*Proc. Natl. Acad. Sci. USA*
Vol. 86, pp. 4071–4075, June 1989
Biochemistry

# Delineation of regulatory domains of early ($\beta$) and late ($\gamma_2$) genes by construction of chimeric genes expressed in herpes simplex virus 1 genomes

**(cis-acting sites/5′ transcribed noncoding domains/promoters/thymidine kinase)**

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ABSTRACT **The expression of the $\gamma_2$ class of viral genes in cells infected with herpes simplex virus 1 requires viral DNA synthesis and functional viral products made earlier in infection. To identify the sequences required for $\gamma_2$ gene expression, we constructed recombinant viruses in which regions of the thymidine kinase gene (*tk*), a $\beta$ gene normally expressed early in infection, were replaced by specific domains of a $\gamma_2$ gene. The phenotypic attributes examined were (*i*) sensitivity or resistance of expression in cells exposed to sufficient phosphonoacetate to block viral DNA synthesis, properties of $\gamma_2$ and $\beta$ genes, respectively; (*ii*) expression early in infection, a property of $\beta$ genes; and (*iii*) expression late in infection, a property of $\gamma_2$ genes. We report that replacement of nucleotides −200 to +51 of *tk* with nucleotides −77 to +104 of the $\gamma_2$ gene conferred upon *tk* all of the tested attributes of $\gamma_2$ genes. The *tk* sequence in the 5′ transcribed noncoding domain downstream of nucleotide +51 played no apparent role in the expression of the chimeric genes. Similarly, *tk* sequence downstream of −16 and $\gamma_2$ sequence upstream of −12, when juxtaposed in correct orientations, yielded a chimeric gene that was poorly expressed. In contrast, the chimeric gene consisting of *tk* sequence upstream of −16 fused to $\gamma_2$ sequence downstream of −12 had the attributes of both $\beta$ and $\gamma_2$ genes in that it was expressed both early and late in infection and was partially resistant to phosphonoacetate. The capacity for expression late in infection encoded in the $\gamma_2$ 5′ transcribed noncoding domain was observed in cells infected with a recombinant virus in which $\gamma_2$ nucleotides +17 to +104 were inserted into the 5′ transcribed noncoding domain of the *tk* gene. We conclude that whereas in the $\beta$ genes exemplified by the *tk* gene the regulatory domains are mainly upstream from nucleotide +51, the sequence(s) that confers $\gamma_2$ regulation is downstream from the TATAA box.**

Herpes simplex virus 1 (HSV-1) genes form several groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion (1–3). The $\alpha$ genes are expressed first. Functional $\alpha$ proteins, but especially infected-cell protein 4 (ICP4, the product of the *α4* gene), are required for the expression of $\beta$ genes, whose products synthesize viral DNA. Lastly, $\gamma$ genes form a heterogeneous group whose expression requires functional $\alpha$ genes and is impeded partially ($\gamma_1$ genes) or completely ($\gamma_2$) by inhibitors of viral DNA synthesis. The mechanisms by which both $\beta$ and $\gamma_2$ genes are induced in cells productively infected by HSV-1 have been the objects of considerable research effort.

The HSV-1 thymidine kinase gene (*tk*) has been thoroughly studied both as a model eukaryotic gene and as a $\beta$ gene (4). *tk* by itself is readily expressed if introduced into cells stably or transiently. This property has allowed detailed mapping of the promoter domains necessary for the expression of *tk* (4–7). In the environment of the viral genome, *tk* expression stringently requires functional $\alpha$ proteins and does not occur at the nonpermissive temperature in cells infected with tight temperature-sensitive mutants in the *α4* gene, which make all other $\alpha$ proteins, or in cells infected under conditions that block expression of genes requiring proteins made after infection (8–11). In cells infected with wild-type virus, *tk* expression is not dependent on accumulation of progeny viral DNA. Extensive analyses of the domain of *tk* by directed mutagenesis revealed that the cis-acting sites required for uninduced expression were also the ones required for efficient ICP4-dependent induction of *tk* (12). These studies led to the conclusion that ICP4 trans-activated *tk* by interacting with host transcriptional factors. However, notwithstanding the failure to detect ICP4 binding sites by immunoprecipitation of DNA–protein complexes by antibody to the protein (13), ICP4 has been reported to bind to a fragment containing the −110 to +50 domain of *tk* (14). An additional site mapping downstream from the nucleotide +51 was also found (unpublished work).

