POST-TRANSCRIPTIONAL REGULATION OF HSV THYMIDINE KINASE EXPRESSION

HERPES SIMPLEX VIRUS THYMIDINE KINASE GENE EXPRESSION UNDER CONTROL OF A LATE VIRAL PROMOTER

by

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ABSTRACT

Herpes simplex virus (HSV) genes are expressed as at least three coordinately regulated gene classes during lytic infection. The delayed-early (DE) and late (L) genes require previous expression of one or more immediate-early (IE) genes for their own expression. The DE genes achieve maximal expression prior to viral DNA synthesis, while the L genes are maximally expressed after DNA replication (Honess and Roizman, 1974). A recombinant strain of HSV-1, X1N17, was used in this study to examine the effect of the gene promoter on the temporal expression of HSV genes. This virus carries a late viral promoter upstream from the coding sequences of a DE gene (thymidine kinase; TK). S1-mapping studies showed that X1N17-TK transcripts initiated under the control of the late promoter and accumulated with L class kinetics. However, the TK activity levels in X1N17-infected cells were not consistent with HSV late gene expression. Western blot analysis of infected cell proteins revealed that despite the high levels of X1N17-TK mRNA present in the cytoplasm late after infection, little TK polypeptide was being synthesized. This suggested that HSV genes are subject to post-transcriptional control mechanisms that modulate the efficiency of translation of viral transcripts. More specifically, it appears as though HSV-TK transcripts are not efficiently translated at late times in infected cells.

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INTRODUCTION

1.1. General Description of Herpes Simplex Virus.

Herpes simplex virus type 1 (HSV-1) is a human herpesvirus of the alpha subfamily, characterized by a variable host range and a short reproductive cycle. Morphological characteristics of the HSV-1 virion include an electron-opaque nucleic acid core enclosed by an icosahedral nucleocapsid 100 nm in diameter, an electron-dense tegument between the nucleocapsid and the envelope and a lipid bilayer envelope derived from the host cell membrane (Spear and Roizman, 1981).

The linear double-stranded DNA genome is approximately 100-megadaltons, and codes for at least 50 polypeptides (Marsden et al., 1976; Honess and Roizman, 1973). HSV DNA consists of two covalently linked segments, the long segment (L) is 82% of the genome length and the short segment (S) is 18% of the genome length. The L and S segments are capable of inverting relative to one another by virtue of recombinational events between inverted repeats flanking the L and S unique sequences (Sheldrick and Berthelot, 1975). This segment inversion results in the production of four equimolar isomers of the HSV genome in the viral population (Hayward et al., 1975).

The replication cycle of HSV-1 begins with fusion of the virion envelope and the cell membrane with subsequent release of the nucleocapsid into the cytoplasm. The nature of ensuing events is unclear, however the DNA is thought to be released from the nucleocapsid and transported to the nucleus for transcription (Spear and

Roizman, 1981).

Viral transcription is thought to be directed by cellular RNA polymerase II. A viral polymerase is precluded by the observation that deproteinized viral DNA is infectious (Spear and Roizman, 1981; Graham et al., 1973; Sheldrick et al., 1973). Transcription of viral DNA is impeded by α-amanitin, an RNA polymerase II inhibitor, only in cells sensitive to the drug (Constanzo et al., 1977). HSV infection drastically reduces the levels of host RNA synthesis, and translation of host mRNAs is barely detectable in infected cells (Wagner and Roizman, 1969; Jacquemont and Roizman, 1975). There are three phases of protein synthesis in infected cells detected by measurement of amino acid incorporation. Shortly after infection there is a rapid decline of cellular protein synthesis followed by a sharp increase in amino acid incorporation, marking the onset of viral protein synthesis. Near the midpoint of the infectious cycle, the rate of protein synthesis reaches a plateau, then begins a steady decline late in infection (Sydiskis and Roizman, 1966, 1967 and 1968; Honess and Roizman, 1973).

1.2. Classification of HSV Genes.

Herpes simplex virus polypeptides are classified into at least three sequentially expressed groups that were identified after quantitative analysis of the rates of protein synthesis in infected cells (Honess and Roizman, 1974). Synthesis of the alpha, or immediate-early (IE), class of infected cell proteins begins early in the infectious cycle and declines about four hours post-infection. This class is defined as the virus-specific polypeptides synthesized

immediately after removal of protein synthesis inhibitors present in culture medium from the time of infection onwards (Honess and Roizman, 1974). The five IE proteins appear to be involved in modifying the host cell to allow DE and L gene expression (Wagner, 1983). The beta, or delayed-early (DE), class of HSV polypeptides are maximally expressed from five to seven hours post-infection (Spear and Roizman, 1981). Many DE polypeptides are enzymes involved in viral DNA replication including TK (Garfinkle and McAuslan, 1974; Leiden et al., 1976) and DNA polymerase (Powell and Purifoy, 1977) The synthesis of this class of proteins requires both prior IE polypeptide synthesis and new RNA synthesis in the presence of functional IE proteins (Honess and Roizman, 1974 and 1975). The gamma, or late (L), class of viral polypeptides consists of structural virion proteins whose rate of synthesis increases until at least twelve hours after infection (Honess and Roizman, 1975). The production of L polypeptides requires previous synthesis of IE and DE proteins. Inhibition of DNA synthesis reduces the level of late class polypeptides in infected cells (Honess and Roizman, 1974).

1.3. HSV Transcription.

HSV DNA is transcribed in the nucleus of the host cell and the transcripts are processed by cleavage, 5'-capping, methylation and polyadenylation much like the transcripts of many eukaryotic cells and other nuclear-replicating DNA viruses (Wagner, 1984). The incidence of splicing is very low, with only two well-characterized HSV mRNAs having recognizable splice sites (Watson et al., 1981; Frink et al.,1981). Each herpes simplex gene appears to be under the control of its own

promoter and transcription is directed off of both strands of the viral genome. Genes belonging to a single temporal class of HSV genes do not appear to be confined to any particular region of the genome (Spear and Roizman, 1981; Wagner, 1984), in contrast to the situation observed in other nuclear replicating DNA viruses such as adenovirus (Berget et al., 1977; Broker et al., 1977) The interspersion of genes of different kinetic classes would not allow coordinate control via regulation of gene expression in defined regions of the HSV genome. It is therefore likely that temporal regulation of HSV gene expression is determined by signals in the individual gene promoters. Evidence to support this view is provided by Post et al. (1981) and by Silver and Roizman (1985). When a chimeric thymidine kinase (TK) gene with the promoter region from a different kinetic class was inserted into the viral genome, cells infected with the recombinant virus expressed TK with the kinetics of its new promoter. Currently, much effort is being expended on attempting to define the cis-acting elements that confer temporal specificity on the various HSV promoter classes. Attempts are also being made to define the mode of action of trans-acting regulatory factors that drive the transition from one phase to the next.

1.4. Regulation of IE Gene Expression.

Transcription of HSV IE genes occurs immediately after infection and requires no de novo protein synthesis (Honess and Roizman, 1974). This led to the initial belief that IE genes are efficiently transcribed by an unmodified host cell machinery. However, more recent results have shown that the HSV virion delivers into the cell a trans-acting protein

that stimulates IE transcription. Batterson and Roizman (1983) have demonstrated that IE gene expression is induced by a virion component located outside of the nucleocapsid. Chimeric genes constructed by fusion of upstream regulatory regions of IE genes with coding regions of various indicator genes such as HSV TK (Mackem and Roizman, 1982a and 1982b; Cordingley et al., 1983; Kristie and Roizman, 1984), bacterial chloramphenicol acetyltransferase (CAT) and human interferon (Mosca et al., 1985) have been used to identify the cis-acting IE regulatory sequences responsible for IE gene regulation. The upstream regulatory regions of IE genes share three common features: TATA homology, multiple G+C-rich sequences and A+T-rich consensus sequences (Mackem and Roizman, 1982a). The TATA homology ,or Hogness sequence, is essential for accurate initiation of transcription (McKnight et al., 1981; Benoist and Chambon, 1981; Grosveld et al., 1982a),and is found 25-30 base pairs (bp) upstream from most eukaryotic protein-coding genes. It is similar in sequence and in function to the Pribnow box of bacterial promoters (McKnight, 1982). A G+C-rich sequence CCCCGCCC and its inverted form are present in multiple copies upstream of the IE genes in HSV types 1 and 2 (Mackem and Roizman, 1982a; Whitton et al., 1983). They have been implicated as general enhancers of RNA polymerase II transcription and have been shown to bind the cellular transcription factor Sp1 in vitro (Jones and Tjian, 1985). The G+C-rich sequence is present in the upstream regulatory region of the HSV DE gene (TK), and nucleotide substitutions in this sequence can lead to significant reductions of TK transcription levels (McKnight and Kingsbury, 1982). Similar G+C-rich sequences modulate transcription levels in the rabbit beta-globin

(Dierks et al., 1983), SV-40 early (Everett et al. 1983), Drosophila heat shock (Pelham, 1982), and mammalian metallothionein (Brinster et al., 1982; Roizman et al., 1975) genes. The A+T-rich consensus sequence TAATGARATTC (R=purine) is present as direct repeats of one to four copies per IE gene (Mackem and Roizman, 1982a). This arrangement resembles other A+T-rich sequences that have been implicated in coordinate induction of other eukaryotic genes such as the silk moth chorion genes and chicken egg-white protein genes (reviewed in Davidson et al. (1983). Experiments by Kristie and Roizman (1984) and by Preston et al. (1984) have implicated this consensus sequence as the cis-acting regulatory element responsible for IE gene induction by a virion component. An upstream region of the IE gene 4 containing two copies of the consensus sequence was able to convert a HSV DE gene into an IE regulated gene in infected cells. A similar result was obtained by Mackem and Roizman (1982c) with a regulatory domain of IE gene 27. The virion component responsible for positive regulation of IE gene expression has been identified by Campbell et al. (1984). A plasmid carrying HSV DNA sequences encoding the major virion polypeptide Vmw65 strongly stimulates TK expression in trans from a chimeric gene with IE regulatory sequences. Thus it appears that virion protein Vmw65 and the consensus sequence TAATGARATTC interact to ensure efficient expression of IE genes. So far, herpesviruses are unique in delivering a positive regulatory protein into cells as a part of the virion. As described below, the virion also contains a negative regulatory factor that shuts off host translation.

Negative regulation of transcription also appears to play a role

in herpes simplex gene regulation. The first indication of this was that IE mRNA continues to accumulate in HSV infected cells in the absence of viral protein synthesis (Roizman et al., 1975; Harris-Hamilton and Bachenheimer, 1985). The IE 175 kilodalton polypeptide (ICP4) was implicated in the negative regulation of IE protein synthesis in experiments using HSV temperature sensitive mutants. When cells infected with a mutant virus carrying a temperature-sensitive ICP4 gene were shifted to the non-permissive temperature late in infection, they began to re-express high levels of IE polypeptides (Dixon and Schaffer, 1980). More recent work by O'Hare and Hayward (1985) has demonstrated a negative regulatory effect of the immediate-early ICP4 protein on its own transcription. Thus it appears as though ICP4 is responsible for the inhibition of IE transcription in the shift from IE to DE gene expression.

1.5. Regulation of DE Gene Expression.

Expression of DE and L genes in HSV-infected cells requires prior synthesis of functional IE polypeptides. Honess and Roizman (1974) have demonstrated that DE and L polypeptides are not synthesized after reversal of a cycloheximide block in the presence of actinomycin D, suggesting that DE and L mRNAs are not synthesized prior to IE polypeptide synthesis. Amino acid analogue incorporation into IE polypeptides in infected cells also inhibited DE and L polypeptide synthesis, indicating that functional IE polypeptides are required for induction of DE and L gene expression (Honess and Roizman, 1975). Experiments with temperature-sensitive mutants of HSV-1 have identified

ICP4 as the IE viral function required for DE and L protein synthesis. Cells infected with tsK, a HSV-1 strain temperature-sensitive for ICP4, at the non-permissive temperature fail to produce detectable levels of DE transcripts (Preston, 1979; Watson and Clements, 1978). Watson and Clements (1980) have demonstrated that DE and L polypeptide synthesis requires continued presence of functional ICP4. TsK infected cells cease production of DE and L RNAs when shifted to the non-permissive temperature at three and seven hours post infection. The role of a second IE gene product, the 110 kilodalton ICP0, in the positive regulation of DE gene expression is unclear. In infected cells, ICP0 appears to be ineffective as a DE gene inducer in the absence of a functional ICP4 (Watson and Clements, 1978; DeLuca et al., 1985). However, in a transient expression system,where genes under control of HSV-DE regulatory regions are cotransfected with intact IE genes, the ICP0 product alone is capable of inducing DE gene expression. The level of induction by ICP4 is higher than that by ICP0, but is augmented by the presence of ICP0 (Quinlan and Knipe, 1985; Everett, 1984b; O'Hare and Hayward, 1985). The experimental evidence demonstrates clearly that these proteins act as trans-activators of DE gene expression, however, their mechanism of action in this capacity is unclear.

The regulatory region of the HSV TK gene has been well characterized. To date, the sequences responsible for promoter function cannot be distinguished from those responsible for induction by IE proteins. Eisenberg et al. (1985) and McKnight and Kingsbury (1982) have identified three subregions essential for accurate and efficient TK transcription in microinjected Xenopus oocytes. The proximal subregion

from 16 to 32 bp upstream from the mRNA start site (-16 to -32) contains the Hogness sequence. The two distal subregions contain hexanucleotide sequences that are inverted complements of one another. Distal signal II (-84 to -105) also contains one of the two CAAT-like sequences found in the TK promoter (Smiley, 1985). Grosveld (1982b) has demonstrated that the CAAT sequence is essential for *in vivo* transcription of the rabbit globin gene. McKnight has reported that single base pair substitutions within the TK CAAT element at -80 reduces the TK promoter activity ten-fold in *Xenopus* oocytes (see Jones *et al.*, 1985). The two distal subregions may function in an analogous manner since mutations in either or both of these signals have the same effect on transcription in microinjected oocytes (McKnight, 1982). An interesting feature of these two essential sequences is that they have the potential to form intrastrand hydrogen bonds by virtue of an inverted repeat sequence. The stem-loop structure thus formed would be sensitive to the single-strand specific nuclease S1, a characteristic that has been associated with transcriptionally active regions in chromatin (Larsen and Weintraub, 1982). Eisenberg *et al.* (1985) report that the three essential subregions of the TK promoter are recognized and activated similarly in microinjected oocytes and in *trans*-induced mouse cells. Similar results have been reported by Everett (1984a) for the HSV glycoprotein D (gD) promoter. All of the promoter elements important for transcription activated in *trans* by viral infection were also important for *cis*-activated transcription by a SV40 enhancer. O'Hare and Hayward (1984) have shown that another HSV DE promoter, that for the HSV-2 38 kilodalton polypeptide, constitutively directs transcription in

uninfected transfected cells, but becomes dependent on IE polypeptide synthesis when the cells are infected. Furthermore, the globin gene can be induced by HSV infection in a transient induction assay (Everett, 1983), demonstrating that this type of induction may not be specific for HSV DE promoters. There appears to be no specific sequence in DE gene promoters responsible for trans-induction by IE gene products. Sequence comparisons show that the only clearly conserved sequences between DE promoters are sequences found in many pol II promoters (Everett, 1983). Although the mechanism of induction of DE genes has not been determined, it is clear that it differs from that seen for specific induction of IE gene expression by the virion component. Whereas IE genes have a consensus sequence in their upstream regulatory region which responds to a specific trans-acting positive regulator, the DE genes appear to be regulated by a more general mechanism of transcriptional modulation.

There are several lines of evidence suggesting that the induction of DE gene expression by IE polypeptides is at the level of transcription initiation. In all situations where it was measured, an increase in TK activity after HSV infection or exposure to IE polypeptides was paralleled by an increase in DE mRNA levels (Harris-Hamilton and Bachenheimer, 1985; Sandri-Goldin et al., 1983; ElKareh et al., 1985). Since the sequences necessary for enhancement of TK activity by IE gene products map upstream from the transcribed region of the TK gene, it seems unlikely that they could act post-transcriptionally. Finally, Leung et. al. (1980), could detect no TK transcripts in the nucleus or cytoplasm of infected cells in the presence of protein synthesis inhibitors or amino acid analogues,

suggesting that IE polypeptides induce new TK RNA synthesis rather than being involved in mRNA processing or transport.

Negative regulation of DE polypeptide synthesis seems to occur after the onset of DNA synthesis. There is a decrease in TK activity and other DE polypeptide levels after twelve hours post-infection in untreated infected cells, but treatment with DNA synthesis inhibitors postpones this late decline (Honess and Roizman, 1974; Harris-Hamilton and Bachenheimer, 1985). Evidence that L polypeptides may be involved in this negative regulation of DE gene expression is provided by Sacks et al. (1985). Cells infected with a ts mutant for IE gene ICP27 overproduce DE polypeptides and viral DNA but drastically underproduce most L polypeptides at the non-permissive temperature. It is not clear whether the repression of DE gene expression by L protein(s) is mediated at the transcriptional or the post-transcriptional level. Harris-Hamilton and Bachenheimer (1985) have reported accumulation of DE mRNAs in infected cells treated with a DNA synthesis inhibitor. This may reflect continued transcription from DE genes or prolonged stabilization of DE mRNAs in the absence of L polypeptides.

1.6. Regulation of L Gene Expression.

The transition from early to late polypeptide synthesis in HSV-infected cells requires the prior production of functional IE and DE proteins (Honess and Roizman, 1974 and 1975; Watson and Clements, 1980). The L class of HSV proteins is divided into two subclasses, the L1 and the L2 subclass, by virtue of their dependence on viral DNA synthesis. Both subclasses show maximal levels of production after viral DNA

replication. The distinction lies in the fact that L1 polypeptides can be detected in infected cells at low levels in the absence of DNA synthesis, while the L2 group of proteins cannot (Conley et al., 1981; Powell et al., 1975; Honess and Watson, 1977). The basis for this dependence on DNA synthesis is not yet clear. It is possible that the increase in L1 expression after DNA replication is simply due to the increased number of templates available for transcription. However, the L2 class may have an additional requirement for conformational changes in the DNA template that result from the DNA replication event itself.

