyacrylamide was removed by centrifugation using an eppendorf centrifuge and the oligonucleotides in the supernatant were recovered by precipitation using 3 volumes of cold 95% ethanol. After pelleting the oligonucleotides by centrifugation they were resuspended in 200 ul 0.3 M sodium acetate pH 5.6 and reprecipitated following the addition of 3 volumes of ethanol. After centrifugation the pellet was washed, dried, and resuspended in TE (10 mM Tris-HCl pH 7.9, 1 mM EDTA).

2.16 Primer Extension

RNA was primer extended according to the method of Smiley et al., 1987. A 10-30 ug sample of cytoplasmic RNA (as extracted above) was precipitated out of 0.3 M sodium acetate pH 5.2. The RNA pellet was then washed with 70% then 95% ethanol and dried for 1 hour in a Speed-Vac lyophilizer without using heat. The pellet was then resuspended in 8 ul TE (10 mM Tris-HClpH 7.9, 1 mM

EDTA) containing 30 000-50 000 cpm of gel purified 5' end labelled oligonocleotide (see above). Two ul of TKE (10 mM Tris-HCL pH 7.9, 1 mM EDTA, 1.25 M KCl) was then added and the mixture was incubated at 60°C for 1 hour to promote hybridization between RNA and the oligonucleotide primer. Upon cooling to room temperature 25 ul of reverse transcriptase buffer (20 mM Tris-HCl pH 8.7, 10 mM $MgCl_2$, 5 mM DTT, 0.33 mM of each dATP, dTTP, dGTP, and dCTP, 10 ug/ml actinomycin D) containing 10 units of avian myeloblastosis virus reverse transcriptase (Life Sciences) was added and the mixture was incubated at 37°C for 1 hour. The extension products were precipitated by adding 0.3 ml cold 95% ethanol. After centrifugation the pellet was washed with 70% then 95% ethanol, dried, and resuspended in 4 ul H_2O. Eight ul of sequencing dye was then added and the samples were run on an 8% sequencing gel containing 7 M urea. The wet gel was then exposed to Kodak XAR-5 film.

2.17 S1 Nuclease Analysis

2.17.1 Preparation of Probes

This protocol details the steps taken to prepare an S1 probe which was used for the detection and

quantification of the novel transcript produced by the synthetic promoters in the recombinant viruses. Approximately 70 ug of lithium chloride prepared p28 plasmid DNA was digested simultaneously with Nru 1 and Sst 1 restriction endonucleases. This digest cut p28 into 3 fragments with approximate sizes 4000 base pairs (bp) 1600 bp and 110 bp. When the double digest had gone to completion the digestion mixture was made to 50 mM Tris-HCl pH 8.0 and 0.5 units of calf intestinal alkaline phosphatase (Boeringer Manheim) was added with subsequent incubation at 37^{o}C for 30 min. Another 0.5 units of calf intestinal alkaline phosphatase was then added with another subsequent 30 min. incubation at 37^{o}C. The entire mixture was then electrophoresed through a 5% polyacrylamide gel and the smallest fragment (110 bp) was isolated as described above and resuspended in 50 ul water.

The isolated fragment was then 5' end labelled according to the protocol of Maxam and Gilbert, 1980, by using 3-5 ul of the resuspended fragment. After precipitation the end labelled fragment was resuspended in 38 ul water. Strand separation was performed by adding 2 ul of 20 X strand separating TBE (2.5 M Tris, 0.8 M boric acid, 53.7 mM EDTA) and 20 ul of dimethylsulphoxide was then added, whereupon the mixture was incubated at

60°C for 15 min. Upon addition of 10 ul of 6 X gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in H_2O) the sample was heated at 90°C for 2 min and electrophoresed through an 8% polyacrylamide gel for 16 hours using 1 X strand separating TBE as running buffer. The following day the gel was exposed to Kodak XAR-5 x-ray film and the desired strand was cut out of the gel, and the DNA was extracted from the gel as described above. After precipitation the probe was counted using Cerenkov counts and resuspended in DEPC treated water to give a final concentration of 6 000 cpm/ul.

2.17.2 S1 Nuclease Protection Assay

Cytoplasmic RNA was extracted from virus infected cells according to the method described above. For this protocol 10-30 ug of RNA and 30 000 cpm of S1 probe (see above) were precipitated out of 0.3 M sodium acetate and the pellet was washed with 70% and then 95% ethanol, dried without using heat, and resuspended in 30 ul of hybridization buffer [0.4 M NaCl, 40 mM piperazine-N-N'-bis(2-ethanesulfonic acid) pH 6.4, 1 mM EDTA, and 50% (v/v) recrystalized, deionized formamide]. Hybridization was allowed to proceed at 42°C for 12-16 hours. After

hybridization 0.3 ml of pre-cooled 1 X S1 buffer (150 mM NaCl, 50 mM sodium acetate pH 4.6, and 5 mM $ZnSO_4$) containing 67 units of S1 nuclease (Boeringer Manheim) was added with subsequent incubation at 37°C for 60 min. The S1 nuclease digestion reaction was then stopped by adding 50 ul of 4 M ammonium acetate and 30 ul 0.2 M EDTA. This mixture was then extracted once with phenol/chloroform and precipitated by adding 10 ug of E.coli carrier tRNA and 3 volumes of cold ethanol. Following centrifugation the pellet was resuspended in 200 ul 0.3 M sodium acetate pH 4.6 and 3 volumes of cold ethanol were then added to reprecipitate the nucleic acids. The precipitate was pelleted by centrifugation and washed with 70% then 95%, dried, and resuspended in 4 ul water. Eight ul of sequencing dye was then added, the samples were heated at 90°C for 2 min., placed immediately on ice and electrophoresed through an 8% sequencing gel. The wet gel was then exposed to Kodak XAR-5 x-ray film.

2.18 DEAE-Dextran Transient Transfections

The protocol followed is a modification of the procedure described in Eisenberg et. al., 1985. LTA cells were plated at a density of 6.25 X 10^6 cells per 150 cm^2

tissue culture plate and allowed to grow for 30 hours at which time the cells were approximately 50% confluent. The cells were then washed 4 times with straight alpha-MEM (supplemented only with 2% $NaHCO_3$). Transfections were carried out by adding 3.75 ml of transfection media [alpha-MEM containing 1% penicillin-streptomycin, 1% L-glutamine, 400 ug/ml diethylaminoethyl-(DEAE)-dextran (molecular weight 500 000, (Pharmacia) (McCutchan and Pagano, 1968, Sompayrac and Danna, 1981) and the appropriate amounts of lithium chloride prepared plasmid DNA] to the cells followed by incubation at 34°C for 1 hour. During the 1 hour incubation the plates of cells were gently rocked at 10 minute intervals to prevent cell death caused by drying. The transfection medium was then removed and the cells were washed twice with straight alpha-MEM with subsequent addition of 25 ml alpha-MEM containing 10% FBS, 1% penicillin-streptomycin, 1% L-glutamine, and 90 uM chloroquine (Sigma) followed by incubation at 37°C for 3 hours. After the chloroquine treatment (Luthman and Magnusson, 1983) the medium was aspirated and the cells were washed 3 times with straight alpha-MEM and 25 ml of alpha-MEM containing 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine was added to each transfected plate. The plates were then allowed to incubate at 37°C for 40 hours at which time cytoplasmic

RNA was harvested according to the protocol described above. RNA was then analysed by primer extension analysis.

Results

3.1 Construction of Recombinant Viruses

To determine if the TAATGARAT motif directs IE kinetics it was necessary to construct minimal promoters containing only desired promoter elements. These promoters were then rescued into the viral genome to determine the kinetics of their transcripts during a viral infection. Essentially, the approach taken was to add the TAATGARAT motif to a promoter which directed synthesis of a leaky late transcript. After addition of the TAATGARAT sequences any change in the kinetics would be directly correlated to the presence of the TAATGARAT motifs.

Previously our lab was interested in determining if a heterologous TATA box alone could function in the context of the viral genome. For this reason a synthetic oligonucleotide containing the sequence of the TATA box (figure 1) of the Ad 2 MLP was cloned into pTKSB, a plasmid harboring a derivative of the HSV-1 tk gene. This tk derivative contains a 200 bp deletion, spanning nucleotides +482 to +682 which renders the gene inactive. The deletion was bridged by a Bam H1 site into which the synthetic Ad 2 MLP TATA box was

inserted to produce plasmid p28. Contained in the 200 bp deletion is the promoter of UL24b, one of the two divergent transcripts that arises from within the tk gene (figure 2). Insertion of the MLP TATA box into the Bam H1 site of pTKSB essentially replaces the UL24b promoter. The resulting plasmid, p28, was then used in a marker transfer experiment (see below) to construct the mutant viral strain, v28, in which the wild type HSV-1 tk gene is replaced by the tk derivative carrying the TATA box of the Ad 2 MLP. Upon infection the Ad 2 MLP TATA box drives transcription of a novel UL24b transcript, UL24b', which is transcribed antisense to tk and accumulates with leaky late kinetics (Keith and Smiley, submitted, and compare figures 12 and 13).

To assess the activity of the TAATGARAT element, synthetic oligonucleotides containing the TAATGARAT sequence were cloned immediately upstream of the Ad 2 MLP TATA box in p28. The resulting plasmids were then used to construct mutant viruses containing the synthetic promoter. Any change in the behaviour of UL24b' in v28 and the TAATGARAT containing viruses should be directly attributed to the presence of the TAATGARAT sequences.

3.1.1 Construction of Synthetic Promoters

The approach taken to construct the synthetic promoters was to place different numbers of tandem repeats of the TAATGARAT motif, in both orientations, immediately upstream (11 bp) of the Ad 2 MLP TATA box. To achieve this end two complementary oligonucleotides were synthesised which, when annealed together, produced a double stranded oligonucleotide containing the sequence of the TAATGARAT motif found between nucleotides -147 and -164 of the ICP0 gene. In addition to the TAATGARAT motif the oligonucleotide contains an overlapping octamer sequence, ATGCTAAT, which has a one base pair mismatch with the consensus octamer sequence, ATGCAAAT. The oligonucleotides were designed to produce one base pair 3' overhangs to allow tandemization of the double stranded structures when annealed together (figure 1). Tandemization of the oligonucleotides produces a structure in which the TAATGARAT motifs are precisely one helix turn (10 base pairs) apart. Prior to annealing, the single stranded oligonucleotides were 5' end labelled with gamma-^{32}P ATP to facilitate ligation of the annealed oligonucleotides, and to monitor the extent of the ligation. End labelled single stranded oligonucleotides were allowed to anneal with subsequent addition of T4 DNA ligase to catalyze end to end ligation of double stranded

oligonucleotides. An aliquot of the ligation mixture was electrophoresed through a 10% polyacrylamide gel. An autoradiogragh of the gel demonstrated that a ladder containing one to twelve copies of the TAATGARAT oligonucleotide were formed (data not shown). The remaining ligation mixture was subjected to a blunting reaction to allow ligation into the blunted Sst 1 site located immediately upstream of the Ad 2 MLP TATA box in p28. The result of this ligation is the placement of different numbers of tandem repeats of the TAATGARAT oligonucleotide, in the same orientation with respect to each other but in both orientations with respect to the TATA box, 11 base pairs upstream of the MLP TATA box.

Recombinant plasmids were screened initially by colony hybridization using a 5' end labelled single stranded TAATGARAT oligonucleotide. Positive clones were then sequenced using the double stranded chain termination technique (see Methods and Materials). After sequencing a series of recombinant plasmids, five were chosen for rescue into virus. The sequences of these plasmids, or subclones of these plasmids as described in the figure legends, are shown in figures 3,4,5 and 6.

Figures 3 illustrates the sequence of the synthetic promoter containing one copy of the TAATGARAT element upstream of the MLP TATA box in plasmid pTG1. In

Figure 1:

A. Structure of the Adenovirus type 2 Major Late Promoter TATA Box

Two oligonucleotides were synthesised which when annealed together give the sequence shown. Both ends of the double stranded structure are Bam H1 compatible. In addition, the oligonucleotide contains a recognition site for the Sst 1 restriction enzyme, which will be the site of insertion of the TAATGARAT oligonucleotides. The TATA sequences are in upper case letters while the lower case letters represent the linker sequences.

B. Structure of the TAATGARAT Element

Two oligonucleotides were synthesised which when annealed together form the structure shown. Both ends contain single base pair 3' overhangs to allow tandemization of the annealed oligonucleotides.

C. Plasmid Map of p28

Plasmid p28 was derived by initially cloning a Pvu II fragment, from HSV-1 KOS strain, containing the thymidine kinase gene into the Pvu II site of pBR322 to give pTK173 (R. McKinnon, Ph.D. Thesis, McMaster). Next, the Bam H1 site at position 375 was eliminated to yield plasmid pTK173 Bam^-. Thymidine kinase sequences from position 2748 to 2948 of pTK173 bam^- were deleted with subsequent addition of a Bam H1 linker at the site of the deletion to yield pTKSB. Finally the double stranded oligonuceotide containing the sequence of the Ad 2 MLP TATA box was inserted into the Bam H1 site bridging the deletion to yield p28. The TATA box was inserted to drive transcription antisense to tk transcription. More details about the construction of p28 can be found elsewere (Keith, B.K., Master's Thesis McMaster, 1988).

A. Structure of Ad 2 MLP TATA box:

5' gatctcgagctcGGGGGGCTATAAAAGGGG 3'
3' agctcgagCCCCCCGATATTTTCCCCCtag 5'

B. Structure of TAATGARAT Element:

5' ATGCTAATGAGATTCT TT 3'
3' ATACGATTACTCTAAGAA 5'

C. Plasmid Map of p28:

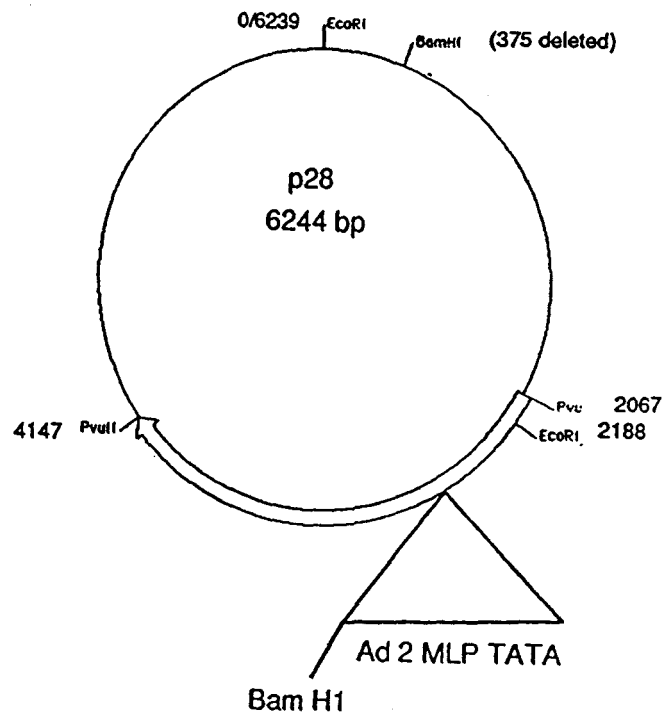
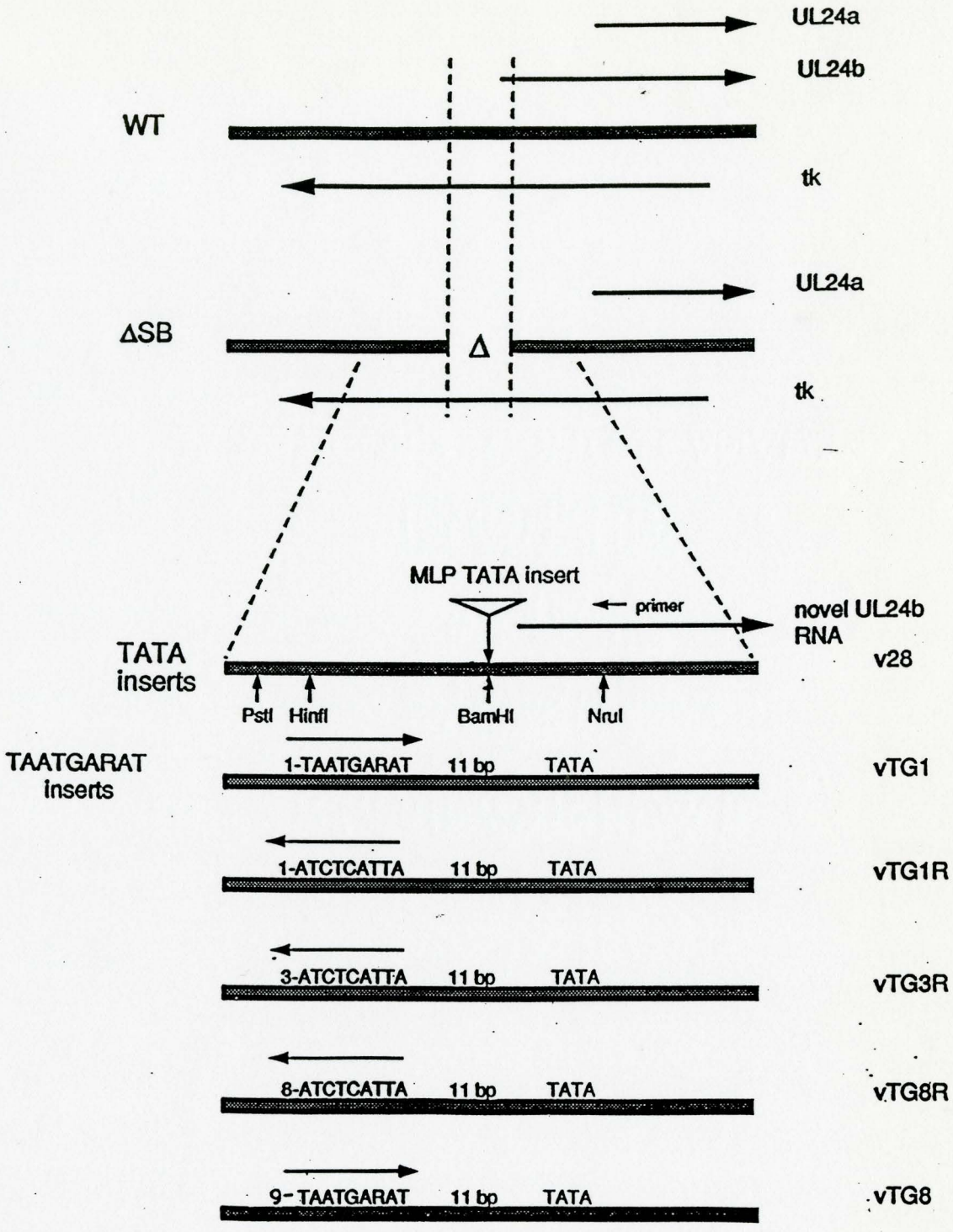


Figure 2 Transcript Map and Structure of Recombinant Viruses

The diagram in figure 2 illustrates the transcript map of the region of tk into which the synthetic promoters were inserted. From the wild type tk sequences two divergent transcripts, UL24a and UL24b, are transcribed antisense to the tk message. The recombinant virus derived from pTKSB, KOS SB, contains a 200 base pair deletion of the tk sequences from position +482 to +682. As a result the promoter of UL24b is removed, hence no UL24b RNA is produced. The recombinant virus v28 contains the TATA box of the Ad 2 MLP inserted at the Bam H1 site bridging the 200 base pair deletion. The MLP TATA box essentially replaces the promoter of UL24b. As a result v28 transcribes a novel UL24b transcript, UL24b'. The position of the primer used to detect the UL24b' transcript is shown. The position of this primer precludes the detection of the UL24a transcript. The structures of the recombinant viruses containing the synthetic promoters with TAATGARAT elements are also shown. The number of tandem TAATGARAT motifs and their orientation with respect ot the TATA box is indicated. In each case the first TAATGARAT motif is located 11 base pairs upstream of the MLP TATA box.


UL24a
UL24b
WT
tk
UL24a
ΔSB
Δ
tk
MLP TATA insert
primer
novel UL24b RNA
v28
TATA inserts
PstI
HinfI
BamHI
NruI
TAATGARAT inserts
1-TAATGARAT 11 bp TATA
vTG1
1-ATCTCATTA 11 bp TATA
vTG1R
3-ATCTCATTA 11 bp TATA
vTG3R
8-ATCTCATTA 11 bp TATA
vTG8R
9-TAATGARAT 11 bp TATA
vTG8

this promoter the TAATGARAT motif is present in the same orientation, relative to the TATA box, as are all TAATGARAT motifs within approximately 300 base pairs of the TATA box in all IE gene promoters (Mackem and Roizman, 1982a). For convention this orientation will be referred to as the forward orientation. The TAATGARAT motif is, however, present greater than 300 base pairs upstream of the TATA box in some IE gene promoters. At this distance the TAATGARAT sequence can exist in either orientation with respect to the TATA box (Smith et al., 1989). The sequence in figure 3 shows that blunting of both the TAATGARAT oligonucleotide and the Sst 1 site in p28 occurred correctly. In addition, figure 3 shows that the TAATGARAT oligonucleotide cloned contains the correct sequence and that it is located in the correct position upstream of the MLP TATA box. Figure 4 illustrates the sequence of the synthetic promoter, contained in plasmid pTG1R, which has one copy of the TAATGARAT oligonucleotide, upstream of the MLP TATA box, in the opposite orientation as in pTG1. For convention this orientation will be referred to as the reverse orientation. The sequence demonstrates that both blunting reactions occurred correctly and that the sequence of the TAATGARAT insert is correct.

The sequences of tandem repeats of the

Figure 3: Sequence of pTG1

The sequence of pTG1 was obtained by the double stranded chain termination method as described in Methods and Materials using AB167 as a primer. Shown in the figure is the sequence of the tk coding strand . The TAATGARAT and the Ad 2 MLP TATA box oligonucleotides are labelled TG and TA respectively.