The significant features of $\gamma_2$ gene regulation are that (*i*) expression initiates after the onset of DNA synthesis and continues late in infection; (*ii*) the requirement for viral DNA synthesis is cis-acting; (*iii*) $\gamma_2$ genes residing permanently or transiently in cells are induced by infection of the cells, but they are regulated as $\beta$ rather than $\gamma_2$ genes; and (*iv*) it has been reported that the sequences required for $\gamma_2$ regulation encompass the nucleotides immediately upstream from the transcription initiation site but particularly the TATAA box (15–20).

Our approach to the mapping of cis-acting domains is not to destroy individual putative cis-acting sites in search of loss of function but rather to identify the sequences of the $\gamma_2$ gene that confer upon *tk*, a $\beta$ gene, the phenotypic properties of the donor gene. We report the results of studies of several recombinants containing chimeric $\gamma_2$–*tk* genes.

## MATERIALS AND METHODS

**Cell and Viruses.** HSV-1(F) is a limited-passage, wild-type virus used in this laboratory (21). Removal of 700 base pairs (bp) from the domain of the *tk* gene yielded HSV-1(F)Δ305 (5). The recombinant R3112 was constructed by insertion of ≈822 bp of the 5′ nontranscribed and 104 bp of the 5′ transcribed noncoding sequence of the *γ₂42* gene next to nucleotide +52 of *tk*; the chimeric *tk* gene is regulated as a bona fide $\gamma_2$ gene (17). Virus stocks and both viral DNA and RNA were made in Vero cells as described (21–23).

**Plasmid Construction.** The cloned HSV-1(F) DNA fragments *Bam*HI Q (pRB103, ref. 24), *Kpn* I–*Bam*HI subfrag-

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Abbreviations: HSV-1, herpes simplex virus 1; ICP, infected-cell protein; *tk*, thymidine kinase gene; TK, thymidine kinase enzyme; pfu, plaque-forming unit(s).

ment of *Bam*HI D′ (pRB3628, ref. 25), and the *Hin*fI subfragment from *Bam*HI D′, carrying the 5′ transcribed noncoding sequence of the $\gamma_2 42$ gene (pRB3865, ref. 25), have been reported. The *Bgl* II–*Bam*HI subfragment from *Bam*HI Q, carrying the coding sequence of the *tk* gene, was cloned as pRB3819 in pUC19. The small *Bam*HI–*Bgl* II subfragment of *Bam*HI Q′, carrying the *tk* promoter, was cloned as pRB3172 in pRB14 (26). Plasmid pRB3732 was made by replacing a portion of the promoter–5′ leader domain of *tk* (*Mlu* I–*Bgl* II fragment of pRB3172) with the *Bss*HII–*Bam*HI $\gamma_2 42$ fragment from pRB3628. Plasmid pRB3799 was made by replacing most of the upstream promoter sequence of *tk* (*Pvu*II–*Mlu* I fragment from pRB3172) with the corresponding sequence of the $\gamma_2 42$ gene (*Eco*RI–*Bss*HII fragment from pRB3628). Plasmid pRB3800 was made by replacing the *Pvu* II–*Bgl* II fragment of *tk* in pRB3172 with the *Ava* I–*Bam*HI fragment of pRB3628 $\gamma_2 42$ sequence. Plasmids pRB3820, pRB3821, and pRB3822 were derived from pRB3189 by cloning the *Xho* I fragment from pRB3732, pRB3799, and pRB3800, respectively, into the *Sal* I site of pRB3819 such that the $\gamma_2$–*tk* chimeric promoter–leader sequences present in the *Xho* I fragments were in the correct transcriptional orientation relative to the structural sequences of *tk*. Plasmid pRB3862 was derived from pRB103 by inserting the *Bam*HI fragment from pRB3865 carrying the $\gamma_2 42$ leader sequence into the *Bgl* II site of pRB103 such that the $\gamma_2$ sequence was in the same transcriptional orientation as *tk*. Plasmid pRB3863 is the same as pRB3862 except that the *Bam*HI fragment from pRB3865 was inserted in the opposite orientation relative to *tk*.

**Construction of Recombinant Viruses.** Plasmids containing the chimeric *tk* genes were cotransfected with intact HSV-1(F)Δ305 DNA and recombinant viruses were isolated and plaque-purified as described (17, 23). The structure of the chimeric $\gamma_2$–*tk* genes was verified by DNA sequencing (27).

