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γ_2-Thymidine Kinase Chimeras Are Identically Transcribed but Regulated as γ_2 Genes in Herpes Simplex Virus Genomes and as β Genes in Cell Genomes

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True γ or γ_2 genes, unlike α, β, and γ_1 (β γ) genes of herpes simplex virus 1 (HSV-1), stringently require viral DNA synthesis for their expression. We report that γ_2 genes resident in cells were induced in *trans* by infection with HSV-1 but that the induction did not require amplification of either the resident gene or the infecting viral genome. Specifically, to test the hypothesis that expression of these genes is amplification dependent, we constructed two sets of γ_2-thymidine kinase (TK) chimeric genes. The first (pRB3038) consisted of the promoter-regulatory region and a portion of 5′-transcribed noncoding region of the domain of a γ_2 gene identified by Hall et al. (J. Virol. 43:594–607) in the HSV-1(F) *Bam*HI fragment D′ to the 5′-transcribed noncoding and coding regions of the TK gene. The second (pRB3048) contained, in addition, an origin of HSV-1 DNA replication. Cells transfected with either the first or second construct and selected for the TK$^+$ phenotype were then tested for TK induction after superinfection with HSV-1(F)Δ305, containing a deletion in the coding sequences of the TK gene, and viruses containing, in addition, a *ts* lesion in the α4 regulatory protein (*ts*502Δ305) or in the β8 major DNA-binding protein (*ts*HA1Δ305). The results were as follows: (i) induction by infection with TK$^-$ virus of chimeric TK genes with or without an origin of DNA replication was dependent on functional α4 protein but not on viral DNA synthesis; (ii) the resident chimeric gene in cells selected for G418 (neomycin) resistance was regulated in the same fashion; (iii) the chimeric gene recombined into the viral DNA was regulated as a γ_2 gene in that its expression in infected cells was dependent on viral DNA synthesis; (iv) the γ_2-chimeric genes resident in the host and in viral genomes were transcribed from the donor *Bam*HI fragment D′ containing the promoter-regulatory domain of the γ_2 gene. The significance of the differential regulation of γ_2 genes in the environments of host and viral genomes by viral *trans*-acting factors is discussed.

Herpes simplex virus 1 (HSV-1) genes form five groups whose expression in permissively infected cells is coordinately regulated in a cascade fashion (18, 19, 36; B. Roizman and W. Batterson, *in* B. Fields, ed., *Virology*, in press). α genes are expressed first. Functional α gene products (19), but particularly α4, are required for the expression of two groups of genes (10, 25, 47), β_1 and β_2, differing solely in the temporal pattern of their appearance (18, 36). One or more as yet unidentified β genes turn off the synthesis of α gene products and induce the expression of γ genes (18, 19). Studies on the requirements for their expression suggest that γ genes form two subgroups, γ_1 and γ_2, differing with respect to their dependence on viral DNA synthesis for their expression. Thus, the expression of γ_2 genes is stringently dependent on viral DNA synthesis, whereas that of γ_1 genes is reduced, but not abolished, in the absence of viral DNA synthesis (7, 17, 18, 21). Inasmuch as HSV-1 genes are transcribed by RNA polymerase II throughout the reproductive cycle (8), a central question concerning the regulation of viral gene expression is the nature of the *cis*-acting sequences and viral *trans*-acting signals which permit the cellular transcriptional machinery to differentiate between the α, β, and γ genes.

To identify the *cis*-acting regulatory and promoter sequences of α genes, we devised a procedure whereby putative promoter-regulatory regions of genes under study were fused to the structural sequences of the thymidine kinase (TK) gene (38). The TK gene served both as an indicator gene for expression of the *cis*-acting sequences and as a selectable marker for the introduction of the chimera into the viral or cellular genome (32, 33, 38). Thus, fusion of noncoding sequences upstream from structural sequences of α genes to the 5′-transcribed noncoding and coding sequences of the TK gene resulted in chimeras that were regulated as α genes when inserted into the virus or introduced as resident genes into TK$^-$ cells converted to the TK$^+$ phenotype (38). This approach permitted identification of distinct promoter and regulatory domains of α genes and differentiation of sequences that determine the constitutive level of expression from those that respond to *trans*-acting viral signals that enhance the expression of the α genes (25a, 28, 29).