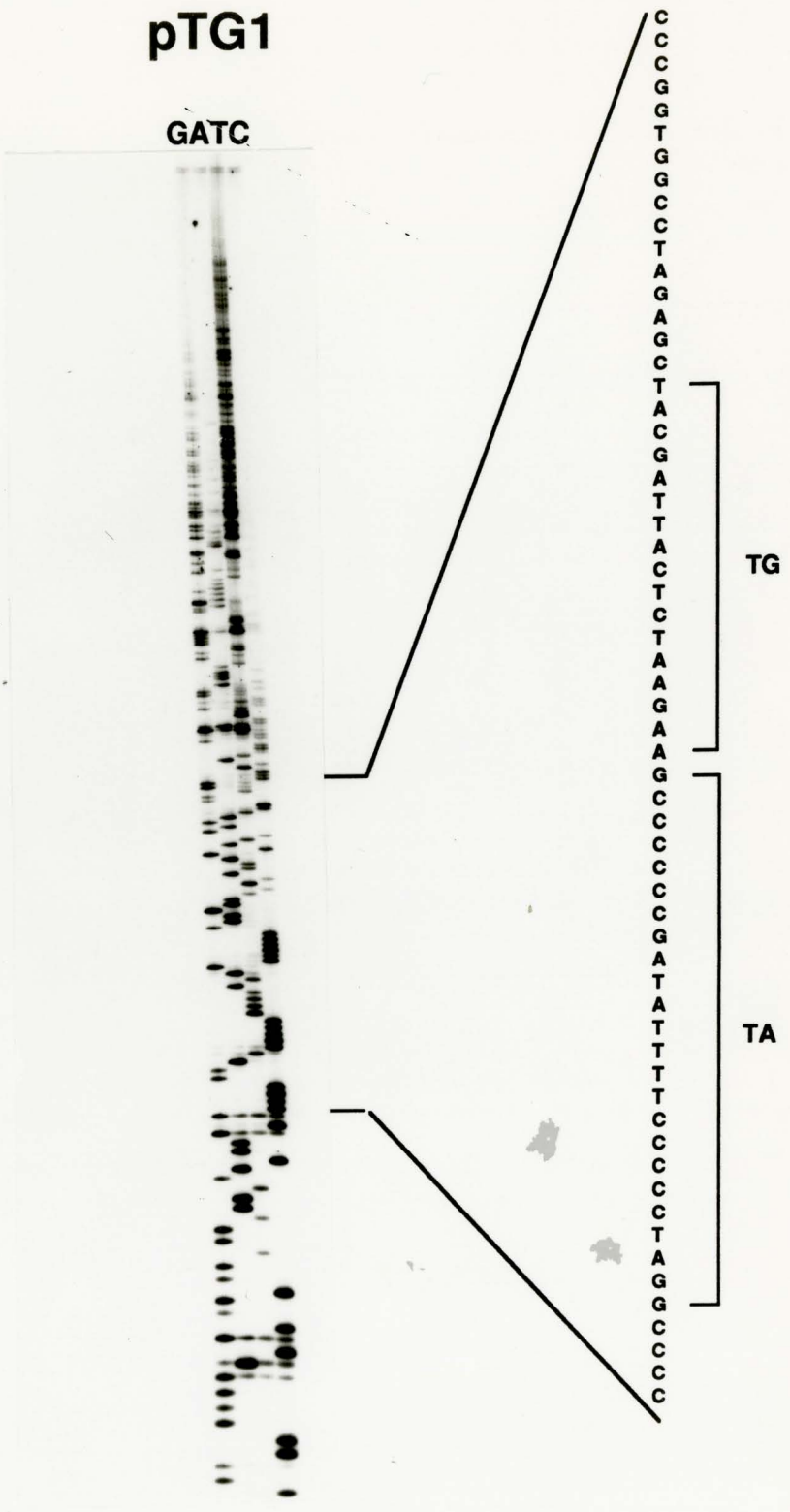
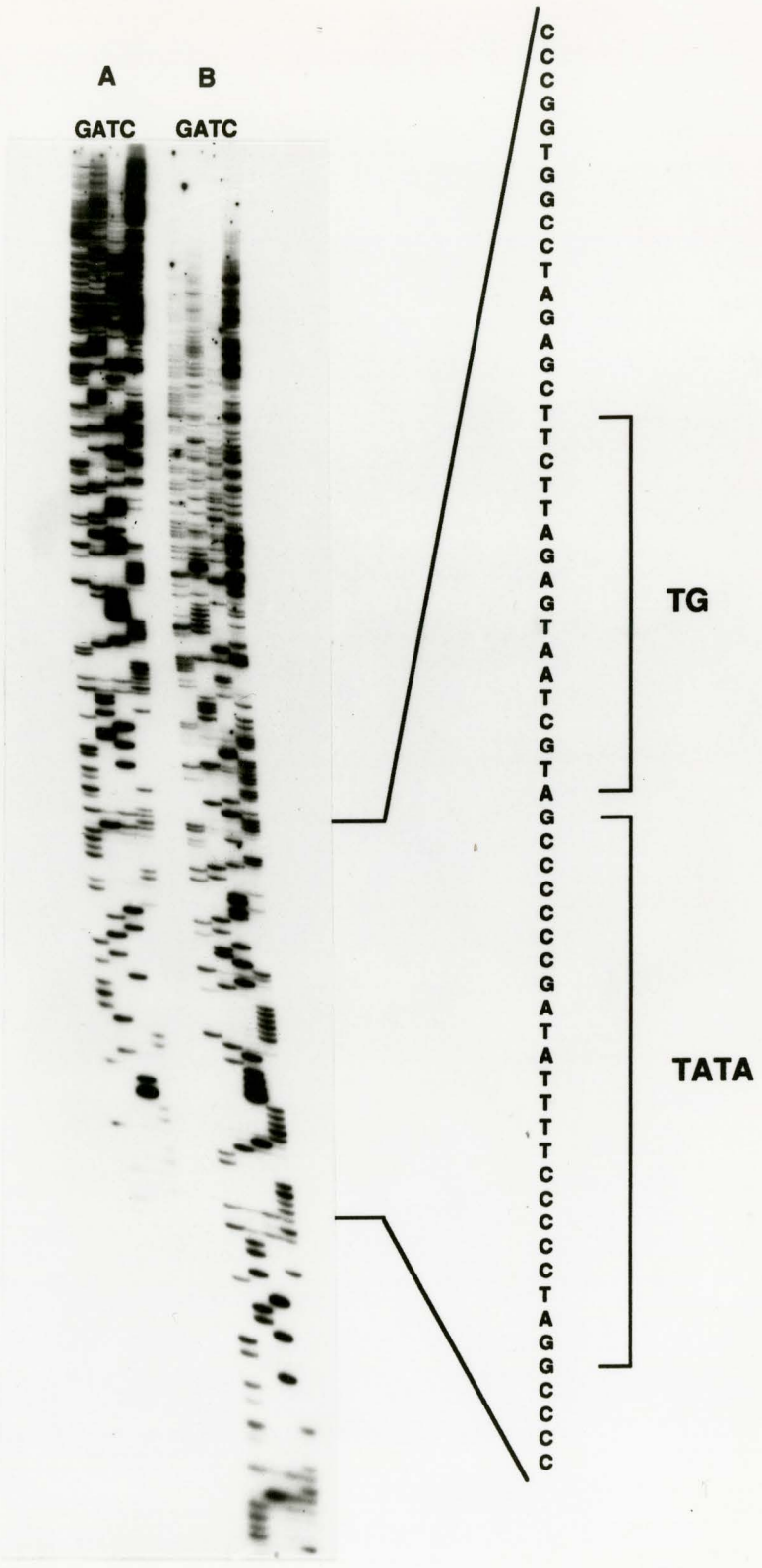

pTG1
GATC
CCCGGTGGCCTAGAGCTACGATTACTCTAAGAAGCCCCCCCCGATATTTTCCCCCCTAGGCCCC
TG
TA

Figure 4: Sequence of pTG1R

The sequence of pTG1R was obtained by the double stranded chain termination technique using AB167 as a primer. Shown is the coding strand of tk. The sequence of the inserted synthetic promoter is shown. The TAATGARAT and Ad 2 MLP TATA box oligonucleotides are labelled TG and TA respectively.

pTG1R


A
B
GATC
GATC
CCCGGTGGCCTAGAGCTTCTTAGAGTAATCGTAGCCCCCCCGATATTTTCCCCCCTAGGCCCC
TG
TATA

TAATGARAT motif are shown in figures 5 and 6. Figure 6 illustrates the sequence of nine tandem repeats of the TAATGARAT motif all in the forward orientation cloned upstream of the MLP TATA box. The sequence in figure 6 demonstrates that during the blunting reaction one base pair, a C from the non coding strand, was eliminated. The deletion of this C occurs 5' to the MLP TATA box at the junction between the MLP TATA box and the first TAATGARAT motif. Figure 5A illustrates the sequence of the synthetic promoter consisting of three tandem repeats of the TAATGARAT oligonucleotide in the reverse orientation immediately upstream of the MLP TATA box. The sequence indicates that during the blunting reaction 2 base pairs ,AT, from the first TAATGARAT oligonucleotide 5' to the MLP TATA box were lost. Deletion of these 2 base pairs results in loss of the first octamer sequence, however all three TAATGARAT sequences remain intact. Finally, figure 5B shows the sequence of the synthetic promoter containing eight tandem copies of the TAATGARAT motif in the reverse orientation present directly adjacent to the MLP TATA box. The sequence demonstrates that one base pair, G, from the first TAATGARAT oligonucleotide 5' to the MLP TATA box is not present. As a result of this deletion the first octamer sequence is disrupted, however the TAATGARAT sequence remains intact.

Figure 5: A) Sequence of pTT3R

B) Sequence of pTT8R

Both sequences were obtained using the double stranded chain termination sequencing technique using the M13 universal primer.

A) Briefly, pTT3R was constructed by isolating the Sau 3A fragment containing the synthetic promoter from pTG3. This Sau 3A fragment was then cloned into the Bam H1 site of pXBA. pXBA is a pUC 18 derivative which contains an Xba 1 linker inserted into the Eco R1 site of the polylinker. The TATA box is indicated by the square brackets and the sequence in the figure is:

5'- CTA GAG GAT CTC GAA GAA TCT CAT TAG CAT AAA GAA TCT CAT TAG CAT AAA GAA TCT CAT TAG C(AT)CG GGG GGC TAT AAA AGG GGG ATC CCC GGG -3'

B) pTT8R was constructed similar to pTT3R. The SAU 3A fragment containing the synthetic promoter was isolated from pTG8R and cloned into the Bam H1 site of pXBA. The TATA box is indicated by the square brackets and the sequence is:

5'- CTA GAG GAT CTC GAA GAA TCT CAT TAG CAT AAA GAA TCT CAT TAG CAT AAA GAA TCT CAT TAG CAT AAA GAA TCT CAT TAG CAT AAA GAA TCT CAT TAG CAT AAA GAA TCT CAT TAG CAT AAA GAA TCT CAT TA(G)C ATC GGG GGG CTA TAA AAG GGG GAT CCC CGG - 3'

For both sequences in A and B the bold letters indicate missing base pairs.

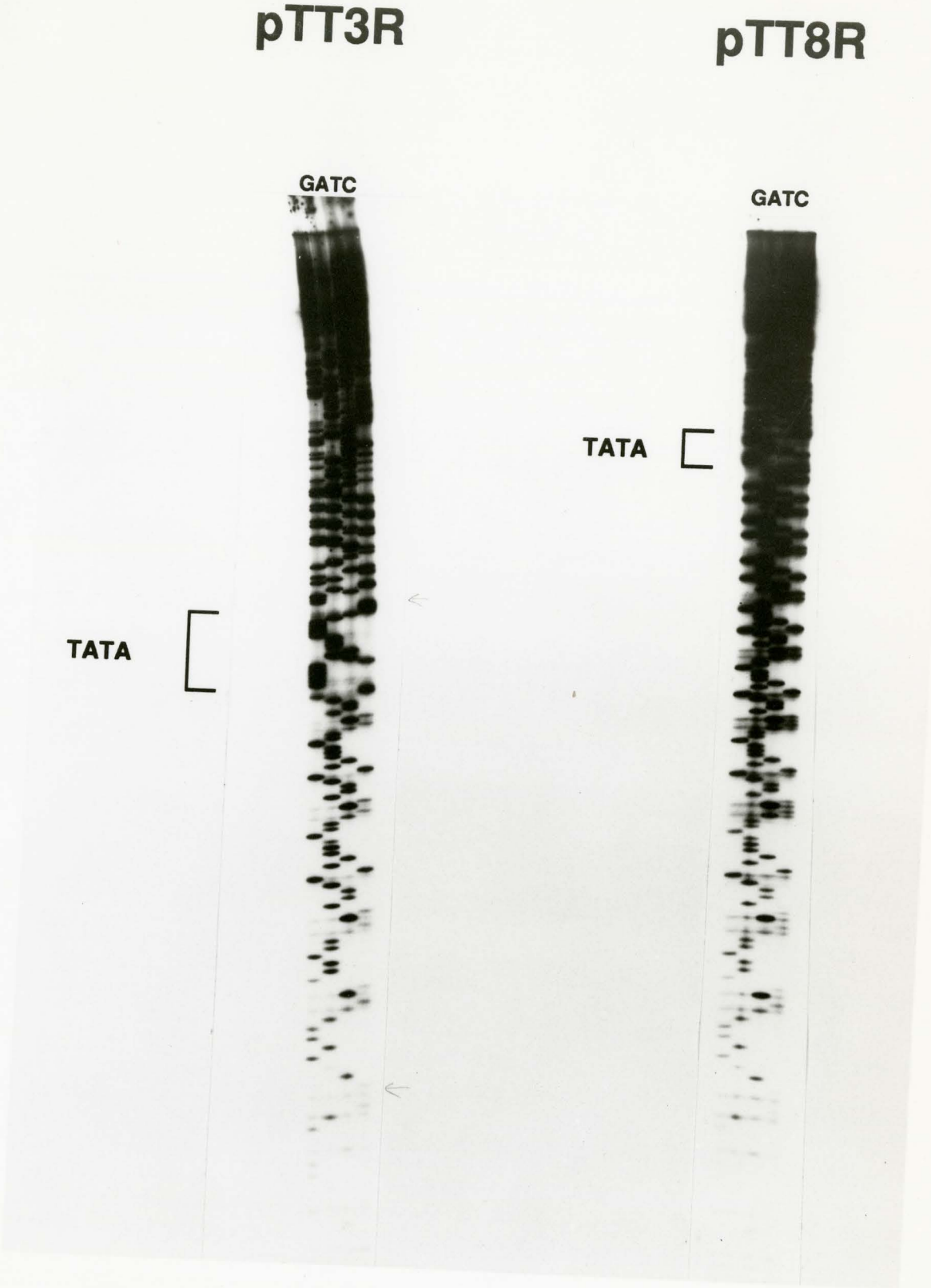

pTT3R
pTT8R
GATC
GATC
TATA
TATA

Figure 6 Sequence of pTTB8

Plasmid pTTB8 was the plasmid used in a transient tranfection assay along with a plasmid encoding Vmw65 (see text). This plasmid contains the rabbit beta-glodin under control of the same synthetic promoter contained in vTG8. This synthetic promoter contains 9 TAATGARAT elements, in the forward orientation, immediately upstream of the Ad 2 MLP TATA box. Briefly pTTB8 was constructed by excising the Sau 3A fragment from pTG8 which contains the synthetic promoter and cloning this fragment into the Bam H1 site of pXBA to yield pTT8. Next, a blunt ended Pvu II and Sst 1 fragment of rabbit beta-globin was cloned into the Sma 1 site of pTT8 to yield pTTB8. The sequence of pTTB8 is therefore indicative of the sequence of the synthetic promoter in vTG8. Figure six demonstrates the sequence of both strands of the synthetic promoter contained in pTTB8. Primer AB 171, the M13 universal primer, was used to generate the sequence of the non-coding strand. The coding strand was obtained by using a primer internal to the beta-globin gene, namely AB 16.

The sequence obtained using primer AB 171 is:

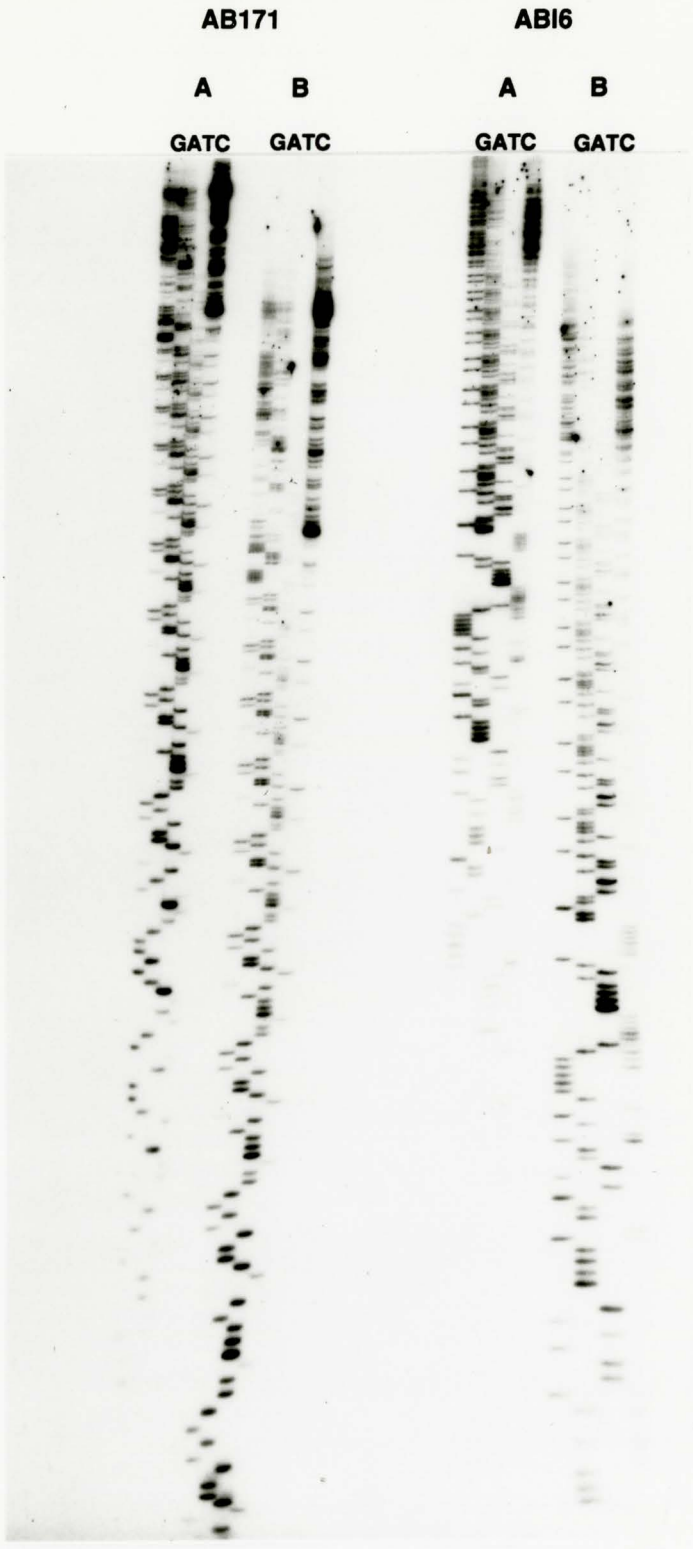
5'- CTA GAG GAT CTC GAT GCT AAT GAG ATT CTT TAT GCT AAT GAG ATT CTT TAT GCT AAT GAG ATT CTT TAT GCT AAT GAG ATT CTT TAT GCT AAT GAG ATT CTT TAT GCT AAT GAG ATT CTT TAT GCT AAT GAG ATT CTT TAT GCT AAT GAG ATT CTT**(C)** GGG GGG CTA TAA AAG GGG G - 3'

The sequence obtained using primer AB 16 is:

5'- AAA AGC CAA GTG TAA GCC AGC AGG GGG ATC CCC CTT TTA TAG CCC CCC**(G)** AAG AAT CTC ATT AGC ATA AAG AAT CTC ATT AGC ATA AAG AAT CTC ATT AGC ATA AAG AAT CTC ATT AGC ATA AAG AAT CTC ATT AGC ATA AAG AAT CTC ATT AGC ATA AAG AAT CTC ATT AGC ATA AAG AAT CTC ATT - 3'

For both sequences the bold letter indicates a missing base pair.

PTTB8



In summary, five synthetic promoters were chosen to be rescued into the tk locus of the intact virus. The choices contain the following sequences cloned 11 base pairs upstream of the MLP TATA box: one TAATGARAT motif in the forward orientation, one in the reverse orientation, three and eight tandem TAATGARAT motifs in the reverse orientation, and nine TAATGARAT motifs in the forward orientation. These choices make it possible to asses the effect of copy number as well as the effect of orientation of the TAATGARAT motif on transcription.

3.1.2 Marker Transfer into Virus

Transfer of the mutant tk gene containing the synthetic promoter in virus was achieved using marker rescue (Smiley, 1980). Mutation of the tk gene is possible since the HSV tk gene is dispensable for viral replication in tissue culture (Summers et al., 1975). One of the five plasmids containing the synthetic promoter (as described above) was linearized with Bgl II to increase the rate of recombination between plasmid and virus. The linearized plasmid DNA was co-transfected with infectious HSV-1 DNA into Vero cells. The infectious DNA was derived from the viral strain PAA^{r}5 which contains an antimutator DNA polymerase that produces fewer

spontaneous tk mutations (Hall et al., 1984). Homologous recombination between the tk sequences in the plasmid and tk sequences in the viral genome facilitates the replacement of the wild type tk gene with the mutant tk derivative containing the synthetic promoter. Viruses with defective tk genes were selected using 5-bromo-2-deoxycytidine. In the presence of 5-bromo-2-deoxycytidine viruses with wild type tk activity are unable to grow, therefore only tk deficient viruses will be able to form plaques (Stow et al., 1978). Plaques from tk deficient viruses were picked and plaque purified at least twice. Southern blot analysis was then used to confirm the presence of the synthetic promoter in the tk deficient viruses.

The wild type tk gene is flanked by 2 Pvu II sites and does not contain an internal Bam H1 restriction site. The tk gene in the desired recombinant virus is 200 base pairs smaller than the wild type tk gene and, in addition, contains a Bam H1 site internal to the flanking Pvu II sites. Simultaneous digestion of wild type virus with Pvu II and Bam H1 would therefore yield only one band, approximately 2000 base pairs, on a Southern blot probed with tk sequences. In contrast a Pvu II and Bam H1 double digest of the desired recombinant virus would yield two bands when probed with tk sequences. One of

these bands would be 686 base pairs in length and the other band would vary in size between 1201 and 1326 base pairs depending on the number of tandem repeats of the TAATGARAT oligonucleotide.

Southern blots of the five recombinant viruses are shown in figures 7,8,9,10, and 11. The names of the viral strains indicate the number and the orientation of the TAATGARAT sequences. For example, vTG3R refers to a virus which contains three tandem repeats of the TAATGARAT oligonucleotide in the reverse orientation. Absence of an "R" in the name of the viral strain indicates TAATGARATs present in the forward orientation. The names apply to all recombinant viruses except vTG8 which contains nine as opposed to eight tandem copies of TAATGARAT in the forward orientation. The figures show plaque isolates of various tk deficient viruses. The control digests in the figures 9,10, and 11 include a Pvu II and Bam H1 double digest of the plasmid which was co-transfected with the infectious PAA^r5 DNA, as well as a Pvu II and Bam H1 double digest of DNA isolated from PAA^r5 infected cells. Upon restriction with Pvu II and Bam H1 the recombinant viruses should yield two bands which co-migrate with the two lower bands of the marker plasmid, and pure isolates of recombinants should not produce a band which co-migrates with wild type tk band

Figure 7 Southern Blot Analysis of vTG1

Thymidine kinase deficient viruses, from 5-bromo-2-deoxycytidine selection, were amplified by infecting confluent monolayers of Vero cells growing in 24 well tissue culture plates. Upon confluent cytopathic effect (cpe) viral DNA was extracted and restricted with Bam H1 and Pvu II simultaneously. To generate marker bands pTG1, the plasmid used in the co-transfection was also digested with both Bam H1 and Pvu II. In addition pTKSB was cleaved with Pvu II alone to generate a non recombinant tk band. All digestion fragments were electrophoresed through a 1% agarose gel with subsequent transfer to nitrocellulose. The Southern blot was probed with nick translated pTKSB. Lanes 1 through 10 contain DNA from tk deficient viruses and control lanes are indicated. The position of pBR322 and the non recombinant tk band derived from pTKSB are shown to the right of the figure. Sizes of the recombinant tk bands are also shown to the right of the figure.