**Assays.** Thymidine kinase enzyme (TK) activity was assayed as described (17). For RNA analyses, Vero cells grown in 150-cm$^2$ flasks were infected at a multiplicity of 10 plaque-forming units (pfu) per cell and cytoplasmic RNA was prepared as described (17). In studies designed to test the stability of the mRNAs, the cells were maintained in medium containing actinomycin D (10 μg/ml). Extraction, electrophoretic separation in denaturing gels, and the procedures for hybridization to specific probes were as described (17, 27). To map the transcription initiation site of the $\gamma_2$–*tk* transcript in the R3820 construct, the method of Berk and Sharp (28) was used except that the probe was not strand-separated. The conditions for hybridization and subsequent S1 nuclease digestion were as described (17).

## RESULTS

**Construction and Properties of Recombinant Viruses.** The structures of the recombinant viruses constructed for these studies are shown in Fig. 1. In recombinant virus R3821, the sequence of −200 to −16 relative to the transcription initiation site of the *tk* gene was replaced with the sequence −179 to −12 of the $\gamma_2 42$ gene relative to the cap site. In recombinant virus R3822, the sequence −200 to +51 of the *tk* gene was replaced by the sequence −77 to +104 of the $\gamma_2 42$ gene. In recombinant virus R3820, the sequence −16 to +51 of the *tk* gene was replaced with the sequence −12 to +104 of the $\gamma_2 42$ gene. In recombinant virus R3862, an 87-bp fragment containing the sequence +17 to +104 of the $\gamma_2 42$ gene was inserted into the +51 position of the *tk* gene in the correct orientation. In the inverted orientation (recombinant R3863), the *tk* gene was barely expressed and this recombinant was not studied further. The construction of the chimeric *tk* genes and of the recombinant viruses is described in *Materials and Methods* and in the legend to Fig. 1.

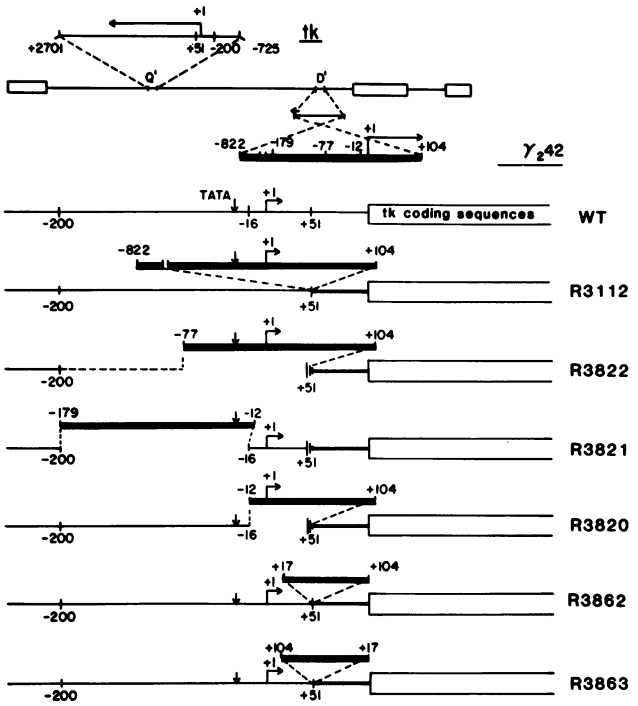


FIG. 1. The DNA sequence arrangement and structure of the natural and chimeric *tk* genes contained in the wild-type and recombinant viruses. The top line indicates the sequence arrangement in the HSV-1 genome and the location in the genome of the *tk* and $\gamma_2 42$ genes (heavy line). The boxes represent the terminal sequences that are repeated in an inverted orientation internally. The expanded regions show the transcription initiation sites (nucleotide +1) and the direction of transcription of the genes. In lines 2 and following the heavy lines represents the $\gamma_2$ gene sequences inserted into *tk*. The numbers above the heavy lines refer to nucleotide numbers relative to the transcription initiation site at +1. The numbers below the thin lines refer to the nucleotide numbers of the *tk* gene in reference to the transcription initiation site (+1) of *tk*. The dashed lines show the sites of insertion of the $\gamma_2$ sequences into the domain of the *tk* gene. The TATAA box (indicated by letters TATA and vertical arrow in line 2) is represented by a vertical arrow in the schematic diagram of recombinant virus *tk* genes. The vertical double lines at the +51 position of *tk* represent the polylinker inserted into that site for ease of cloning.