In this paper we describe the construction of TK chimeras designed to identify the promoter-regulatory regions of genes which impart γ_2 regulation. As indicated earlier in the text, γ_2 genes are operationally defined as HSV-1 genes stringently dependent on viral DNA synthesis for their expression. We report that a γ_2-TK chimeric gene was regulated as a γ_2 gene when inserted into the viral genome. However, γ_2-TK chimeras could not be differentiated from the natural β-TK gene when resident in the genome of the cell.

MATERIALS AND METHODS

Viruses and cells. The properties of HSV-1 strain F [HSV-1(F)] were described elsewhere (11). The construction of a 700-base-pair (-bp) deletion in the TK genes of HSV-1(F) [HSV-1(F)Δ305] and of HSV-1*ts*502 (HSV-1*ts*502Δ305), a temperature-sensitive mutant in α4, was previously described (38). The same procedure was used to construct the Δ305 deletion in HSV-1*ts*HA1 (HSV-1*ts*HA1Δ305), a tem-

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perature-sensitive mutant in the major DNA-binding protein (β8)(7).

Virus stocks were prepared and titrated in Vero cells. Rabbit skin cells, originally obtained from J. McLaren, were used for transfection with viral DNA. The procedures for the cultivation of viruses and cells and the composition of growth and maintenance media have been published elsewhere (42). Ltk^- cells (24), $LVtk^+$ cells, and 143 TK^- cells (6) were maintained as described by Post et al. (38). G418-selected Ltk^- cells were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum and G418 (420 μg/ml; GIBCO Laboratories, Grand Island, N.Y.) (43). To inhibit viral DNA synthesis, cells were maintained for 1 h before, during, and after infection in medium containing 300 μg of phosphonoacetate (PAA; Abbott Laboratories, North Chicago, Ill.) per ml of medium (21).

Plasmids. The properties of recombinant plasmid pSV2neo conferring resistance to neomycin and kanamycin have been described elsewhere (43). The plasmids reported previously from this laboratory and their properties are as follows. pRB142 and pRB103 contain the HSV-1(F) *Bam*HI fragments D′ and Q, respectively, cloned into pBR322 (37). pRB408 consists of pKC7 carrying the 501-bp *Bgl*II-*Sac*I fragment from the coding sequences of the TK gene that was deleted from HSV-1(F)Δ305 (33). pRB407 contains the *Bgl*II-*Bam*HI fragment containing the coding sequences of TK cloned in pKC7 (28). pRB316 contains *Bam*HI-N inserted into the *Bgl*II site of pRB103, placing TK under the control of the α4 gene promoter (38). The plasmids constructed for the studies reported here and their properties were as follows. pRB3038 contained *Bam*HI fragment D′ cloned into the *Bgl*II site of pRB407 such that the γ_2 promoter identified by Hall et al. (15) was in the correct transcriptional orientation relative to the structural sequence of the TK gene. pRB3037 was cloned in the same manner as pRB3038 except that the *Bam*HI-D′ was inverted so that the γ_2 promoter was distal and pointed away from the TK gene. pRB3048 and pRB3047 contained a 1,500-bp fragment derived from HSV-1(F) *Bam*HI-N cloned into the *Bam*HI sites of pRB3038 and pRB3037, respectively, using T4 polymerase and blunt-end ligation. This fragment contains the HSV-1(F) left S component origin of replication (33, 45). pRB3112 contained *Bam*HI-D′ inserted into the *Bgl*II site of pRB103.

DNA preparation. The procedures for preparation of HSV and plasmid DNAs were described elsewhere (23, 32, 37).

Transformation and selection for TK^+ expression or G418 resistance in Ltk^- cells. Ltk^- cells were converted to the TK^+ phenotype by transfection of flasks containing 4×10^6 cells with 5 μg of plasmid DNA containing the TK gene, by the calcium phosphate precipitation method followed by incubation in hypoxanthine-methotrexate-thymidine (HAT) medium as previously described (38, 49). Ltk^- cells were converted to G418 (neomycin) resistance by cotransfection with an artificial mixture consisting of 5 μg of plasmid DNA containing the TK gene and 0.4 μg of pSV2neo plasmid DNA and incubation in medium containing G418 as previously described (25a, 43).