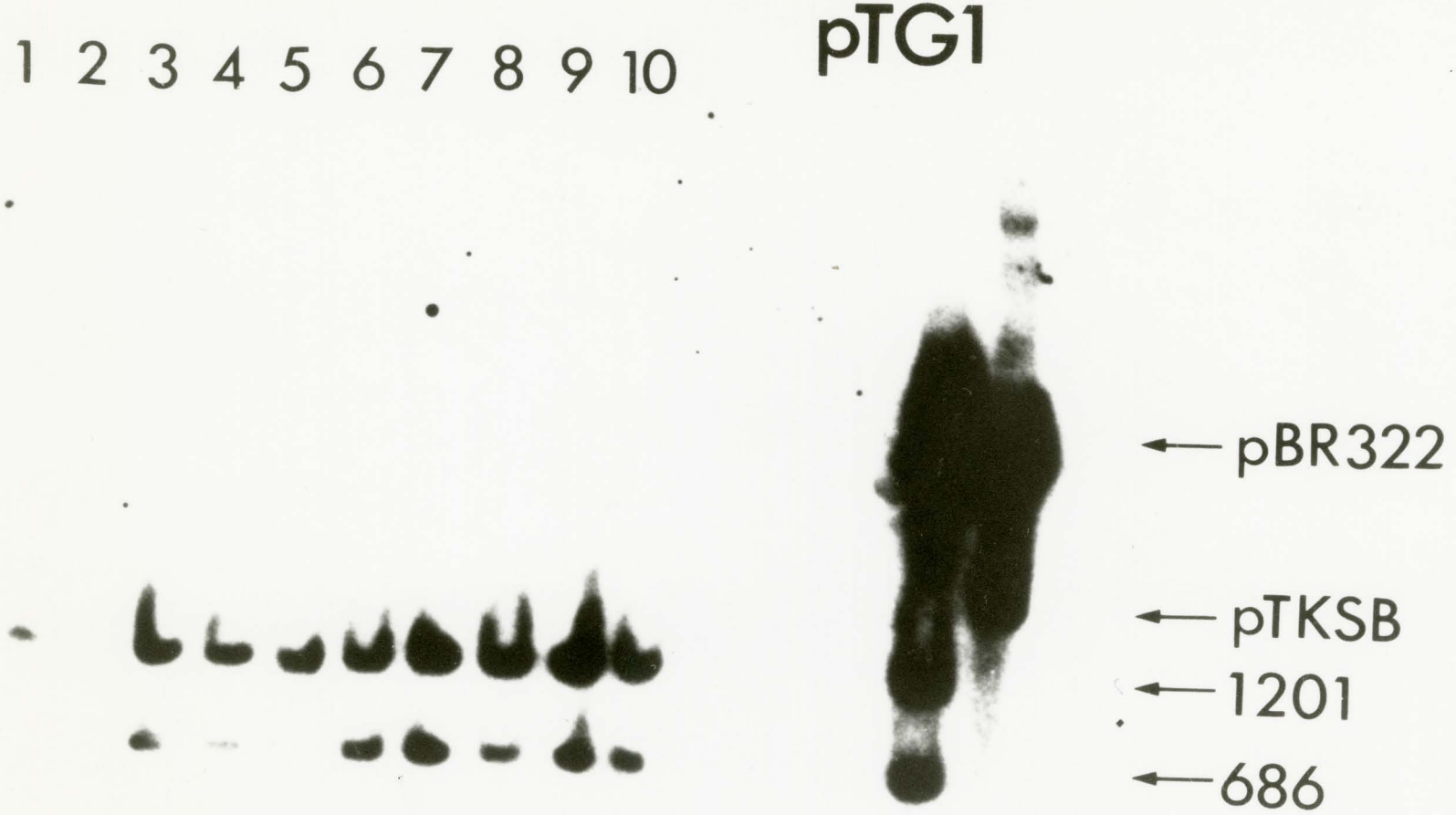

1 2 3 4 5 6 7 8 9 10
pTG1
pBR322
pTKSB
1201
686

Figure 8 Southern Blot Analysis of vTG1R

Thymidine kinase deficient viruses arising from the co-transfection of pTG1R and PAA^{r}5 infectious DNA were used to infect confluent monolayers of Vero cells. Viral DNA was extracted and cleaved with both Bam H1 and Pvu II. Marker bands were derived from pTG1R simultaneously restricted with Bam H1 and Pvu II. A non - recombinant tk band was obtained by digesting pTKSB with Pvu II alone. DNA fragments were electrophoresed through a 1% agarose gel, transfered to nitrocellulose, and probed with nick translated pTKSB. DNA from tk deficient viruses is present in lanes 1 through 5. Control lanes are as indicated in the figure. The locations of pBR322 and the non recombinant tk band are shown to the right of the figure. The sizes of the recombinant tk bands are also shown to the right of the figure.

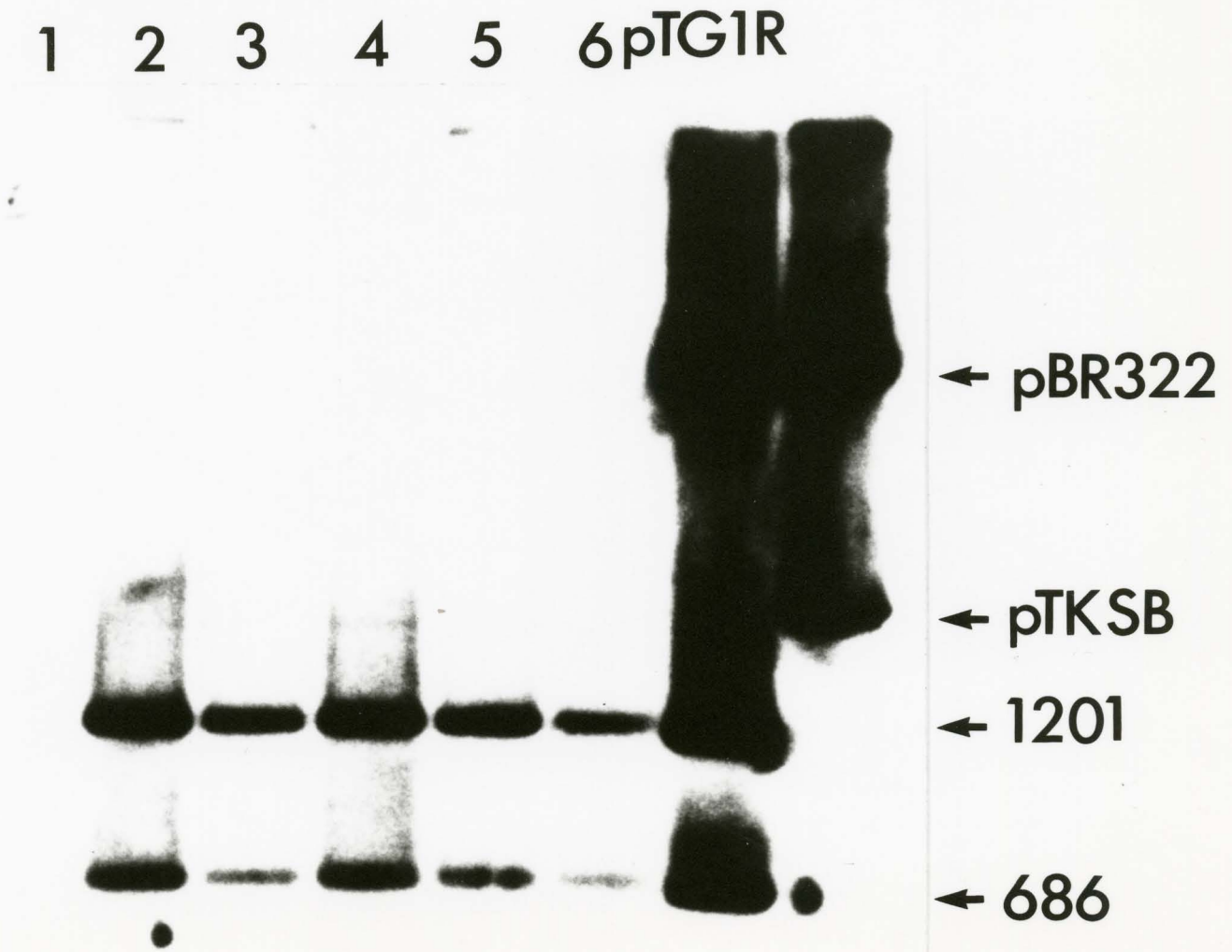

1 2 3 4 5 6 pTG1R
pBR322
pTKSB
1201
686

Figure 9 Southern Blot Analysis of vTG3R

Confluent monolayers of Vero cells growing in 24 well tissue culture plates were infected with tk deficient viruses isolated by 5-bromo-2-deoxycytidine selection. Viral DNA was extracted when cpe became confluent and restricted with Bam H1 and Pvu II simultaneously. Marker bands were generated by digesting pTG3R, the plasmid co-transfected with pAA^r5 infectous DNA, with both Bam H1 and Pvu II. A wild type tk band was obtained by digesting viral DNA extracted from PAA^r5 infected cells with both Bam H1 and Pvu II. Restriction fragments were size fractionated by electrophoresis through a 1% agarose gel. DNA fragments were detected by Southern blot analysis using nick translated pTKSB as a probe. DNA from tk deficient viruses is present in lanes 1 through 12 and control lanes are indicated. The positions of pBR322 and wild type tk bands are indicated to the right of the figure. The sizes of the recombinant tk bands are also indicated to the right of the figure.

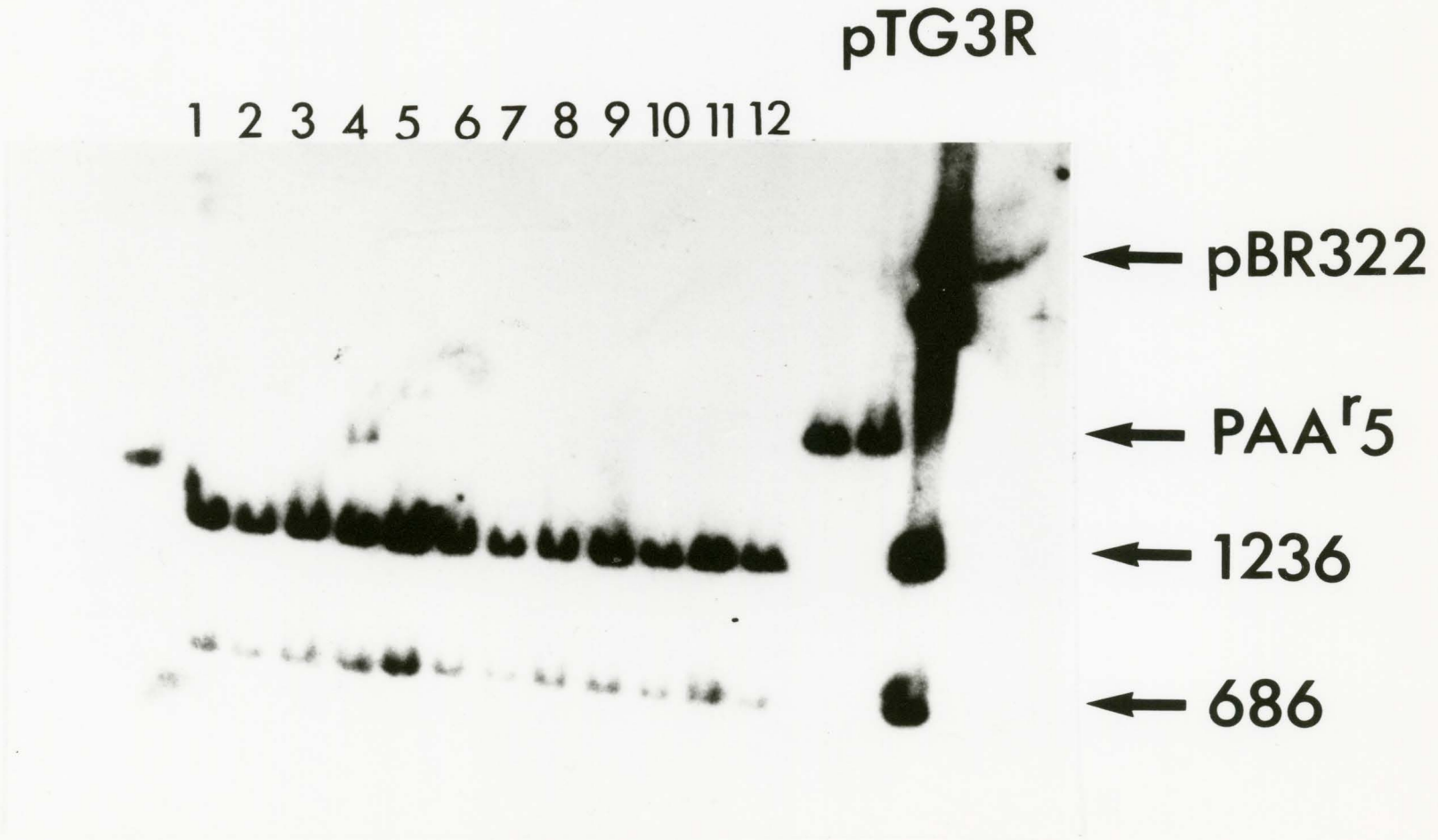

pTG3R
1 2 3 4 5 6 7 8 9 10 11 12
pBR322
PAA^r5
1236
686

Figure 10 Southern Blot Analysis of vTG8R

Viruses obtained from plaques produced by the co-transfection of pTG8R and PAA^{r}5 infectious DNA were amplified on confluent monolayers of Vero cells. Viral DNA was extracted and simultaneously digested with Bam H1 and Pvu II. Control digests producing size markers include a double digestion of pTG8R and viral DNA extracted from PAA^{r}5 infected cells with Bam H1 and Pvu II. Restriction fragments were analysed by Southern blot analysis using nick translated pTKSB as a probe. Lanes 1 through 9 contains viral DNA from tk deficient viruses and lanes with control digests are indicated. The positions of pBR322 and wild type tk gene are shown to the left of the figure. The sizes of the recombinant tk bands are also shown on the left of figure 10.

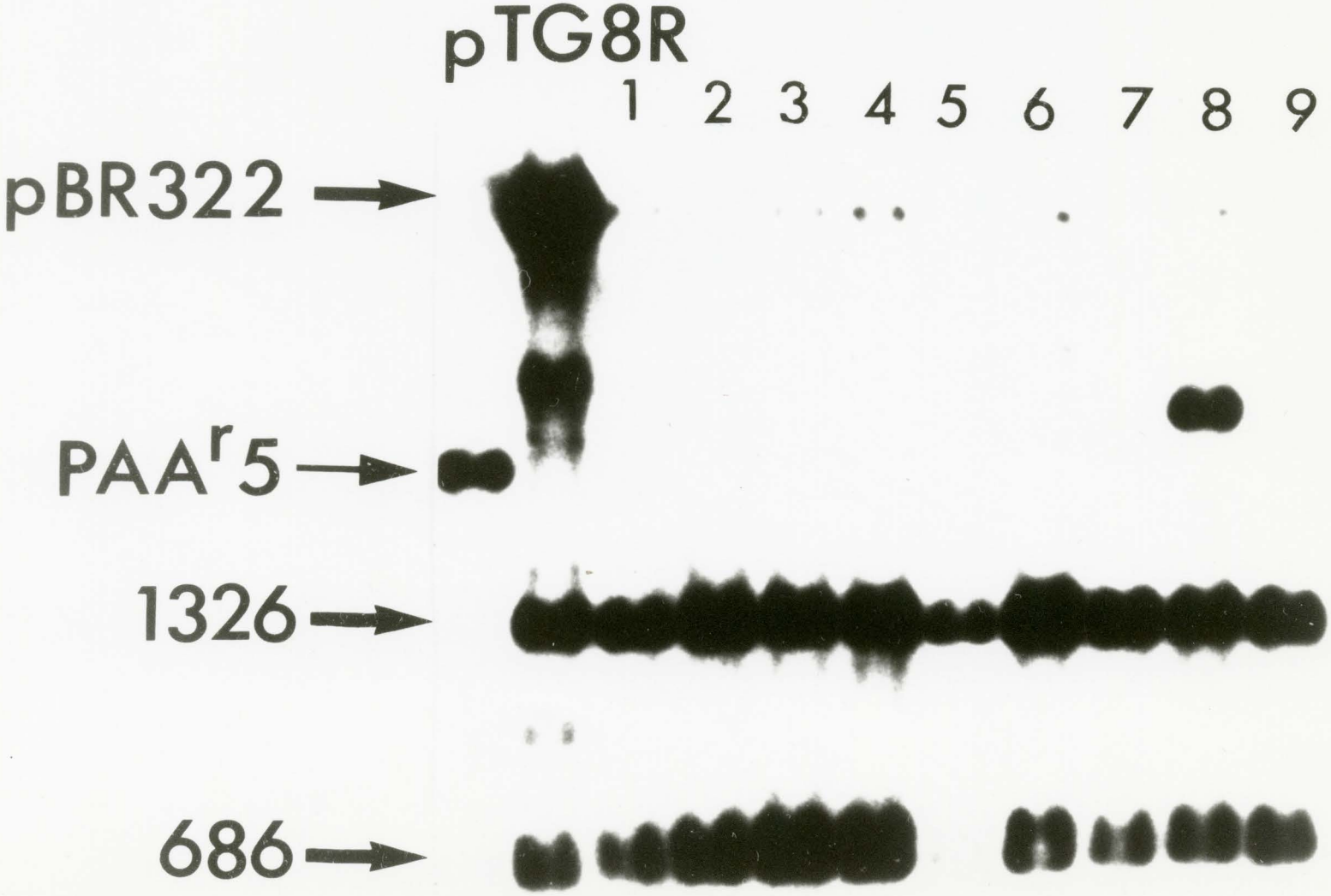
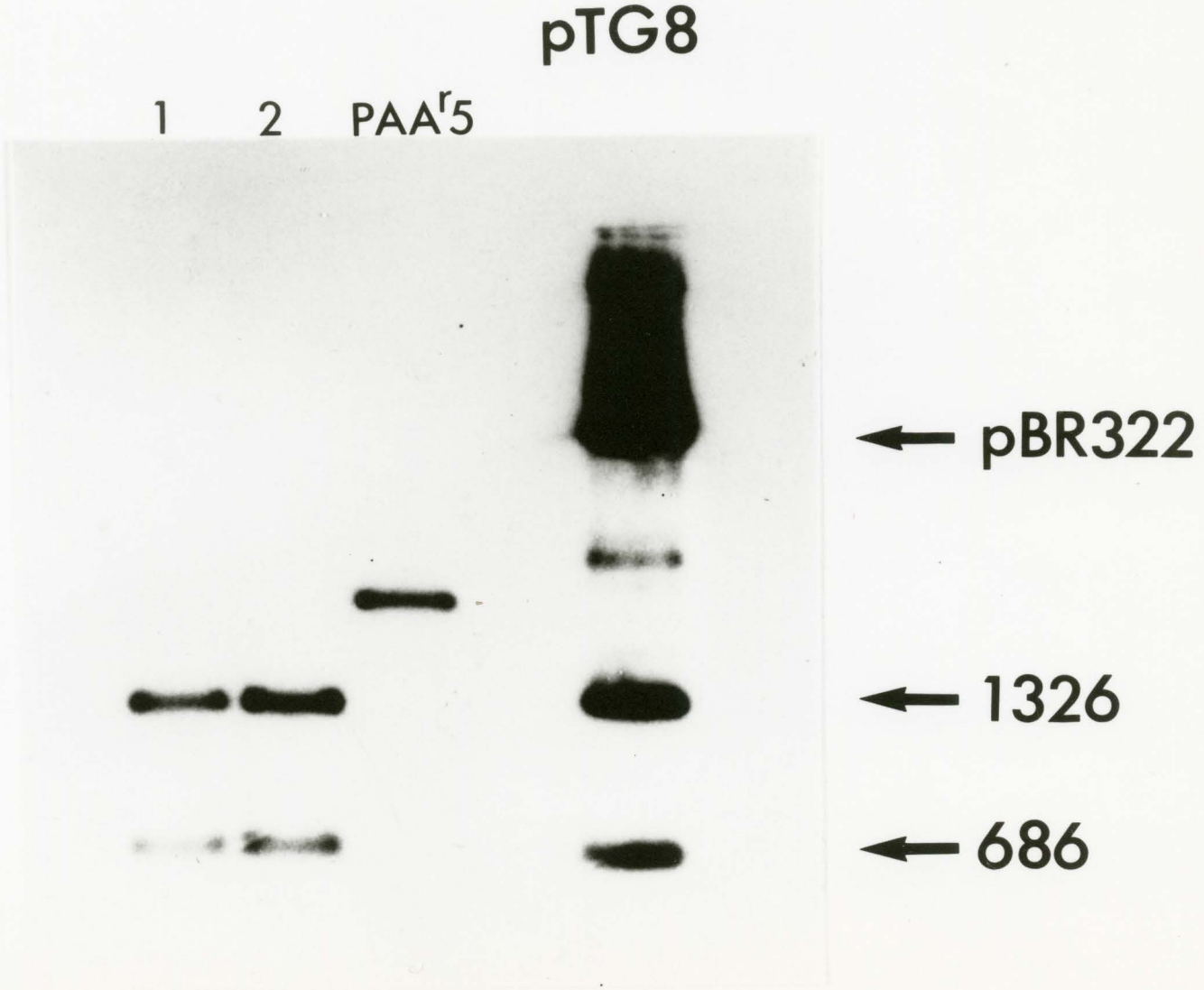

pTG8R
1 2 3 4 5 6 7 8 9
pBR322
PAA^r5
1326
686

Figure 11 Southern Blot Analysis of vTG8

Confluent monolayers of Vero cells were infected with tk deficient viruses isolated from 5-bromo-2-deoxycytidine selection. Viral DNA was harvested when cpe was confluent and digested with Bam H1 and Pvu II. Size markers were produced by restricting pTG8, the plasmid co-transfected with PAA^r5 - infectious DNA, and PAA^r5 viral DNA with Bam H1 and Pvu II. All restriction fragments were electrophoresed through a 1% agarose gel and blotted onto nitrocellulose. The nitrocellulose filter was probed using nick translated pTKSB. Lanes 1 and 2 contains DNA from tk deficient viruses and control lanes are indicated. The right side of the figure indicates the positions of pBR322 and wild type TK as well as the sizes of the recombinant tk bands.


pTG8
1
2
PAA^r5
pBR322
1326
686

from PAA^{r}5 infected cells. In figures 7 and 8, instead of using PAA^{r}5 infected cell DNA to generate a wild type tk marker, pTKSB was digested with Pvu II alone to generate a non recombinant tk marker. The viruses containing the desired structure, as determined by the criteria above, were amplified on Vero cells and viral stocks of each were made.