To characterize the regulation of the chimeric $\gamma_2 42$–*tk* genes resident in these viruses, replicate cultures of 143TK$^-$ cells were infected with HSV-1(F) or with the recombinant viruses in the presence or absence of phosphonoacetate. The infected cells were harvested 7, 10, 13, and 16 hr postinfection and assayed for TK activity. The results (Fig. 2) were as follows.

(*i*) The TK activity expressed by R3821 was low throughout infection. These results suggest that the $\gamma_2 42$ sequence upstream from nucleotide −12 and the *tk* sequence downstream from −16 are not sufficient to enable efficient expression of *tk*.

(*ii*) The TK activity induced by R3822 was similar, with respect to the level attained and the sensitivity to phosphonoacetate, to that of the chimeric $\gamma_2$–*tk* gene in the recombinant R3112. These results suggest that the cis-acting sites contained in the sequence −77 to +104 are sufficient to confer on the *tk* reporter gene the capacity to be regulated as a $\gamma_2$ gene.

(*iii*) The TK activity expressed by the chimeric $\gamma_2$–*tk* gene in R3820 exhibited the properties of both β and $\gamma_2$ genes. Thus, the TK activity induced by R3820 appeared at early times postinfection like that of the wild-type HSV-1(F) gene, but it continued to accumulate at late times postinfection like that of a bona fide $\gamma_2$ gene and in contrast to a β-regulated

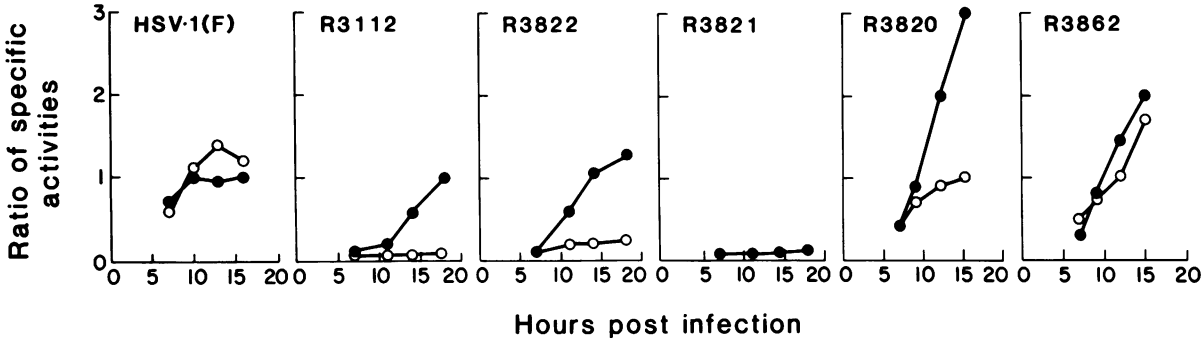


FIG. 2. The relative specific activities of the TK made in cells infected with wild-type and recombinant viruses in the presence or absence of phosphonoacetate. Replicate cultures of human 143TK$^-$ cells were infected at a multiplicity of 5 pfu per cell with the parental HSV-1(F) and various recombinant viruses and incubated in the presence (○) or absence (●) of phosphonoacetate (gift of Abbott Laboratories; 300 μg/ml of medium). The cells were harvested at times shown, the cytoplasmic fractions were assayed for TK activity as described (17), and the specific activities of each extract were determined. The results of these studies are shown as the specific activities normalized to that of the maximum activity seen in wild-type virus-infected cells in the absence of phosphonoacetate.

gene. Moreover, in the presence of phosphonoacetate, the TK activity induced by R3820 was reduced by a factor of 3–4—i.e., to the level of the TK activity expressed by the wild-type HSV-1(F) *tk* gene, which was unaffected by the drug. These results indicate that DNA sequences downstream from the TATAA box of the $\gamma_2 42$ gene contain cis-acting sites that impart to the chimeric gene two phenotypic characteristics of the $\gamma_2$ genes: the capacity to be expressed late in infection and a dependence on DNA synthesis for maximal expression. Taken together with those obtained with R3821 and R3822, the data suggest that the sequence upstream from nucleotide −16 of the *tk* gene is responsible for the β phenotype of the expression—i.e., a slightly earlier onset of expression than that seen in the case of the *tk* gene of recombinant R3112 and a partial insensitivity to inhibition of DNA synthesis by phosphonoacetate.