Construction of virus recombinants carrying a γ_2-regulated TK gene. pRB3112 DNA (ca. 0.1 to 0.2 μg) containing the γ_2-TK chimeric gene was linearized with *Bam*HI and cotransfected with 0.5 μg of intact HSV-1(F)Δ305 DNA into rabbit skin cells. The TK^+ recombinant virus was selected from the progeny of transfection by plating on $143tk^-$ cells in HAT medium followed by plaque purification in Vero cells as described by Post et al. (38). Recombinant viral DNA was purified by centrifugation in NaI density gradients, digested with *Bam*HI, subjected to electrophoresis in agarose gels, transferred to nitrocellulose sheets, and hybridized to nick-translated, ^{32}P-labeled pRB142 and pRB408, as previously described (38).

TK activity. The assay for TK activity with [^3H]thymidine was as previously described (26, 38).

DNA dilution dot blot hybridization. DNA from L3048 TK^+-selected cells was extracted, denatured, and spotted onto nitrocellulose by the method of Palmiter et al. (35). The quantity of DNA spotted onto nitrocellulose ranged from 5 to 0.7 μg. Ltk^- cellular DNA was added to samples containing less than 5 μg of DNA so that the total DNA concentration in each sample was 5 μg. Nick-translated, ^{32}P-labeled pRB408 DNA served as the hybridization probe.

Genomic DNA Southern blot hybridization. DNA was extracted from mock-infected and HSV-1(F)Δ305-infected L3038 and L3048 TK^+ cells as well as Ltk^- cells, as previously described (33). In addition, 2.9×10^{-5}, 2.9×10^{-4}, or 2.9×10^{-3} μg of pRB3038 DNA was mixed with 10 μg of Ltk^- DNA to yield 1, 10, or 100 plasmid copy equivalents per cell, respectively. For pRB3048 DNA, 3.5×10^{-5}, 3.5×10^{-4}, or 3.5×10^{-3} μg was mixed with 10 μg of Ltk^- DNA to yield 1, 10, or 100 copy equivalents per cell, respectively. The DNA (10 μg each) was digested with *Eco*RI, subjected to electrophoresis in a 0.8% agarose gel, transferred to nitrocellulose paper, and hybridized to the *Bgl*II-*Sac*I DNA fragment from pRB408 labeled with ^{32}P by nick translation.

S1 nuclease mapping of transcription initiation sites. Vero cells were infected with recombinant virus R3112 (20 PFU per cell) and harvested for RNA analysis at 15 h postinfection. Transformed cell lines were infected with HSV-1(F)Δ305 (5 PFU per cell) and harvested at 10 h postinfection. Mock-infected, transformed cell lines were maintained in maintenance medium for 10 h before harvesting for RNA analysis. Cytoplasmic RNA was prepared from infected and mock-infected cells as described previously (3). Cytoplasmic RNA from Ltk^- cells was also extracted for use as a negative control. To map the transcription initiation sites in infected and mock-infected, transformed cell lines as well as in cells infected with recombinant virus R3112, we used the S1 nuclease mapping techniques of Berk and Sharp (4) as modified by Weaver and Weissman (48). The probes were 5′ end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (31) and then subjected to electrophoresis on 5% polyacrylamide gels under strand-separating conditions (30). To detect γ_2-TK transcripts, we used two probes. The *Mlu*I-*Kpn*I DNA fragment, ca. 350 bp in length (probe A), was prepared from pRB3038 by digestion with *Mlu*I, located 83 bp from the *Bgl*II site within the TK gene, 5′ end labeling, cleavage with *Kpn*I, and purification by gel electrophoresis. The *Kpn*I site was within *Bam*HI-D′ ca. 160 bp upstream from the γ_2 transcription initiation site. The 301-nucleotide *Ava*I-*Bgl*II DNA fragment (probe B) was prepared from pRB408 by digestion with *Ava*I, 5′ end labeling, cleavage with *Bgl*II, and purification by gel electrophoresis. ^{32}P (ca. 0.02 pmol) 5′ end-labeled probe was hybridized to 150 μg of cytoplasmic RNA in 20 μl of 80% recrystallized formamide–0.4 M NaCl–0.04 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.5)–1 mM EDTA at 59°C for 12 h after a 10-min incubation at 78°C to ensure denaturation of nucleic acids. For S1 nuclease digestions, 0.20 ml of ice-cold S1 buffer containing 250 mM NaCl, 4.5 mM $ZnSO_4$, and 50 mM sodium acetate (pH 4.5) was quickly added to hybridization samples. The hybrids were digested with 5×10^3 U of S1 nuclease (Boehringer Mannheim Biochemicals, Indianap-