Experiments with temperature-sensitive (ts) mutants have implicated at least two IE gene products as trans-acting positive regulators of late gene expression. Watson and Clements (1980) reported that ongoing late transcription, in cells infected with a temperature sensitive ICP4 mutant, ceased following a shift from the permissive to the non-permissive temperature. DeLuca et al. (1984) have identified a ts mutant of HSV-1 that is defective for the IE protein ICP4. At the non-permissive temperature, cells infected with the mutant virus produce DE polypeptides and viral DNA but fail to produce detectable levels of L polypeptides. This mutant differs from most ts ICP4 mutants which fail to produce DE and L polypeptides as well as viral DNA at the non-permissive temperature. Cells infected with a HSV ts mutant with a lesion in the gene for IE polypeptide ICP27 also fail to produce L polypeptides at the non-permissive temperature, while DE polypeptide and viral DNA synthesis are unimpaired. Presumably, DE gene products and viral DNA replication alone are not sufficient for late gene expression, there is an obvious direct requirement for functional ICP4 and ICP27.

The expression of HSV late genes in uninfected mammalian cells differs somewhat from that in infected cells. Silver and Roizman (1985) have demonstrated that a chimeric TK gene under the control of a HSV L2 promoter is expressed with L2 kinetics when it is introduced into cells as part of a recombinant HSV genome. In contrast, when the same construct is used to stably transform mammalian cells, it expresses TK with DE kinetics since maximal TK expression relies on the presence of functional ICP4 but not on viral DNA replication. In uninfected cells, TK transcripts are produced at low levels from the L2 promoter. These results differ from those presented by Dennis and Smiley (1984) where the L1 promoter for the major capsid protein VP5 was tested in a similar manner. In uninfected cells stably transformed by the VP5 promoter-TK chimeric gene, no transcription from the VP5 promoter could be detected. Low levels of a truncated TK mRNA that initiates within the TK coding sequence were detected. In infected cells, transcription from the VP5 promoter was induced and TK activity accumulated with early kinetics. These results were interpreted to indicate that the VP5 promoter does not function in uninfected cells, and that the gene is *trans*-induced by one or more factors supplied by the superinfecting virus. Another study by Costa *et al.* (1985) further demonstrated the inactivity of the VP5 promoter in uninfected cells. A chimeric gene composed of VP5 promoter and bacterial CAT coding sequences was not expressed in uninfected cells, but could be induced by HSV infection or by cotransfection with cloned HSV IE genes for ICP0, ICP4 and ICP27. The requirement for these inducers was lost if a 50 bp 'silencer' sequence between -75 and -125 was removed from the VP5 promoter. It is possible that this 'silencer'

sequence is involved in the late control of VP5 expression. Perhaps viral infection inhibits some repressor function related to this sequence, thereby accounting for the lack of expression in uninfected cells. The presence of such a 'silencer' sequence in other L1 or L2 promoters has not yet been reported.

1.7. Translational Regulation.

Translational regulation also appears to play a role in control of gene expression in HSV-infected cells. The expression of cellular polypeptides diminishes rapidly after infection due to disaggregation of polyribosomes (Sydiskis and Roizman, 1966 and 1967). This early shutoff mechanism appears to be mediated by a component of the infectious viral particle since de novo protein synthesis is not required (Nishioka and Silverstein, 1978; Arsenakis and Roizman, 1984). Read and Frenkel (1983) have isolated mutants defective in the virion-associated host shutoff. These mutants also showed overproduction of IE polypeptides, apparently due to a defect in the post-transcriptional shutoff of IE gene expression. Nishioka and Silverstein (1978) have reported that host mRNAs are physically degraded in Friend erythroleukemia cells after infection and that this degradation is dependent on viral protein synthesis. More recent studies by Schek and Bachenheimer (1985) suggest that in other cell systems, host mRNA degradation occurs even in the absence of viral protein synthesis. This virion-associated cell mRNA degradation is absent in the host shutoff mutants of Read and Frenkel (Bachenheimer, personal communication with Dr. J. R. Smiley). Although some host transcripts continue to associate

with polyribosomes throughout infection (Stringer et al., 1977; Kozak and Roizman, 1974), their translation is suppressed by some unknown mechanism (Silverstein and Engelhardt, 1979).

The cascade regulation of HSV polypeptide synthesis is also affected at the translational level. The prolonged expression of IE and DE polypeptides in infected cells that are unable to express L polypeptides, due to a transcriptional block by actinomycin D, suggests that L gene product(s) may by involved in repression of IE and DE translation (Honess and Roizman, 1974). Harris-Hamilton and Bachenheimer (1985) have reported the continued presence of ICP4 and ICP0 mRNAs in the cytoplasm of infected cells at late times after infection, long after IE polypeptides have ceased being produced. Clearly, translational regulation plays a role in the shutoff of IE polypeptide synthesis, possibly mediated by one or more L gene products. Johnson and Spear (1984) have also shown that translatable transcripts for the L1 polypeptide gD continue to accumulate in the cytoplasm of infected cells until at least 16 hours post-infection. The gD polypeptide itself, however, stops being synthesized between four and six hours after infection. The factors involved in this inhibition of gD mRNA translation are as yet unknown.

Little is known about the factors affecting differential translation of mRNAs present in HSV-infected cells. It may involve differential modification of the transcripts. Bartoski and Roizman (1978) demonstrated that viral and cellular mRNA made early in HSV-infected cells are internally methylated while late mRNAs are not. The addition of amino acid analogues prior to L polypeptide synthesis

resulted in continued methylation of transcripts made late in infection. It is therefore possible that one or more late proteins are responsible for inhibiting the internal methylation of transcripts made at late times post-infection and that this methylation somehow affects the translatability of the mRNAs. Another factor possibly affecting translation is the modification of ribosomal proteins in infected cells. Fenwick and Walker (1979) have identified a 48 kilodalton ribosomal protein that becomes phosphorylated after HSV-2 infection. The phosphorylation event is dependent on DE or L polypeptide synthesis which would be consistent with the inhibition of cellular viral IE polypeptide synthesis described previously.

1.8. Present Research.

Existing evidence suggests that the transcriptional pattern of a HSV gene is governed by the kinetic class of its promoter. The present study set out to determine if the DE TK gene could by converted to L1 kinetics by fusion to the L1 class VP5 promoter. At the time that the VP5-TK chimeric gene was constructed, such studies had only been carried out on IE-TK fusion genes. The technique of marker rescue allows the VP5-TK construct to be analyzed in the context of the infecting viral genome. This system is ideal for the study of viral gene expression in light of the evidence that viral genes behave differently in transformed or transfected cells. The results of this study confirmed that the VP5 promoter conferred L1 transcriptional control on TK. However, despite efficient transcription of the chimeric gene as an L1 gene, the polypeptide was inefficiently expressed,

indicating that another level of control modulates the expression of HSV genes at the polypeptide level.

MATERIALS AND METHODS

2.1 Tissue Culture

African green monkey kidney cells (Vero), initially obtained from Flow Laboratories, were grown in Dulbecco's modified Eagle's medium (α-MEM) supplemented with 5% (v/v) calf serum. Mouse TK^- $Aprt^-$ L-cells (LtA), initially obtained from Dr. R. Hughes (Roswell Park Memorial Institute), were grown in modified α-MEM containing 10%(v/v) fetal bovine serum (FBS). α-MEM was modified by adding 100 units/ml penicillin, 100 micrograms/ml (μg/ml) streptomycin, 0.03%(w/v) l-glutamine (Sigma) and 0.15% (w/v) $NaHCO_3$ (Analar). All media and supplements except glutamine and bicarbonate were from Gibco. Cells were maintained in monolayers in 150 cm^2 Corning plastic flasks and sub-cultured twice weekly by washing in phosphate-buffered saline (PBS;Gibco) and treatment with trypsin-EDTA (Gibco) to loosen the cells from the flask wall. Cells were incubated at 37° Celsius (°C) in a humidified environment containing 5% CO_2.

2.2 Virus Strains

HSV-1 strain KOS, initially obtained from Dr. W. E. Rawls (McMaster University) was used as the wild type virus in this study. The TK-deficient deletion mutant, lacking the 800 bp Pst I C fragment, was HSV-1 strain d2 (Smiley, 1980). X1N17 was plaque-purified from a partially purified TK^+ virus stock, prepared by Helen Rudzroga and Dr.

J. R. Smiley by DNA-mediated marker rescue, as outlined in Figure 4. The TK^+ viral progeny were recovered from Vero cells co-transfected with HSV-1 strain d2 DNA and purified pX1N17 DNA (Dennis and Smiley, 1984), which carries a VP5-TK chimeric gene (see Figure 3).

2.2.1. Viral Stocks

Virus stocks were prepared by infection of sub-confluent monolayers of Vero cells, in 150 cm^2 plastic flasks, with virus at a multiplicity of infection of 0.05 in 10 millilitres (ml) of modified α-MEM. After adsorption of the virus for two hours at 37°C, 20 ml of pre-warmed α-MEM containing 5% FBS was added. Infected cells were collected after two to three days by tapping the flask to loosen the infected cells. The cells were pelleted by centrifugation and resuspended in a small volume of α-MEM containing 5% FBS. The cells were disrupted by sonication and cellular debris was separated from the infectious virions by centrifugation. The supernatant, containing virus particles, was aliquotted into screw-capped NUNC (Inter-Med) vials and stored at -70°C.

2.2.2. Determination of Virus Titres

Titres of virus stocks were determined by plaque assay in 24-well tissue culture microtitre trays (Corning). Serial dilutions of thawed virus stock were made in α-MEM, and small aliquots of each dilution were used to infect sub-confluent monolayers of Vero cells in the wells of the titre tray. After two hours incubation at 37°C, the infected cells were overlaid with α-MEM containing 5% FBS and 0.05% human immune serum (Canadian Red Cross Society). Plaques were counted

after two to three days incubation.

2.3. Enzymes

Restriction endonucleases, T4 polynucleotide kinase and DNA polymerase I were obtained from Boehringer-Mannheim Canada (BMC) or Bethesda Research Laboratories (BRL). Deoxyribonuclease I and nuclease S1 were from BMC.

2.3.1. Restriction Endonuclease Digestion

Reactions were performed in a general restriction digestion buffer of 10 millimolar (mM) Tris-HCl, pH7.5, 10mM $MgCl_2$ and 10 μg/ml bovine serum albumen (BSA). The salt concentrations were adjusted according to the supplier's recommended conditions. Digestions were carried out at 37°C (60°C for Bst NI) for 1 to 16 hours and were arrested by adjusting to 10mM EDTA.

2.3.2. DNA Polymerase I

This enzyme was used to prepare high specific-activity radiolabelled DNA probes for use in Southern blot analysis. Plasmid DNA (250-500 nanograms (ng)) was incubated for ten minutes at 37°C in the presence of 25mM Tris-HCl, pH 7.4, 2.5mM $MgCl_2$, 5mM β-mercaptoethanol, 50μg/ml BSA and 2 ng deoxyribonuclease I to introduce multiple nicks in the DNA. E.coli DNA polymerase I (4 units) and 50 microcuries (μCi) of α^{32}P-dCTP (800 Ci/mmole; New England Nuclear (NEN)) were added to the reaction mixture and incubation was continued at 12°C for 80 minutes. The reaction was terminated by adjusting to 20mM EDTA. Unincorporated

nucleotides were removed by chromatography through a G-50 Sephadex column (Pharmacia). The radiolabelled DNA was boiled for five minutes, to denature the DNA, then hybridized to Southern blots as described in Section 2.5.2.

2.3.3. T4 Polynucleotide Kinase

T4 kinase was used to prepare the 5' end-labelled DNA fragments used as probes in the transcript mapping experiments. After restriction endonuclease digestion with the appropriate enzyme, the terminal phosphates were removed from all DNA fragments by incubation with calf intestinal alkaline phosphatase (BMC). The dephosphorylation was carried out for 30 minutes at 37°C in 50 mM Tris-HCl, pH 8.0 with 1.25 units of phosphatase per 10 μg of DNA. After dephosphorylation, the DNA fragments were separated by electrophoresis through a preparative polyacrylamide gel and the appropriate fragment was eluted from the gel, as described later in Section 2.4.2. The isolated DNA was labelled at the 5' ends using the forward reaction conditions described by Maxam and Gilbert (1980). DNA was first denatured at 90°C for two minutes in 35 microlitres (μl) of 20 mM Tris-HCl, pH 9.5, 1 mM spermidine and 0.1 mM EDTA, then quick-chilled in ice water. Reaction conditions were adjusted by adding 5 μl of buffer containing 500 mM Tris-HCl, pH 9.5, 100 mM $MgCl_2$, 50 mM dithiothrietol and 50% (v/v) glycerol, followed by 50 μCi of γ^{32}P-ATP (3000 Ci/mmole; NEN) and 20 units of T4 polynucleotide kinase. The reaction was at 37°C for 15 minutes, and was terminated by adding 200 μl of 2.5 M NH_4 acetate. The radiolabelled DNA was precipitated twice with ethanol to remove excess salt. After

labelling, the DNA was either digested with another restriction endonuclease or was directly strand-separated on a polyacrylamide gel as described in Section 2.4.3.

2.3.4. Nuclease S1

This enzyme is a single strand-specific nuclease used in this study to analyze single-stranded end-labelled DNA fragments that hybridize to viral TK transcripts. DNA-RNA hybridization mixtures, set up as described in Section 2.7.3., were treated with 2000 units of S1 nuclease in 0.3 ml of 150 mM NaCl, 50 mM Na- acetate and 5 mM $ZnSO_4$, pH 4.6 for 60 minutes at 37°C. The digestion was arrested on ice with the addition of 20 μl of 200 mM EDTA and 50 μl of 4 M NH_4-acetate. The reaction products were extracted with phenol-chloroform (1:1), and ethanol precipitated in the presence of 10 μg of E. coli tRNA (BMC) as carrier. The DNA fragments protected from nuclease S1 digestion by hybridization to viral mRNA were analyzed on polyacrylamide sequencing gels as described later.

2.4. Gel Electrophoresis of DNA

2.4.1. Analytical Gels

Restriction endonuclease digested DNA fragments were separated by electrophoresis through agarose (Sigma) or polyacrylamide (BioRad) vertical gels. Agarose gels were prepared by melting 0.5 grams of agarose powder in 50 ml of electrophoresis buffer. Polyacrylamide gels were prepared by mixing the appropriate amount of an acrylamide stock solution (40% (w/v) acrylamide, 1.08% (w/v) N,N' methylene bis

acrylamide (BIS) with electrophoresis buffer, 0.06% (w/v) ammonium persulfate and 0.001% (v/v) tetramethylethylenediamine (TEMED). Electrophoresis buffer was 89 mM Tris base, 89 mM boric acid and 25 mM EDTA, pH 8.3. Gels were cast between two glass plates (17 X 15 centimetres (cm)) separated by spacers 0.1 cm wide (for acrylamide) or 0.3 cm wide (for agarose). DNA samples (generally 20 µl) were mixed with 10 µl of loading buffer containing 7.5% (w/v) Ficoll, 10% (v/v) glycerol, 1% (w/v) sodium dodecyl sulfate (SDS), 0.3% (w/v) bromophenol blue, 0.3% (w/v) xylene cyanol and 10 mM EDTA. The samples were loaded onto the gels and electrophoresed at 2-6 volts/cm. After electrophoresis, the gel was soaked for 15-30 minutes in electrophoresis buffer containing 0.45 µg/ml of ethidium bromide. Gels were photographed under ultraviolet (UV) light with a mounted Polaroid camera using Polaroid type 667 film.

2.4.2. Isolation of DNA Fragments from Polyacrylamide Gels

Preparative polyacrylamide gels were used to obtain purified restriction endonuclease fragments for use as probes in transcript mapping experiments. The gels were longer (30 cm) and thicker (0.3 cm) than those used for analytical purposes to achieve better separation of fragments and to allow more DNA to be loaded in a single well. After electrophoresis, the DNA fragments were stained with ethidium bromide and visualized under UV light, or radioactive DNA fragments were detected by exposing the gel to Kodak XAR-5 or XRP x-ray film. The desired fragment was excised from the gel with a razor blade and crushed to a paste in a microcentrifuge tube. The gel paste was mixed with 0.6

ml of 500 mM NH_4 acetate, 10 mM Mg acetate, 1 mM EDTA, 0.1% (w/v) SDS and incubated overnight at 37°C. The acrylamide was pelleted by centrifugation for 5 minutes, and the supernatant was passed through siliconized glass wool to remove traces of acrylamide. The DNA was precipitated twice with ethanol to remove salt, then dried under vacuum. The recovered DNA could then be end-labelled as described in Section 2.3.3.

2.4.3. Strand Separation of Duplex DNA

The denatured complementary strands of some DNA fragments can be separated by electrophoresis, probably as a consequence of secondary structure due to intrastrand base pairing. This property was used to isolate single-stranded DNA fragments for use as probes in transcript mapping experiments. End-labelled DNA fragments were denatured in 150 mM NaOH for 10 minutes at room temperature and prepared for electrophoresis by adjusting to 0.05% bromophenol blue, 0.05% xylene cyanol and 5% (v/v) glycerol. The denatured DNA samples were loaded onto a 30 cm long strand separation gel that was pre-run for one hour at 200 volts. The acrylamide stock used to prepare these gels was 40% (w/v) acrylamide, 0.8% (w/v) BIS and the electrophoresis buffer was 44.5 mM Tris base, 44.5 mM boric acid, 12.5 mM EDTA, pH 8.3. Electrophoresis was at 200 volts with recirculation of the buffer between the upper and lower buffer chambers by a peristaltic pump. The single stranded bands were located by exposing the gel to x-ray film and aligning the film to the gel. The desired single-stranded fragment was cut out of the gel and eluted as described in Section 2.4.2.