3.2 Kinetics of Expression of UL24b'

To determine the kinetics of expression of UL24b' Vero cells were infected with various recombinant viruses and cytoplasmic RNA was isolated at different times post infection. Cytoplasmic RNA was then probed for the presence of the UL24b' transcript by primer extension analysis using a probe, AB167, which is the sequence of the non coding strand of tk between nucleotides +391 and +425 (see figure 2). In an initial experiment Vero cells were infected with vTG8 at a multiplicity of infection (MOI) of 10 plaque forming units (pfu) per cell and cytoplasmic RNA was harvested at 0,3,6 and 12 hours post infection. As controls Vero cells were also infected with either v28 or KOS SB using the same MOI. KOS SB is a viral strain which has the identical 200 base pair deletion at the tk locus, but it does not contain the MLP

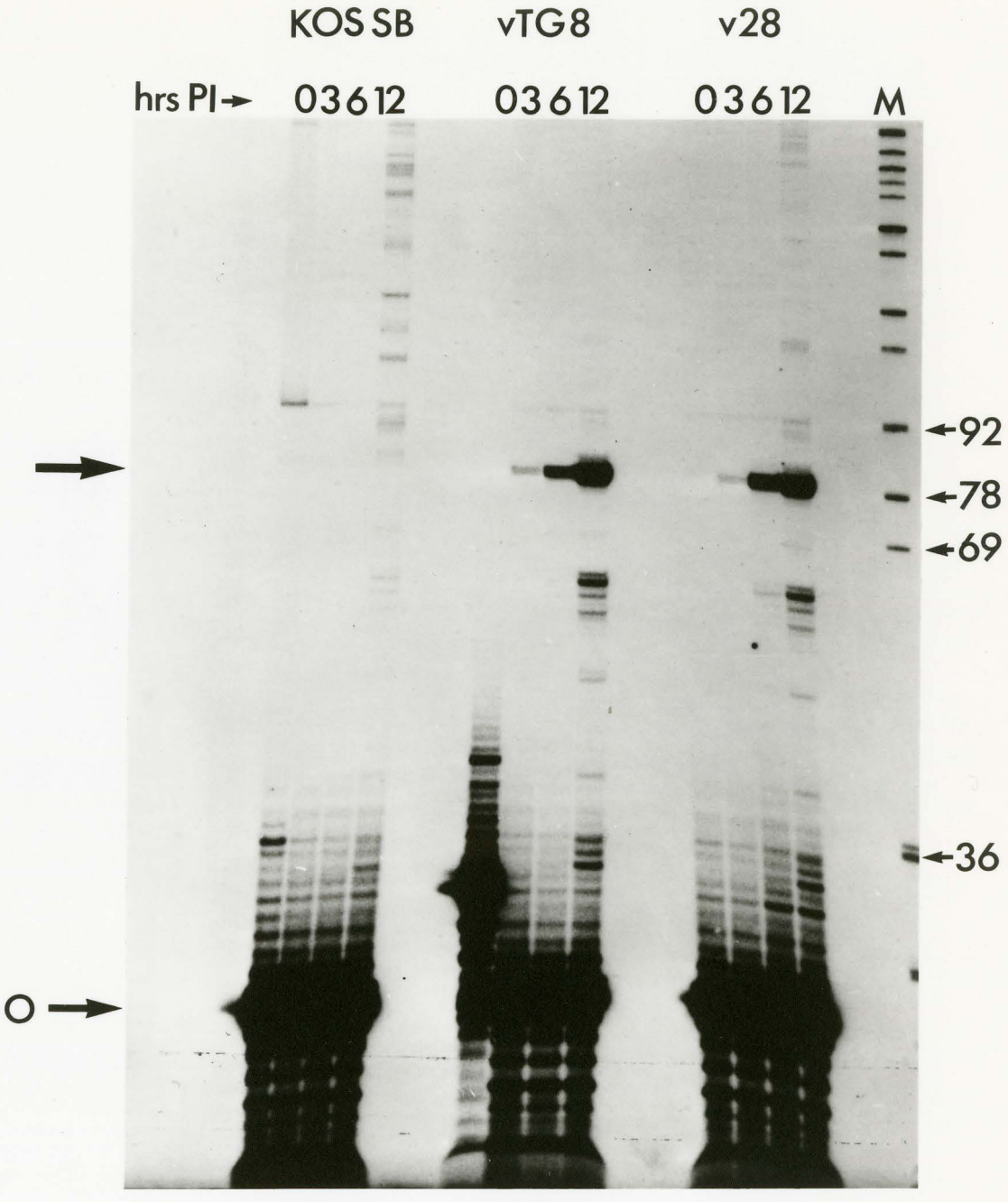
TATA box or any TAATGARAT sequences.

Figure 12 illustrates the results of this experiment. Firstly, no UL24b' transcript is made in cells infected with KOS SB indicating that the 200 base pair deletion effectively removed the UL24b promoter. The presence of a UL24b' transcript in both v28 and vTG8 infected cells demonstrates that the synthetic promoters inserted in these viruses are competent to promote significant levels of transcription. Secondly, the size of the primer extension products, indicated by the large arrow to the left of figure 12, indicates that in both viruses, v28 and vTG8, transcription initiation occurred approximately 30 nucleotides downstream of the MLP TATA box. Both synthetic promoters therefore produce exactly the same transcript, UL24b'. Thirdly, UL24b' produced by both v28 and vTG8 accumulates with the same kinetics. Expression of UL24b' is low at 3 hours post infection and is maximal at late times post infection (see also figure 14). The addition of nine tandem TAATGARAT elements in the forward orientation to the MLP TATA box therefore had no effect on the kinetics of expression of UL24b'.

Additional bands, indicated by the small arrows to the left, are seen in figure 14. These bands are not present in the S1 nuclease protection analysis of the

Figure 12 Kinetic Analysis of UL24b' Produced from v28 and vTG8

UL24b' RNA was quantitated by primer extension using AB167 as a probe. Confluent monolayers of Vero cells, approximately 1.5 X 10^7 cells per dish, were infected at an MOI of 10 pfu per cell with v28 or vTG8. As a control Vero cells were also infected with KOS SB at the same MOI. Cytoplasmic RNA was harvested by the method of Berk and Sharp at 0,3,6, and 12 hours post infection. RNA was then analysed by primer extension. The molecular mass markers, in the lane labelled M, were derived by cleaving pBR322 with Hpa II and end labelling the resulting fragments by filing in the 3' end with alpha-^{32}P-dCTP and E.coli DNA polymerase I. The size, in base pairs, of the relevant size markers are shown to the right of the figure. The large arrow to the left of the figure indicates the primer extension product of UL24b' RNA. The arrow labelled with an O indicates the position of the free primer.

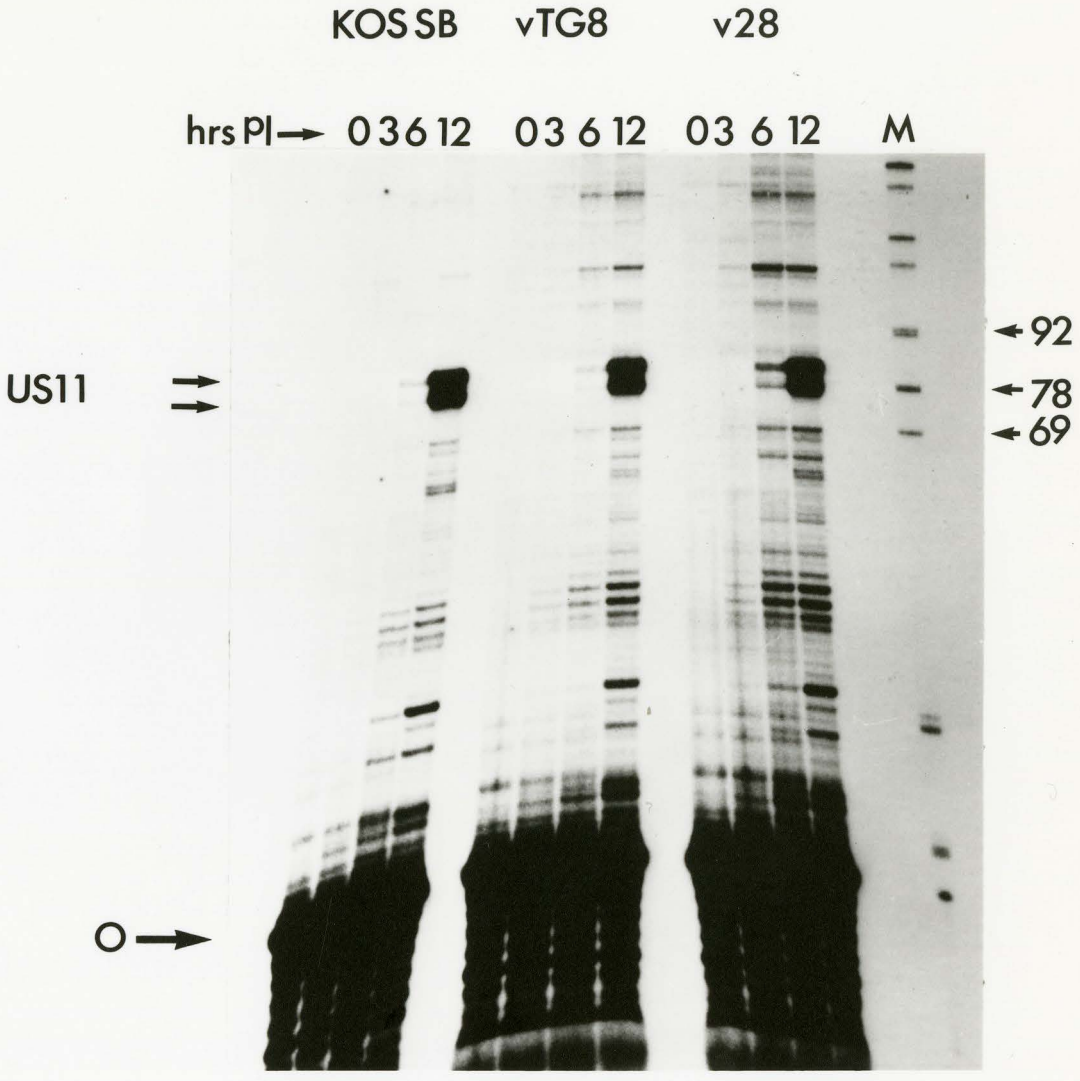

KOS SB
vTG8
v28
hrs PI→
0 3 6 12
0 3 6 12
0 3 6 12
M
←92
←78
←69
←36
O

UL24b' transcript (see figures 17 and 18) and probably represent partial reverse transcriptase products arising from pausing of the reverse transcriptase.

Primer extension of the US11 transcript, a true late HSV-1 transcript, was used as in internal control to control for: consistency in the RNA harvest, the amount of RNA used in the primer extension reaction, and differences in MOI. The same RNA used for analysis of the UL24b' transcript was primer extended using a primer specific for the US11 gene. Figure 13 shows that the levels of US11 produced by KOS SB, vTG8, and v28 are all similar indicating that the MOIs, RNA harvest, and amount of RNA primer extended are comparable. Any differences in the amount of UL24b' transcript between KOS SB, vTG8, and v28 therefore reflect true differences in the expression of UL24b'. Furthermore, the presence of the US11 transcript in KOS SB infected cells indicates that the viral infection did proceed, therefore the absence of the UL24b' transcript in KOS SB infected cells (figure 12) is a real result. Moreover, US11 is expressed very weakly during early times post infection and maximum expression occurs at late times post infection. A comparison of the kinetics of expression between US11 and UL24b' demonstrates that UL24b' accumulates earlier than US11, indicating that UL24b' belongs to the leaky late kinetic

Figure 13 Primer Extension Analysis of US11

As an internal control to the experiment described in figure 11 the same RNA used for the analysis of UL24b' RNA was primer extended using a probe specific for the US11 gene. The time post infection that the RNA was harvested is indicated at the top of the figure. The US11 primer extension products as well as the postion of the free probe is indicated on the left of the figure. Marker lanes were derived as previously described. Relative sizes, in base pairs, are indicated to the right of the figure.


KOS SB
vTG8
v28
hrs PI→ 0 3 6 12 0 3 6 12 0 3 6 12 M
US11
O→
92
78
69

class (see below).

3.3 Expression of UL24b' in the Presence of Cycloheximide

Kinetic analysis of UL24b' in cells infected with v28 and vTG8 indicated that the TAATGARAT sequences in vTG8 did not alter the pattern of appearance of UL24b' from late to IE. Immediate early genes are identified not only according to the criterion of temporal appearance but are also identified according to their behaviour in the presence of protein synthesis inhibitors. In the continuous presence of a protein synthesis inhibitor, such as cycloheximide, the only HSV genes to be expressed are the IE genes. For this reason, it was feasible to test for the expression of UL24b' in the presence of cycloheximide.

Figure 14 demonstrates the expression of UL24b' in the presence of cycloheximide. Vero cells were infected at an MOI of 10 with vTG8 either in the continuous presence or the absence of cycloheximide. Cytoplasmic RNA was harvested at 0,3,6,9, and 12 hours post infection and analyzed by primer extension, as mentioned above. As a control Vero cells were also infected at an MOI of 10 with v28 in the continuous presence or absence of cycloheximide. Figure 14 shows

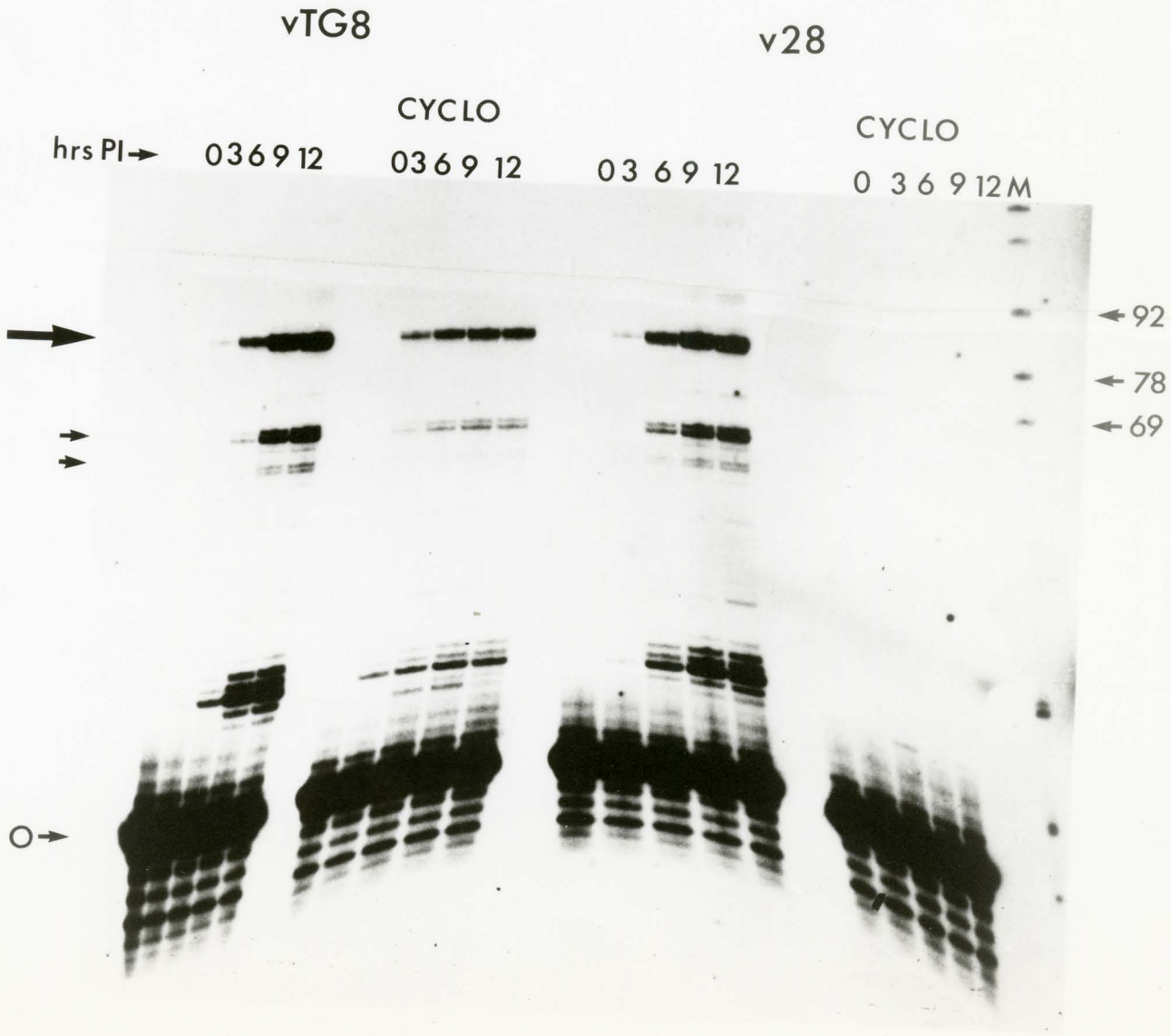
that in the absence of cycloheximide the expression of UL24b' in cells infected with v28 and vTG8 is nearly identical and is characteristic of the leaky late kinetic class ,therefore confirming earlier experiments (figure 12). In contrast, in the presence of cycloheximide, only cells infected with vTG8 express UL24b'.

As internal controls the same RNA used for the analysis of UL24b' was also primer extended using oligonucleotides specific for the IE gene ICP4 and the true late gene US11. Figure 15 illustrates primer extension analysis of ICP4 mRNA. ICP4, a true IE gene, is expressed maximally at early times post infection and expression declines at late times due to autoregulation by the ICP4 protein (Roberts et al., 1989). Comparison of the kinetics of appearance of ICP4 and UL24b' RNA clearly demonstrates that UL24b' is not expressed with the kinetics characteristic of IE genes. In contrast comparing the kinetics of expression of UL24b' with US11 (figure 16) confirms the conclusion that UL24b' is expressed with leaky late kinetics.

In the presence of cycloheximide no ICP4 protein is synthesised. ICP4 mRNA is therefore greatly over produced due to the removal of autoregulation. The over production of ICP4 mRNA in cells infected in the presence of cycloheximide with v28 and vTG8 indicates

Figure 14 Expression of UL24b' in the Presence of Cycloheximide

Confluent monolayers of Vero cells, approximately 1.5 X 10^7 cells per plate were infected with an MOI of 10 pfu per cell with either vTG8 or v28 in the presence or absence of 100 ug/ml cycloheximide. Cells to be infected in the presence of cycloheximide were preadsorbed with 100 ug of cycloheximide per ml of tissue culture media for one half hour prior to infection. Cytoplasmic RNA was harvested at 0,3,6,9, and 12 hours post infection as indicated at the top of the figure. Lanes marked CYCLO indicate RNA harvested from cells treated with cycloheximide. On the left the large arrow indicates the position of the UL24b' primer extension product and the small arrow, labelled 'O' marks the position of the free primer. Other arrows indicate products produced by pausing of the reverse transcriptase. Size markers in the lane labelled M are as previously indicated and sizes, in base pairs, are indicated on the right.


vTG8
v28
CYCLO
CYCLO
hrs PI →
0 3 6 9 12
0 3 6 9 12
0 3 6 9 12
0 3 6 9 12 M
92
78
69
O →

that the dose of cycloheximide was efficient in inhibiting de novo viral protein synthesis. This point is further confirmed by the absence of a US11 transcript in cells infected in the presence of cycloheximide (figure 16). Cycloheximide indirectly inhibits viral DNA replication by preventing the synthesis of the early class viral proteins which are required for viral DNA replication (Olivo et al., 1989). US11, being a true late gene is strictly dependent on viral DNA replication for maximal expression. Lastly, the similar levels of ICP4 and US11 RNA produced in cells infected with v28 or vTG8 indicates that the MOI, RNA harvest, and amount of RNA primer extended were comparable. The differences in the behaviour of UL24b' produced in v28 and vTG8 infected cells therefore reflect true differences in the expression of UL24b'.

In summary, UL24b' RNA encoded by vTG8 and v28 accumulates with a temporal pattern consistent with the leaky late kinetic class. The nine forward TAATGARAT elements therefore had no effect on temporal appearance of the UL24b' transcript. In the presence of cycloheximide UL24b' was only expressed in cells infected with vTG8. The expression of UL24b' in vTG8 infected cells is therefore characteristic of the IE pattern of HSV gene expression since expression is independent of

Figure 15 Primer Extension Analysis of ICP4

As an internal control to the experiment described in figure 14 the same RNA used to quantitate UL24b' RNA was primer extended with a probe specific for the ICP4 gene. The times post infection at which the RNA was harvested are indicated at the top of the figure. Lanes marked CYCLO indicate RNA harvested from cells treated with cycloheximide. The positions of the ICP4 primer extension product and the free probe are indicated on the left. Sizes, in base pairs, of the markers in lane M are indicated on the right.

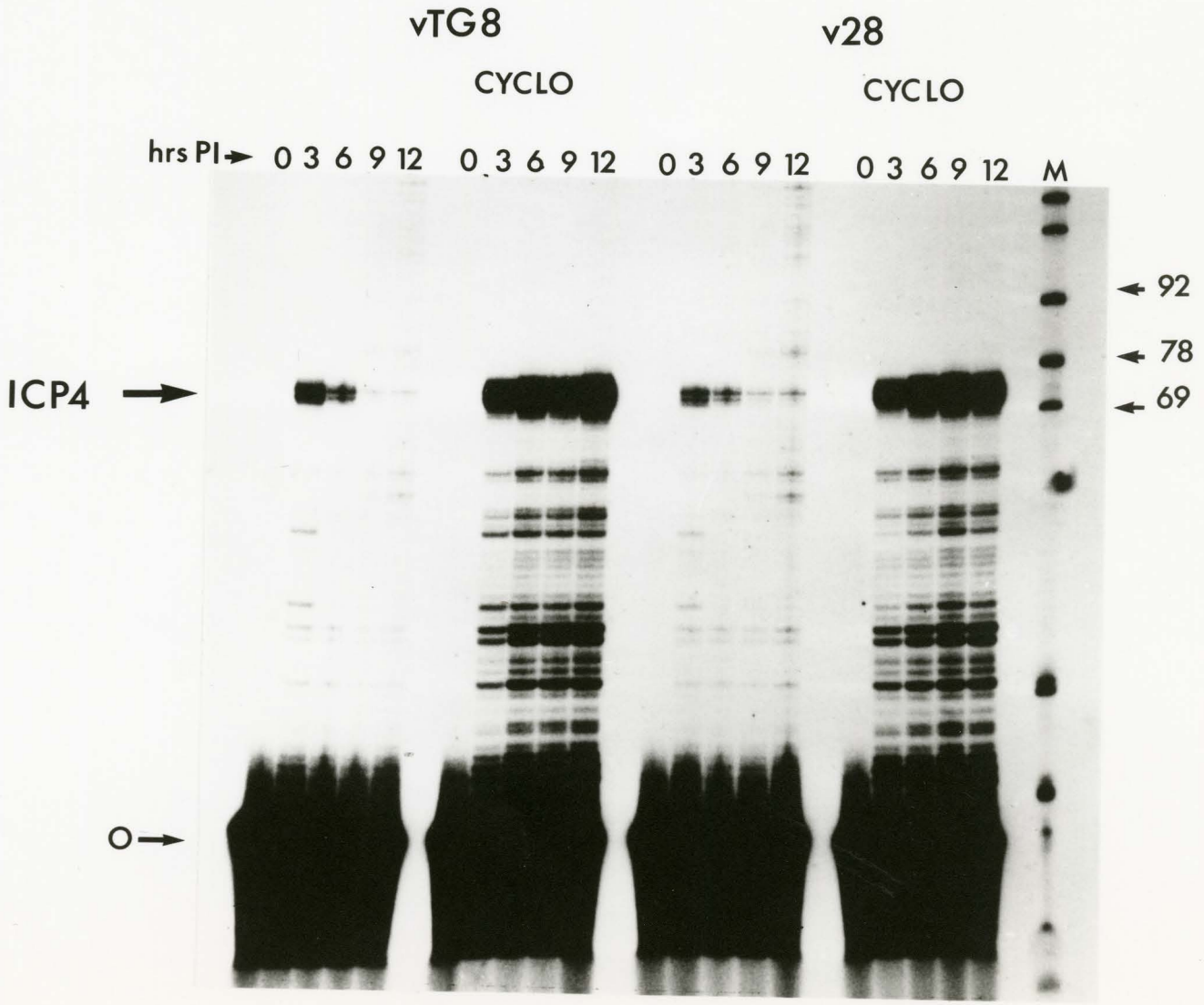
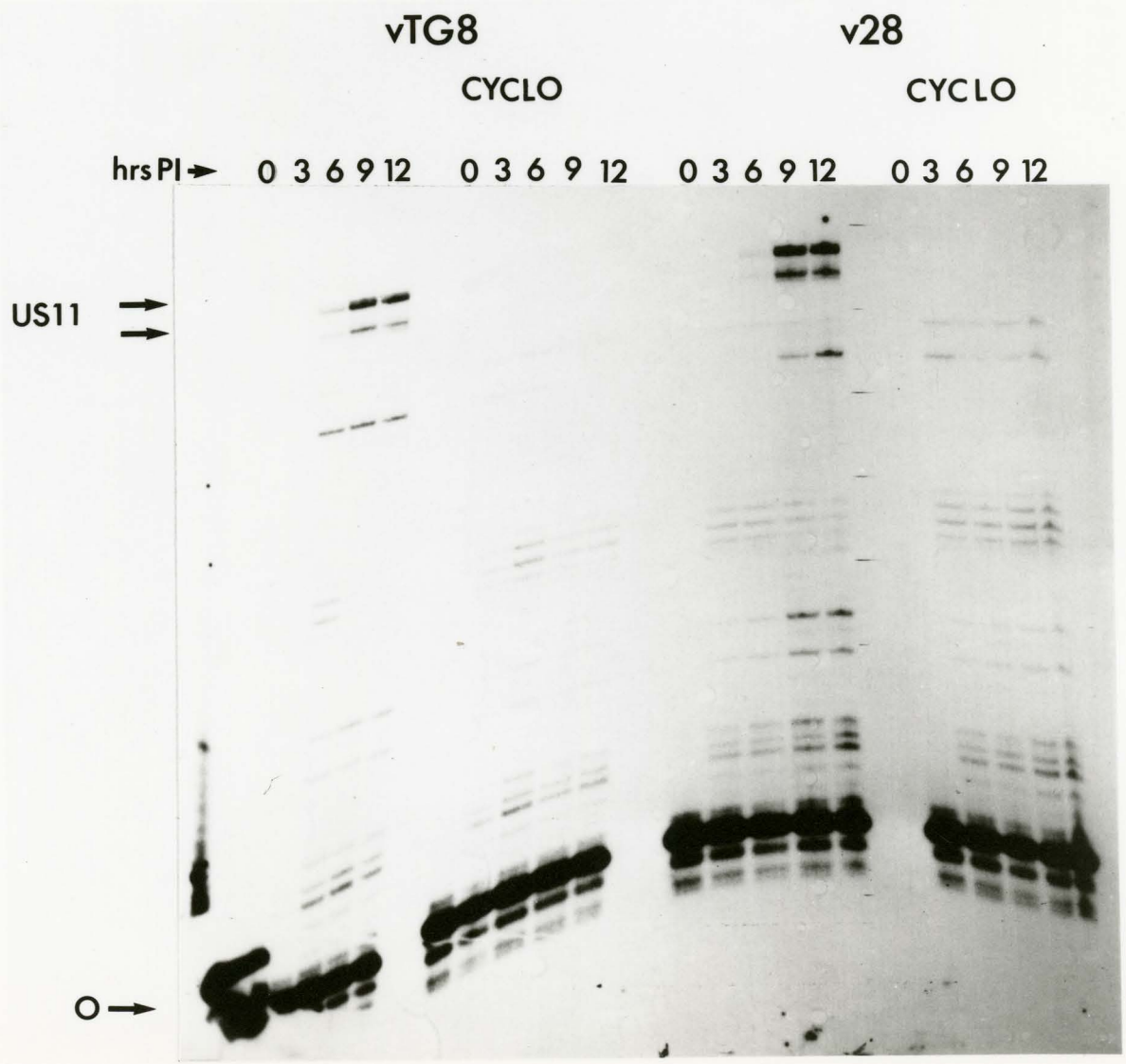

vTG8
CYCLO
v28
CYCLO
hrs PI
0 3 6 9 12
0 3 6 9 12
0 3 6 9 12
0 3 6 9 12
M
92
78
69
ICP4
O