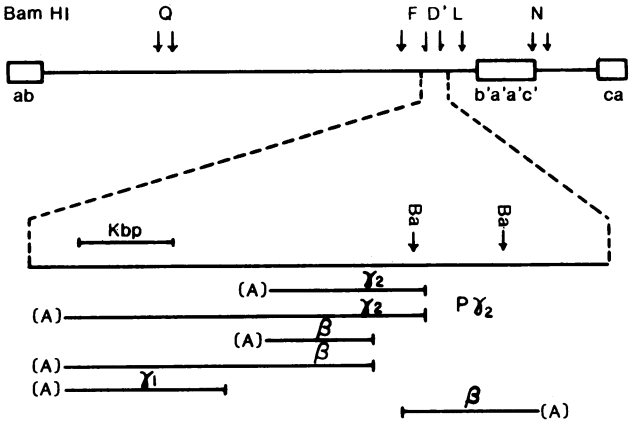

FIG. 1. Sequence arrangement of HSV-1 DNA and origin of relevant mRNA species. A schematic diagram of the HSV-1 genome (prototype arrangement) shows the positions of the reiterated sequences *ab* and *ca* (40) and the map positions of restriction endonuclease fragments *Bam*HI-Q, -F, -D', -L, and -N. The expanded map of the region including and surrounding *Bam*HI fragment D' shows the positions and classifications of HSV-1 mRNA species mapped by Hall et al. (15). (A), Polyadenylated tail; $p\gamma_2$, γ_2 promoter; Ba, *Bam*HI.

olis, Ind.) at 45°C for 30 min. The reactions were stopped by the addition of EDTA (20 mM); the protected DNA was extracted with phenol-chloroform, precipiated with ethanol, dissolved in sequencing-gel loading buffer, heated to 100°C for 2 min, and electrophoretically separated on 8.3 M urea–8% polyacrylamide gels (4, 31). *Hpa*II-digested pBR322 labeled with polynucleotide kinase and [γ-^{32}P]ATP served as size markers.

RESULTS

Location of a γ_2-promoter and transcription initiation site within the *Bam*HI-D' restriction endonuclease DNA fragment. Hall et al. (15) have shown that two γ_2 mRNA species of 3.8 and 1.8 kilobases located within *Eco*RI fragment I of HSV-1 DNA have identical 5' ends and, when translated in vitro, each encodes a 42,000-molecular-weight polypeptide identified as a true γ or γ_2 (15). Transcription is from right to left on the prototype arrangement of HSV-1 DNA. S1 nuclease and DNA sequencing analyses showed that the transcription initiation site of the RNAs is 107 bp from the left terminus of the 900-bp *Bam*HI fragment D' (15; Fig. 1). The sequences downstream from the transcription initiation site compose a portion of the 5'-transcribed noncoding sequences. It could be predicted, therefore, that the putative promoter-regulatory region of the gene is contained within DNA sequences upstream from the transcription initiation site.

Fusion of the putative γ_2 promoter to the TK gene. To verify the presence of the predicted γ_2 promoter-regulatory region, we fused *Bam*HI-D' to the 5'-transcribed noncoding and the downstream coding sequences of the HSV-1(F) TK gene. The plasmids constructed for this purpose are shown in Fig. 2. pRB3038 lacks the wild-type β-TK promoter and places the γ_2 promoter and cap site in the correct transcriptional orientation to the structural TK gene, whereas in pRB3037, the γ_2 is distal and in the incorrect transcriptional orientation relative to the TK gene. In pRB3112, *Bam*HI-D' was inserted in the correct transcriptional orientation between the TK promoter and capping site and the downstream 5'-transcribed noncoding and coding sequences of the TK gene. To test the hypothesis that efficient expression of the γ_2 genes requires amplification of the gene, we cloned into these constructs an origin of DNA replication; thus, pBR3048 and pBR3047 derived from pRB3038 and pRB3037, respectively, each carried a 1,500-bp fragment containing an HSV-1(F) S component origin 3' to the TK gene.