2.4.4. Sequencing Gels

The products of S1 nuclease digestion of RNA-DNA hybrids (see Section 2.3.4) were sized on polyacrylamide sequencing gels 40 cm long and 0.3 mm thick. The procedures for sequencing electrophoresis have been thoroughly reviewed by Maxam and Gilbert (1980). Briefly, gels consisted of 8.3 M urea, 89 mM Tris base, 89 mM boric acid, 25 mM EDTA, pH 8.3 0.06 % (w/v) ammonium persulfate, 0.001% TEMED and the appropriate amount of deionized acrylamide stock solution (38% (w/v) acrylamide, 2% (w/v) BIS). The gel was clamped to a vertical electrophoresis apparatus with a 3mm thick pressed aluminum sheet, coated with a thermal compound (Wakefield Inc.), against the front glass plate for heat distribution. The dried samples were dissolved in sequencing dye (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% (w/v) bromophenol blue and 0.1% xylene cyanol) and loaded onto a gel that had been pre-run for one hour at a constant current of 25 milliamperes (mA). Electrophoresis was also at 25 mA for varying lengths of time, depending on the sizes of the fragments to be resolved. Following electrophoresis, the gels were transferred onto used x-ray film, covered with plastic wrap, and exposed to x-ray film at -45°C for 18 hours to two weeks.

2.5. Isolation of Plaque Purified Recombinant Virus

2.5.1. Extraction of Viral DNA

Viral DNA was extracted as described by Hirt (1967). Vero cell monolayers in 25 cm^2 Corning flasks were infected with 10^5 plaque-forming units (pfu) of virus in 1 ml of modified α-MEM. After two

hours the cells were overlaid with 4 ml of α-MEM containing 5% FBS. The cells were harvested after three days incubation at 37°C and pelleted by centrifugation for 10 minutes at 850 g. The cell pellet was resuspended in 0.5 ml 10 mM Tris-HCl, pH 7.6, 10 mM EDTA and transferred to a 1.5 ml plastic tube. The cells were lysed by adjusting to 0.55% SDS. The mixture was adjusted to 1M NaCl and stored on ice overnight. The proteins and high molecular weight DNA were pelleted by centrifugation at 4°C for 5 minutes. The supernatant was transferred to a clean tube and extracted 2-3 times with phenol. The volume was adjusted to 0.5 ml with (TE) 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, followed by ethanol precipitation with 2.5 volumes of 95% ethanol. The nucleic acids were pelleted by centrifugation for 5 minutes. RNA was removed by ribonuclease treatment (100 μg/ml) in 50 μl of TE. The DNA could then be digested with restriction endonucleases as described in Section 2.3.1.

2.5.2. Southern Transfer

DNA restriction fragments that have been separated by agarose gel electrophoresis can be transferred to a solid support (nitrocellulose or nylon membranes) for detection of specific sequences by hybridization with radiolabelled nucleic acid probes. Viral DNA extracted from different TK^+ plaque isolates of the VP5-TK marker rescue was tested in this manner for the presence of the desired insert in TK sequences. After electrophoresis, DNA in agarose gels was transferred onto nitrocellulose filters by a modification of the technique described by Southern (1975). The gel was treated for two 10 minute periods in

250 mM HCl, followed by two rinses in distilled water. The DNA was denatured by soaking the gel for two 15 minute intervals in 500 mM NaOH, 1 M NaCl, again followed by rinsing twice with distilled water. The final treatment involved soaking the gel for one hour in 3 M NaCl, 500 mM Tris-HCl, pH 7.4, with one change of the soaking solution. The transfer of DNA from the gel onto the nitrocellulose membrane was carried out overnight by placing a piece of nitrocellulose (Schleider and Schuell, Inc.) presoaked in 3 M NaCl, 300 mM Na citrate, on top of the agarose gel and covering with a layer of paper towels weighted down with lead weights. After transfer, the DNA was permanently bound to the filter by baking at 80°C for two hours.

2.5.3 Southern Hybridization

The nitrocellulose filter was pre-treated for 5-24 hours at 42°C in a mixture of 50% formamide, 750 mM NaCl, 75 mM Na citrate, 0.1% (w/v) ficoll, 0.1% polyvinyl pyrolidone, 0.1% BSA, 50 mM NaH_2PO_4, 7% (w/v) dextran sulfate and 100 μg/ml denatured sheared salmon sperm DNA (Sigma). The radiolabelled probe DNA, prepared as outlined in Section 2.3.2, was mixed with 50% formamide, 750 mM NaCl, 75 mM Na citrate, 0.02% ficoll, 0.02% polyvinyl pyrolidone, 0.02% BSA, 20 mM NaH_2PO_4, 10% dextran sulfate and 100 μg/ml salmon sperm DNA. The filter was sealed into a plastic bag with the probe and incubated at 42°C for 18-24 hours. After hybridization, the filters were washed for 20 minutes with gentle agitation in 300 mM NaCl, 30 mM Na citrate, 0.1% SDS, with four changes of washing solution. A more stringent wash in 30 mM NaCl, 3 mM Na citrate, 0.1% SDS at 50°C was carried out four times for 10 minutes

each. The washed filter was dried at room temperature and exposed to x-ray film at -70°C.

2.5.4. Plaque Purification

One of the TK^+ recombinant virus isolates from the original marker rescue experiment was further plaque purified after southern blot analysis of the DNA confirmed the presence of the VP5-TK chimeric gene. Vero cell monolayers in 150 cm^2 Corning dishes were infected with 100 pfu of the recombinant virus in modified α-MEM. After two hours, the infected cells were overlaid with α-MEM containing 5% FBS and 0.05% human immune serum. Individual plaques were picked up with sterile wooden sticks and used to infect Vero cells in the wells of a microtitre tray. After four days growth, the infected cells were collected by repeated pipetting of the media over the cells to loosen them from the plastic. The cells were stored at -45°C in individual NUNC vials. The titres of two of these re-purified virus isolates were determined by the method described in Section 2.2.2. The virus with the higher titre was tested again by Southern blot analysis, and subsequently used in the remaining experiments in this study. This re-purified isolate was named X1N17.

2.6. Plasmids

pTK173 (Enquist *et al.*, 1979) and pX1N17 (Dennis and Smiley, 1984) were obtained from Dr. J. R. Smiley. The plasmids were maintained in *E. coli* strain LE392, stored at -45°C in 7% (v/v) dimethylsulfoxide in Luria broth (10 mM Tris-HCl, pH 7.6, 0.4% (w/v) glucose, 0.5% (w/v)

NaCl, 1% (w/v) bactotryptone (Difco), 0.5% (w/v) yeast extract (Difco)).

2.6.1. Large Scale Isolation of Plasmid DNA

One litre cultures of bacteria in Luria broth were grown at 37°C with vigorous shaking until the culture reached an optical density at 660 nm of 0.4-0.6. The plasmid copy number was amplified by continued incubation overnight in the presence of 200 μg/ml chloramphenicol. The cells were pelleted by centrifugation at 5000 r.p.m. for 20 minutes in a Sorvall GS-A rotor. Cell pellets were resuspended in 10 ml of 50 mM Tris-HCl, pH8.0, 25% sucrose, and divided between four Oak Ridge tubes (Nalgene). To each tube was added 0.25 ml of lysozyme solution (15 mg/ml lysozyme (Sigma) in 0.25 M Tris-HCl, pH 8.0), followed by gentle mixing and five minutes on ice. Incubation on ice was repeated after addition of 1 ml 0.2 M EDTA, and again after 2.5 ml of lysis solution (50 mM Tris-HCl, pH 8.0, 0.1% (v/v) Triton X-100 (BioRad), 50 mM EDTA) was added. Genomic DNA and cellular debris were removed by centrifugation for one hour at 30,000 r.p.m. in a Beckman 50-Ti rotor at 4°C. Plasmid DNA was further purified by cesium chloride (CsCl) density gradient centrifugation. The supernatants from the above preparations were combined with 200 μg/ml ethidium bromide and 0.9 g/ml CsCl. The mixtures were transferred to Beckman polyallomer ultracentrifuge tubes, topped off with H_2O-saturated mineral oil and capped. The samples were centrifuged for 60 hours at 35,000 r.p.m. in a Beckman 50-Ti rotor at 20°C. Plasmid DNA bands were visualized with UV light and withdrawn by puncturing the side of the tube with a hypodermic needle. The ethidium bromide was removed by several extractions with isoamyl alcohol. The

aqueous phase was transferred to a dialysis sack and dialyzed for 12 hours against TE, pH 7.6 with three changes of dialysis buffer. The DNA was ethanol precipitated in the presence of 150 mM NaCl, and pelleted at 8000 r.p.m. for 20 minutes at 4°C in a Sorvall SS34 rotor. The pellet was resuspended in TE, pH 7.4 and the concentration of the DNA suspension was determined from the optical density at 260 nm in a Beckman DU-7 spectrophotometer.

2.7. Transcript Mapping

This technique was used to determine the initiation sites of TK transcripts in infected cells and to measure the levels of TK transcripts in KOS and X1N17-infected cells at different times after infection.

2.7.1. RNA Extraction

Infected cell RNA was isolated by the method of Berk and Sharp (1977). Monolayers of LtA cells in 150 cm^2 flasks were infected with 5 pfu/cell of HSV-1 strain KOS or X1N17. At the appropriate time after infection, the cells were harvested by scraping, and pelleted by centrifugation at 2000 r.p.m. for 10 minutes at 4°C in a Beckman TJ-6 Chillspin. The cells were washed once with ice cold PBS and repelleted. The pellet was resuspended in ice cold isotonic buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.8, 1.5 mM $MgCl_2$) to a final cell concentration of 4-8x10^7 cells/ml. An equal volume of isotonic buffer containing 2% (v/v) Nonidet P-40 (NP-40; BRL) was added, and the suspension was held on ice for ten minutes. The nuclei were pelleted by centrifugation at 5000

r.p.m. for five minutes in a Sorvall SS34 rotor at 4°C. The supernatant was transferred to a fresh tube and combined with an equal volume of urea solution (7M urea, 350 mM NaCl, 10 mM Tris-HCl, pH 7.8, 10 mM EDTA, 1% (w/v) SDS). The mixture was held at room temperature for five minutes, followed by two phenol and three phenol-chloroform extractions. The final aqueous phase was precipitated with 2.5 volumes of ethanol at -20°C overnight. The RNA was pelleted by centrifugation in a Sorvall SS34 rotor at 12,000 r.p.m. for 30 minutes at 4°C. The RNA pellet was resuspended in 300 mM Na acetate and reprecipitated with ethanol. The final pellet was washed twice with 70% ethanol, 400 mM Na acetate, pH 5.2 and dried under vacuum. The RNA was resuspended in 0.5-1.0 ml of diethyl pyrocarbonate-treated water and its concentration was determined by measuring the optical density at 260 nm.

2.7.2. Isolation of Probe DNA

pX1N17 DNA (100 μg) was digested to completion with endonuclease Rsa I, dephosphorylated with calf intestinal alkaline phosphatase, and the fragments were separated by electrophoresis through an 8% preparative polyacrylamide gel. The 400 bp fragment, which spans the site of fusion of VP5 promoter sequences and TK transcribed sequences, was cut out of the gel after ethidium bromide staining and eluted by the method described in Section 2.4.2.

pTK173 DNA (100 μg) was digested to completion with Ava I, dephosphorylated, and the fragments were separated on a 3.5% polyacrylamide preparative gel. The 1200 bp fragment that traverses the wild-type initiation site of TK mRNA was cut out of the gel and eluted

as described above.

The eluted DNA fragments were end-labelled as described in Section 2.3.3. After labelling, the Rsa I fragment of pX1N17 was denatured and strand separated on a 5% polyacrylamide gel as outlined in Section 2.4.3. The gel was run for 14 hours at 200 volts to achieve sufficient separation of the two single stranded fragments. The DNA bands were visualized by autoradiography (see Figure 1)for 10 minutes with Kodak XAR-5 film, and the faster migrating strand was cut out and eluted from the gel for hybridization to infected cell RNA.

The end-labelled Ava I fragment of pTK173 was digested with Pvu II and run on a 5% preparative polyacrylamide gel till the xylene cyanol dye had reached the bottom of the gel. The DNA was visualized under UV light after ethidium bromide staining (see Figure 2). The smaller Ava I-Pvu II fragment, which contains the TK mRNA start site, was cut out and eluted from the gel, followed by denaturation and strand separation on a 5% gel. Only a single band was visible by autoradiography since the Ava I-Pvu II fragment was labelled at one terminus. This band was eluted from the gel for use in hybridization and S1 nuclease treatment.

Approximately 5% of each probe was retained in the double stranded form for use as size markers alongside the S1 digestion products in sequencing gels. The yield of single stranded probe and double stranded marker DNA was measured as Cerenkov counts per minute (cpm) in a Beckman LS-1801 scintillation counter.

2.7.3. Hybridization

For each sample, 10 μg of infected cell RNA was combined with

FIGURE 1: Strand separation of the 400 base pair Rsa I fragment.

Purified pX1N17 400 nucleotide pair Rsa I fragment was radiolabelled as described in Section 2.3.3, then denatured in 150 mM NaOH. The labelled strands were separated on a 5% polyacrylamide strand separating gel. The DNA bands were visualized by autoradiography and the faster migrating strand (arrow) was cut out of the gel and eluted for use as a probe in S1 mapping experiments with HSV RNA.

FIGURE 2: Pvu II digest of 1200 bp Ava I fragment of pTK173.

The end-labelled Ava I fragment of pTK173 was further digested with Pvu II, followed by separation on a 5% polacrylamide gel (LANE 2). The smaller fragment (arrow) was eluted from the gel to be strand-separated and used as a probe in the S1-mapping experiments of Section 3.2.

LANE 1 is pBR322 digested with restriction endonuclease Bst N1. The sizes of the fragments in LANE 1 are indicated in base pairs to the left of the gel.


1 2
1857
1060
928
383

10,000-20,000 Cerenkov cpm of single stranded, end-labelled probe DNA. The nucleic acid mixture was adjusted to 300 mM Na acetate and precipitated with two volumes of 95% ethanol. After centrifugation, the pellet was washed with cold 70%, then 95% ethanol and dried under vacuum in a Savant Speedvac for 5-10 minutes. The co-precipitates were resuspended in 30 µl of a hybridization solution containing 50% (v/v) formamide, 400 mM NaCl, 40 mM Pipes, pH 6.4 and 1 mM EDTA. The mixture was incubated at 42°C for 16 hours. The samples were then treated with nuclease S1 as described in Section 2.3.4, followed by electrophoresis on a polyacrylamide sequencing gel alongside the appropriate molecular weight marker.

2.7.4. Generation of Molecular Weight Markers for Transcript Mapping

Double stranded, end-labelled DNA fragments were subjected to another restriction endonuclease digestion to generate the appropriate size markers for transcript mapping. The Rsa I fragment of pX1N17 was digested with Sau 3A, which cuts at the junction between VP5 and TK sequences. The Ava I-Pvu II fragment of pTK173 was further digested with Bgl II, which cuts downstream from the wild-type TK mRNA start site. After digestion, the DNA was extracted with phenol-chloroform and precipitated with ethanol. The DNA was pelleted by centrifugation, washed with 70% ethanol, and dried in the Speedvac. The dried pellet was dissolved in sequencing dye to a final concentration of 250 cpm/µl, and 2 µl was loaded onto the sequencing gel alongside the S1 nuclease digestion products.

2.8. Measurement of TK Activity in Infected Cells

Subconfluent LtA cell monolayers in 75 cm^2 Corning plastic flasks were infected with 5 pfu/cell of either KOS or X1N17 in 5 ml of α-HT (modified α-MEM supplemented with 13.2 g/ml hypoxanthine and 9.7 g/ml thymidine). After two hours incubation at 37°C, the cells were overlaid with 20 ml α-HT containing 10% FBS. At the appropriate time post-infection, the cells were harvested by scraping, and washed in ice-cold PBS. Cell extracts were prepared by the method of Summers et al. (1975). Washed cell pellets were resuspended in 0.3 ml of 20 mM Tris-HCl, pH 7.8, 1mM β-mercaptoethanol, 0.05 mM thymidine and sonicated for 90 seconds on ice. The broken cell suspensions were centrifuged for 15 minutes at 4°C in a microcentrifuge and the supernatants were stored on ice.

The TK enzymatic assay was a modification of the method of Post et al. (1981). Cell extracts (40 μl) were combined with 120 μl of TK assay buffer containing 150 mM Tris-HCl, pH 7.8, 16 mM $MgCl_2$, 25 mM NaF, 16 mM ATP, 8 mM creatine phosphate, 1 unit/ml creatine kinase and (20 μCi/ml) ^{14}C-thymidine (50 mCi/mmole; NEN). The reactions were incubated at 37°C, and 10 μl aliquots were removed at 0, 10, 20, 30, 60 and 120 minutes. The aliquots were spotted onto Whatmam DE81 filter squares that were previously saturated with unlabelled thymidine (see Leiden et al., 1976). The filters were washed in 1 mM NH_4 formate, pH 3.6 for three 30 minute washes, followed by two rinses in distilled water and one in 95% ethanol. Filters were dried under a heat lamp and the bound radioactivity was measured by liquid scintillation. TK activity was obtained by calculating the slope from a plot of the average

radioactivity retained on duplicate filters versus the incubation time. The TK activity values were corrected for the total amount of protein in each cell extract, measured by the Lowry method (Lowry, 1951), to give TK specific activity.

2.9. Detection of TK Polypeptides in Infected Cell Extracts

2.9.1. Preparation of Protein Extracts.

LtA cell monolayers in 10 cm^2 plastic dishes were infected with 5 pfu/cell of HSV-1 strain KOS or X1N17 in α-HT medium. After two hours, 7 ml of α-HT containing 10% FBS was added and incubation was continued at 37°C. At the appropriate time after infection, cells were harvested by scraping, and pelleted at 850 g for 10 minutes at 4°C. Cell pellets were washed three times with cold PBS, then sonicated for 30 seconds in ice-cold RIPA (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.1% (w/v) SDS, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100) containing 10 units/ml aproteinin (Sigma) and 35 g/ml phenylmethyl-sulfonylflouride (PMSF; Sigma) as protease inhibitors. Cellular membranes were pelleted by centrifugation at 15,000 r.p.m. in a Sorvall SS34 rotor for 30 minutes at 4°C. The supernatant, containing proteins, was stored at -70°C for analysis by SDS-polyacrylamide gel electrophoresis.