(*iv*) The chimeric *tk* gene of R3862 was expressed at early times postinfection like the wild-type HSV-1(F) gene but the level of TK activity late in infection was consistently higher than that induced by the wild-type *βtk* gene. R3862 induced very low TK activity both early and late in infection.

**Analyses of the *tk* RNA of Recombinant R3820.** The central question is the molecular basis of the $\gamma_2$ phenotype of *tk* expression by R3820—i.e., whether it reflects stabilization of the mRNA late in infection or continued transcription at late times, long after transcription of the *βtk* gene ceases. Several experiments were done to establish whether the TK activity at late times postinfection in R3820-infected cells was determined at the transcriptional or at the posttranscriptional level. In the first, the cytoplasmic mRNAs (20 μg) extracted from Vero cells 7 and 22 hr after infection with HSV-1(F), R3112, or R3820 were denatured in formaldehyde, electrophoretically separated according to size in an agarose gel, and transferred to a nitrocellulose membrane. The immobilized RNA was then hybridized with $^{32}$P-labeled BamHI Q DNA fragment of HSV-1. This probe hybridized with two transcripts, a 1.5-kilobase (kb) RNA encoding the viral TK and a 3.3-kb RNA encoding glycoprotein H. The 3.3-kb transcript served as an internal control to allow standardization of the infection and of the RNA preparation. As shown in Fig. 3, at 7 hr postinfection the 1.5-kb *tk* RNA was detected in all RNA specimens, whereas at 22 hr postinfection the *tk* RNA was detected in the RNA extracted from R3820- and R3112-infected cells but not in the RNA extracted from HSV-1(F)-infected cells. These results indicate that the kinetics of accumulation of the *tk* transcript in R3820-infected cells differed from that in the HSV-1(F)-infected cells and followed the pattern of an RNA transcribed from a γ-regulated gene.

To determine whether the transcription of the chimeric *tk* gene resident in R3820 virus utilized the transcription initiation site of the $\gamma_2 42$ gene, the cytoplasmic RNA extracted from Vero cells infected with R3820 was hybridized to the ≈910-bp *Mlu* I–*Hin*dIII fragment derived from pRB3820 and 5′ labeled at both ends, and digested with S1 nuclease. The *Mlu* I site is located at nucleotide +134 within the *tk* gene, 83 bp downstream from the *Bgl* II site at +51, whereas the *Hin*dIII site not represented in the mRNA; it is located in the polylinker of the vector sequence and is not present in the viral genome. As shown in Fig. 4, the RNA protected from S1 digestion by the $\gamma_2$–*tk* probe was ≈195 bases long, indicating that the transcription of the chimeric gene initiated at the predicted cap site of the $\gamma_2 42$ gene, 104 bases from the *Bam*HI site of the BamHI D′ fragment.

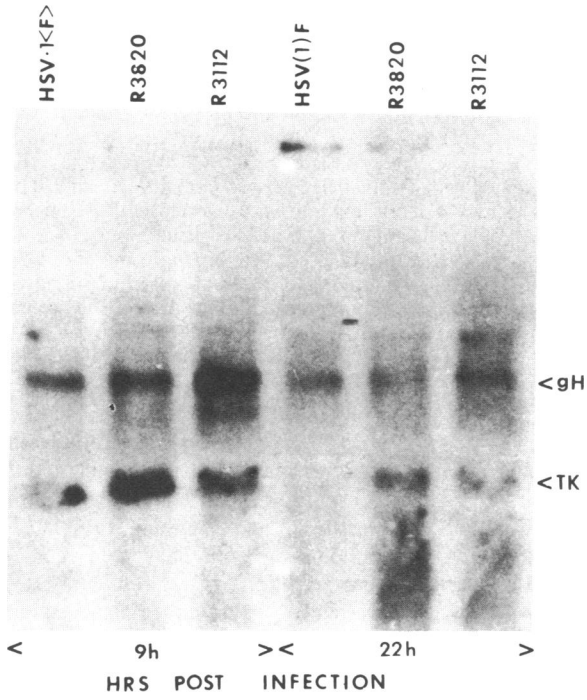


FIG. 3. Steady-state mRNA levels in cells infected with wild-type and recombinant viruses. Replicate cultures of Vero cells were infected with 10 pfu of wild-type or recombinant virus per cell. The cultures were harvested at 9 and 22 hr postinfection. Cytoplasmic RNA was denatured in formaldehyde, electrophoresed in agarose gels, transferred to nitrocellulose, and hybridized with $^{32}$P-labeled BamHI Q DNA fragment. The two mRNAs detected in these experiments are 1.5 kb and 3.3 kb in length and correspond to the *tk* and glycoprotein H (gH) mRNAs, respectively. The left three lanes show the RNA harvested 9 hr postinfection, whereas the right three lanes show the RNA harvested 22 hr postinfection.