Induction of TK activity in converted TK$^+$ cells. To verify the presence in *Bam*HI-D' of a γ_2 promoter in the predicted orientation, Ltk$^-$ cells were transfected with pRB3038, pRB3037, pRB3048, and pRB3047 and selected for the TK$^+$ phenotype. All transfections yielded stable TK$^+$ convertants, although less efficiently than with pRB103. The cell lines were designated by the plasmid with which they were

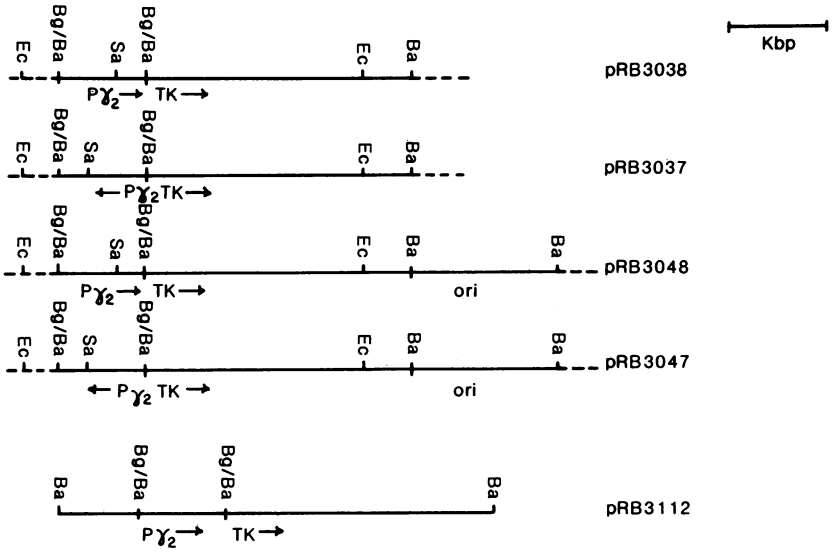

FIG. 2. Structure of chimeric γ_2-TK genes used in this study. The constructions of the various plasmids are described in the text. The transcriptional orientation of the γ_2 promoter is indicated by an arrow for each construct. $p\gamma_2$, γ_2 promoter contained within *Bam*HI fragment D'; ori, sequences containing an origin of DNA replication from the S component of HSV DNA (33, 45); Ba, *Bam*HI; Sa, *Sal*I; Bg, *Bgl*II; Ec, *Eco*RI cleavage site. The solid line represents HSV-1 sequences, whereas the dashed line represents vector sequences.