Figure 16 Primer Extension Analysis of US11 in the Presence of Cycloheximide

As an internal control to the experiment described in figure 14 the same RNA used for the analysis of UL24b' RNA was primer extended with a probe specific for the US11 gene. The time at which RNA was harvested post infection is indicated at the top of the figure. Lanes marked CYCLO indicate RNA harvested from cells that were treated with cycloheximide during the infection. The position of the US11 primer extension products and the free probe are indicated on the left.


vTG8
v28
CYCLO
CYCLO
hrs PI
0 3 6 9 12
0 3 6 9 12
0 3 6 9 12
0 3 6 9 12
US11
O

any de novo viral protein synthesis. Expression of UL24b' encoded by vTG8 displays characteristics of both IE and late kinetic classes. Differences in the pattern of expression of UL24b' between v28 and vTG8 can be directly attributed to the presence of the nine tandem repeats of the TAATGARAT oligonucleotide linked upstream of the MLP TATA box in vTG8.

3.4 Effect of Orientation and Number of TAATGARAT Motifs

A direct comparison of the level of UL24b' expression in cells infected with v28, vTG1, vTG1R, vTG3R, vTG8R, and vTG8 is presented in figures 17 and 18. Vero cells were infected with the desired recombinant virus using an MOI of 10 in the continuous presence or absence of specific metabolic inhibitors. The metabolic inhibitors used were cycloheximide and aphidocolin which inhibit viral protein synthesis and viral DNA replication respectively. Viral DNA replication was inhibited to examine transcription of UL24b' off of the same number of viral DNA templates in the cell. A direct comparison of the strength of each promoter could therefore be performed. Cytoplasmic RNA was harvested at 0 and 6 hours post infection and the UL24b' transcript was quantitated by an S1 nuclease protection assay. Details on the

preparation of the probe used for the analysis is provided in Methods and Materials. The large arrow to the left of figures 17 and 18 indicates the fragment of the probe protected by the UL24b' transcript and the small arrow indicates the position of the full length probe. The size of the protected fragment indicates that the site of UL24b' transcription initiation is similar to the site predicted by primer extension analysis. All of the results illustrated in figures 17 and 18 were quantitated by densitometry and are presented in graphic form in figures 19 and 20.

A comparison of the UL24b' levels in cells infected with vTG1R, vTG3R, and vTG8R allows one to determine the effect of different numbers of tandemly repeated TAATGARAT elements on transcription. Figure 17 shows that in the presence or absence of metabolic inhibitors UL24b' expression is similar in cells infected with vTG1R and v28. In the absence of metabolic inhibitors addition of 3 or 8 TAATGARATs increased the amount of UL24b' expression compared to vTG1 and v28. Comparing the expression of UL24b' from the same number of DNA templates, in the presence of ahidocolin, further confirms the observation that addition of 3 or 8 TAATGARAT elements increase the strength of the promoter while one TAATGARAT element has no significant effect.

Furthermore, unlike the synthetic promoter containing one TAATGARAT, the synthetic promoters containing 3 or 8 TAATGARATs were competent in alleviating the requirement for de novo viral protein synthesis prior to the expression of UL24b'. Both vTG3R and vTG8R expressed UL24b' in the presence of cycloheximide while no UL24b' RNA is evident in cells infected with vTG1R.

Comparing the expression of UL24b' in cells infected with vTG1 and vTG8 confirms the observation that TAATGARAT motifs must be present as tandem repeats to promote transcription in the absence of any viral protein synthesis. Figure 18 illustrates that vTG8, but not vTG1, was competent in transcribing UL24b' in the presence of cycloheximide. All three viruses, v28, vTG1, and vTG8 transcribed similar amounts of UL24b' in cells infected without any metabolic inhibitors and in cells infected in the presence of aphidocolin.

The effect of orientation of the TAATGARAT element on transcription was determined by comparing the levels of UL24b' in cells infected with vTG8R and vTG8. Figure 17 demonstrates that without metabolic inhibitors 8 TAATGARATs in the reverse orientation are more active than 9 TAATGARATs in the forward orientation. In fact, 3 TAATGARATs in the reverse orientation are more active than 9 TAATGARATs in the forward orientation in the

absence of inhibitors. Both promoters containing 8 TAATGARAT motifs in the reverse or 9 TAATGARAT motifs in the forward orientation were able to direct transcription of UL24b'in the presence of cycloheximide. Interestingly, both vTG8 and vTG8R express more UL24b' in the presence of cycloheximide than in the presence of aphidocolin. Both orientations of the TAATGARAT motifs, with respect to the MLP TATA box, are therefore similar in the ability to promote transcription of UL24b' in the absence of de novo viral protein synthesis.

As an internal control the same RNA used to quantitate the UL24b' transcript was also primer extended using a probe specific for the early glycoprotein D (gD) gene. Figure 21 indicates relatively constant levels of gD produced by all of the recombinant viruses. The gD gene is classified as an early gene despite a decrease in gD expression when viral DNA replication is inhibited (Smith and Sandri-Goldin, 1988). Figure 20 demonstrates that gD levels do decrease in the presence of aphidocolin. In addition, no gD expression is expected in the presence of cycloheximide since expression of gD is dependent on IE polypeptides (Everett, 1983). Relative consistency in the gD signal indicates that MOI, RNA harvested, and the amount of RNA probed by S1 analysis was constant. Any differences in the UL24b' signal

Figure 17 Comparison of UL24b' Expressed From Different TAATGARAT Recombinant Viruses

Confluent monolayers of Vero cells were infected with an MOI of 10 pfu per cell with one of the following viruses: vTG1R, vTG3R, vTG8R, vTG8, or v28, as indicated at the top of the figure. Cells were infected in the absence of metabolic inhibitors or in the presence of 100 ug/ ml cycloheximide (lanes labelled with a 'c') or in the presence of 10 ug/ml aphidocolin (lanes labelled with an 'a'). Cytoplasmic RNA was harvested at 0 and 6 hours post infection. UL24b' RNA was quantitated by S1 nuclease analysis using the 110 base pair 5'- ^{32}P end labelled Nru I and Sst I fragment of p28 as probe (for more details on the construction of the S1 probe refer to the Methods and Materials). The position of the UL24b' protected fragment is indicated by the large arrow on the left. The position of the intact probe is indicated by the arrow labelled 'P'. Size markers were derived as previously described and sizes are indicated in base pairs on the right.

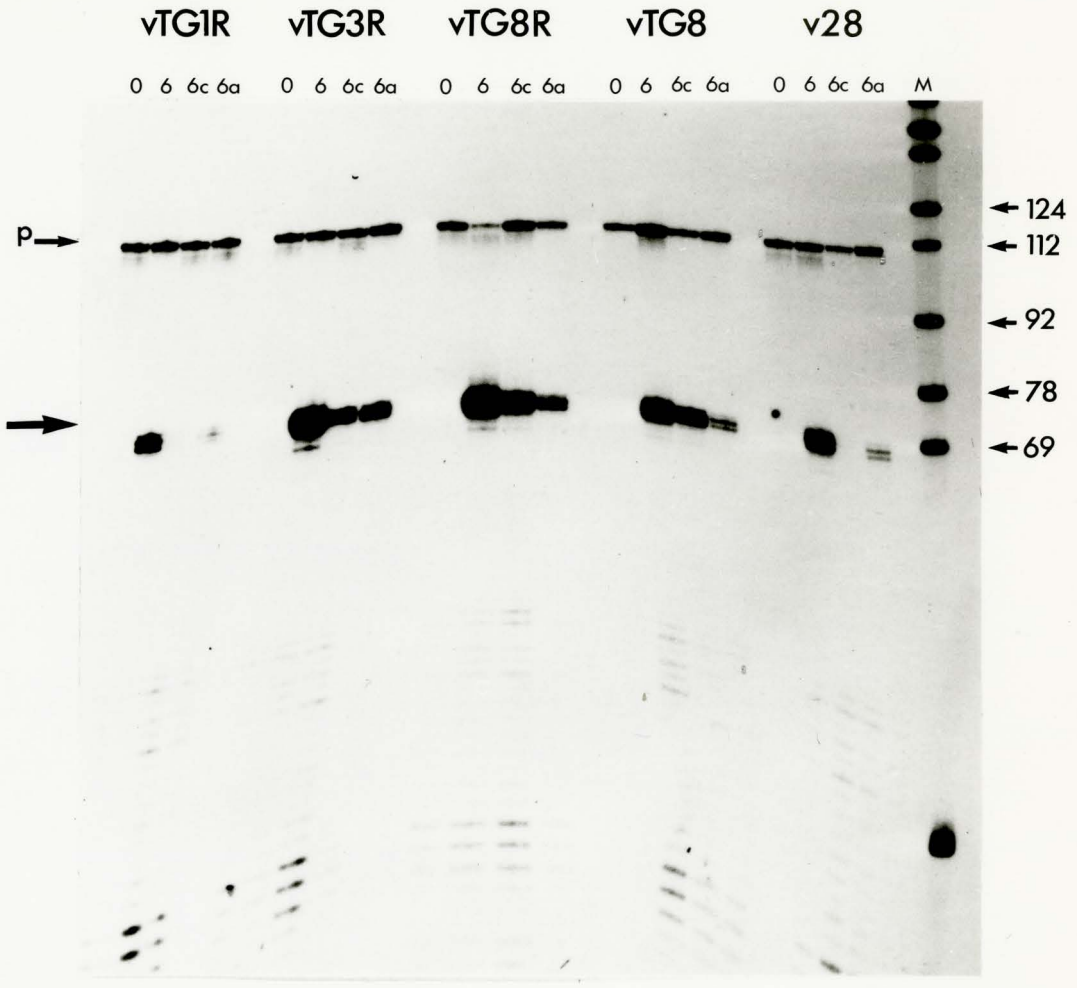

vTG1R
vTG3R
vTG8R
vTG8
v28
0 6 6c 6a
0 6 6c 6a
0 6 6c 6a
0 6 6c 6a
0 6 6c 6a
M
P
124
112
92
78
69

Figure 18 Comparison of UL24b' Expressed From vTG1 and vTG8

Monolayers of Vero cells, approximately 1.5 X 10^7 cells per plate were infected at an MOI of 10 with one of the following viruses: vTG1, vTG8, or v28. Cells were infected in the absence of any metabolic inhibitors or in the presence of either 100 ug/ml cycloheximide (lanes labelled with a 'c') or 10 ug/ml aphidocolin (lanes labelled with an 'a'). Cytoplasmic RNA was harvested at 0 and 6 hours post infection. RNA was analyzed by S1 nuclease protection using a probe specific for the UL24b' transcript as described in the figure legend of figure 17. The large arrow on the left indicates the position of the UL24b' protected fragment and the arrow labelled with a 'P' indicates the position of the intact probe. Sizes of the markers, in base pairs, are indicated to the right.

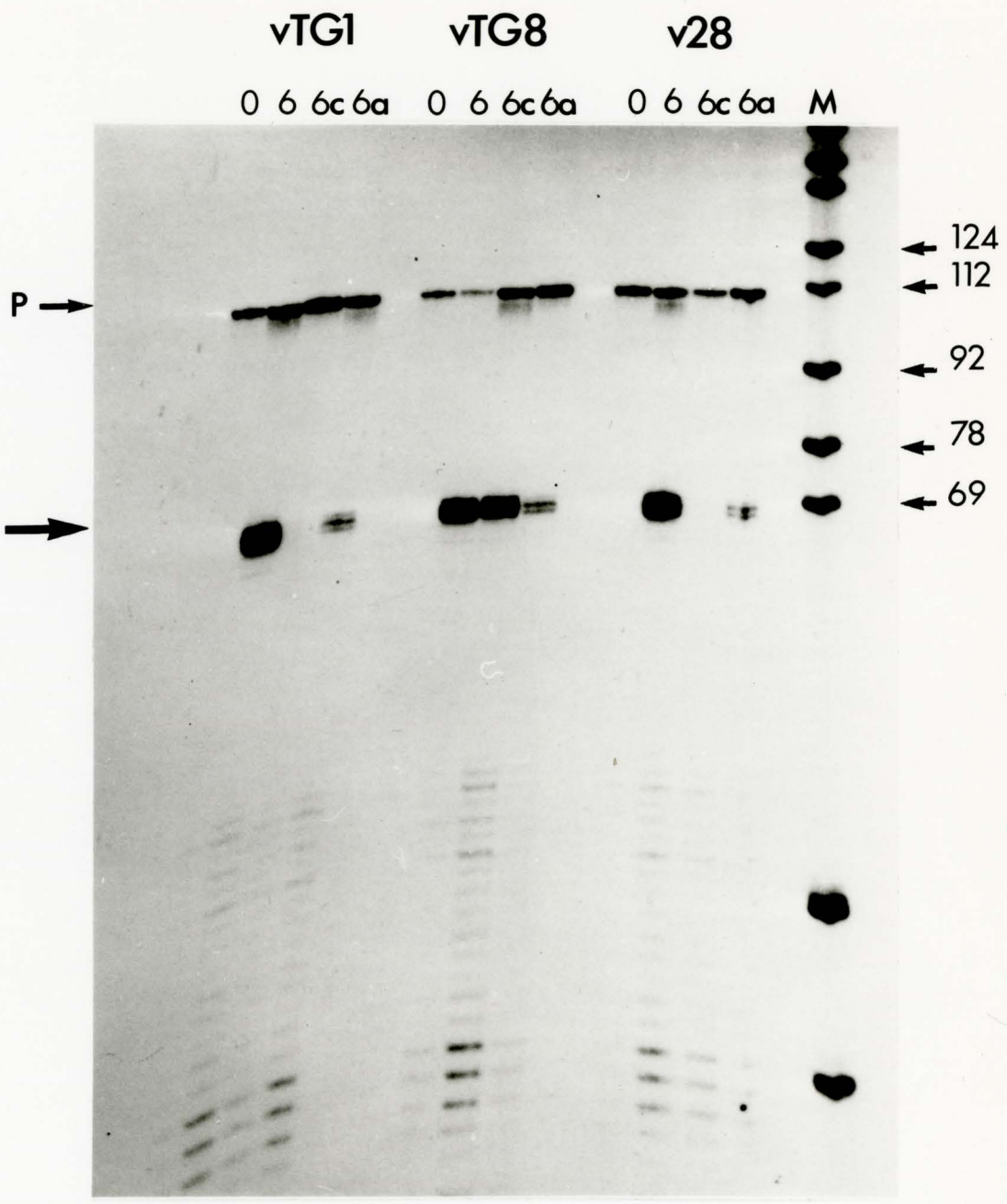

vTG1
vTG8
v28
0 6 6c 6a 0 6 6c 6a 0 6 6c 6a M
P
124
112
92
78
69

Figure 19 Densitometric Analysis of Figure 17

The autoradiogram of figure 17, not exposed with intensifying screens, was analysed by densitometry. Values on the ordinate, levels of cytoplasmic RNA, were obtained by weighing the peaks obtained by the densitometry.

CYTOPLASMIC LEVELS OF THE NOVEL TRANSCRIPT PRODUCED BY DIFFERENT "TAATGARAT" RECOMBINANT VIRUSES IN DRUG BLOCKED AND UNBLOCKED INFECTIONS

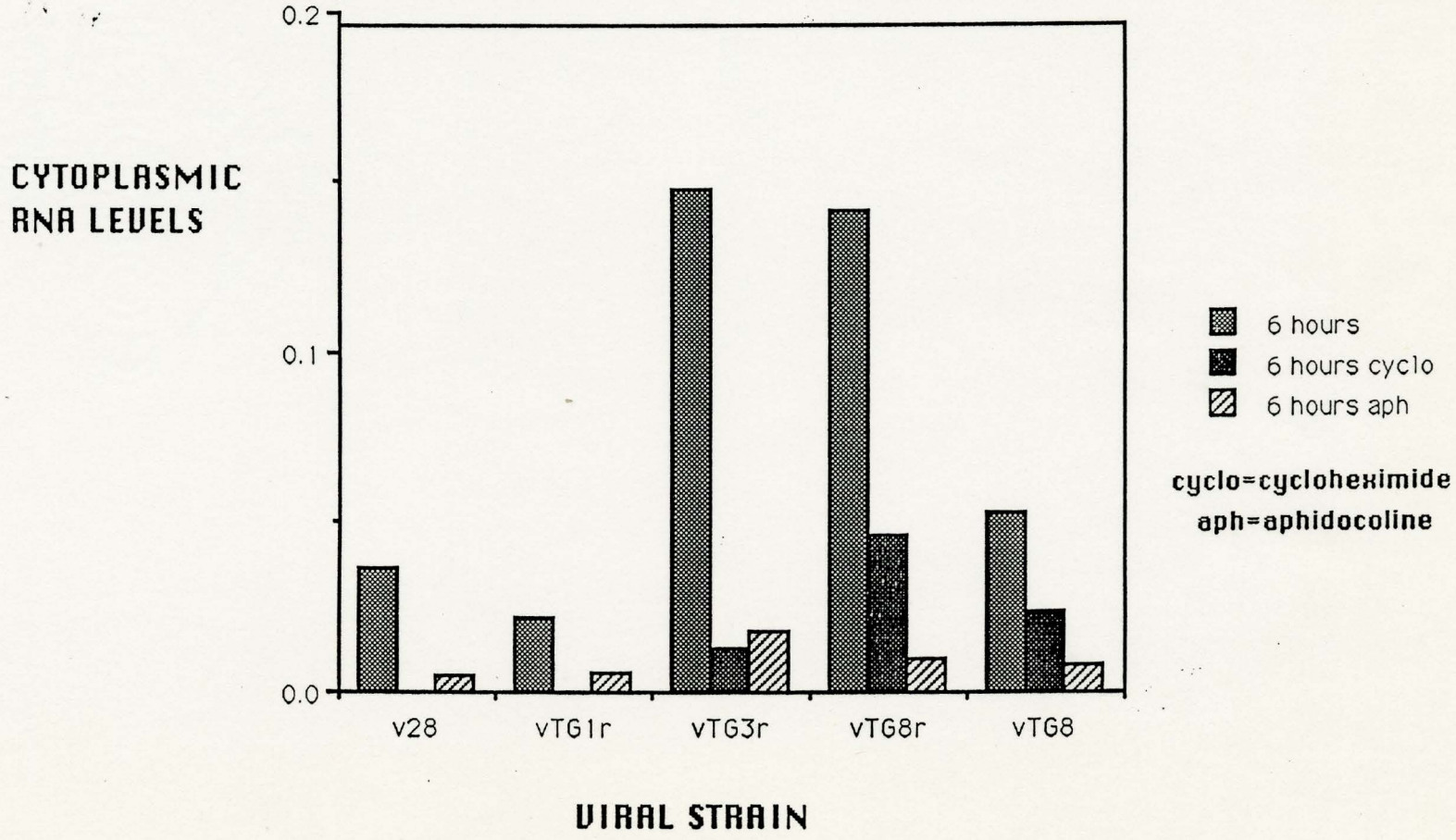
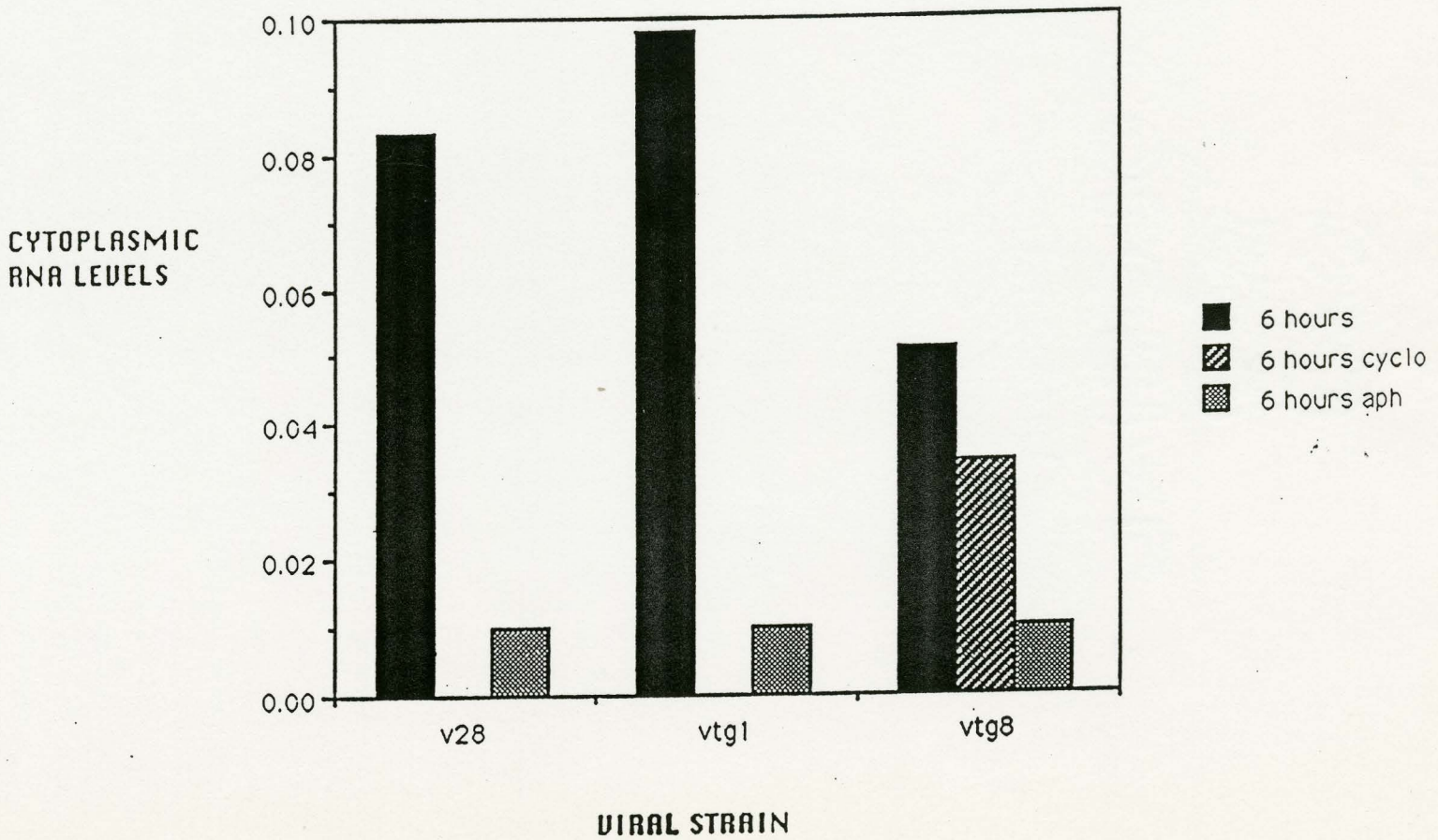


Figure 20 Densitometric Analysis of Figure 18

The autoradiogram of figure 18, not exposed with intensifying screens, was analysed by densitometry. Values on the ordinate, levels of cytoplasmic RNA, were obtained by weighing the peaks produced by the densitometry.