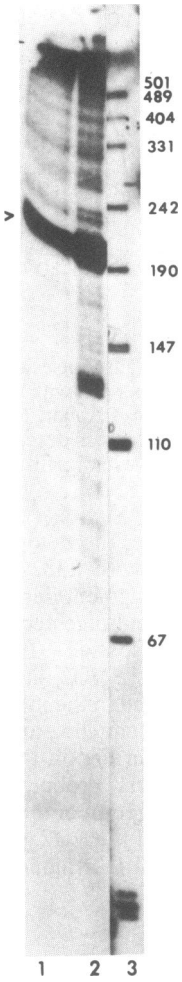


FIG. 4. Transcription initiation site of $\gamma_2 42$–*tk* gene in R3820. mRNA extracted from the cytoplasm of Vero cells harvested 8 hr after infection with 10 pfu of recombinant R3820 per cell. The RNA was hybridized with a $^{32}$P-labeled *Mlu* I–*Hin*dIII fragment 910 bp in length. The hybridization mixture was digested with S1 nuclease, denatured, and electrophoresed in polyacrylamide gels. Lane 1, 20 μg of RNA; lane 2, 100 μg of RNA; lane 3, *Hpa* II digest of pUC19 5′ labeled with $^{32}$P, denatured, and electrophoresed to provide size markers (lengths indicated in bases). The position of the protected labeled band is indicated by an arrowhead.

To obtain a measure of the relative stability of the *tk* mRNAs of the natural (β) *tk* gene of HSV-1(F) and the chimeric $\gamma_2$–*tk* gene of R3820, we performed actinomycin D chase experiments. Actinomycin D (10 μg/ml of medium) was added 4 hr postinfection to replicate Vero cell cultures infected with HSV-1(F) or with the recombinant R3820. The cytoplasmic RNA extracted from cells harvested at 2, 3.5, 5.5, and 6.5 hr after the addition of actinomycin D was denatured, electrophoretically separated in agarose gels, transferred to nitrocellulose, and hybridized to a mixture of two $^{32}$P-labeled probes designed to detect *tk* and *α0* mRNAs, respectively. The assays for *α0* RNA, which is expressed before *tk*, served as an internal control. The amount of label hybridized to each band was measured in a Betascope blot analyzer (Betagen, Waltham, MA). The results of the experiment (Table 1) indicated that over a 6.5-hr interval the mRNA of the $\gamma_2$–*tk* gene in R3820 was only slightly more stable than the mRNA of the wild-type *tk* of HSV-1(F). Other experiments were consistent with this result and suggest that accumulation of RNA late in infection and continued increase in TK activity late in infection reflect continued synthesis of *tk* RNA.

Table 1. Stability of HSV-1 mRNA after exposure of infected cells to actinomycin D

| Virus | Time, hr | cpm in mRNA | | *tk*/*α0* ratio |
|---|---|---|---|---|
| | | *tk* | *α0* | |
| HSV-1(F) | 2 | 18,000 | 14,300 | 1.26 |
| (wild-type *tk*) | 3.5 | 12,100 | 12,000 | 1.01 |
| | 5.5 | 8,800 | 9,800 | 0.90 |
| | 6.5 | 8,200 | 8,900 | 0.92 |
| R3820 | 2 | 12,500 | 9,900 | 1.26 |
| ($\gamma_2$–*tk*) | 3.5 | 10,700 | 10,000 | 1.07 |
| | 5.5 | 9,900 | 8,800 | 1.13 |
| | 6.5 | 8,400 | 7,600 | 1.11 |

Replicate cultures of Vero cells were infected at a multiplicity of 10 pfu of HSV-1(F) or recombinant R3820 per cell and were treated with actinomycin D (10 μg/ml) 4 hr after infection. The RNA was extracted from infected cells harvested 2, 3.5, 5.5, and 6.5 hr after addition of actinomycin D. The RNA was denatured, electrophoresed in agarose gels, transferred to nitrocellulose, and hybridized with $^{32}$P-labeled probes from *α0* (4.3-kb *Bam*HI–*Sac*I fragment encoding α0 protein and cloned as pRB420; ref. 19) and *tk* (BamHI Q fragment cloned as pRB103) genes. The nitrocellulose sheet was scanned by a Betascope blot analyzer (Betagen), which measured the radioactivity in each band.