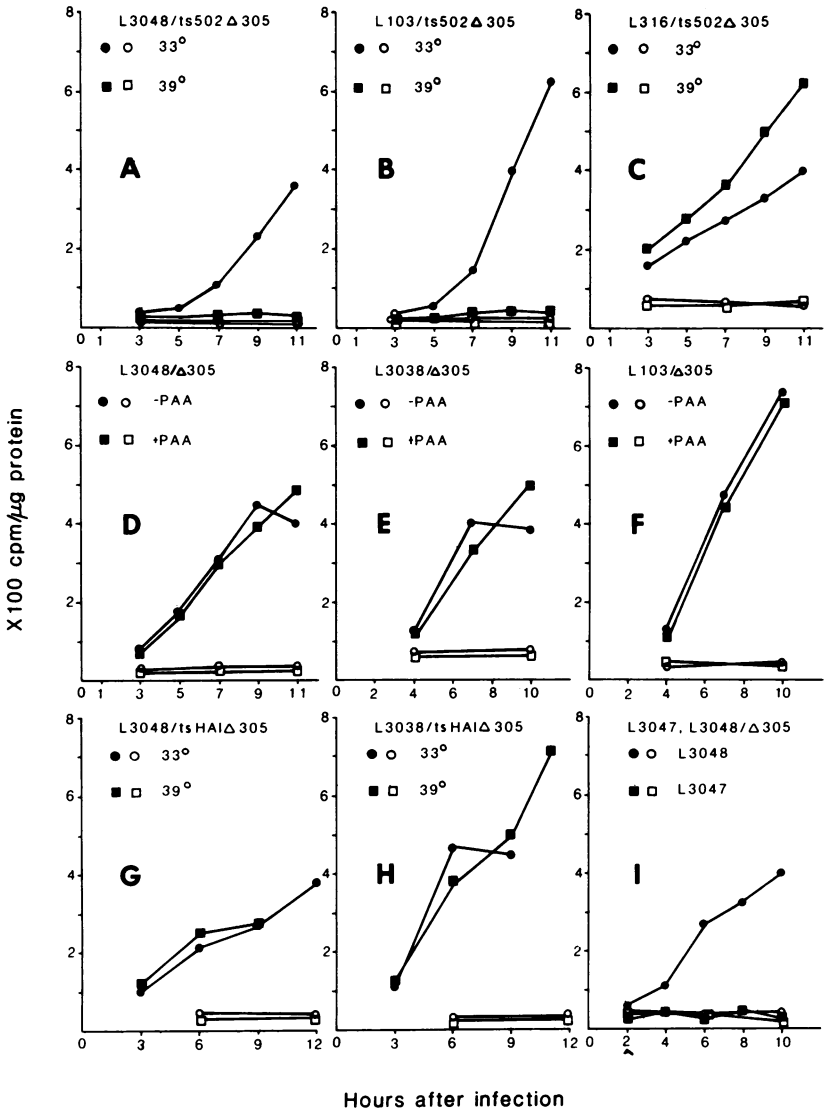

FIG. 3. TK activity in cells selected for TK$^+$ expression. Solid symbols (● and ■) represent infected cells. Open symbols (○, and □) represent mock-infected cells. Panels: A through C, L3048 (A), L103 (B), and L316 (C) cells infected with HSV-1*ts*502Δ305 at a permissive (33°C) or nonpermissive (39°C) temperature; D through F, L3048 (D), L3038 (E), and L103 (F) cells infected with HSV-1(F)Δ305 in the presence or absence of PAA (300 μg/ml); G through H, L3048 (G) and L3038 (H) cells infected with HSV-1*ts*HA1Δ305 at a permissive (33°C) or nonpermissive (39°C) temperature; I, L3047 and L3048 cells infected with HSV-1(F)Δ305. The viruses used for infections are described in the text. In all experiments, cells were infected with 5 PFU per cell.

transfected. The following experiments were done to characterize the regulation of the resident chimeric γ_2-TK genes in cells converted to the TK$^+$ phenotype.

(i) In cell lines infected with HSV-1(F)Δ305 and maintained at 37°C, the expression of resident chimeric TK genes linked to the γ_2 promoter in the proper orientation (L3038 and L3048) was enhanced (Fig. 3D, E, and I), whereas that of resident TK genes linked in the incorrect orientation, (e.g., L3047 [Fig. 3I]) was not. The pattern of accumulation of TK activity in the infected L3038 and L3048 cells was similar to that of infected L103 cells except that at comparable times the level of TK activity accumulating in infected L3038 and L3048 cells was lower than that in infected L103 cells (Fig. 3D, E, and F). These results indicate that *Bam*HI-D′ contains at its left terminus the predicted viral promoter and that the chimeric genes respond to *trans*-acting viral regulatory signals.

(ii) To identify the regulatory group to which the *trans*-acting regulatory signal belongs, three series of experiments were done. The first series was based on the observation that γ_2 genes contained in viral DNA and introduced into the cell during infection required DNA synthesis for their expression. In these experiments, L3038, L3048, and L103 cells were treated with 300 μg of PAA per ml of medium and infected with HSV-1(F)Δ305 as described above. The drug had no significant effect on the accumulation of TK in any of these cell lines (Fig. 3D, E, and F). Previous studies (7, 21) have shown that this concentration of PAA totally inhibits DNA synthesis, reduces the accumulation of γ_1 proteins, and prevents the expression of γ_2 genes. To verify that PAA is effective in blocking DNA synthesis and that the resident chimeric TK gene in L3048 cells is linked to a functional origin of DNA replication, we infected L3048 cells with HSV-1(F)Δ305 in the presence or absence of PAA. DNA

was extracted from cells 7, 9, and 11 h postinfection and probed by the dot blot hybridization procedure with the ^{32}P-labeled pRB408 DNA containing the *Bgl*II-*Sac*I fragment of the TK gene deleted from HSV-1(F)Δ305.

The salient features of the results shown in Fig. 4 rest on comparisons of the TK gene dosage in mock-infected L3048 cells and in L3048 cells infected with HSV-1(F)Δ305 and treated with PAA. The results show that there was no amplification of the TK gene during infection in the presence of PAA even when the TK gene was linked to an origin of viral DNA synthesis. In the absence of the drug, the TK template was amplified ca. fourfold. The presence of PAA had no appreciable effect on the induction of TK; i.e., the induction neither required nor reflected the amplification of the resident TK gene (Fig. 3D).