2.9.2. <u>In Vivo</u> Protein Labelling

Infected cell polypeptides were labelled <u>in vivo</u> with ^{35}S-methionine to be used in immunoprecipitations with anti-TK antiserum for comparison with Western blot immunoassay using the same antiserum.

LtA cells in 10 cm^2 dishes were infected at a multiplicity of infection (moi) of 10 with KOS or X1N17 in α-HT medium. After one hour, excess virus was washed away with two PBS washes, and the medium was replaced with 3 ml of 199-met medium (Gibco), supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 0.3% (w/v) l-glutamine, 0.15% (w/v) $NaHCO_3$, 5% (v/v) FBS, 10 mM Hepes, and 60 μCi/ml ^{35}S-methionine (1100 Ci/mmole; Amersham). The cells were harvested by scraping and proteins were extracted as described in the previous section.

2.9.3. Immunoprecipitation

Radiolabelled proteins were immunoprecipitated with a rabbit antiserum prepared against purified HSV-1 TK (generously provided by Sheldon Girvitz, McMaster University). Different amounts of cellular protein extracts were combined with 15 μl of anti-TK antiserum and 100 μl of pre-swelled Protein A sepharose CL4B (Pharmacia). The samples were rotated slowly end-over-end at 4°C for 5 hours. The sepharose beads were pelleted by centrifugation for 60 seconds at 4°C. The supernatant was discarded and the beads were washed three times with cold RIPA. The dry beads were resuspended in protein sample loading buffer (50 mM Tris-HCl, pH 8.0, 2.5% (w/v) SDS, 15% (v/v) glycerol, 0.05% β-mercaptoethanol and 0.0015% (w/v) pyronin-Y (Sigma) and boiled for 3 minutes. The beads were pelleted by centrifugation for 60 seconds and the supernatant was loaded onto a SDS-polyacrylamide gel for electrophoresis.

2.9.4. SDS-Polyacrylamide Gel Electrophoresis

Immunoprecipitated proteins or total cellular protein extracts were separated by electrophoresis on vertical SDS-polyacrylamide gels. Gels consisted of a 4% acrylamide stacking gel, 3 cm in length, over a 10% or 12% acrylamide separating gel, 14 cm long. The acrylamide stock solution was 30% (w/v) acrylamide and 0.8% (w/v) BIS. The separating gel was made up of the appropriate amount of acrylamide stock, 1% (v/v) glycerol, 0.1% (w/v) SDS, 375 mM Tris-HCl, pH 8.8, 0.05% ammonium persulfate and 0.001% TEMED. The gel was poured between two glass plates, separated by 0.1 cm thick plastic spacers, to a level 3 cm from the top of the plates. A solution of 0.1% (w/v) SDS was gently added to the top of the acrylamide solution before polymerization to ensure an even surface along the top of the separating gel. After polymerization, the liquid was poured off the top of the gel, and the stacking gel was added. The stacking gel consisted of acrylamide stock solution, 4% glycerol, 0.1% (w/v) SDS, 125 mM Tris-HCl, pH 6.8, 0.05% ammonium persulfate, and 0.001% TEMED. The sample wells were formed in the stacking gel. Protein samples were boiled in protein sample buffer for 3 minutes prior to loading. Electrophoresis was at 2-6 volts/cm in electrophoresis buffer containing 25 mM Tris base, 190 mM glycine and 0.1% (w/v) SDS. After electrophoresis, gels containing radiolabelled proteins were fixed in 5% (v/v) methanol, 7% (v/v) acetic acid, and dried under vacuum onto Whatman 3MM paper. Gels containing non-labelled protein samples were electroblotted onto nitrocellulose or nylon (Genescreen; NEN) filters and subjected to Western immunoassay as described in the following sections.

2.9.5. Electrophoretic Transfer of Proteins

Proteins separated on the basis of molecular weight by SDS-polyacrylamide gel electrophoresis were transferred onto nitrocellulose or nylon membranes by a procedure based on techniques described by Towbin et al. (1979) and Burnette (1981). After electrophoresis, the gel was removed from the electrophoresis apparatus and excess polyacrylamide was trimmed from the bottom and sides of the gel. The stacking gel was also removed. The gel was soaked for 30 minutes in electroblot buffer (25 mM Tris base, 192 mM glycine, 20% (v/v) methanol) to remove SDS. A 'sandwich' was assembled inside the plastic gel holder of a BioRad Transblot Cell, consisting of a Scotch-Brite fiber pad, 2 sheets of Whatman 3MM paper, the pre-equilibrated gel, nitrocellulose or nylon membrane, 2 sheets of 3MM paper and a final fiber pad. Everything was pre-wet in electroblot buffer. Transfer took place in a BioRad Transblot Cell containing three litres of pre-cooled electroblot buffer. A magnetic stir bar was used inside the unit during transfer to ensure homogeneous pH across the gel. Transfer was at 50 volts and 240 mA constant current for 4-6 hours in a cold room. Efficiency of transfer was monitored by the transfer of pre-stained protein molecular weight markers (BRL). After transfer, the TK polypeptides were detected by immunoassay as described below.

2.9.6. Immunodetection of Protein on Transfer Membranes

Electrophoretically transferred polypeptides on membranes were detected by a 'sandwich' of antigen-specific antibody and labelled second antibody. The second antibody, goat anti-rabbit IgG, was either

enzymatically labelled with horse radish peroxidase (BioRad) or radioactively labelled with ^{125}I (NEN). Prior to reaction with the TK specific antiserum, the filter was 'blocked' in a 3% (w/v) solution of BSA in TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl). This blocking reduces the amount of non-specific binding of antibody to the filter. After 60 minutes of gentle agitation in blocking solution, the filter was transferred to a 1% (v/v) solution of anti-TK antiserum in blocking solution. The antibody was reacted with the filter for 3-18 hours with gentle agitation. After use, the diluted antibody was recovered into a 50 ml centrifuge tube (Corning) and stored at -70°C for future use. The filter was washed for two 10 minute periods in TBS. The second antibody was diluted 1:3000 in blocking solution and reacted with the filter for 1 hour with gentle shaking. The filter was again washed twice for 10 minutes with TBS. If the second antibody was ^{125}I-labelled, the filter was dried at room temperature and exposed to x-ray film at -70°C. If the second antibody was enzymatically labelled, the filter was reacted with a mixture of 30 mg HRP Color Development Reagent (BioRad), 17% (v/v) methanol, 0.015% H_2O_2, 17 mM Tris-HCl, pH 7.5 and 416 mM NaCl for 5-20 minutes until the colour reaction was complete. The filter was rinsed well with distilled water and air-dried.

RESULTS

The following studies were undertaken to test the hypothesis that the temporal pattern of HSV gene transcription is mediated by the gene promoter regions. This hypothesis stems from earlier evidence that HSV TK expression can be changed from DE to IE kinetics by linking TK coding sequences to an IE gene promoter. More specifically, this research sets out to determine whether inserting an L1 promoter in front of the coding region for the DE TK gene would confer late kinetics on that gene's expression.

For these studies, the promoter of the late gene for viral capsid protein, VP5, was chosen. As described in the introduction, the L1 genes of HSV are transcribed at low levels early after infection and at high levels late in the infectious cycle, after the onset of viral DNA replication. The TK gene, in contrast, is transcribed at high levels before viral DNA synthesis and is shut off late in infection. Previously in this laboratory, Douglas E. Dennis constructed the plasmid pX1N17 which contains HSV TK structural gene sequences downstream from the VP5 promoter. This construct was generated by inserting a 600 bp Bgl II-Bam H1 fragment carrying VP5 promoter sequences into the Bgl II site within the 5' non-translated leader of the TK transcribed region. As seen in Figure 5, the VP5-TK chimeric gene lacks any VP5 coding sequences, but carries the intact VP5 TATA homology and upstream region. Dennis and Smiley (1984) demonstrated that in mammalian cells transformed with pX1N17 and infected with TK-deficient HSV-1, this

FIGURE 3: Construction of the VP5-TK chimeric gene.

The plasmid pX1N17 was constructed previously by Dennis and Smiley (1980) according to the outline in the figure. The VP5 promoter sequences (P_{VP5}) contained within the 600 bp Bgl II-Bam HI fragment of plasmid pIBN1 were inserted upstream from the TK coding region of pX1.

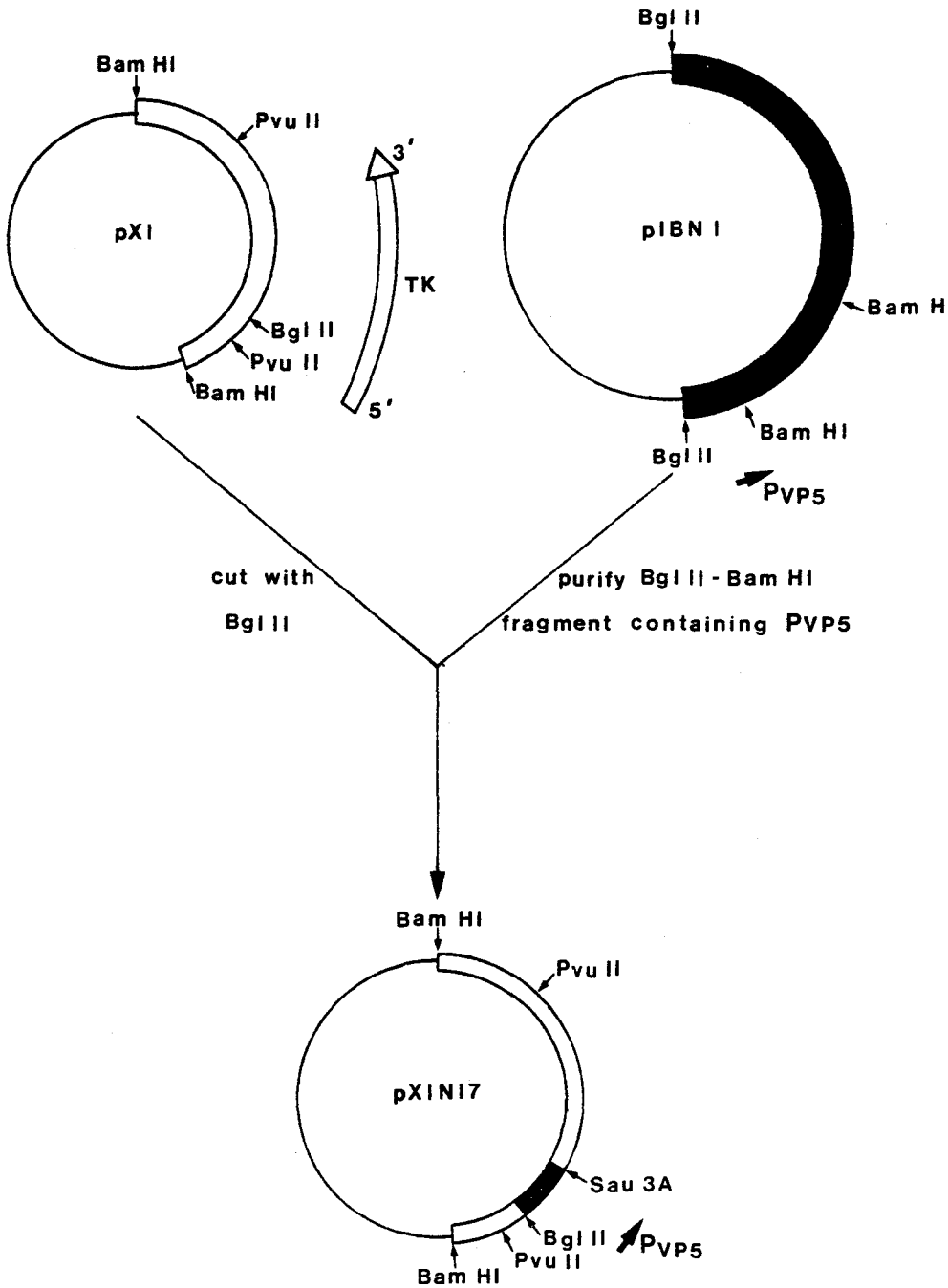

Bam HI
Pvu II
pXI
Bgl II
Pvu II
Bam HI
3'
TK
5'
Bgl II
pIBN I
Bam HI
Bam HI
Bgl II
PVP5
cut with
Bgl II
purify Bgl II - Bam HI
fragment containing PVP5
Bam HI
Pvu II
pXINI7
Sau 3A
Bgl II
Pvu II
Bam HI
PVP5

FIGURE 4: The production of HSV strain X1N17 by DNA-mediated marker rescue.

A double-reciprocal recombination event between the homologous sequences (·····) and (⋈⋈⋈⋈) of HSV TK-deletion strain d2 and plasmid pX1N17 results in the production of a phenotypically TK^+ recombinant virus, X1N17, which carries VP5 promoter sequences.

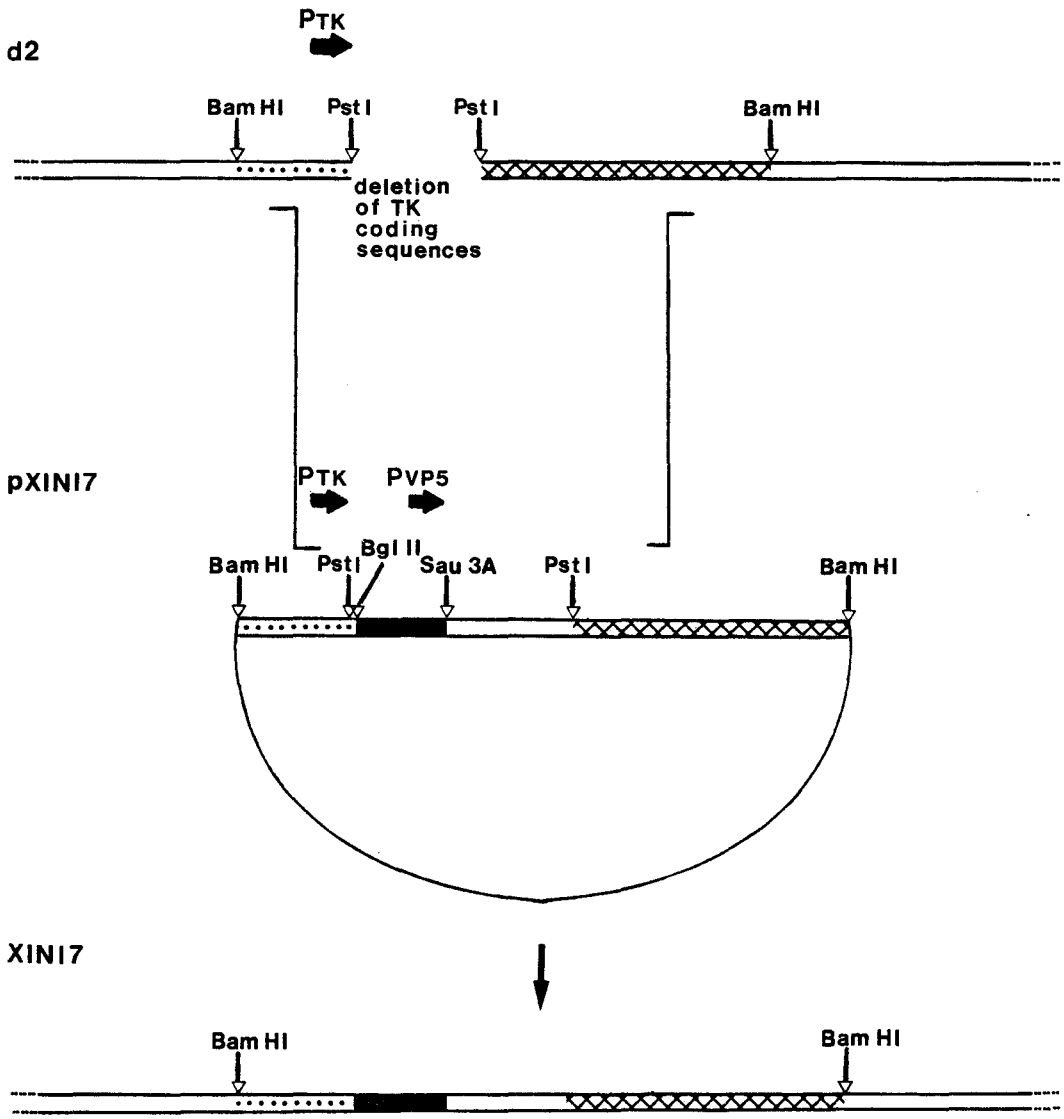

d2
PTK
Bam HI
Pst I
Pst I
Bam HI
deletion
of TK
coding
sequences
pXIN17
PTK
PVP5
Bam HI
Pst I
Bgl II
Sau 3A
Pst I
Bam HI
XIN17
Bam HI
Bam HI

FIGURE 5A: Schematic diagram of KOS and X1N17 TK genes.

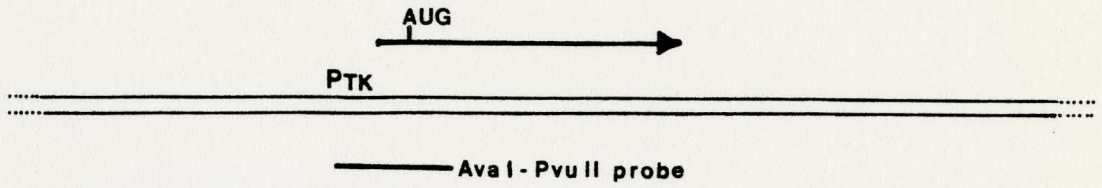
The long arrows indicate the positions of the KOS and X1N17-TK transcripts relative to the promoter regions (P_{TK}, P_{VP5}) and the DNA probes used in transcript mapping experiments.

FIGURE 5B: Nucleotide sequences of the VP5 and TK promoter regions.

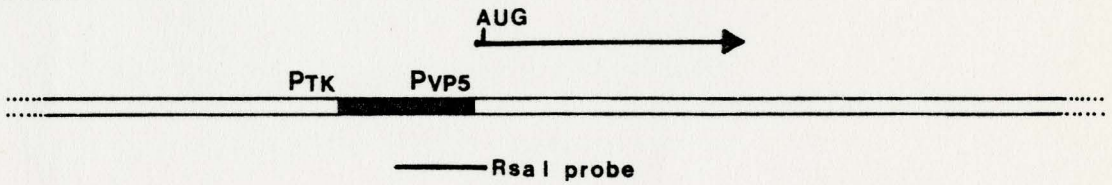
The sequences provided in this figure (Dennis and Smiley, 1984) span the promoter regions of the wild type VP5 and TK genes as well as the VP5-TK chimeric gene. The transcript initiation sites are indicated by +1.