CYTOPLASMIC LEVELS OF THE NOVEL TRANSCRIPT PRODUCED BY vTG1, vTG8 and v28



therefore reflect true differences in the amount of UL24b' expressed by the different viruses.

To determine whether the TAATGARAT sequence functions downstream of a promoter the same RNA used for the analysis of UL24b' RNA was primer extended using a probe specific for tk. Within the recombinant viruses the TAATGARAT elements are located approximately 600 base pairs downstream of the tk transcription initiation site. Figure 22 shows that none of the recombinant viruses alter the characteristics of the tk transcript. Thymidine kinase transcription is inhibited by exposure to cycloheximide which suggests that tk expression is still strongly dependent on the presence of IE proteins. In addition, primer extension of tk RNA provides a second internal control. The similar levels of tk expression in all of the recombinant viruses confirm the conclusion that the differences in the amounts of UL24b' RNA reflect true differences in the expression of UL24b' between the different recombinant viruses.

In summary, the result presented in figures 17, 18, and 19 and 20 demonstrate the following: insertion of one TAATGARAT motif, in either orientation upstream of the MLP TATA box had no detectable effect on the expression of UL24b' under any condition tested. Addition of 3 and 8 TAATGARAT motifs in the reverse orientation or

Figure 21 Primer Extension Analysis of gD

As an internal control for the experiment described in figures 17 and 18 the same RNA used for the S1 nuclease protection assay was primer extended with a probe specific for the HSV gD gene. The viral strain producing the gD transcript is indicated at the top of the figure. The superscripts 1 and 2 added to the name of the viral strains indicate RNA obtained from the experiments presented in figure 17 and 18 respectively. Cytoplasmic RNA was harvested at 0 and 6 hours post infection. Lanes labelled with a 'c' or an 'a' indicate RNA harvested from cells treated with cycloheximide or aphidocolin respectively. The position of the gD primer extension product as well as the position of the free probe is indicated on the right. The length of the marker bands, in the lane labelled M, are indicated on the left.

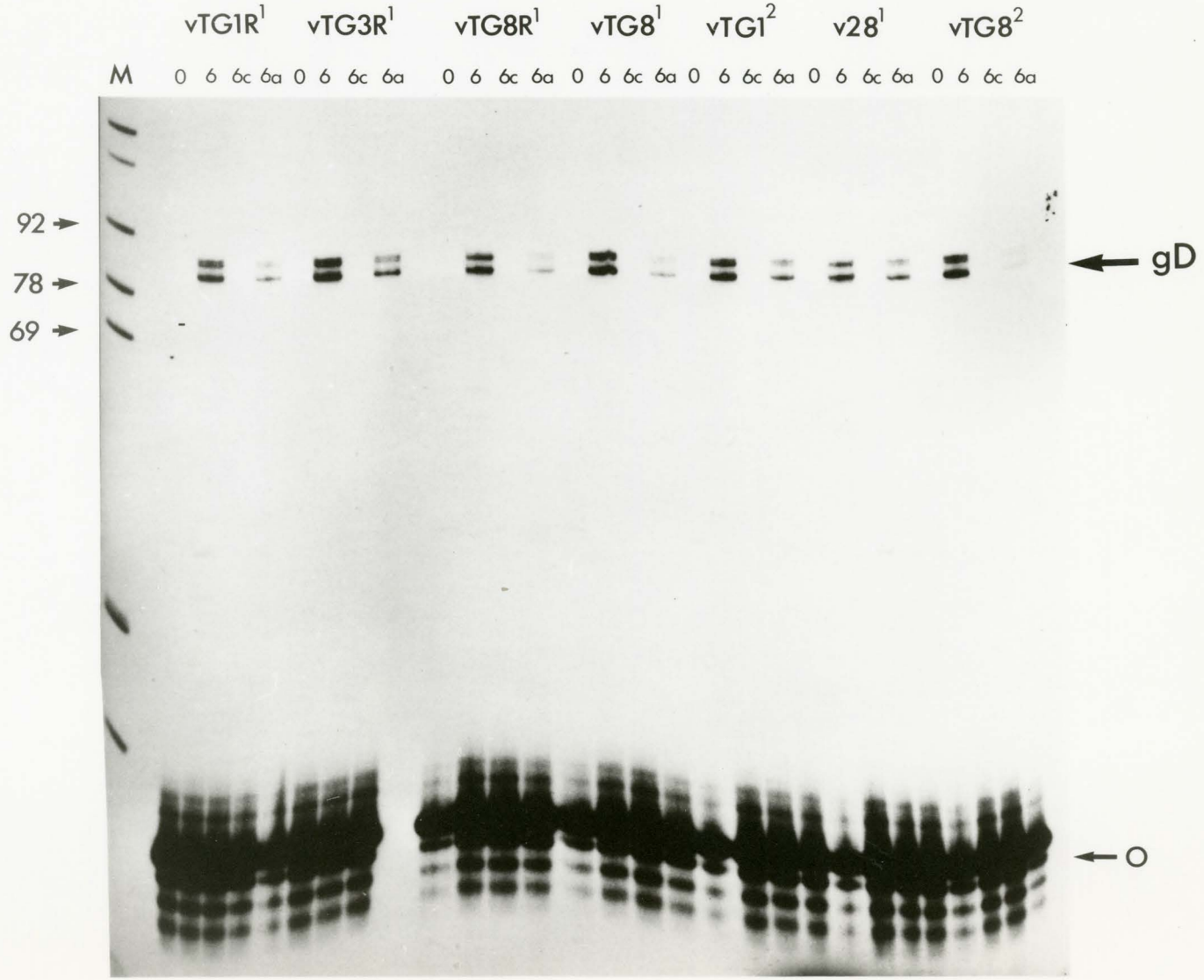
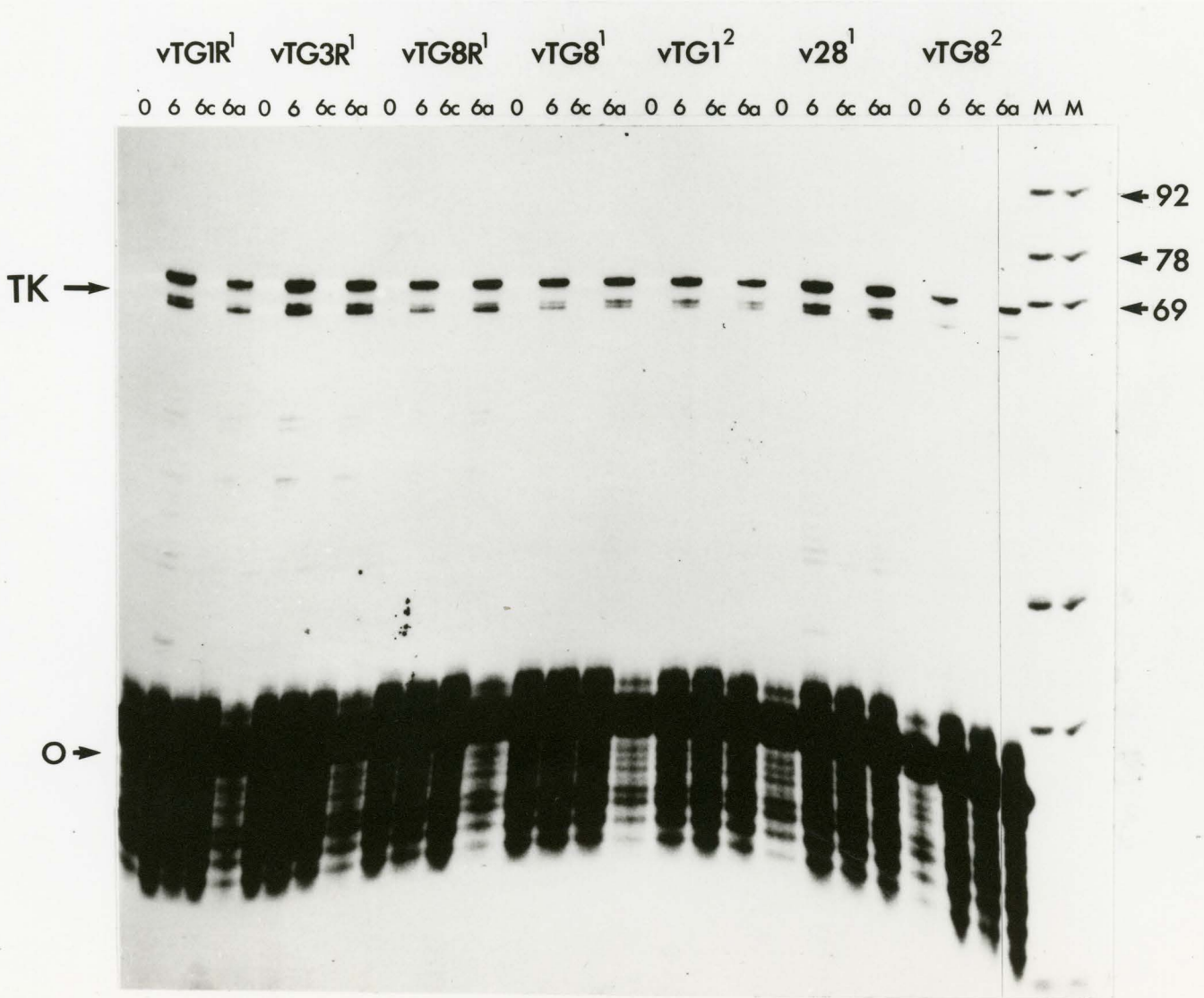

vTG1R[1]
vTG3R[1]
vTG8R[1]
vTG8[1]
vTG1[2]
v28[1]
vTG8[2]
M
0 6 6c 6a 0 6 6c 6a 0 6 6c 6a 0 6 6c 6a 0 6 6c 6a 0 6 6c 6a 0 6 6c 6a
92
78
69
gD
O

Figure 22 Primer Extension Analysis of Thymidine Kinase

To determine the effect of the TAATGARAT motifs on the expression of tk the same RNA used for the S1 nuclease analysis of UL24b' presented in figures 17 and 18 was primer extended with a probe specific for the tk gene. Names of the viral strains are indicated at the top of the figure. The superscripts 1 or 2 added to the name of the viral strain indicate RNA obtained from the experiments presented in figure 17 or 18 respectively. Cytoplasmic RNA was harvested at 0 and 6 hours post infection. Lanes labelled with a 'c' or with an 'a' indicate RNA harvested from cells treated with cycloheximide or aphidocolin respectively. The position of the tk primer extension product and the free primer is indicated on the left. The lengths of the marker bands, in base pairs, are indicated on the right.


vTG1R[1]
vTG3R[1]
vTG8R[1]
vTG8[1]
vTG1[2]
v28[1]
vTG8[2]
0 6 6c 6a 0 6 6c 6a 0 6 6c 6a 0 6 6c 6a 0 6 6c 6a 0 6 6c 6a 0 6 6c 6a M M
92
78
69
TK
O

9 TAATGARAT motifs in the forward orientation relieved the requirement for do novo viral protein synthesis for the expression of UL24b'. Lastly, promoters containing 3 or 8 TAATGARAT motifs, in the reverse orientation, expressed greater amounts of UL24b' in an unblocked infection than did any other recombinant virus tested.

3.5 Transient Expression Assay

The experiments in which Vero cells were infected with the different recombinant viruses carrying various synthetic promoters demonstrated that some promoters were able to direct transcription of UL24b' in the presence of cycloheximide. Transcription of UL24b' in the absence of de novo viral protein synthesis in cells infected with vTG3R, vTG8R, and vTG8 but not in cells infected with v28 suggests that the virion component Vmw65 is responsible for activating the tandem TAATGARAT containing synthetic promoters. At least two alternative explanations can be offered. Firstly, in addition to Vmw65 the virus particle is a vehicle for other viral proteins such as vhs, a protein responsible for degrading host cell RNA as well as various glycoproteins. Other virion proteins brought into the cell other than Vmw65 may be responsible for activating

UL24b'. Secondly, the tandem repeats of the TAATGARAT motif may constitute a constitutive enhancer which no longer requires Vmw65 for activation.

To determine if the synthetic promoter containing tandem TAATGARAT motifs serves as a target for Vmw65 a transient transfection assay was developed. A derivative of the rabbit beta-globin gene was used as a reporter. The normal rabbit beta-globin promoter was replaced by the same synthetic promoter cloned into vTG8. The reporter plasmid, pTTB, therefore contained a rabbit beta-globin gene driven by a synthetic promoter consisting of nine tandem repeats of the TAATGARAT motif in the forward orientation located immediately upstream of the Ad2 MLP TATA box. A control plasmid, pMLB, containing the rabbit beta-globin gene under control of the MLP TATA sequence alone was also constructed. The reporter plasmid or the control plasmid were co-transfected into mouse LTA cells with pMC1, a plasmid containing the Vmw65 gene (Campbell et al., 1984).

Mouse LTA cells were transfected with the appropriate plasmids using DEAE-dextran followed by chloroquine treatment (as described in Methods and Materials). Cytoplasmic RNA was harvested, as previously described, 40 hours after transfection, and RNA was analyzed by primer extension using a probe specific for

beta-globin RNA. Specifically, mouse LTA cells were transfected with pTTB alone, or with pTTB and pMC1, or with pTTB and pDsal. The plasmid pDsal contains a Vmw65 derivative with a stop codon in place of the fourth amino acid, therefore no functional Vmw65 is produced. As controls LTA cells were transfected with pMLB alone, or pMLB and pMC1, or pMLB and pDsal. To control for the transfection efficiency plasmid pRED5 was included in the transfection mixture. This plasmid contains a rabbit beta-globin gene placed under control of the HSV gD gene promoter and linked to the SV40 72 base pair repeat enhancer. Upon transfection of pRED5, beta-globin is constitutively expressed (Everett, 1984a). The internal control plasmid, pRED5, and reporter plasmids pTTB and pMLB contain the same beta-globin gene, therefore any beta-globin RNA transcribed from these plasmids can be detected by primer extension using the same oligonucleotide primer. Beta-globin encoded by pRED5 uses the transcription start site specified by the gD promoter and therefore produces a transcript which is longer than the transcript produced by pTTB or pMLB. The beta-globin RNA produced by pRED5 and pTTB or pMLB can therefore be readily distinguished.

Results of a co-transfection experiment are demonstrated in figure 23. Clearly, Vmw65 activated

transcription only from pTTB and not pMLB (compare lanes pTTB + and pMLB +). The nine tandem TAATGARAT motifs present in the promoter which drives the expression of globin in pTTB are therefore targets for transactivation by Vmw65. Beta-globin was not detectably expressed from pTTB in the absence of a functional Vmw65 gene product. The presence of significant levels of a pRED5 encoded beta-globin transcript in all of the lanes indicates that all of the cells were transfected with comparable efficiency, therefore any differences observed are not due to differential transfection efficiency. Furthermore, in cells transfected with pRED5 alone (labelled, R5) no transcript other than the pRED5 transcript is produced. The pTTB transcript (in the pTTB + lane) is therefore not the result of reverse transcriptase pausing during the reverse transcription of the pRED5 transcript. Comparison of the pTTB signal and the signal produced by the reverse transcription of purified rabbit beta-globin (labelled B) reveals that the beta-globin transcript from pTTB does not use the normal beta-globin cap site but rather uses two closely spaced start sites approximately 30 base pairs downstream of the MLP TATA box.

The transient transfection experiment clearly demonstrated that nine tandem TAATGARAT motifs acted as targets for transactivation by Vmw65. Most importantly,

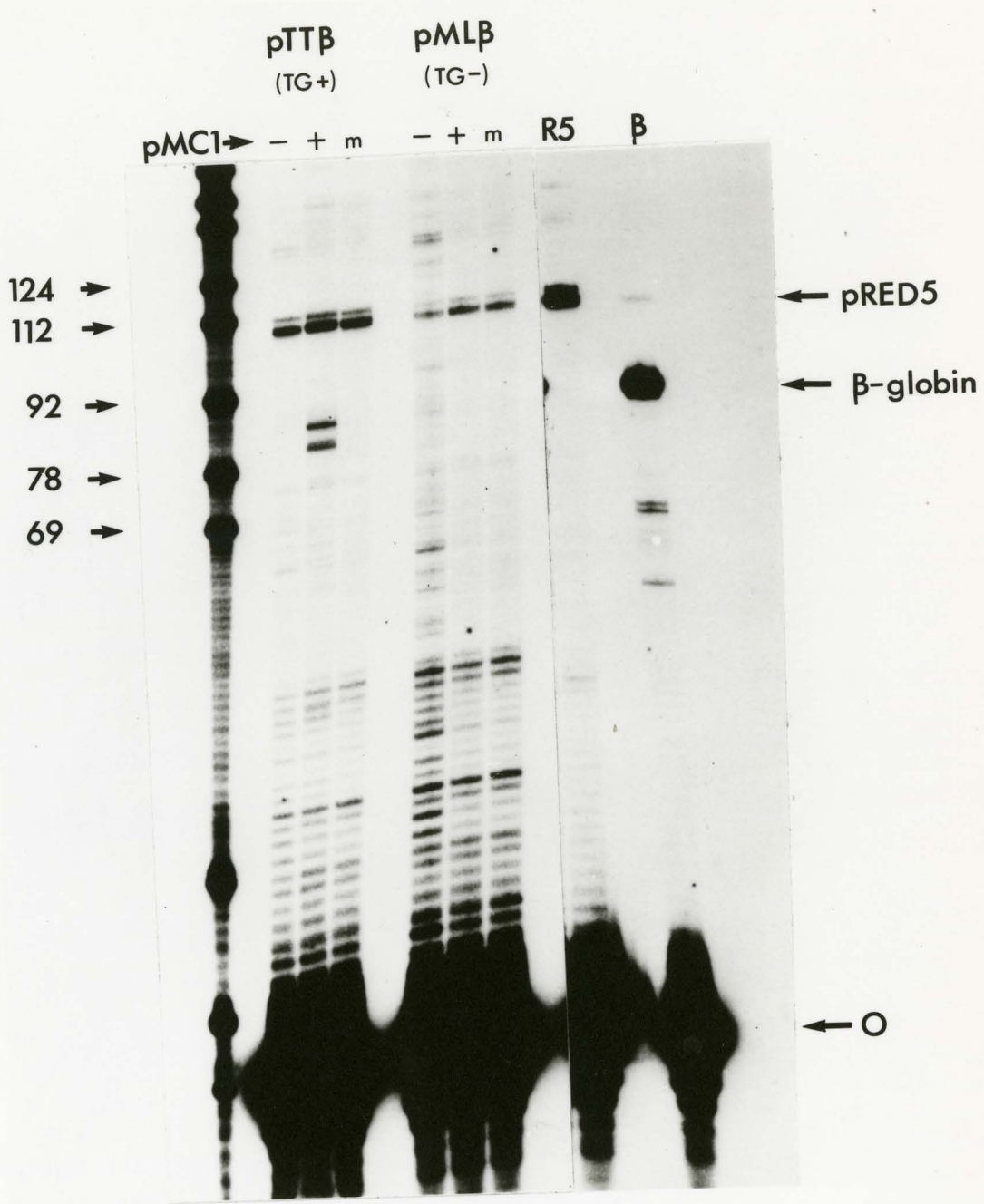
no other sequence motif other than the TAATGARAT motif and the TATA box was required for the activation of the linked beta-globin gene by Vmw65.

3.6 Expression of UL24b' in Vero and Mouse LTA Cells

The series of experiments in which Vero cells were infected with various recombinant viruses demonstrated that synthetic promoters containing tandem TAATGARAT elements were active independent of de novo viral protein synthesis. These observations suggested that the TAATGARAT elements were targets for a transcription activating protein brought into the cell as part of the virus particle, namely Vmw65. Transient transfection experiments clearly demonstrated that the synthetic promoter in vTG8 was a direct target for Vmw65 transactivation. Collectively, both types of experiments strongly suggest that the tandem TAATGARAT elements, of the synthetic promoters in the recombinant viruses, serve as targets for Vmw65 brought into the cell during progressive viral infection. The infections with the different recombinant viruses and the transient transfections were performed using different cell types. Vero cells were used in the infection experiments and mouse LTA cells were used in the transient transfection

Figure 23 Transient Transfection Assay

Mouse LTA cells were transfected using DEAE Dextran followed by a chloroquine chase. Cytoplasmic RNA was harvested 40 hours post transfection and primer extended using a probe specific for rabbit beta-globin. As indicated on the figure, the cells were transfected with the following DNA: pTTB without pMC1 (-), pTTB with pMC1 (+), pTTB with pDSAL (m), pMLB without pMC1 (-), pMLB with pMC1 (+), pMLB with pDSAL (m). Plasmid pTTB contains a synthetic promoter with TAATGARAT motifs (TG+) whereas pMLB does not contain any TAATGARAT (TG-). To control for transfection pRED5 was included in all transfection cocktails. The lane labelled R5 indicates RNA harvested from cells transfected with pRED5 alone. As a marker rabbit beta-globin RNA obtained from Bethesda Research Laboratories was also primer extended and is included in the lane labelled B. Sizes of the marker bands (pBR322 digested with Hpa II) are indicated to the left of the figure. Locations of the pRED5 and rabbit beta-globin primer extension products are indicated to the right. The arrow labelled 'O'marks the position of the free primer.


pTTβ
(TG+)
pMLβ
(TG−)
pMC1
− + m
− + m
R5
β
124
112
92
78
69
pRED5
β-globin
O

experiments. Mouse LTA cells were transfected instead of Vero cells because of the greater potential of LTA cells to take up DNA during a transfection. To demonstrate the relevance of the transfection assays in relation to the infection experiments, it was necessary to demonstrate that the recombinant viruses express UL24b' similarly in both Vero and LTA cells.