The second series of experiments was based on the observation that the synthesis of viral DNA and accumulation of γ_2 gene products did not occur at nonpermissive temperatures in cells infected with *ts*HA1Δ305, an HSV-1 mutant carrying a temperature-sensitive lesion in the β8 gene (7). Infection of L3038 and L3048 cells with *ts*HA1Δ305 resulted in the enhancement of TK expression at both permissive and nonpermissive temperatures (Fig. 3G and H).

The experiments described above indicated that the expression of TK by the 3038 and 3048 chimeric TK genes was independent of viral DNA synthesis and could not be differentiated from that of the natural β-TK gene resident in L103 cells. The third series of experiments was then designed to test the hypothesis that the induction of the resident TK gene in L3038 and L3048 cells, like that of L103 cells, is dependent on the expression of the α4 gene. *ts*502Δ305, a mutant carrying a temperature-sensitive lesion in the α4 gene, induced TK expression in L3048 and L3038 at the permissive temperature (33°C), but not at the nonpermissive temperature (39°C) (Fig. 3A, B, and C). In contrast,

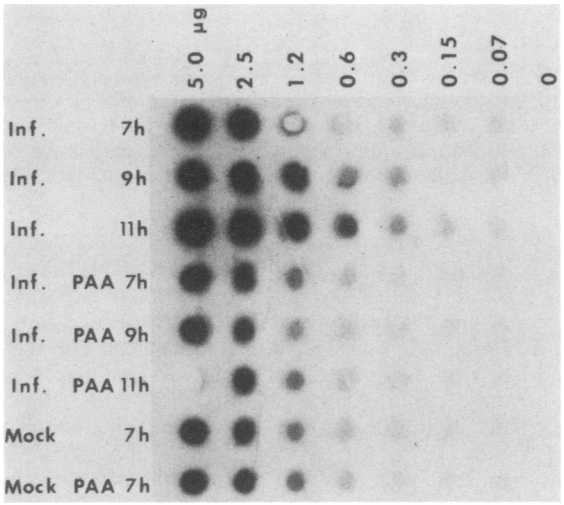

FIG. 4. Dot hybridization of DNA from L3048 TK$^+$ cells mock-infected (Mock) or infected with HSV-1(F)Δ305 (Inf.) and maintained in the presence or absence of PAA. DNA was extracted at 7, 9, and 11 h postinfection. The indicated amounts of DNA at each time point were spotted onto nitrocellulose, baked, and hybridized with pRB408 probe. pRB408 contains the 501-bp *Bgl*II-*Sac*I fragment within the domain of the TK gene. This DNA sequence is deleted in HSV-1(F)Δ305.