A

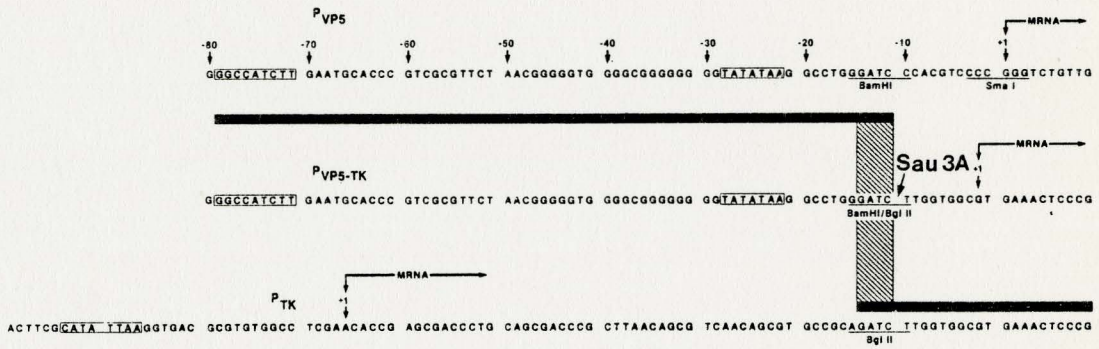
KOS


AUG
PTK
Ava I - Pvu II probe

XINI7


AUG
PTK
PVP5
Rsa I probe

B


PVP5
MRNA
-80
-70
-60
-50
-40
-30
-20
-10
+1
GGGCCATCTT GAATGCACCC GTCGCGTTCT AACGGGGGTG GGGCGGGGGG GGTATATAAG GCCTGGGATC CCACGTCCCC GGGTCTGTTG
BamHI
Sma I
PVP5-TK
Sau 3A
GGGCCATCTT GAATGCACCC GTCGCGTTCT AACGGGGGTG GGGCGGGGGG GGTATATAAG GCCTGGGATC TTGGTGGCGT GAAACTCCCG
BamHI/Bgl II
PTK
ACTTCGCATA TTAAGGTGAC GCGTGTGGCC TCGAACACCG AGCGACCCTG CAGCGACCCG CTTAACAGCG TCAACAGCGT GCCGCAGATC TTGGTGGCGT GAAACTCCCG
Bgl II

construct directs the transcription of a truncated TK mRNA. The transcript initiates just downstream from the VP5-TK fusion site, within sequences that form part of the non-translated leader of wild-type TK mRNA.

The VP5-TK chimeric gene of pX1N17 was introduced into the HSV genome in order to study the control of its expression in the context of a lytic viral infection. The genome of HSV is 150 kb in length, making direct insertion by *in vitro* methods impossible. Consequently, the technique of DNA-mediated marker rescue, which relies on *in vivo* recombination, was adopted. The VP5-TK fusion gene was transferred into the genome of the TK-deficient deletion mutant d2 (Smiley, 1980). The d2 virus bears a deletion of the 840 bp Pst I C fragment of TK DNA, extending from +16 to +856 in the TK mRNA. This deletion removes a large portion of the TK protein coding region. Since the pX1N17 plasmid bears sequences homologous to d2 DNA both 5' and 3' to the Pst I deletion, then a double reciprocal recombination event between the plasmid and the viral DNA would result in the elimination of the d2 deletion at the TK locus by replacing it with the pX1N17 insertion (see Figure 4). The resulting recombinant virus should be phenotypically TK^+. As seen in Figure 4, the d2 deletion spans the Bgl II site at +56, thus d2 physically lacks the sequences immediately flanking the site of VP5-TK fusion in pX1N17. Consequently, a double reciprocal recombination event that inserts the TK gene at the TK locus in d2 must also insert the VP5 promoter.

The marker rescue was carried out by Helen Rudzroga of this laboratory by transfecting Vero cells with a mixture of infectious d2

viral DNA and circular pX1N17 DNA. The infectious virions emerging from the Vero cells were used to infect human TK-deficient 143 cells in HAT selection medium. Under these conditions, plaque formation depends on the presence of viral TK enzymatic activity.

3.1. Recovery of Purified Recombinant Virus

Seven different TK^+-plaque isolates were tested for the presence of the VP5-TK chimeric gene in the viral genome by Southern blot analysis. Pvu II digests of the viral DNA and plasmids pX1 and pX1N17 were analyzed by agarose gel electrophoresis and Southern blot hybridization to radiolabelled pTK173. Plasmid pTK173 carries the 2.0 kb Pvu II fragment of HSV TK cloned into the Pvu II site of pBR322 (see Figure 6A). Five of the seven isolates (4,6,7,8 and 9) were identified as carriers of the VP5-TK construct, due to the detection of a single Pvu II fragment in the viral genome that co-migrates with the Pvu II fragment of pX1N17 (Figure 7, Lane 4). The other two virus isolates, 1 and 2, also appear to carry the desired insert, but its presence could not be positively confirmed from Figure 7 due to the presence of background radioactivity on the filter. The possibility that the VP5-TK gene integrated elsewhere in the genome is excluded by the fact that none of the positive isolates carried the deleted TK-Pvu II fragment of d2 viral DNA (Figure 8, Lane 4). Therefore, plasmid DNA must have physically replaced the deleted TK gene of d2, suggesting recombination at the TK locus. Also, insertion of the VP5-TK fusion gene elsewhere in the viral genome would have produced novel-sized Pvu II fragments representing the junctions flanking TK. No novel Pvu II fragments are

seen in Figures 7 and 8. Since only a single band is detected by the probe in all of the viral DNA samples, it is obvious that none of the positive recombinants contain pBR322 DNA sequences. A single cross-over event between pX1N17 and d2 DNA would result in the integration of the entire pX1N17 plasmid, including pBR322 sequences, into the viral genome. Since pBR322 sequences are not detected in Southern blot analysis of the recombinants, they must have arisen by a double reciprocal recombination event. This observation is consistent with recent findings by Margaret Howe and J.R. Smiley in this laboratory. Although single crossovers and plasmid integration events occur during marker rescue, these events are less frequent than direct sequence replacement by double reciprocal crossovers (personal communication). Thus the data presented in Figure 7 demonstrates that positive isolates 4, 6, 7, 8 and 9 carried the desired insert and were suitable for further study.

One of the positive plaque isolates, #8, was selected for a further plaque purification to ensure the purity of the virus stocks that would be used in subsequent experiments. Southern blot analysis of Pvu II digested viral DNA from one of these re-purified plaque isolates also confirmed the presence of the VP5 insertion in the TK gene (Figure 8, Lane 2). This re-purified recombinant virus strain was named X1N17 and was amplified in Vero cells, as described in Materials and Methods, for use in the remainder of this study.

3.2. Analysis of TK Transcripts Produced in Infected Cells

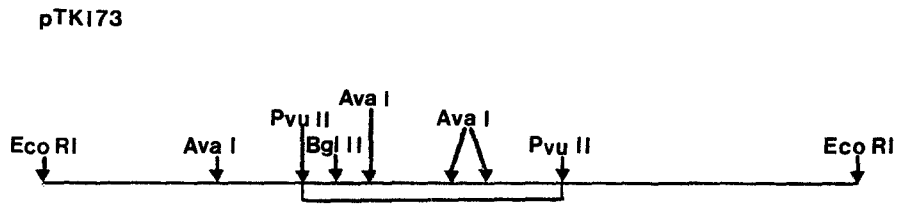
X1N17 virus contains a TK gene with VP5 promoter sequences

FIGURE 6A: Restriction map of pTK173.

FIGURE 6B: Restriction map of pX1N17.

The restriction endonuclease cleavage sites relevant to this study are indicated. The open bars specify the extent of HSV sequences in the plasmids. The closed bar specifies the VP5 promoter sequences contained within the TK gene of pX1N17.

A


pTKI73
Ava I
Pvu II
Ava I
Eco RI
Ava I
Bgl II
Pvu II
Eco RI

B

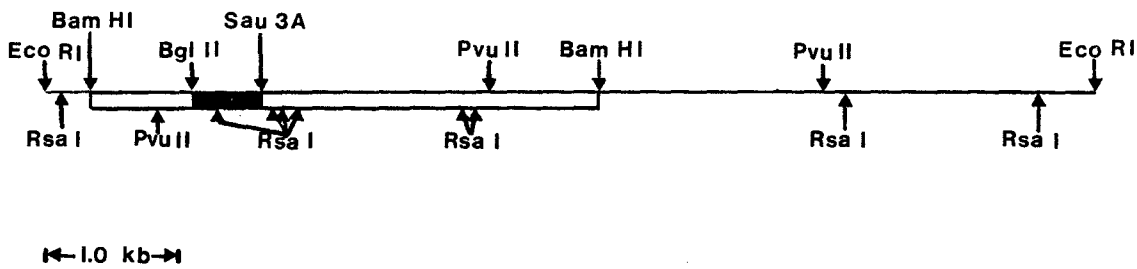

pXINI7
Bam HI
Sau 3A
Eco RI
Bgl II
Pvu II
Bam HI
Pvu II
Eco RI
Rsa I
Pvu II
Rsa I
Rsa I
Rsa I
Rsa I
1.0 kb

FIGURE 7: Southern blot analysis of recombinant HSV-TK^+ plaque isolates.

DNA from seven different TK^+ plaque isolates was digested with restriction endonuclease Pvu II along with plasmids pX1 and pX1N17. The DNA fragments were separated by electrophoresis through a 1% agarose gel and transferred to nitrocellulose. TK DNA fragments were detected by hybridization to radiolabelled pTK173 DNA.

LANES 1 through 3: TK^+ plaque isolates #1, #2, and #4 respectively
LANE 4: pX1N17
LANE 5: pX1
LANES 6 through 9: TK^+ plaque isolates #6 through #9 respectively

The arrow indicates the position of the recombinant Pvu II fragment carrying the VP5 insert.

1 2 3 4 5 6 7 8 9

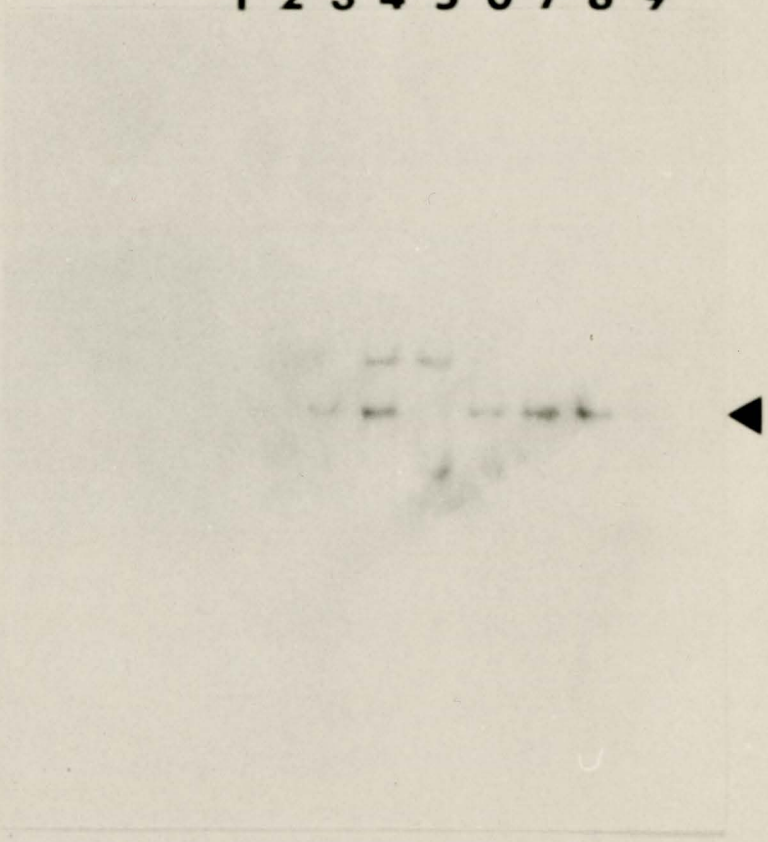


FIGURE 8: Southern blot analysis of X1N17 DNA.

TK^+ plaque isolate #8 (renamed X1N17) was plaque-purified and re-analyzed by Pvu II digestion and Southern blot hybridization with radiolabelled pTK173.

LANE 1: pX1N17
LANE 2: X1N17 viral DNA
LANE 3: pX1
LANE 4: D2A viral DNA
LANE 5: KOS viral DNA

All DNA samples were digested with endonuclease Pvu II. The arrow indicates the VP5-containing Pvu II fragment of X1N17 DNA.


1 2 3 4 5

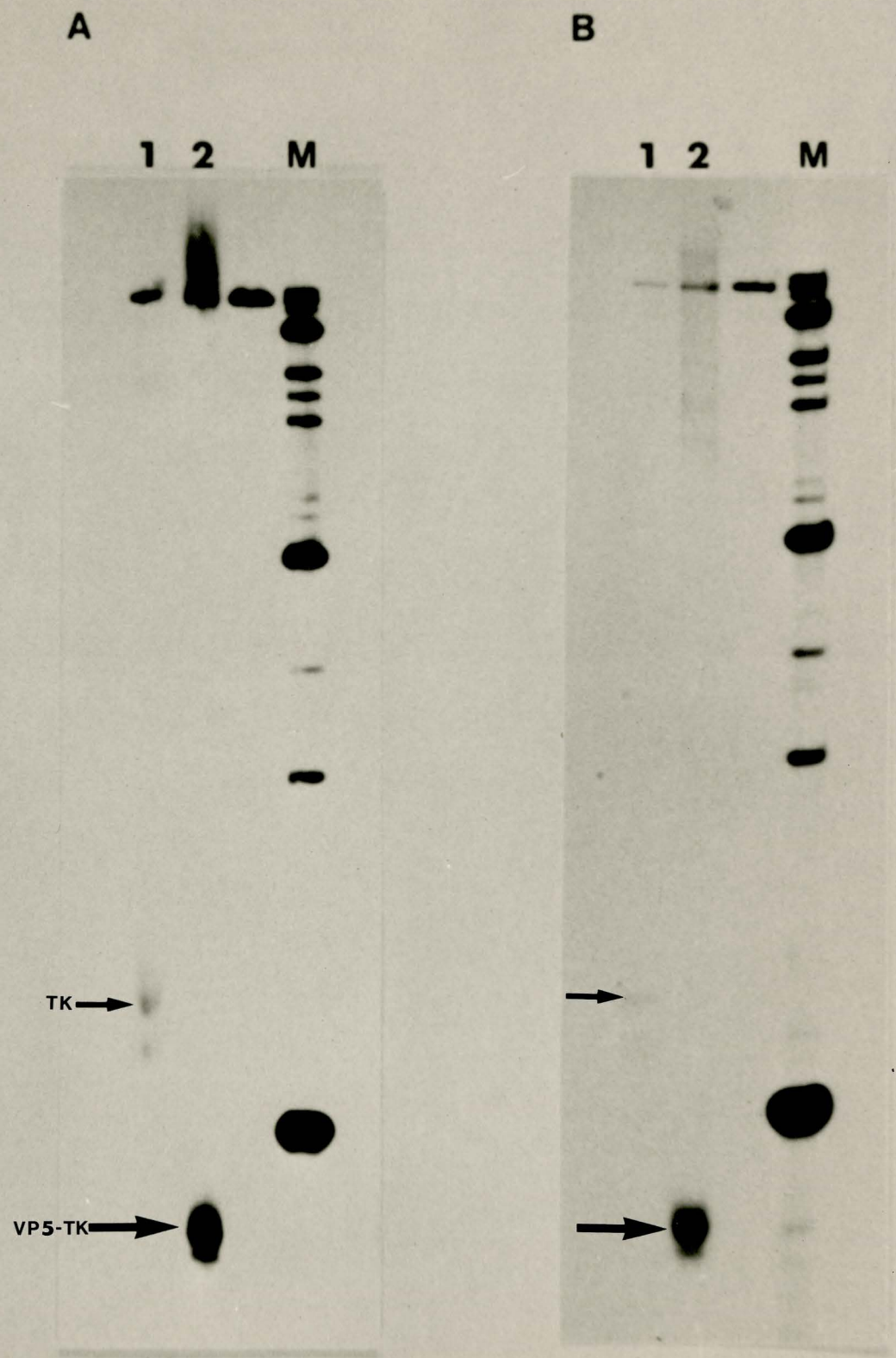
inserted between the wild type TK promoter and the TK coding sequences. Due to the presence of two promoters upstream from TK coding sequences, it was necessary to determine which promoter was actively transcribing TK in X1N17-infected cells. The initiation sites of TK transcripts in KOS and X1N17-infected cells were determined by the transcript mapping procedure outlined by Berk and Sharp (1977). As described in Materials and Methods, total cytoplasmic RNA from LtA cells infected for nine hours with KOS or X1N17 was hybridized to a single-stranded, uniquely end-labelled Rsa I fragment of pX1N17. The Rsa I fragment extends from a site within the VP5 promoter to position +123 in the TK transcript. The resulting hybrids were subjected to S1 nuclease digestion, and the products were sized on DNA sequencing gels alongside an end-labelled molecular weight marker generated by Sau 3A digestion of the Rsa I probe. As seen in the restriction map of pX1N17 (Figure 6B), restriction endonuclease Sau 3A cleaves at the junction between VP5 and TK coding sequences. The results of two separate transcript mapping experiments are shown in Figure 9. KOS-infected cell RNA (Lane 1) protected a probe fragment several nucleotides longer than the 66 nucleotide Sau 3A-Rsa I marker fragment (Lane M). This is consistent with the nucleotide sequence data shown in Figure 5B. The wild-type TK and VP5-TK sequences diverge upstream from the G residue in the Bgl II site of wild-type TK, four residues upstream from the site of Sau 3A cleavage. RNA from X1N17-infected cells (Lane 2) protects a probe fragment slightly smaller than the Sau 3A marker fragment, indicating TK transcript initiation downstream from the site of VP5-TK fusion. The exact site of TK transcript initiation in X1N17-infected cells was not determined,

however, the size of the protected probe fragment is consistent with initiation at or near the site identified by Dennis and Smiley (1984) for pX1N17 TK mRNA (Figure 5B). The pX1N17-TK mRNA initiates 27 bp downstream from the Hogness box of the VP5 promoter, within sequences that are normally part of the 5' non-translated leader of the TK transcript. If the TK transcripts in X1N17-infected cells were transcribed under the control of the upstream TK promoter, then initiation 25-30 bp downstream from the TK Hogness sequence would be within the TK region upstream from the VP5 insert. The schematic diagram of KOS and X1N17 TK genes in Figure 5A, showing the location of the Rsa I probe, indicates that transcript initiation from the TK promoter of X1N17 would result in protection of the full length Rsa I probe in the S1 mapping experiment. As described above, the results in Figure 9 are consistent with X1N17 TK mRNA initiation downstream from the VP5 insert.