Vero and LTA cells were infected at an MOI of 10 with vTG8 in the presence or absence of cycloheximide. As a control Vero and LTA cells were also infected with v28 under the same conditions. Cytoplasmic RNA was harvested at 6 and 12 hours post infection and UL24b' RNA was quantitated by S1 nuclease analysis as previously described. Figure 23 shows that UL24b' was expressed similarly but not identically in both Vero and LTA cells infected with v28. No UL24b' transcript was produced in Vero cells infected with v28 in the presence of cycloheximide, however UL24b' was expressed weakly in LTA cells infected with v28 under the same conditions. Expression of UL24b' was similar in both Vero and LTA cells infected with vTG8. Infection of both cell types with vTG8 produced a UL24b' transcript in the presence of cycloheximide, however greater amounts of UL24b' RNA are produced in LTA cells in the presence of cycloheximide. Most importantly, UL24b' RNA is present in far greater

amounts in LTA cells infected with vTG8 in the presence of cycloheximide than in LTA cells infected with v28 under the same conditions. The comparison of Vero and LTA cells infected with v28 or vTG8 demonstrates that both viruses express UL24b' with similar characteristics in both cell types.

As an internal control the same RNA used to quantitate UL24b' RNA was primer extended with a probe specific for gD RNA. Figure 24 shows that similar amounts of gD RNA was expressed in Vero cells infected with either vTG8 or v28. Slightly less gD was produced in LTA cells infected with either vTG8 or v28 than in Vero cells. The comparable levels of gD expressed by both vTG8 and v28 indicate that any differences observed in the amount of UL24b' RNA reflects true differences in the expression of UL24b' by these viruses.

Figure 24 Expression of UL24b' in Vero and Mouse LTA cells

Confluent monolayers of Vero and LTA cells were infected at an MOI of 10 pfu per cell with either v28 or vTG8 in the continuous presence or absence of 100 ug/ml cycloheximide. Cytoplasmic RNA was harvested at 6 and 12 hours post infection and UL24b' RNA was quantitated by an S1 nuclease protection assay. The time at which RNA was harvested post infection is indicated above each lane. Lanes labelled with a 'c' indicates RNA harvested from cells exposed to cycloheximide. On the left of the figure the large arrow marks the position of the UL24b' protected fragment and the arrow labelled 'P' indicates the position of the full length probe. The sizes of the marker bands, in base pairs, is indicated on the right of the figure.

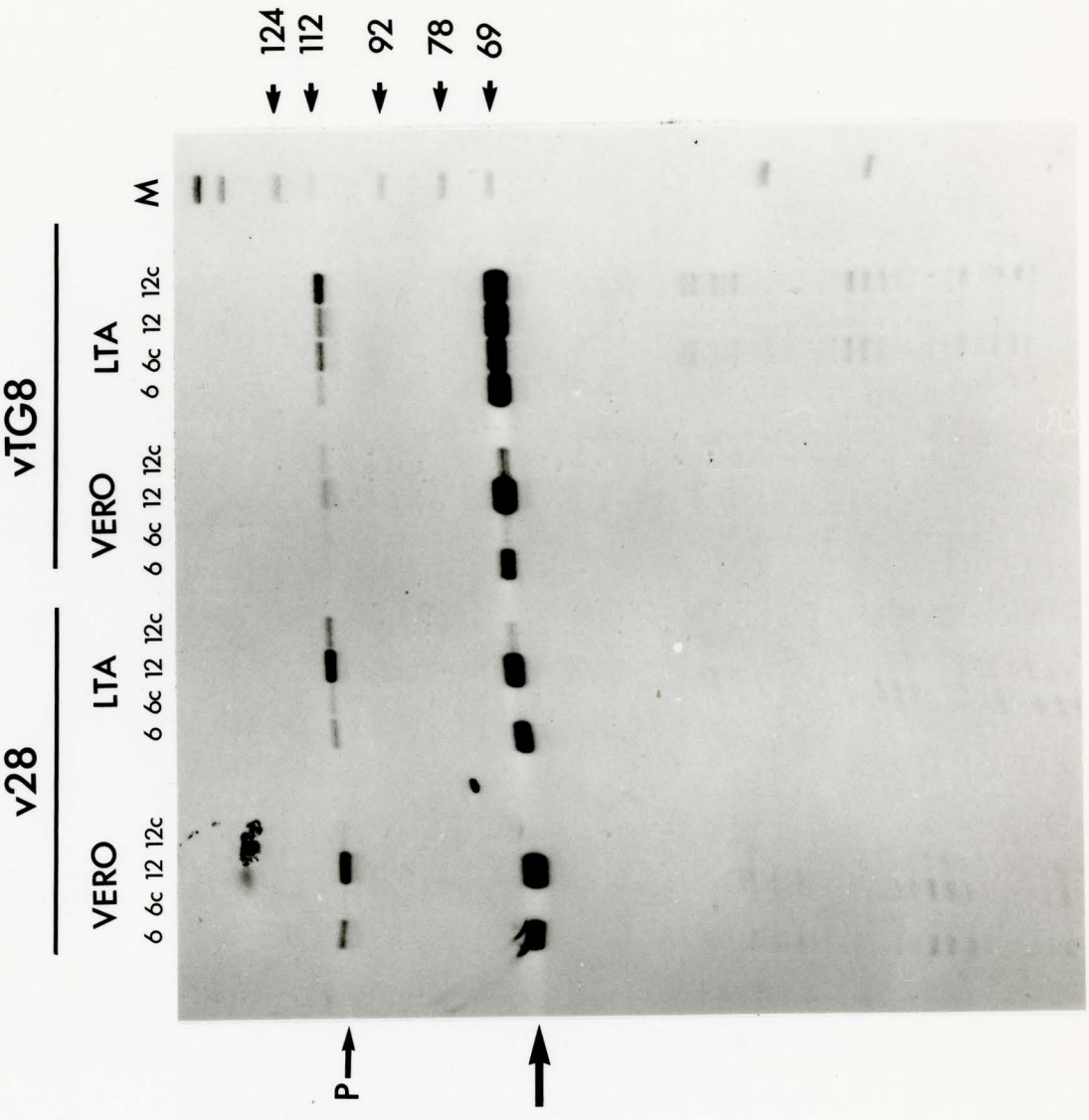
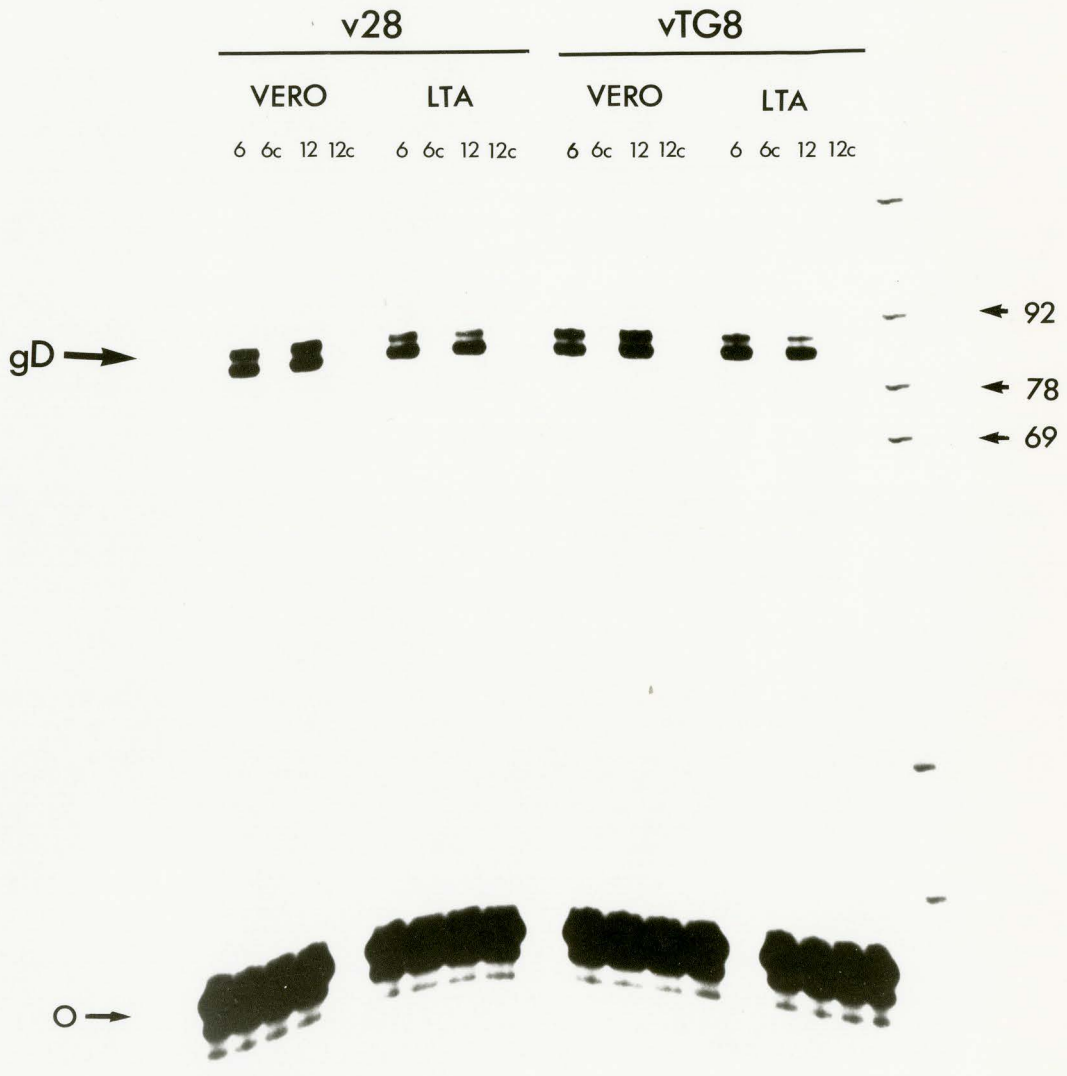

v28
vTG8
VERO
LTA
VERO
LTA
M
6 6c 12 12c
6 6c 12 12c
6 6c 12 12c
6 6c 12 12c
124
112
92
78
69
P

Figure 25 Expression of gD in Vero and Mouse LTA Cells

As an internal control to the experiment presented in figure 24 the same RNA used to quantitate UL24b' RNA was primer extended with a probe specific for gD. The time at which RNA was harvested post infection is indicated above each lane. Lanes labelled with a 'c' indicated that RNA was harvested from cells treated with cycloheximide. The positions of the gD primer extension product and the free probe is indicated on the left. Sizes of the marker bands, in base pairs, is indicated on the right.


v28
vTG8
VERO
LTA
VERO
LTA
6 6c 12 12c
6 6c 12 12c
6 6c 12 12c
6 6c 12 12c
gD
92
78
69
O

Discussion

From the experiments described in this thesis two main conclusions can be drawn. Firstly, the TAATGARAT motif, by itself, when linked to a TATA box provides an adequate target for transcriptional activation by Vmw65. Secondly, even though the TAATGARAT motif is recognised by Vmw65 the transactivation which ensues is not sufficient to induce the IE pattern of transcription on the linked sequences. Both of these conclusions were deduced from experiments involving minimal synthetic promoters containing only certain defined sequence elements.

The construction of transcription regulatory regions using synthetic oligonucleotides is an excellent strategy to determine the importance of individual sequences in the control of gene transcription. This strategy has many advantages over the classical deletion analysis of promoter and enhancer sequences. For example, the construction of synthetic promoters ensures that only the desired sequences are present, therefore any contribution that these sequences have in modulating transcription can be readily determined. Finally, in designing synthetic oligonucleotides the correct spacing between individual sequences can be controlled. For these

reasons, synthetic promoters were designed to examine the mechanisms that control HSV IE gene transcription by RNA pol II.

Specifically, synthetic promoters were designed to answer two main questions. Firstly, does the TAATGARAT sequence, by itself, direct IE kinetics. All HSV IE genes contain at least one copy of the TAATGARAT motif in their far upstream transcription regulatory regions (Mackem and Roizman, 1982a). Furthermore, the TAATGARAT motif serves as a target for transactivation by the structural virion protein Vmw65 (Campbell et al., 1984). Together these observations strongly suggest that IE genes are coordinately induced and are the first genes expressed upon infection because of transactivation by Vmw65 through the conserved TAATGARAT motif. This hypothesis, however, has not been stringently tested. The requirement of the TAATGARAT motif for activation of IE gene transcription by Vmw65 was elucidated mainly through transient transfection assays in which a Vmw65 gene was co-transfected into cells along with a target gene controlled by a promoter containing a TAATGARAT element (Cordingly et al., 1983, Preston et al., 1984, Gaffney et al., 1986, Bzik and Preston, 1986). Kinetics of expression can only be determined when the test gene is present in the context of the viral genome during a

productive infection. Transient transfection assays therefore cannot determine if transactivation of IE gene promoters, by Vmw65, results in the IE pattern of gene expression.

The second question asks if the TAATGARAT element, by itself, is sufficient to act as a target for transactivation by Vmw65, when it is present immediately upstream of a TATA box. The TAATGARAT motif was functionally defined by deletion analysis of IE gene promoters, or by promoter reconstruction experiments (Cordingly et al., 1983, Kristie and Roizman, 1984, Preston, 1984, Gaffney et al., 1985, Bzik and Preston, 1986). In each case the TAATGARAT motif was found together with other sequence elements, therefore the individual role that Vmw65 played in activating transcription could not be determined.

Vmw65 belongs to the class of proteins which activate transcription through a negatively charged acidic activating domain (Triezenberg et al., 1988a, Ace et al., 1988, Sadowski et al., 1988). Other proteins which belong to this class of transcription factors include the yeast transcription activating proteins GAL4 and GCN4 (Ptashne, 1988). The acidic activating domain functionally interacts with other components of the transcription machinery. By using minimal synthetic

promoters the number of protein binding sequences are reduced, therefore the number of proteins which may be contacted by the acidic domain of Vmw65 is greatly reduced. Determining the minimal functional contacts between Vmw65 and other essential proteins will lead to a further understanding of how acid domains function to activate transcription.

4.1 Synthetic Promoters and Recombinant Viruses

Minimal promoters were constructed with synthetic oligonucleotides and contained only two defined sequence motifs. The first oligonucleotide contained the sequence of the TAATGARAT motif located at -147 to -164 relative to the ICP0 transcription start site. The second oligonucleotide contained the sequence of the Ad 2 MLP TATA box. Specifically, five promoters were constructed and assayed. The five promoters consisted of 1,3 or 8 tandemly repeated TAATGARAT motifs cloned 11 base pairs immediately upstream of the Ad 2 MLP TATA box in the reverse orientation, as well as 1 or 9 tandem TAATGARAT motifs 11 base pairs upstream of the MLP TATA box in the forward orientation. The effect of copy number as well as orientation of the TAATGARAT motifs could therefore be determined.

Subsequently, five recombinant viruses were constructed, by marker rescue, in which the synthetic promoters were inserted into a derivative of the tk gene. This derivative contains a 200 bp deletion wich simultaneously removes the UL24b promoter and inactivates tk activity. The UL24 is an open reading frame (ORF) which overlaps tk in a head to head arrangement and transcription is therefore in the opposite orientation as tk transcription (figure 2). The promoters were inserted so that they essentially replace the promoter of the UL24b gene. A novel UL24 transcript, UL24b', is therefore produced. Southern blot analysis of the five recombinant viruses shown in figures 7,8,9,10, and 11 demonstrate that each virus contains the desired structure and there is no detectable contamination by PAA^r5 virus.

Primer extension and S1 nuclease analysis of RNA extracted from cells infected with each of the recombinant viruses demonstrated that they all expressed UL24b' in an unblocked infection. (see figures 12, 17, and 18). Furthermore, each recombinant virus produced the same transcript starting at the same transcription initiation site. Any differences in the pattern of expression of UL24b' can therefore be directly correlated with differences in the promoter sequences. In contrast no UL24b is produced from cells infected with the KOS SB

viral strain (figure 12). KOS SB is a recombinant strain of PAA^{r}5 which contains the same 200 base pair deletion within the tk locus as do all the recombinant viruses, but does not contain a synthetic promoter. The deletion, therefore, effectively removes the promoter of the UL24b gene. The production of a UL24b' transcript in cells infected with the recombinant viruses demonstrates that the synthetic promoters functionally replace the UL24b promoter.

4.2 Kinetic Analysis of UL24b'

Previously our lab determined that the Ad 2 MLP TATA box, by itself, could function to promote transcription in the context of the HSV genome (Keith and Smiley, manuscript in preparation). The recombinant virus constructed, v28, contains the Ad 2 MLP TATA box in place of the UL24b promoter and therefore has a structure similar to the TAATGARAT recombinant viruses. Upon infection with v28 expression of UL24b' is maximum after DNA replication at approximately 12 hours post infection (figures 12 and 14). Expression of UL24b' is however not strictly dependent on DNA replication (figures 17 and 18) as compared to the true late gene US11 (figure 16). UL24b' therefore falls into the category of a leaky late

gene. By adding TAATGARAT sequences upstream of the Ad 2 MLP in v28 the effect of these sequences on the kinetics of UL24b' can be readily determined. The UL24b' transcript produced by v28 and the different TAATGARAT recombinant viruses is the same (see figures 17 and 18) therefore any differences in the patterns of expression of UL24b' can be directly attributed to the presence of the TAATGARAT sequences.

A direct comparison of the expression of UL24b' produced by v28 and vTG8 over a 12 hour time course reveals that addition of 9 tandem TAATGARAT elements, in the forward orientation with respect to the TATA box, did not change the kinetics of appearance of UL24b' (see figures 12 and 14). Both viruses expressed UL24b' with leaky late kinetics in comparison to the true late gene, US11, used as an internal control (figures 13 and 16). Furthermore, all of the TAATGARAT recombinant viruses expressed UL24b' with leaky late kinetics indistinguishable from v28 (data not shown). Taken together, these experiments clearly demonstrate that the TAATGARAT motifs, by themselves, present either singly or as tandem repeats and in either orientation relative to the TATA box do not alter the kinetics of expression of the linked DNA sequences.

Recently, Ace et al. (1989) constructed an HSV 1

mutant strain, in1814, which contained a 12 base pair insertion in the Vmw65 gene. This insertion abolished the ability of Vmw65 to transactivate IE gene expression in transient expression assays. In addition, the mutant Vmw65 polypeptide was unable to form a TAATGARAT binding complex together with OCT-1 and other cellular proteins, a condition necessary for IE gene activation (Ace et al., 1988). The mutant Vmw65 protein was however still able to function adequately as a structural virion component. Characterization of in1814 demonstrated that the virus was viable in a non complementing cell line. Plaquing efficiency was impaired at low moi but unaffected at high moi. Furthermore IE genes were the first genes expressed even in the absence of Vmw65 transactivation (Ace et al., 1989). This result, consistent with the results presented above (figures 12 and 14), demonstrates that the TAATGARAT sequence, and transactivation by Vmw65, do not determine the IE pattern of gene expression. In the absence of Vmw65 transactivation all HSV genes are expressed according to the inherent strengths of their promoters and IE genes are expressed first. These results taken together with the results presented in figure 12 suggest that transactivation of IE promoters by Vmw65, through the TAATGARAT motifs, does not determine the IE pattern of gene expression.

The synthetic promoters contain only two types of sequence elements; TAATGARAT and TATA box elements. Addition of the TAATGARAT element to the Ad 2 MLP TATA box did not alter the pattern of expression of UL24b' which suggests that within the promoter series the TATA element appears to be the dominant contributer to temporal control. This raises an interesting possibility: perhaps TATA sequences generally play dominant roles in dictating the different patterns of expression of HSV genes.

Evidence consistent with this hypothesis includes the following observations: Firstly, the tk gene promoter consists of two Sp1 binding sites, a CCAAT box, and an immunoglobulin octamer sequence linked upsteam of a TATA box. Amongst these sequences only the TATA box is required for induction of tk transcription during an infection (Boni and Coen, 1989). If the TATA box is the key sequence required for induction perhaps it is also the key sequence which determines the kinetics of expression. Secondly, correctly regulated expression of the true late gene gC requires only a 15 base pair sequence containing the TATA box sequence (Homa et al., 1988). These two observations suggest that the TATA box sequence determines the kinetics of expression of two genes belonging to different kinetic classes, namely

early and true late.

In support of the theory that the TATA box sequence determines the kinetics of expression of some HSV genes are the observations that not all TATA sequences function equivalently. For example, the adenovirus E1A protein is able to activate transcription from the hsp70 promoter but not from the SV40 early promoter. The promoter sequences responsible for this differential activation mapped to the TATA box (Simon et al., 1988). Similarly, ICP4 can transactivate the HSV gD promoter but not the SV40 early promoter. Changing the SV40 early promoter TATA sequence to the gD TATA sequence makes the SV40 early promoter responsive to ICP4 transactivation (Everett, 1988). In addition, within the context of the HSV genome TATA boxes do not function equivalently. Recombinant viruses in which three different TATA boxes, the true late gene US11 TATA box, the tk TATA box, and the Ad 2 MLP TATA box replace the promoter of the UL24b ORF demonstrate that these three TATA boxes are not functionally equivalent (Keith, Kibler, and Smiley, manuscript in preparation). Furthermore, Homa et al., (1988) demonstrated that the TATA box of an early gene, tk, could not functionally replace the TATA box of the true late gene gC. Taken together, these results demonstrate that all TATA

sequences are not functionally equivalent. Differences in the TATA sequences of some HSV genes could contribute to different temporal patterns of expression during the course of an infection.

Consistent with the theory that not all TATA boxes are equivalent is the evidence that there are alternate forms of TF11D or that a single TF11D can interact differently with various TATA sequences (Chen and Struhl, 1988, Nakajima et al., 1988). A diversity TATA sequences could interact with different TFIID proteins which in turn would interact differently with RNA pol II itself or regulatory components of the transcription machinery. If the same TFIID protein interacts with various TATA sequences different domains on TFIID may interact with the transcription machinery. Such interactions between the transcription machinery and alternate or the same TFIID proteins could result in modified patterns of expression of HSV genes. Alternatively, the activity of TFIID could be modified by viral transactivators such as ICP4 to recognize class specific TATA elements during the course of infection.

Evidence not in aggreement with the model that HSV temporal kinetics is determined by the sequence of the TATA box is derived from the following experiment: When the TATA box of the true late US11 gene promoter is

inserted in the HSV genome, in place of the UL24b promoter, a novel UL24b' transcript is made with leaky late kinetics. Similarily, when a promoter consisting of an SP1 binding site linked to the tk TATA box replaces the UL24b promoter a transcript is produced with leaky late kinetics. Both TATA boxes, each belonging to a different kinetic class, promote the same patterns of expression on the linked sequences (Smiley, personal communication). Perhaps the kinetics of some HSV genes are determined by the specific TATA box sequence. The kinetics of other genes may determined by the modular arrangement of sequence elements present in their transcription regulatory regions.

4.3 Expression of UL24b' in the Presence of Cycloheximide and Vmw65 Transactivation

The experiments presented above clearly demonstrated that the TAATGARAT element, by itself, did not determine the kinetics of UL24b'. Despite the presence of the TAATGARART elements, UL24b' was expressed with leaky late kinetics. Another criterion, other than the kinetics of expression post infection, used to classify IE genes is their expression in the presence of protein synthesis inhibitors. IE genes are the only class

of genes which do not require de novo viral protein synthesis prior to their expression and therefore are the only genes expressed in the presence of protein synthesis inhibitors such as cycloheximide. To determine if UL24b', expressed from the TAATGARAT recombinant viruses, shared this unique property of IE genes the expression of UL24b' was investigated in the presence of cycloheximide.