## DISCUSSION

We have examined the expression of several chimeric $\gamma_2$–*tk* genes with respect to (*i*) sensitivity or resistance to phosphonoacetate properties of $\gamma_2$ and β genes, respectively; (*ii*) expression early in infection, a property of β genes; and (*iii*) expression late in infection, a property of $\gamma_2$ genes. The salient features of the results are as follows.

The sequence downstream from +51 of *tk* does not confer any regulatory signals in the absence of signals located upstream. This conclusion is based on the observation that both R3112 and R3822 are regulated as $\gamma_2$ genes as defined by phosphonoacetate sensitivity and late gene expression. The conclusion is significant, since an ICP4 binding site exists downstream from nucleotide +51 (N. Michael and B.R., unpublished studies). The conclusion that this site does not confer inducibility as a β gene is consistent with the report that a mutant lacking the sequence −12 to +189 exhibited wild-type *tk* expression (29).

The $\gamma_2$ sequence −77 to +104 (R3822) imparted all of the properties of the $\gamma_2$ gene to the recipient *tk* gene. The contribution of this sequence can be deduced from the regulation of the *tk* gene in R3820 and R3821. The $\gamma_2$ sequence −179 to −12 (R3821) impaired the capacity of the *tk* gene to be expressed efficiently. We conclude from these results that either the $\gamma_2$ sequence upstream of −12 plays no role in the regulation of $\gamma_2$ genes, in that the sequence does not promote expression, or that it acts only in cooperation with $\gamma_2$ sequence downstream from −12. Conversely, the β *tk* sequence downstream from nucleotide −16 does not contribute to the regulation of *tk* or cannot contribute in the absence of the upstream sequence. The possibility that this sequence contributes little can be deduced from the reciprocal recombinant, R3820. In this recombinant the early expression and partial resistance to phosphonoacetate to the level of the response of the wild-type *tk* gene, correlate with the presence of β *tk* sequence upstream of nucleotide −16. The transcription of the *tk* gene late in infection, and the sensitivity of the added expression of *tk* beyond the level attained by the wild-type gene, correlates with the presence of $\gamma_2$ sequence downstream from nucleotide −12. Lastly, at least some of the attributes of late gene expression appear to be encoded in the sequence +17 to +104 of the $\gamma_2$ gene inasmuch as R3862 retained the phosphonoacetate-resistant phenotype of the β

genes but acquired the γ-gene phenotype of late gene expression.

In other reports we postulated that the cis-acting effect of DNA synthesis on the $\gamma_2$ genes could reflect an unblocking of the transcribed domains of these genes (19, 30). The observation that the sequences that confer $\gamma_2$ attributes are downstream from the TATAA box and are either at the transcription initiation site or downstream suggests that the $\gamma_2$ cis-acting sites requires for trans-activation of the transcription of these genes are blocked in the absence of viral DNA synthesis but that the block does not affect the transcription of the gene initiated by the upstream sequences of α or β promoters fused upstream from the cap site of the $\gamma_2$ gene.

Finally, we note that both the β *tk* and the $\gamma_2$*42* gene contain ICP4 binding sites in the 5′ transcribed noncoding domains (25). This report and that of Halpern and Smiley (29) indicate that the ICP binding site plays little if any role in the *tk* gene that is expressed, but it could be responsible for the lack of expression of the *tk* gene in the absence of functional ICP4. The function of the two ICP4 binding sites in the 5′ transcribed noncoding domain of the $\gamma_2$*42* gene is not known. ICP4 binding sites have been correlated with both turn-on and shutoff of viral gene expression. It is not inconceivable that ICP4 has a similar function in the case of $\gamma_2$ genes (31, 32).

We thank Sussana Rudofsky for technical assistance, Minas Arsenakis for the gift of the pRB3172, and Abbott Laboratories for the gift of phosphonoacetate. These studies were aided by U.S. Public Health Service grants from the National Cancer Institute (CA47451) and the National Institute for Allergy and Infectious Diseases (AI24009 and AI15488-11) and by a grant from the Davis Foundation.

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