The signals corresponding to TK mRNA initiation sites in Figures 9A and 9B, appear to vary in length by one to several nucleotides. This is due to incomplete digestion with S1 nuclease, resulting in short, variable-length, undigested overhangs. Another observation made from these two transcript mapping experiments was that the level of TK transcripts in X1N17-infected cells appears to be higher than the wild-type TK mRNA levels. This is consistent with the predicted transcription kinetics for the two genes since viral DNA replication would have begun by nine hours post-infection, resulting in the shut-off of DE transcription and the enhancement of L1 transcription.

FIGURE 9: Determination of X1N17 and KOS TK transcript initiation sites by S1 mapping.

Ten μg of KOS (LANE 1) and X1N17 (LANE 2) cytoplasmic RNA, extracted at nine hours post-infection, was hybridized to a single-stranded end-labelled Rsa I fragment of pTK173. After S1 nuclease digestion, the products were separated on 8% polyacrylamide sequencing gels alongside the Rsa I fragment of pTK173 digested with Sau 3A (LANE M). The large arrows indicate the protected fragment corresponding to the VP5-TK transcript in X1N17-infected cells. The small arrows indicate the DNA fragment protected by the TK transcripts in KOS-infected cells. The gels were exposed to x-ray film for two weeks. Figures 9A and 9B are the results of two separate transcript mapping experiments.


A
1 2 M
TK
VP5-TK
B
1 2 M

3.3. Comparison of TK Transcript Levels in Infected Cells

The results presented in the preceding section showed that the VP5-TK fusion gene initiates transcription at the same site identified by Dennis and Smiley (1984) in virally infected, pX1N17-transformed cells. In addition, they suggested that the hybrid gene was transcribed to high levels. These results indicate that TK transcription in X1N17 is driven from the VP5 promoter element, and that the VP5 promoter works efficiently in an anomalous genomic site. The next step in the analysis of the VP5-TK fusion gene was to determine whether the TK transcripts accumulated with L1 or DE kinetics. The kinetics of TK expression were examined by comparing the levels of TK transcripts present in KOS and X1N17-infected cells at various times after infection. Cytoplasmic RNA from infected LtA cells was extracted every three hours until 15 hours post-infection and hybridized to a single stranded, end-labelled Ava I-Pvu II probe derived from pTK173. The probe, which spans the wild-type TK transcription start site, extends from -196 to +356 in the TK gene. The protected DNA fragments resulting from S1 nuclease treatment were separated on sequencing polyacrylamide gels alongside a 301 nucleotide marker fragment generated by Bgl II digestion of the probe DNA. Wild-type TK mRNA initiates 52 nucleotides upstream from the Bgl II site, thereby protecting a probe fragment 353 bases in length. X1N17-TK mRNA starts approximately 12 bases downstream from the corresponding site in the VP5-TK fusion gene, producing a signal 289 nucleotides long. The levels of TK transcripts present in infected cells throughout the infectious cycle were represented by the intensities of the bands on autoradiographs of the gels. Figures 10A and 10B are autoradiographs of

two separate transcript mapping experiments. TK mRNA in KOS-infected cells is visible by three hours post-infection (Lane 8), and appears to peak at or before nine hours post-infection. In these gels, the most intense wild-type TK signal is at nine hours (Lane 6), however, the RNA sample for the six hour time point was degraded in one experiment, leaving no signal in Lane 7. Wild-type TK transcripts were barely detectable at 15 hours post-infection (Lane 5), suggesting shutoff of TK transcription and mRNA turnover. These results are consistent with the kinetics of DE transcription identified by others (Honess and Roizman, 1973; 1974). DE gene expression peaks early, before viral DNA replication, and declines late. The kinetics of accumulation of X1N17-TK transcripts are different from wild-type kinetics. X1N17-TK mRNA is not detectable at three hours post-infection (Lane 4), but appears by six hours (Lane 3) and continues to accumulate through nine and fifteen hours (Lanes 2 and 1). The intense signal observed at 15 hours post-infection indicates that the TK gene in X1N17 is being transcribed with late kinetics. This pattern of expression is consistent with previous information on VP5 RNA accumulation (Harris-Hamilton and Bachenheimer, 1985). The transcription of the VP5-TK chimeric gene does not appear to be impaired, since X1N17 produces TK transcript levels equal to or greater than wild type levels. The results indicate that the 600 base pair insert carrying VP5 promoter sequences was sufficient to confer the L1 pattern of transcription on TK. This is consistent with the idea that it is not the TK structural sequence, but the promoter region that determines the kinetic class of a HSV transcription unit. Recently, Silver and Roizman (1985) published the results of similar work on an

FIGURE 10: Examination of TK transcript levels in KOS and X1N17-infected cells throughout HSV infection.

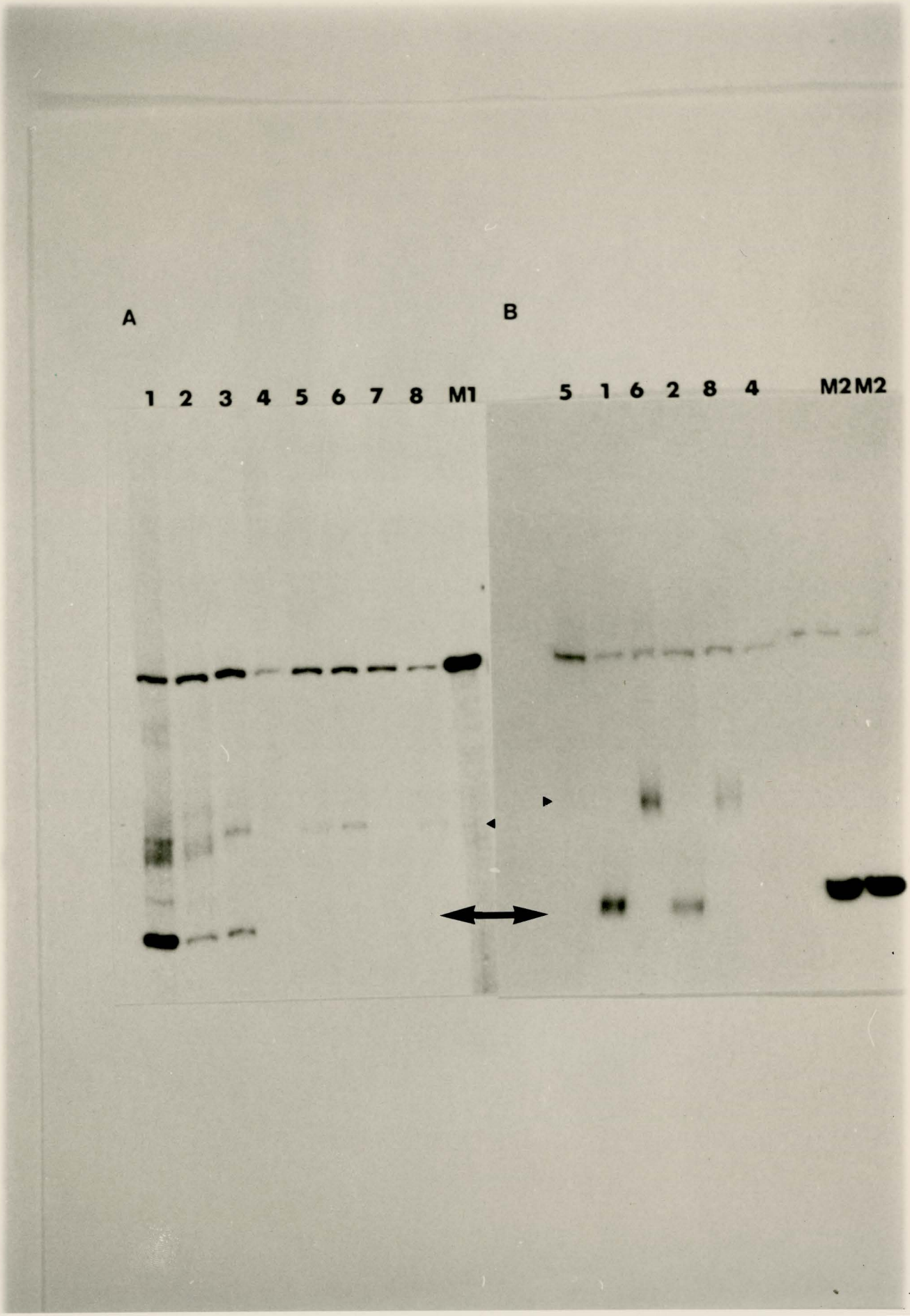
Ten μg of cytoplasmic RNA, extracted from KOS or X1N17-infected LtA cells at various times after infection, was hybridized to an end-labelled Ava I-Pvu II fragment of pX1N17. The hybrids were subjected to S1 nuclease digestion and electrophoresis on 6% polyacrylamide sequencing gels. The gels were exposed to x-ray film for two weeks. Figures 10A and 10B are replicates of the experiment described above.

LANE M1: full length Ava I-Pvu II probe DNA
LANE M2: Bgl II digested Ava I-Pvu II probe DNA

The probe DNA was hybridized to infected cell RNA as follows:

LANES 1 through 4: X1N17 RNA at 15, 9, 6, and 3 hours post-infection respectively
LANES 5 through 8: KOS RNA at 15, 9, 6, and 3 hours post-infection respectively

The small arrows indicate the position of the probe fragment protected by TK transcripts from KOS-infected cells. The large arrows indicate the DNA fragment protected by VP5-TK transcripts from X1N17-infected cells.


A
1 2 3 4 5 6 7 8 M1
B
5 1 6 2 8 4 M2 M2

L2-TK chimeric gene in which they describe late kinetics of TK expression under L2 promoter control.

3.4. Measurement of TK Enzymatic Activity Levels in Infected Cells

Previous experiments by Post et al. (1981) and by Silver and Roizman (1985) have demonstrated that chimeric TK genes in viral genomes are expressed at the protein level with the kinetics of their promoter class. If HSV gene regulation is mediated solely by the gene promoters, then TK enzymatic activity should be expressed with L1 kinetics in X1N17-infected cells since its transcription is under the control of the VP5 promoter. We have already shown that TK is transcribed with late kinetics in X1N17 (Section 3.3), the next step was to determine if the late kinetics were being conserved at the protein level. The TK activity levels in infected LtA cells were measured every three hours for 18 hours by the TK assay described in Section 2.8. Figures 11 and 12 show the TK activity levels at different times after infection in LtA cells infected at the same m.o.i. with HSV-1 strain KOS or X1N17. It is important to note the difference in scale of the vertical axes in Graph A and B in each figure. The TK activity in X1N17-infected cells is lower than wild type activity at any time after infection. The magnitude of the difference between KOS and X1N17-TK activity levels varies with the time after infection. For example, the experiment in Figure 11 shows almost nine times more TK activity in KOS-infected cells than in X1N17-infected cells at nine hours post-infection, while the difference at 12 hours is 67-fold. The relatively low level of TK activity in X1N17-infected cells is not likely due to an altered or

truncated TK polypeptide. The construction of the VP5-TK chimeric gene resulted in transcripts that initiate within the non-translated leader sequence of TK, 45 bp upstream from the first AUG translation start codon. Therefore, the TK polypeptides produced by KOS and X1N17 should be identical. As described below in Section 3.5, analysis of KOS and X1N17-infected cell proteins by denaturing polyacrylamide gels show no detectable size difference between wild-type and X1N17-TK proteins. Thus the low TK enzymatic activity levels presumably reflect low polypeptide levels.

Another interesting observation is that TK activity in X1N17-infected cells appears to follow DE kinetics. There is a drop in TK activity late in infection that is not consistent with the high levels of TK transcripts detected at that time by the transcript mapping experiments discussed in Section 3.3. These results suggest that although the VP5-TK chimeric gene is being transcribed from an L1 promoter, there is some other mechanism responsible for preserving DE kinetics of TK activity in these cells. Both KOS and X1N17-infected cells display a drop in TK activity around 12 to 15 hours after infection, followed by an increase at 18 hours. The reason for this increase in TK activity so late in the infectious cycle is unclear, however it has been observed consistently in TK assays performed in this laboratory by Helen Rudzroga and myself.

3.5. Measuring TK Polypeptide Levels in Infected Cells.

TK polypeptide levels were compared in KOS and X1N17-infected cells at different times after infection to determine whether the low TK

FIGURE 11: Thymidine kinase activity levels in KOS and X1N17-infected LtA cells.

The TK enzymatic activity levels in infected cells were assayed every three hours for eighteen hours by the method described in Section 2.8. Graph A represents TK activity levels in KOS-infected LtA cells. Graph B represents TK activity levels in X1N17-infected LtA cells.

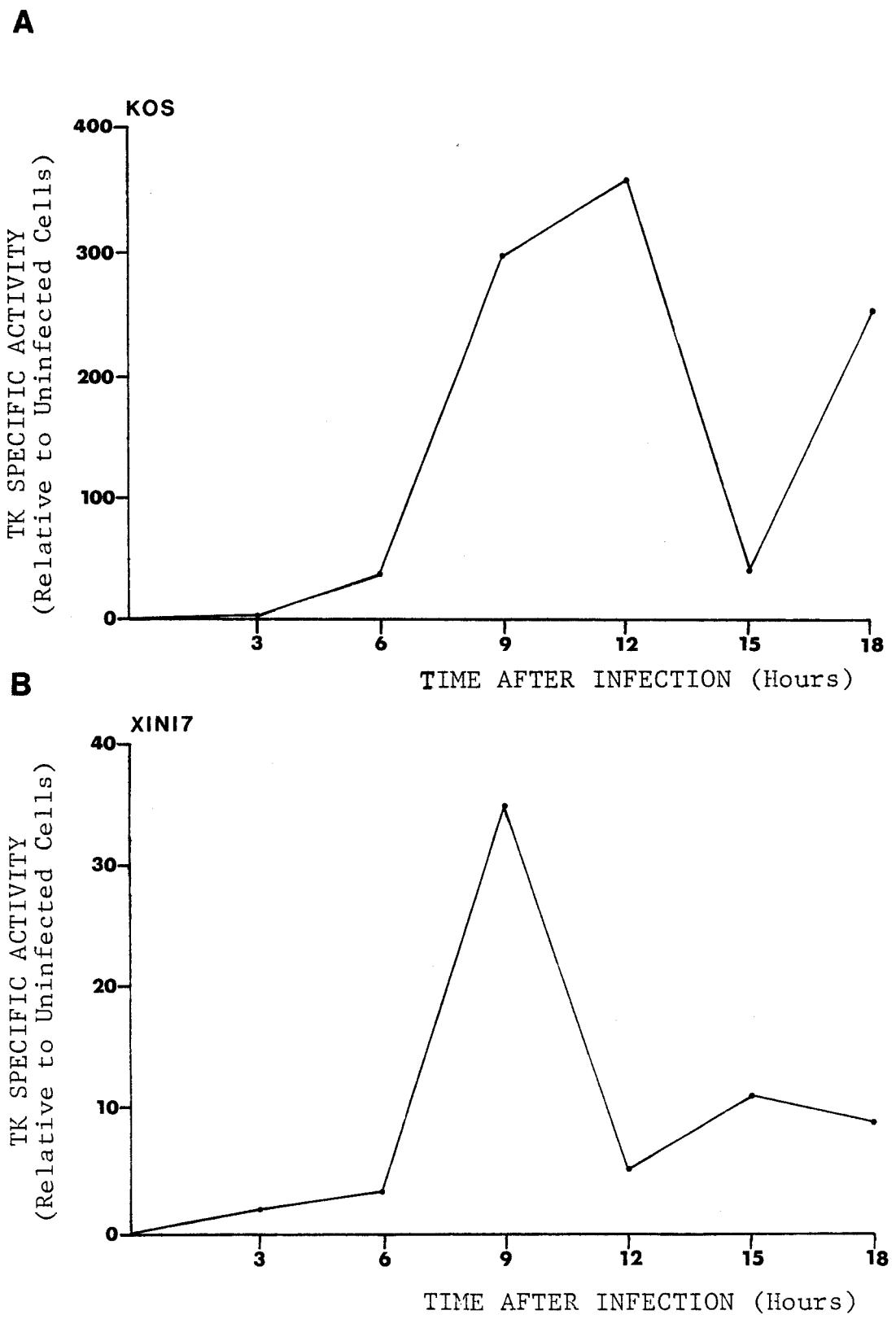
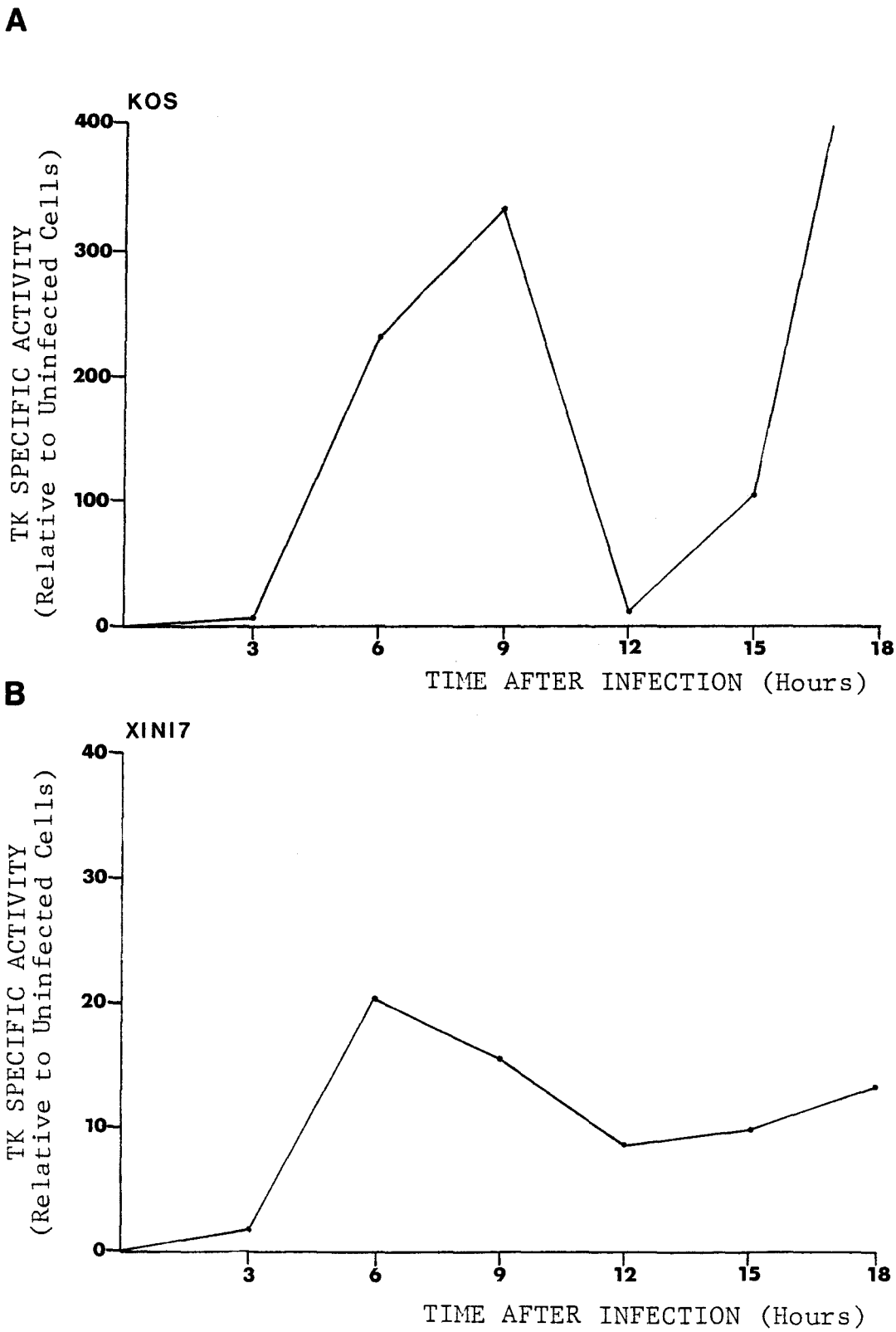