Figures 14 and 18 demonstrate that UL24b' is expressed from vTG8 but not from v28 in the continuous presence of cycloheximide. The nine tandem TAATGARAT motifs therefore relieved the requirement for de novo viral protein synthesis prior to the expression of UL24b'. Furthermore, UL24b' was also expressed from vTG3R and vTG8R in the absence of de novo viral protein synthesis (figure 17). Three and eight tandem TAATGARATS in the reverse orientation also enable UL24b' to be expressed independent of prior viral protein synthesis. In contrast, both vTG1R (figure 17) and vTG1 (figure 18) were unable to express UL24b' in the presence of cycloheximide. A single copy of the TAATGARAT motif linked one helical turn upstream of the Ad 2 MLP TATA box in either orientation was therefore not active in promoting transcription in the absence of any other viral proteins. Taken together these results demonstrate that tandem repeats of the TAATGARAT motif are able to confer

on the TATA box the ability to promote expression in the absence of de novo viral protein synthesis.

A number of explanations can be offered to interpret the results that tandem TAATGARAT motifs relieve the requirement for viral IE proteins for the expression of UL24b'. Firstly, it is well established that the TAATGARAT motif serves as a target for Vmw65 transactivation. The tandem TAATGARAT motifs in the promoters of UL24b' may therefore also function as targets for Vmw65. Secondly, proteins other than Vmw65 are brought into the cell as part of the virus particle. Some of these proteins include the vhs host shut off protein and the IE protein ICP4 (Yao and Courtney, 1989). The function of ICP4 as a virion component is not known, however it is possible that the ICP4 carried by the virion could transactivate UL24b' through the tandem TAATGARAT motifs. Thirdly, the structure of the tandem TAATGARAT motifs could constitute a constitutive enhancer. Consistent with the modular organization of enhancers (Ondek and Herr, 1988, Fromental et al., 1988), each TAATGARAT motif could function as an enhancer element which on its own is inactive. Duplication of enhancer elements, TAATGARAT motifs, results in the production of an enhancer which functions constitutively.

To determine if the TAATGARAT motif, by itself,

can function as a target for Vmw65 transactivation and to distinguish between the above three explanations a transient transfection assay was developed. The assay involved co-transfection of a plasmid carrying a reporter gene, pTTB, with a plasmid encoding Vmw65, pMC1 (Campbell et al., 1984). The reporter gene chosen was rabbit beta-globin driven by the same promoter present in vTG8. Promoter sequences therefore include nine tandem TAATGARAT motifs in the forward orientation linked one helical turn upstream of the Ad 2 MLP TATA box. A control plasmid, pMLB, contained rabbit beta-globin placed under control of the Ad 2 MLP TATA box by itself. Figure 23 clearly demonstrates that the nine tandem TAATGARAT motifs present in pTTB functioned as targets for Vmw65 transactivation. In contrast the MLP TATA box, by itself, did not respond to Vmw65. The tandem TAATGARAT elements also do not form a constitutive enhancer. The promoter of beta-globin in pTTB could not be activated by co-transfection with pDsal, a plasmid which encodes a non functional Vmw65 protein (Werstuk and Capone, personal communication). The results of the transient transfection experiment demonstrate that the TAATGARAT element, by itself, when linked to a TATA box functions as a target for Vmw65. These results, however, do not dismiss the possibility that the virion bound ICP4 modifies

transactivation by Vmw65 during infection.

The results of the transient transfection demonstrates that the TAATGARAT elements in the promoter of vTG8 serve as targets for Vmw65 transactivation, when linked to the rabbit beta-globin gene. These results suggest that the expression of UL24b' by vTG8, vTG8R, and vTG3R in the presence of cycloheximide is the result of transactivation by Vmw65. Virion contained Vmw65 therefore recognizes the TAATGARAT motifs in the promoter of UL24b', encoded by these three viruses. The transient transfections, however, were performed using mouse LTA cells and the viral infection experiments were performed using African green monkey kidney cells (Vero cells). To correlate the results of the transfection experiment, with LTA cells, and the viral infection experiment, with Vero cells, it was necessary to demonstrate that the recombinant viruses expressed UL24b' similarly in both cell types.

Figure 24 demonstrates that vTG8 behaves similarly but not identically in both Vero and LTA cells with respect to the expression of UL24b'. UL24b' encoded by v28 is also expressed similarly but not identically in both cell types. In LTA but not Vero cells, v28 expresses a small amount of UL24b' in the presence of cycloheximide. In addition, LTA cells are far more

competent in expressing UL24b' in the presence of cycloheximide during an infection with vTG8. The observed differences between Vero and LTA cells may reflect differences in the activity or availability of the host cell transcription factors, such as TFIID, required for the expression of UL24b'. For instance, the expression of UL24b', by v28 in the presence of cycloheximide, in LTA cells may be explained by the presence a species of TFIID which has a higher affinity for the MLP TATA box than does the corresponding TFIID in Vero cells. The high affinity TFIID may not require the presence of other viral transactivators such as Vmw65 or the IE proteins to activate transcription. In the presence of a transactivator, such as Vmw65, the high affinity TFIID is able to promote transcription to higher levels in LTA cells than in Vero cells. Most importantly, in both cell types vTG8 expresses far more UL24b' than does v28 in the presence of cycloheximide suggesting that the mechanism responsible for activating transcription of UL24b' in the absence of de novo viral protein synthesis occurs in both cell types.

The transient transfection experiment demonstrated that in LTA cells nine tandem TAATGARAT elements in the forward orientation functioned as targets for Vmw65 transactivation. A comparison of the expression

of UL24b' in both Vero and LTA cells demonstrated that similar mechanisms were responsible for the activation of UL24b' in both cell types. Taken together, these two experiments strongly suggest that the expression of UL24b' in Vero cells infected with vTG8, vTG8R, and vTG3R, in the presence of cycloheximide, is the result of Vmw65 transactivation through the TAATGARAT sequences present in the promoters of UL24b' in these recombinant viruses. Furthermore, the absence of UL24b' RNA in Vero cells infected with vTG1 and vTG1R, in the presence of cycloheximide, reflects the inability of the single TAATGARAT sequences to be recognized by Vmw65 (see below). Rescue of the synthetic promoters into a viral strain containing a Vmw65 which is transactivation deficient, will definitively determine if the TAATGARAT elements in the promoters function as targets for Vmw65.

The effect of TAATGARAT elements when present downstream of a TATA box was also investigated. Within the recombinant viruses the TAATGARAT sequences are approximately 600 base pairs 3' to the tk transcription initiation site. Thymidine kinase is expressed indistinguishably among all the TAATGARAT recombinant viruses and v28 indicating that the TAATGARAT elements had no effect on tk expression (figure 22). The TAATGARAT elements therefore did not relieve the requirement for IE

polypeptides for the expression of tk. This result demonstrates that in the context of a viral infection the TAATGARAT elements do not function downstream of a TATA box.

In agreement, Preston and Tannahill (1984) reported that the far upstream region of the ICP4 gene could not function to enhance transcription when placed downstream of the ICP4 gene TATA box in a transient-transfection assay. The far upstream region contains at least one copy of the TAATGARAT consensus. In contrast, when the far upstream region of the ICP4 gene is placed at the farthest 3' end of the tk gene transactivation was observed (Lang et al., 1984). Two explanations can be offered to resolve the discrepancy. Firstly, the far upstream region was placed downstream of two different TATA boxes, the ICP4 TATA box in the first case and the tk TATA box in the second. Differential functional contacts between Vmw65 and the proteins bound at the TATA box could result in activity in one situation but not the other. Secondly, Lang et al., (1984) cloned the far upstream region downstream of the entire tk gene which was contained within a circular plasmid. The activity observed may therefore represent the activity of the far upstream region present at a distance upstream as opposed to downstream of the TATA box.

Recently, Sadowski et al. (1988) demonstrated that a chimeric protein containing the yeast GAL4 binding domain and the carboxyl terminal 78 amino acid acidic transcription activating domain of Vmw65 could transcativate a promoter containing GAL4 binding sites. Activation could be achieved even when the GAL4 binding sites were located 1850 base pairs downstream of the transcription initiation site. In contrast to the results presented above, the Vmw65 activation domain could function downstream of a TATA box. Perhaps the presence of the remaining amino terminal portion of the Vmw65 polypeptide restricts the activity of the acidic domain so that it can only function upstream of a TATA box. Alternatively, the interaction between Vmw65 and OCT-1 may form a complex which can activate transcription only when present upstream of a TATA box.

4.4 Effect of Copy Number

Infection of Vero cells with the TAATGARAT recombinant viruses demonstrated that at least three tandem TAATGARAT sequences are required to express UL24b' in the absence of de novo viral protein synthesis. Furthermore, the transient transfection experiment demonstrated that tandem copies of TAATGARAT function as

targets for Vmw65. Taken together, these two experiments suggest that at least three consecutive copies of the TAATGARAT sequence are required for Vmw65 transactivation. This conclusion is contradictory to a number of observations which indicate that only one TAATGARAT element is sufficient to serve as a target for Vmw65. For example, the TAATGARAT element is never found tandemly repeated in any IE gene promoters (Mackem and Roizman, 1982a). Moreover, mutation analysis of IE gene promoters has demonstrated that only one intact TAATGARAT is required for Vmw65 responsiveness (O'Hare and Hayward, 1987, apRhys et al., 1989). Secondly, oligonucleotides or short IE gene promoter restriction fragments ,containing only one TAATGARAT consensus sequence, are able to form complexes with the host cell factors, including OCT-1, and Vmw65 (Kristie and Roizman, 1987, O'Hare et al, 1988, Preston et al., 1988, Baumruker et al., 1988, Gerster and Roeder, 1988, ApRhys et al., 1989, Stern et al., 1989). Thirdly, single TAATGARAT oligonucleotides can confer Vmw65 responsiveness, when linked to previously unresponsive promoters (Gaffney et al., 1985, O'Hare et al, 1988).

A number of explanations can be offered to explain the discrepancy between the results presented in this thesis and the observations that single TAATGARAT

elements respond to Vmw65. Even though vTG1 and vTG1R did not express UL24b' in the presence of cycloheximide it cannot be concluded that one TAATGARAT alone does not respond to Vmw65. It is possible that the expression of UL24b' in cells infected with vTG3R, vTG8R, and vTG8 reflects the activity of only one TAATGARAT element which is located the correct distance from the TATA box, with the remaining TAATGARAT oligonucleotides serving only a spacer function. The inability of vTG1 and vTG1R to express UL24b' in the presence of cycloheximide may therefore reflect the inappropriate position of the TAATGARAT element with respect to the TATA box. Vmw65 interacting at this single TAATGARAT may not be in the correct stereospecific alignment with TFIID or other proteins, required for transcription initiation, bound at the TATA box. Consistent with this hypothesis, single TAATGARAT elements are functional when present at least 60-70 base pairs upstream of the TATA box (Gaffney et al., 1985, O'Hare et al., 1988) To test this hypothesis it is necessary to construct a series of synthetic promoters in which the position of a single TAATGARAT element is varied with respect to the TATA box. The position can be varied by using oligonucleotides containing a randomly generated sequences which do not contain any previously identified binding sites for

cellular transcription factors.

An alternative explanation is that the promoter consisting of a single TAATGARAT element immediately upstream of a TATA box is competent to respond to Vmw65 transactivation. Binding of Vmw65 to the single TAATGARAT, facilitated by OCT-1 and other host protein(s), may not be strong enough to activate transcription to detectable levels by the assay used. In agreement, tandemly repeated TAATGARAT motifs were more active in promoting Vmw65 dependent transcription than single copies when linked to a heterologous promoter. (Gaffney et al., 1985). The presence of tandem copies of the TAATGARAT motif may provide a higher affinity binding site for the Vmw65 and host cell protein complex. Alternatively, a tandemly repeated binding site may increase transcription activity by allowing cooperative binding of functional Vmw65 host cell protein complexes. Consistent with this hypothesis, OCT-2 binds cooperatively to adjacent DNA binding sites (Lebowitz et al., 1989). The cooperative binding of OCT-2 is mediated by the POU domain. Given the high degree of homology, 87%, between the POU domains of OCT-1 and OCT-2 (Herr et al., 1988) and that the DNA binding specificity of OCT-1 and OCT-2 is indistinguishable (Staud et al., 1986, Scheidereit et al., 1987), OCT-1 may also be able to bind

cooperatively to repeated binding sites.

In support of the cooperative binding hypothesis other transcription factors have been shown to bind cooperatively to adjacent DNA binding sites. For example, the yeast transcription activator GAL4 bind cooperatively to adjacent sites and the degree of transcription activation is proportional to the number of GAL4 molecules bound. An increase in the number of closely opposed acidic activation domains enhances transcription (Giniger and Ptashne, 1987). In addition, steroid hormone receptors may also bind cooperatively to adjacent sites (Tsai et al., 1989).

Another explanation can be offered to explain the inability of vTG1 and vTG1R to express UL24b' in the presence of cycloheximide. Enhancers consist of a modular array of individual sequence elements organized in a hierarchical arrangement. The basic unit, an enhanson, is defined as a short DNA sequence which constitutes a binding site for a transcription factor. The second level of organization consists of enhancer elements which consist of two repeated enhancons which can be identical or non identical. Both enhancons and some enhancer elements fail to activate transcription. Enhancer activity ensues when two enhancer elements, either identical or non identical, are juxtaposed (Ondek and

Herr, 1988).

The TAATGARAT element, present in the oligonucleotide (figure 1), can be considered as an enhancer element. The octamer homology (ATGCTAAT) present at the 5' end of the oligonucleotide can be considered one enhancon since it constitutes a binding site for OCT-1 (Baumruker et al., 1988). The second enhancon consists of the GARAT sequence which may be a binding site for an unidentified cellular protein(s) (McKnight et al., 1987, O'Hare et al., 1988, Gerster and Roeder, 1988). Alone the TAATGARAT enhancer element has no enhancer function. Tandemly repeated TAATGARAT elements would restore enhancer function, as was observed. Alternatively, linking the TAATGARAT to other DNA sequences which bind cellular transcription factors would also restore function. Perhaps the activity of single TAATGARAT elements linked to heterologous promoters (Gaffney et al., 1985, O'Hare et al., 1988) reflects the restoration of activity resulting from the juxtaposition of the TAATGARAT enhancer element to other non identical enhancer elements. Furthermore, the activity of single TAATGARAT sequences in the natural context of IE gene promoters may also result from the juxtaposition of non identical enhancer elements. Consistent with this hypothesis GA rich sequences, present in all IE far

upstream regions in close proximity to the TAATGARAT elements, increase the activity of Vmw65 transactivation (Bzik and Preston, 1986, Triezenberg, 1988b). These GA rich sequences may therefore constitute enhancer elements which cooperate with the single TAATGARAT enhancer element to activate transcription to higher levels.

4.5 Orientation of the TAATGARAT Element

A comparison of UL24b' expression in cells infected with the various recombinant viruses demonstrated that the orientation of the TAATGARAT element affected transcription. Both vTG1 and vTG1R behaved identically to v28 under all conditions tested and therefore will not be considered in this discussion (figures 17 and 18). Figure 17 demonstrates that in the presence of cycloheximide vTG3R, vTG8R, and vTG8 all expressed UL24b' similarly. Consistent with earlier conclusions (Gaffney et al., 1985) both orientations of the TAATGARAT are therefore targets for Vmw65 transactivation. In an unblocked infection, however, vTG3R and vTG8R, expressed significantly more UL24b' than did v28 and vTG8. All of the recombinant viruses make the same UL24b' transcript, therefore any differences in the amount of cytoplasmic UL24b' RNA cannot be attributed to

differential RNA processing, transport, or half life. Any differences must therefore be related to promoter strength. These experiments demonstrate that tandem TAATGARAT elements in the reverse orientation create a promoter which is more active than a promoter with TAATGARAT elements in the forward orientation.

Under conditions which preclude the synthesis of any viral proteins the recombinant viruses express UL24b' similarly. The orientation effect seen in an unblocked infection can therefore not be caused by differential transactivation by Vmw65 or by constitutive enhancer activity of the TAATGARAT elements in the reverse orientation. One possibility to explain the orientation effect is that the TAATGARAT elements in the reverse orientation serve as a target for transactivation by a viral encoded protein other than Vmw65. Tandem TAATGARAT elements in the forward orientation would not be recognised by this viral transactivator. Alternatively, nine TAATGARAT elements in the forward orientation may be a target for a virus encoded transcription repressor, whereas TAATGARAT elements in the reverse orientation would not be recognised by the repressor.

Figure 17 also demonstrates that levels of UL24b' are greater when the infection by vTG8R and vTG8 is blocked by cycloheximide than when the infection is

blocked by ahidocolin. Two explanations can be used to account for these differences. Firstly, in the absence of DNA replication IE, early and leaky late proteins are produced. A virus protein included in one of these classes may act as a transcription repressor and inhibit transcription from the synthetic promoters. In the presence of cycloheximide this potential viral repressor is not translated and will not inhibit transcription from the synthetic promoter. Secondly, cycloheximide may stabilize RNA, perhaps by inhibiting the production of a labile cellular protein which degrades RNA. Both of these explanations can account for the differences observed in a cycloheximide versus an ahidocolin blocked experiment.

4.6 Role of Vmw65 Transactivation

The results presented in this thesis clearly demonstrate that transactivation by Vmw65 through the TAATGARAT element is not a determinant of the IE pattern of gene expression. The role that Vmw65 plays during a viral infection can be inferred by observing the phenotype of a Vmw65 mutant viral strain which is unable to transactivate IE gene expression.

Mutant virus , in1814, contains a 12 base pair insertion in the acidic domain of Vmw65 required for

transactivation. The resulting Vmw65 protein is competent to serve as a structural component of the virion but is unable to transinduce expression of IE genes. Phenotypically, the virus is able to grow on a non complementing cell line however viral replication is severely impaired at low moi. Furthermore, the particle to pfu ratio is high compared to wild type virus (Ace et al., 1989). These characteristics demonstrated that in1814 behaved phenotypically very similar to an ICP0 deletion mutant (Sacks and Schaffer, 1987). Recently Cai and Schaffer (1989) demonstrated that ICP0 plays a key role in stimulating viral gene expression early in infection with intact virions. In addition, ICP0 is the earliest viral regulatory protein active during a de novo infection, infection with transfected viral DNA only, in the absence of virion proteins (Cai and Schaffer, 1989). Furthermore, ICP0 plays an essential role in the reactivation of HSV from the latent state (Harris et al., 1989). Taken together, these observations demonstrate that ICP0 plays a key role in promoting the lytic cycle during early stages of infection.

Considering the key role that ICP0 plays in promoting lytic infection, perhaps the most important function of Vmw65, early in infection, is to ensure adequate levels ICP0. A number of observations are

consistent with this hypothesis. Firstly, viruses containing transactivation minus mutations in Vmw65, in1814, or null mutations in ICP0 both display very similar phenotypes. Secondly, the phenotypic defects observed during infection with in1814 can be significantly reduced by supplying functional ICP0 (Ace et al., 1989). Finally, the TAATGARAT sequence in the far upstream region of ICP0 contains an overlapping sequence which is a close consensus to the immunoglobulin octamer sequence (Machem and Roizman, 1982a). The TAATGARAT element, by itself without an overlapping octamer sequence, is able to bind the cellular protein OCT-1. The octamer sequence, however, functions as a higher affinity binding site for OCT-1 (Baumruker et al., 1988). These differences in the affinity for OCT-1 and the presence of a nearly perfect octamer sequence in the ICP0 promoter suggest that ICP0 is the first gene to be activated by Vmw65 during infection. Consistent with this hypothesis, ICP0 gene expression is more severely reduced compared to other IE gene expression during infection with in1814 (Ace et al., 1989). Altogether, these observations suggest that an key role of Vmw65 during infection is to ensure adequate levels of IE proteins, namely ICP0.

During a physiological infection each cell receives a very small number of virus particles, a

situation which compares to infection of tissue culture cells using a low moi. The inefficient viral growth of in1814 observed during infection of tissue culture cells using a low moi suggests that Vmw65 may be important in an in vivo infection. In support of this hypothesis, in1814 is avirulent in mice (Ace et al, 1989). In light of this observation it is interesting to speculate on the fate of Vmw65 in establishment of latency. In a neuronal infection the HSV nucleocapsid is transported by retrograde transport from the axon to the cell body, sometimes traversing long intracellular distances. During this time Vmw65 may be degraded by cellular proteases, or be lost in the cytoplasm of the neuron. In the absence of sufficient Vmw65 threshold levels of ICP0, and other IE proteins will not be reached possibly resulting in the establishment of latency (Ace et al., 1989).

4.7 Targets for Vmw65

The results of the transient transfection experiment clearly demonstrate that tandem TAATGARAT elements located immediately upstream of the Ad 2 TATA box serve as a target for Vmw65. Given the minimal complexity of the synthetic promoter the possible protein contacts for Vmw65 are limited. Possible functional

contacts include RNA pol II itself or proteins which bind to RNA polymerase such as RAP30/74 (TFIIE) (Sopta et al., 1989). Additional possible contacts include the proteins which bind at or near the TATA sequence. These proteins include TFIIA, TFIIB, or TFIID (Buratowski et al., 1989). Other contacts may include unidentified cellular proteins which may or may not bind to specific DNA sequences. Despite the number of possible contacts with Vmw65 the construction of the minimal promoter significantly reduced the number of functional contacts compared to a more complex promoter.

Within the group of proteins able to contact Vmw65 a likely candidate is TFIID. This general transcription factor plays a central role in the molecular events leading to transcription initiation. Binding of TFIID is the initial step in the assembly of a functional transcription complex. Subsequent to TFIID binding the remaining general transcription factors and RNA polymerase II complete a functional initiation complex (Buratowski et al., 1989). Following transcription initiation TFIID remains bound at the promoter and may catalyze multiple rounds of initiation (Van Dyke et al., 1988). Taken together, this information demonstrates the key role that TFIID plays in the early stages of transcription initiation and illustrates that

TFIID is a prime target for other transcription regulatory proteins.

A number of recent observations demonstrate that TFIID serves as a target for various cellular and viral transcription modifying proteins. These proteins include the: adenovirus E1A protein (Wu et al., 1987, Leong et al., 1988), pseudorabies virus IE protein (Abmyer et al., 1988), HSV IE protein ICP4 (Everett, 1988, Boni and Coen, 1989), USF, and ATF, cellular proteins required for maximum expression of the Ad MLP and the Ad E4 promoter respectively (Sawadago and Roeder, 1985a, Horikoshi et al., 1988b).

Recently, the gene encoding yeast TFIID has been cloned (Horikoshi et el., 1989, Hahn et al., 1989). This gene encodes an essential function for both <u>in vitro</u> transcription and for yeast viability (Eisenmann et al., 1989). Sequence analysis of yeast TFIID has revealed a central cluster of basic amino acids. This cluster of positive charges on the protein may represent a domain which interacts with the negatively charged domains contained on a number of transcription factors including GAL4 and Vmw65 (Ptashne, 1988, Sadowski et al., 1988). In fact, GAL4 modifies the TFIID footprint over the TATA box region suggesting functional contact between the transcription activating acidic domain of GAL4 and TFIID

(Horikoshi et al., 1988a). Furthermore, the acidic domain of Vmw65 can functionally replace the acidic domain of GAL4 (Sadowski et al., 1988). From this latter experiment it follows that a possible target for acidic domain contained on Vmw65 is TFIID.

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