A
KOS
400
300
200
100
0
TK SPECIFIC ACTIVITY
(Relative to Uninfected Cells)
3
6
9
12
15
18
TIME AFTER INFECTION (Hours)
B
XINI7
40
30
20
10
0
TK SPECIFIC ACTIVITY
(Relative to Uninfected Cells)
3
6
9
12
15
18
TIME AFTER INFECTION (Hours)

FIGURE 12: Thymidine kinase activity levels in KOS and X1N17-infected LtA cells.

The results shown in Figures 11A and 11B are supported by the data presented here from a replicate experiment. Graph A is the TK activity curve from KOS-infected LtA cells. Graph B is from X1N17-infected cells.


A
KOS
400
300
200
100
0
TK SPECIFIC ACTIVITY
(Relative to Uninfected Cells)
3
6
9
12
15
18
TIME AFTER INFECTION (Hours)
B
XINI7
40
30
20
10
0
TK SPECIFIC ACTIVITY
(Relative to Uninfected Cells)
3
6
9
12
15
18
TIME AFTER INFECTION (Hours)

activity levels observed in the recombinant virus reflected an enzymatic deficiency of the TK polypeptide or reduced levels of translation of a normal TK polypeptide.

The anti-TK antiserum was first tested for effectiveness in the Western blot immunoassay by comparison to a standard immunoprecipitation. The anti-TK antiserum was a rabbit antiserum prepared by Sheldon Girvitz (Department of Biology, McMaster University) against HSV TK purified by affinity chromatography on thymidine agarose columns. Radiolabelled proteins were isolated from LtA cells infected at a m.o.i. of 15 with HSV strain KOS. The cells were harvested at 3, 6 and 9 hours post-infection following a two hour incubation in the presence of ^{35}S-methionine. The proteins were extracted as described in Section 2.9.2. and different amounts of extracts were immunoprecipitated with anti-TK antiserum and Protein-A Sepharose beads. The products of the immunoprecipitation were separated on a 12% SDS-polyacrylamide gel and visualized by autoradiography of the dried gel. Unlabelled proteins extracted from LtA cells infected as above but in the absence of ^{35}S-methionine were prepared in the same manner as above. The proteins were separated on a 12% SDS-polyacrylamide gel, then electroblotted onto a nitrocellulose membrane as described in Section 2.9.5. The TK protein bound to the membrane was detected by reacting the filter to anti-TK antiserum, followed by a horse radish peroxidase (HRP) labelled goat anti-rabbit second antibody and the 4-chloronapthol colour reaction for HRP (Figure 13B). HSV-TK, indicated by the arrows, is a 42 kilodalton polypeptide that migrates near the 43,000 dalton (ovalbumin) marker. Lanes 2-7 in Figure 13A and Lanes 2-4 and 6-8 in Figure 13B show that

identical 42 kilodalton polypeptides were detected by the antiserum in both tests.

The Western blot immunoassay appeared to be a better assay for TK polypeptide levels than immunoprecipitation of radiolabelled proteins for several reasons. First, the Western blots could detect overall TK polypeptide levels in infected cells at different times after infection while the *in vivo* labelling of proteins limited detection to those polypeptides being synthesized during the labelling period. The method used to attain high specific activity of labelled polypeptides is to introduce the radiolabelled amino acid(s) into the culture medium in the presence of little or none of the corresponding unlabelled amino acid(s). Since the radiolabelled amino acid available in the medium would be quickly incorporated into the cells, the continued labelling of polypeptides would require frequent media changes to replenish the radiolabelled amino acid supply. This would introduce a new variable into the experiment, making comparison with the experiments described in Sections 3.3 and 3.4 difficult. Therefore, the efficiency of incorporation of radioactivity into proteins could not be kept steady over a long period. The alternative labelling method, using a mixture of the radiolabelled and unlabelled amino acid, results in low specific activity labelled polypeptides and therefore reduces the signal intensity. If, as suspected, the low TK activity levels observed in X1N17-infected cells are due to low TK polypeptide levels, then low specific activity radiolabelling would be insufficient to allow detection of TK from X1N17-infected cell extracts. Also, as seen in Figure 13B, the Western blot immunoassay appears to provide a more

sensitive assay for subtle differences in protein levels than the immunoprecipitation. Lanes 3-7 in Figure 13A are immunoprecipitations of 100, 200, 300, 400, and 500 microlitres of protein extract from cells infected for six hours with KOS. The differences in signal intensities between these samples were not as obvious as the difference observed between Lanes 3 and 7 in Figure 13B. These lanes represent 20 and 40 microlitres respectively of proteins extracted at six hours from KOS-infected cells.

One technical observation that was made during the course of these studies was that nitrocellulose was a more favourable solid support for Western blot immunoassay by the method described than Genescreen nylon membranes. After electrophoretic transfer, proteins were retained better on nitrocellulose than on Genescreen membranes throughout the immunoassay procedure. This was monitored by the retention of the pre-stained molecular weight protein standards on the filters.

TK polypeptide levels in KOS and X1N17-infected cells were compared by extracting proteins from infected LtA cells every three hours for 15 hours. The total protein concentrations in each sample were measured by the method of Lowry et al (1951). Equal amounts of total protein were run on 12% SDS polyacrylamide gels followed by immunodetection with anti-TK antiserum and ^{125}I-labelled goat anti-rabbit IgG. The autoradiographs of two separate time course experiments are shown in Figures 14 and 15. There is no detectable size difference between wild type and X1N17 TK proteins (Figures 14 and 15; Lanes 6 and 7). TK protein is produced in barely detectable levels in X1N17-

FIGURE 13A: Immunoprecipitation of KOS-infected cell proteins with anti-TK antiserum.

Uninfected and KOS-infected LtA cells were grown in the presence of ^{35}S-methionine for two hours prior to harvesting. The cells were harvested as described in Section 2.9.1 and TK was immunoprecipitated with anti-TK antiserum as described in Section 2.9.3. The immunoprecipitated products were separated on a 12% SDS-polyacrylamide gel and detected by autoradiography.

The positions of the molecular weight markers are indicated by bars to the left of the autoradiograph and the molecular weights are indicated in kilodaltons. TK is indicated by an arrow.

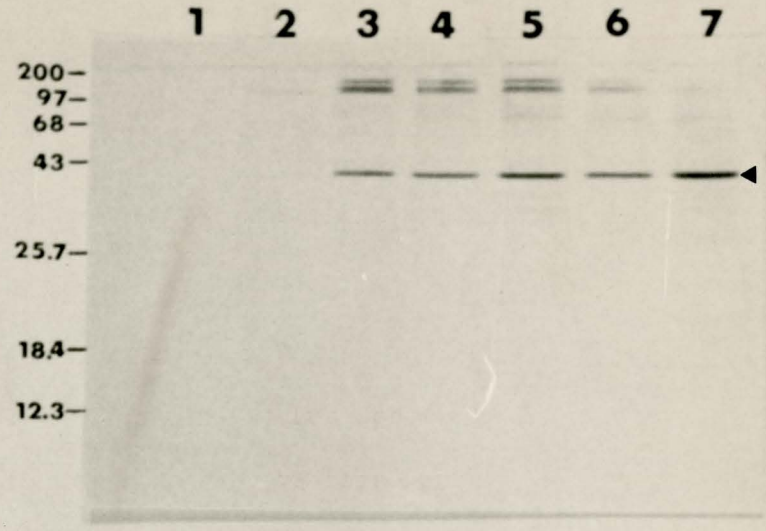
LANE 1: Uninfected LtA cells
LANE 2: 500 µl of protein extract from KOS-infected cells at 9 hours post-infection
LANES 3 through 7: 100, 200, 300, 400, and 500 µl respectively from KOS-infected cells at 6 hours post-infection

FIGURE 13B: Western blot analysis of KOS-infected cell proteins.

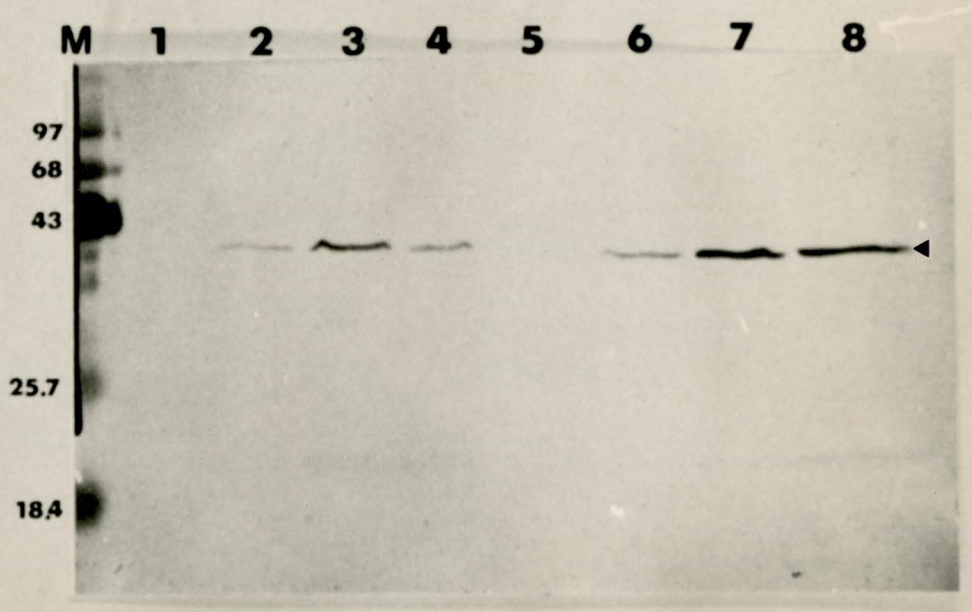
Uninfected and KOS-infected LtA cell proteins were extracted at various times post-infection as described in Section 2.9.1. Proteins were separated on a 15% SDS-polyacrylamide gel and electroblotted onto nitrocellulose as described in Section 2.9.5. HSV TK (arrow) was detected by reacting the filter to anti-TK antiserum, followed by horse radish peroxidase-linked goat anti-rabbit IgG and the HRP colour reaction with 4-chloronapthol.

LANE M: molecular weight standards (sizes are in kilodaltons)
LANE 1: 20 µl of protein extract from uninfected LtA cells
LANES 2 through 4: 20 µl of protein extract from KOS-infected cells at 3, 6 and 9 hours respectively
LANE 5: 40 µl of protein extract from uninfected LtA cells
LANES 6 through 8: 40 µl of protein extract from KOS-infected cells at 3, 6 and 9 hours respectively

A



B



infected cells throughout the infectious cycle. TK is first detected in X1N17-infected cells at six hours post-infection (Figures 14 and 15; Lane 5). Slightly higher levels are detected at nine hours (Figures 14 and 15; Lane 7), after which the levels appear to remain stable (Figure 14B; Lanes 10 and 12). TK is detectable earlier in KOS-infected cells where a faint signal is visible at three hours (Figures 14 and 15; Lane 2). Lanes 6, 9 and 11 (Figure 14B) show that TK levels plateau by nine hours post-infection and remain stable through 15 hours. From these observations it appears as though TK accumulates with similar kinetics in X1N17 and KOS-infected cells. Stronger signals for TK from X1N17 infected cell extracts could not be achieved due to limitations on the total amount of protein that could be loaded into a sample well. This experiment confirms that reduced TK activity in X1N17-infected cells is a direct result of depressed levels of TK polypeptide.

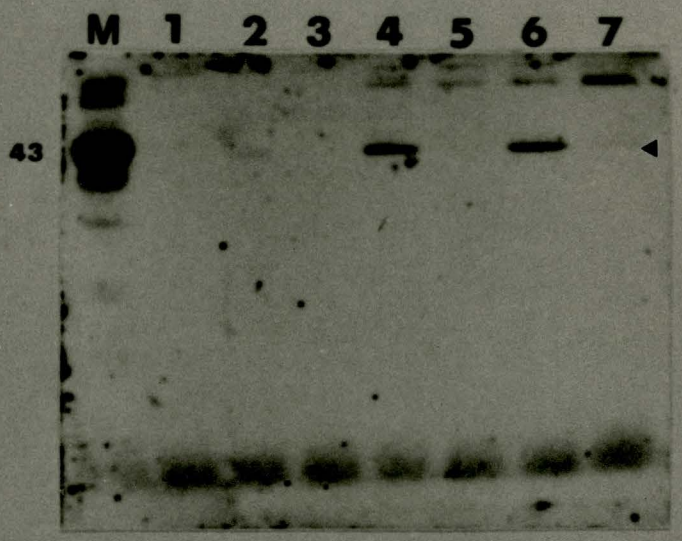
A second observation that can be made from Figures 14 and 15 is that the TK polypeptide levels remain stable in infected cells at late times post-infection, long after the TK activity levels have dropped (see Section 3.4). Perhaps there is another level of TK regulation that represses the enzymatic activity of intact TK polypeptides present in cells at late times after infection. Or possibly, the TK enzyme undergoes a physical change at late times after infection making it resistant to extraction by the methods used for TK assays. A similar observation has been made for HSV ribonucleotide reductase, another DE gene. The reductase activity levels decline at late times post-infection despite continued presence of the enzyme (Dr. S. Bacchetti, personal communication). Finally, both the Western blot immunoassay and

FIGURES 14A and 14B: Examination of TK polypeptide levels in KOS and X1N17-infected LtA cells.

Proteins were extracted from KOS and X1N17-infected LtA cells every three hours for fifteen hours as described in Section 2.9.1. The protein content in each sample was determined by the method of Lowry et al. (1951) and 20 μg of protein from each sample was separated on 12% SDS-polyacrylamide gels. The gels were electroblotted onto nitrocellulose and immunoassayed with anti-TK antiserum and ^{125}I-labelled goat anti-rabbit IgG as described in Section 2.9.6. The filters were exposed to x-ray film for 24 hours. TK is indicated by arrows.

LANE M: molecular weight standards (sizes are in kilodaltons)
LANE 1: uninfected LtA cells
LANES 2, 4, 6, 9 and 11: KOS-infected cells at 3, 6, 9, 12 and 15 hours respectively
LANES 3, 5, 7, 10, and 12: X1N17-infected cells at 3, 6, 9, 12 and 15 hours respectively
LANE 8: TK-deficient HSV strain D2A-infected cells at 9 hours

A



B

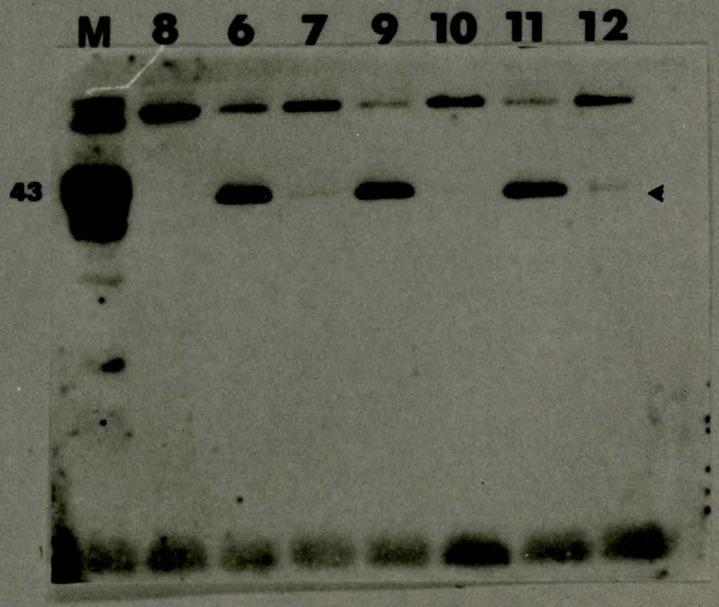
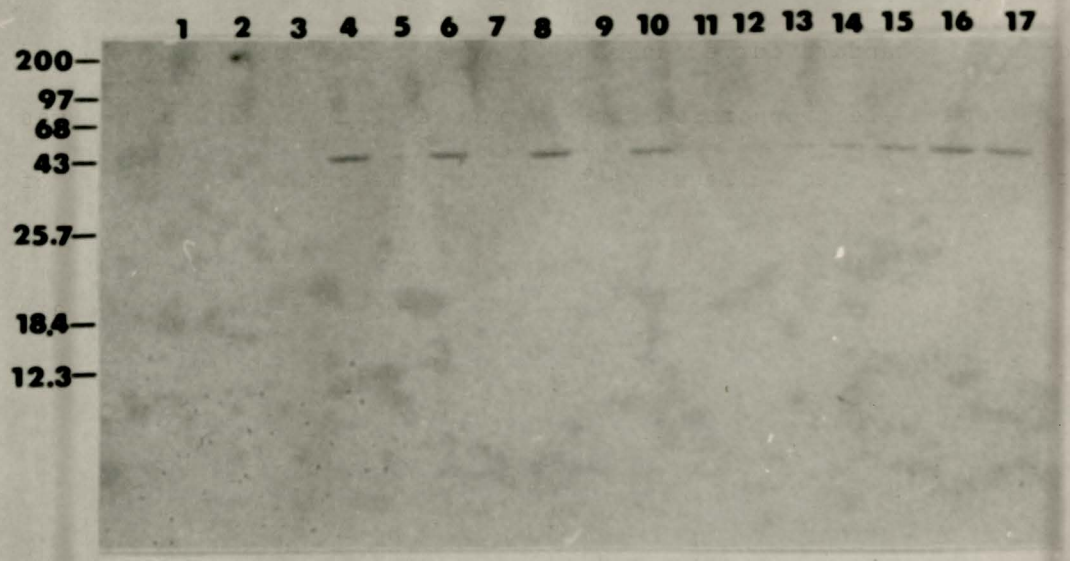


FIGURE 15: Examination of TK polypeptide levels in KOS and X1N17-infected cells.

This autoradiograph represents a repeat of the experiment shown in Figures 14A and 14B with the following changes. All of the samples, including a standard curve generated with proteins from KOS-infected cells, were electrophoresed on a single gel. The gel was electroblotted onto Genescreen nylon membrane instead of nitrocellulose.

The molecular weights (in kilodaltons) of protein markers are indicated to the left of the gel.

LANE 1: uninfected LtA cells
LANES 2, 4, 6, 8 and 10: KOS-infected cells at 3, 6, 9, 12 and 15 hours respectively
LANES 3, 5, 7, 9 and 11: X1N17-infected cells at 3, 6, 9, 12 and 15 hours respectively
LANES 12 through 17: 1, 2, 5, 10, 20 and 25 μl respectively of KOS-infected cell extracts at 9 hours


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
200
97
68
43
25.7
18.4
12.3

the immunoprecipitation detected a second signal in SDS gels. A larger viral polypeptide, presumably the large 140 kd subunit of ribonucleotide reductase, is efficiently precipitated by this anti-TK serum (Dr. S. Bachetti, personal communication). Its appearance is first detected at six hours post-infection, and its levels stabilize by nine hours, just as the wild-type TK polypeptide. The accumulation of this protein follows similar DE kinetics in KOS and X1N17, and the levels of this protein present in X1N17-infected cells at any particular time after infection are equal to or greater than wild-type levels. Comparison of the levels of this second polypeptide, present in KOS and X1N17-infected cells throughout infection, suggests that infection by these two HSV strains proceeds at similar rates. Thus there is no obvious lag in the progression of the X1N17 infectious cycle and no general reduction in viral polypeptide synthesis that could be responsible for the low levels of TK activity detected in cells infected with this recombinant virus.

Altogether, the data presented here indicate that the VP5 promoter in X1N17 confers late kinetics on the transcription of the TK gene. However, despite high levels of TK mRNA accumulation at late times in the infectious cycle, the TK polypeptide fails to accumulate significantly in X1N17-infected cells. Some possible mechanisms for this apparent translational defect will be presented in the Discussion.

DISCUSSION

The results of this study provide further evidence that transcriptional control of HSV gene expression is determined by the kinetic class of the gene promoter. Transcription of the TK gene of recombinant HSV-1 strain X1N17 was directed by the VP5 promoter situated upstream from TK coding sequences. The resulting transcripts initiated approximately 27 nucleotides downstream from the Hogness box of the VP5 promoter. This is consistent with other eukaryotic transcription units in which it has been demonstrated that mRNAs initiate 25 to 30 bases downstream from the Hogness box (McKnight et al., 1981; Benoist and Chambon, 1981; Grosveld et al., 1982a). The initiation site for X1N17-TK transcripts maps at or near the site previously identified by Dennis and Smiley (1984) for TK transcripts directed off of plasmid pX1N17. These transcripts are 65 nucleotides shorter than wild type TK mRNA, conserving 45 bases of the 110 base long non-translated leader sequence.

The levels of TK transcripts present in KOS or X1N17-infected cells at various times throughout infection revealed typical DE kinetics for wild type TK and L1 kinetics for the VP5-TK chimeric gene. TK mRNA was more abundant at late than early times post infection in X1N17-infected cells (see Figure 10, Lanes 1 and 3), as expected for a gene under late temporal control. Thus, the insertion of approximately 600 base pairs of VP5 upstream sequences between the TK promoter and TK coding regions successfully converted the TK gene from DE to L1 kinetics. Gene conversions of this type have been previously reported by Mackem and Roizman (1982a; 1982b) using HSV IE promoter regions.

Silver and Roizman (1985) have recently reported the conversion of TK to L2 kinetics by marker rescue of an L2 promoter-TK construct into the HSV genome. Taken together, these results show that the determinants that confer temporal expression on HSV genes lie in the upstream regulatory regions of HSV genes. There does not appear to be any obvious sequence differences between the early and late promoters, other than the 'silencer' sequence identified in the VP5 upstream region (Costa et al, 1985). If other late genes are found to carry similar silencer sequences, it could be postulated that this sequence is responsible for the template effect on late gene expression. Thus the late genes would be low level expressers that depend on increased template number to boost transcription levels. More detailed analysis of late promoter regions will be necessary to precisely map the cis acting regulatory regions responsible for L1 and L2 gene expression.

The TK enzymatic activity levels in KOS and X1N17-infected cells were measured at various times after infection to determine if the conversion of TK in X1N17 to a late transcription unit resulted in a corresponding shift in translational kinetics. According to the TK activity curves in Figures 11 and 12, X1N17 generally expresses less than 20% of wild type TK activity levels after three hours post infection. Decreasing TK activity levels in X1N17-infected cells after nine hours contradict the increasing amounts of TK mRNA detected in the cytoplasm at that time (Figure 10). In KOS-infected cells, the decrease in TK activity after nine hours reflects the decrease in TK mRNA levels in the cytoplasm at that time (Figure 10). Clearly, some post-transcriptional control mechanism must be responsible for the opposing

trends observed for TK mRNA and TK activity levels in X1N17-infected cells. The following sections will present some potential explanations for the aforementioned observations.

4.1. Possibility of an Altered TK Polypeptide in X1N17

Perhaps the low TK activity levels observed in X1N17-infected cells were the result of synthesis of a truncated TK polypeptide with reduced enzymatic activity or higher turnover rate than wild type TK. This is unlikely since the construction of the VP5-TK chimeric gene resulted in a TK transcript that initiates within the non-translated leader sequence of wild type TK mRNA. The first translation start codon (AUG) remains intact 45 nucleotides downstream from the X1N17-TK mRNA initiation site. Therefore, the truncated transcript should direct translation of an intact, unaltered TK polypeptide. If translation from the first AUG was inhibited by the presence of a truncated leader sequence, then the TK polypeptide resulting from initiation at the second AUG would be 45 amino acids shorter than wild type TK. Results presented in Section 3.5 indicate that TK translation in X1N17 initiates at the same site as wild type TK translation since denaturing polyacrylamide gel electrophoresis of infected cell proteins revealed no detectable size difference between X1N17 and KOS-TK polypeptides (see Figures 14A and B).

4.2. Inefficient Translation of X1N17-TK Transcripts

Since the evidence suggests that X1N17-TK is identical to wild type TK, then the low enzymatic activity levels observed in Figures 11

and 12 must be due to low TK polypeptide levels rather than to reduced enzymatic activity of the X1N17-TK enzyme. Western blot immunoassays of KOS and X1N17-infected cell proteins show this to be the case. The TK polypeptide from X1N17-infected cell extracts was barely detectable throughout the infectious cycle (see Figures 14 and 15). The kinetics of accumulation of both KOS and X1N17-TK polypeptides closely resembled their respective TK activity curves (Figures 11 and 12) until about nine hours after infection. After this time, TK polypeptide levels in wild type and recombinant HSV-infected cells remained high (Figures 14 and 15) despite falling enzymatic activity and TK transcript levels. This indicates that there is no obvious difference in the turnover rates of wild type and X1N17-TK polypeptides in infected cells. The observed deficiency of TK polypeptide in X1N17-infected cells at late times, despite the abundance of TK mRNA suggests some sort of translational defect. This situation can be compared to the regulation of HSV glycoprotein D (gD) expression (Johnson and Spear, 1984) which will be discussed below.

4.3. HSV gD Expression

An investigation into the levels of two HSV late gene products gC and gD, revealed that gC was expressed with L2 kinetics at the transcriptional and translational levels while gD is maximally transcribed late but translated most efficiently early (Johnson and Spear, 1984). Thus, at late times after infection, gD mRNA was abundant, while gD polypeptide levels had declined. Further investigation revealed no detectable differences in turnover rates of gD

and gC polypeptides at late times post-infection, suggesting that the translation efficiency of gC was higher than that of gD at late times after infection. In vitro translation of infected cell RNA revealed that gD and gC mRNAs are translated with equal efficiency. These results were interpreted to indicate a post-transcriptional regulation of gD that results in inefficient gD synthesis at late times in HSV-infected cells. Since this pattern parallels the observations made for TK in the present study, any explanations offered by Johnson and Spear (1984) for this finding could also be applied to the late shutoff of TK synthesis observed in this study.

Johnson and Spear suggested that differential processing of viral transcripts early and late in infected cells could affect translation efficiency at particular times post-infection. Northern blot analysis of infected cell RNA revealed no change in splicing or degradation patterns of gD transcripts present at early or late times post-infection. Differential 5'-capping and internal methylation of viral transcripts could also affect their translation efficiency. To date, no comparisons of cap structures on single HSV mRNA species isolated at different times post infection have been reported. Bartoski and Roizman (1978) have reported that viral transcripts made late in the HSV replication cycle lack internal methylation. It is unclear how the absence of internal methylation of HSV transcripts would affect translation, but if involved, it must affect gD and other viral late transcripts differently. If any of the above mentioned differential processing events could influence translation, the effects should be detected in vitro as well as in vivo. It has been reported that

methylated reovirus and vesicular stomatitis virus mRNAs are translated more efficiently in vitro than unmethylated mRNAs (Both et al., 1975). Johnson and Spear compared in vitro translation products of gD and gC mRNA and found no difference in their translatability at early and late times post-infection. Presumably, there is some factor or factors in the infected cell that affects the relative translatability of gD and gC mRNAs late in infection. Johnson and Spear present the possibility that ionic or metabolite imbalances or disruption of cellular membrane systems brought about by viral infection could have a selective inhibitory effect on the translation of certain viral mRNAs. Carrasco and Smith (1976) have reported that picornavirus mRNAs are translated more efficiently than cellular mRNAs at high salt concentrations. This led to the proposal of a model that explains the shutoff of host protein synthesis in picornavirus infected cells by an influx of sodium ions into infected cell cytoplasm. Increased salt concentrations stabilize mRNA secondary structure, therefore transcripts with potential 5'-end secondary structure may be translated less efficiently at high salt concentrations (Sonenberg, 1981; Gehrke et al., 1983). Perhaps HSV transcripts have a similar dependence on ion or metabolite concentrations for optimal translation efficiency, and the requirements for each kinetic class of HSV genes follow the changing environment in the infected cell as infection proceeds.

4.4. Possible Mechanisms Underlying the Inefficient Translation of X1N17-TK

As described above for HSV gD gene regulation, a potential

explanation for the translational defect observed for X1N17-TK could be differential processing of the VP5-TK transcript, resulting in a transcript that is inefficiently translated. Some of the processes that could be affecting X1N17-TK transcripts include splicing, 5'-capping, internal methylation and degradation. Splicing is rare in HSV and TK is an unspliced transcript (McKnight, 1980; Wagner et al., 1981), therefore this mode of post-transcriptional processing is not likely to be responsible for affecting the translatability of the X1N17-TK transcript. Differential 5'-capping and internal methylation of wild type and X1N17-TK mRNAs could be responsible for their differences in translation efficiency. Since the majority of X1N17-TK transcripts are made late in the infectious cycle, as opposed to wild type TK which is transcribed early, the post-transcriptional modifications may be different. If internal methylation of mRNA affects efficiency of translation, then perhaps the TK transcripts made late in infection are poorly translated because they are poorly methylated in comparison to the wild type TK transcripts made early in KOS-infected cells. Differences in the translatability of wild type and X1N17-TK mRNAs could be tested by in vitro translation. Another possible reason for the inefficient translation of X1N17-TK mRNA could be selective degradation of the truncated TK transcripts. Perhaps the presence of an early viral transcript at late times post-infection is not tolerated by the infected cell and therefore TK transcripts are destroyed in X1N17-infected cells. The probe used in the S1 mapping experiment described in Section (3.3) protects about 300 nucleotides at the 5' end of the X1N17-TK transcript, approximately one-fourth of the length of the TK mRNA. The signal

detected for X1N17-TK in the sequencing gels (Figures 10A and B) is a discrete band, indicating that at least the 5' end of the transcript is intact throughout infection. Therefore, mRNA degradation does not appear to be the mechanism by which TK translation is blocked in X1N17-infected cells.

Another factor that could be affecting translation of the VP5-TK transcript is the presence of a truncated leader sequence. It is unlikely that the sequences missing from the non-translated leader are directly responsible for reducing translation efficiency of X1N17-TK mRNA since a HSV TK deletion mutant Δ1, lacking the entire non-translated leader is efficiently translated in infected cells (Smiley et al., 1983). However, it is possible that the remaining portion of the leader sequence forms a secondary structure, by intrastrand hydrogen bonding, that reduces the efficiency of ribosome binding (Sonenberg, 1981; Gehrke et al., 1983). This possibility could be tested by constructing a variant of X1N17 that lacks the entire non-translated leader sequence. If the truncated leader of X1N17-TK is responsible for inhibiting translation, then the TK activity should be higher in this variant than in X1N17.

A further possibility for the observed translational defect in X1N17 is the specific inhibition of TK translation at late times post-infection. Certain viral gene product(s) could interact with TK mRNA or with the translation machinery to suppress TK translation. This type of translational control could explain the low TK polypeptide levels observed in X1N17-infected cells throughout the infectious cycle. At early times, when the VP5 promoter directs low levels of TK

transcription, the infected cell can efficiently translate TK mRNA. At late times post-infection, when the VP5 promoter is producing high levels of TK mRNA, the protein synthesizing machinery can no longer efficiently translate TK transcripts. This virus-directed late shutoff of translation could be directed toward TK mRNA specifically, or it could involve early viral transcripts in general. It could also explain the observed suppression of HSV gD translation at late times post infection. As with adenovirus late transcripts, the non-translated leader sequences of HSV transcripts may carry signals that temporally affect the efficiency of translation.

The majority of adenovirus late transcripts are derived from the major late transcription unit and share a common 5' non-translated region. This region is called the tripartite leader sequence because it is transcribed from three distinct segments on the viral chromosome. Logan and Shenk (1984) have shown that a recombinant adenovirus type 5 variant that produces E1A transcripts carrying most of the tripartite leader are translated more efficiently at late times post-infection than wild type E1A, an immediate-early RNA. The tripartite leader is not responsible for a general enhancement of translation since it does not affect the efficiency of translation at early times after infection or in vitro. Two types of changes that occur from early to late times in infected cells could affect translation. The first is altered permeability of infected cell membranes resulting in changes in ion or metabolite concentrations. These changes could conceivably affect the sequence and structural requirements for efficient translation. The second change is the presence of new viral gene products in the infected

cell. Viral gene product(s) could interact with the adenovirus tripartite leader region or the translation machinery altering the specificity of efficient translation. One particular adenovirus gene product, VA1 RNA, has been shown to be required for efficient translation of all adenovirus late transcripts (Thimmappaya et al., 1982). Similarly, the non-translated leaders of HSV mRNAs may prove to be important in determining the efficiency of their translation at particular times during the infectious cycle. This possibility could be tested by switching the non-translated leader sequences of HSV genes of different kinetic classes. Thus an X1N17-TK transcript that carried the leader sequence of a true late gene might be expected to produce high levels of TK polypeptide at late times in HSV-infected cells. Conversely, a late gene carrying the non-translated leader region of a DE gene might be poorly translated late in infection.

The low levels of TK polypeptide present in X1N17-infected cells after nine hours post infection are not consistent with the abundance of TK transcripts detected in those cells. Presumably, there is some mechanism at work which inhibits the translation of the TK mRNA at late times in infection when TK enzymatic activity is no longer required for viral replication. In the wild type virus, the late shutoff of TK expression occurs at the transcriptional level, as seen in Figure 10 (Lane 5),by the near absence of TK transcripts at 15 hours post infection. In the X1N17 recombinant virus, VP5 control of TK transcription results in the overproduction of TK mRNA late in infection. That this overproduction is not reflected at the protein level may indicate another level of gene regulation in HSV.

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