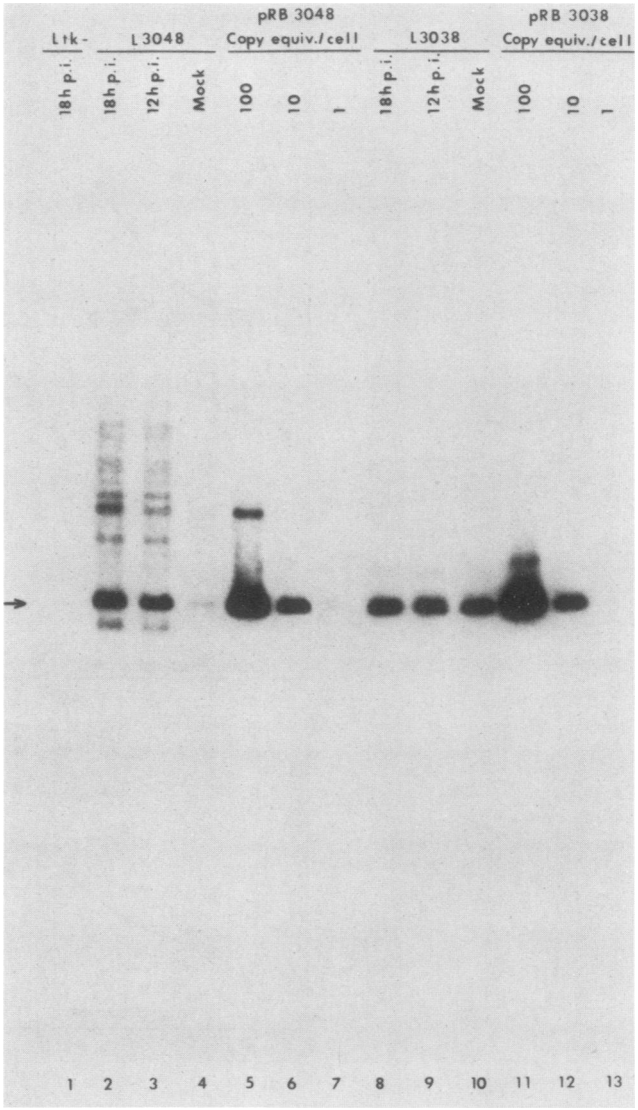

FIG. 5. Autoradiographic images of *Eco*RI digests of pRB3038, Ltk$^-$, L3038, and L3048 DNAs electrophoretically separated in agarose gels, transferred to a nitrocellulose sheet, and hybridized with ^{32}P-labeled *Bgl*II-*Sac*I probe from pRB408. The *Eco*RI fragments expected to hybridize with the *Bgl*II-*Sac*I probe contained within the domain of the TK gene are shown in Fig. 2. The *Bgl*II-*Sac*I fragment had been deleted from HSV-1(F)Δ305. Lanes: 1, Ltk$^-$ DNA extracted at 18 h postinfection with HSV-1(F)Δ305; 2 through 4, L3048 TK$^+$-selected genomic DNA extracted from cells at 18 and 12 h postinfection with HSV-1(F)Δ305 and from mock-infected cells, respectively; 5 through 7, mixtures of pRB3048 and Ltk$^-$ DNA yielding 100, 10, and 1 pRB3048 copy equivalents per cell, respectively; 8 through 10, L3038 TK$^+$-selected genomic DNA extracted from cells at 18 and 12 h postinfection with HSV-1(F)Δ305 and from mock-infected cells, respectively; 11 through 13, mixtures of pRB3038 and Ltk$^-$ DNA yielding 100, 10, and 1 pRB3038 copy equivalents per cell, respectively. The arrow points to the 3,530-bp *Eco*RI fragment containing the γ_2-TK and hybridizing to the ^{32}P-labeled *Bgl*II-*Sac*I DNA fragment from pRB408.

*ts*502Δ305 induced TK activity in L316 cells at both temperatures (Fig. 3C). These cells carry a TK gene regulated by an α4 promoter-regulatory region that does not require a functional α4 protein for its induction (38).

(iii) To ascertain that the cells transformed to the TK$^+$ phenotype contained nonrearranged copies of the chimeric

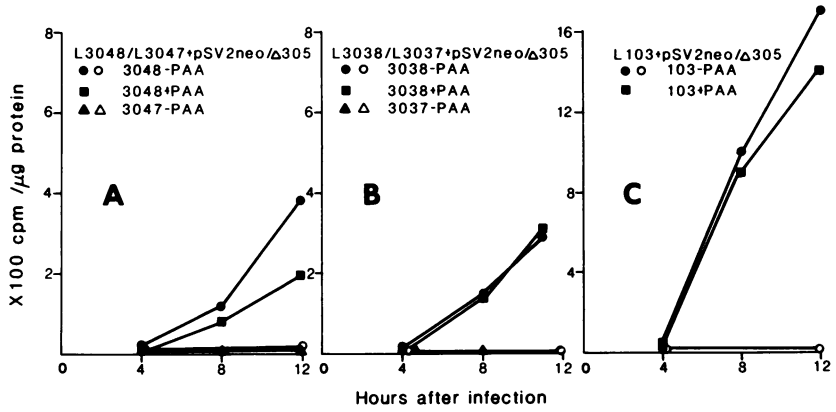

FIG. 6. TK activity in TK-containing cell lines selected for resistance to G418. Solid symbols (●, ■, and ▲) represent infected cells. Open symbols (○ and △) represent mock-infected cells. TK-containing, G418-resistant cell lines were obtained by cotransfection of pRB3048, pRB3047, pRB3038, pRB3037, or pRB103 DNA with pSV2neo plasmid DNA as described in the text. The following G418-resistant cell lines were infected with HSV-1(F)Δ305 in the presence or absence of PAA: A, L3048 and L3047; B, L3038 and L3037; C, L103.

γ_2-TK genes and to measure precisely the gene copy number at the time of superinfection and at subsequent intervals, cells from replicate cultures were harvested after mock infection and at 12 and 18 h postinfection. The DNA was extracted, cleaved with *Eco*RI, electrophoretically separated in agarose gels, transferred to a nitrocellulose sheet, and hybridized with the *Bgl*II-*Sac*I fragment of the TK gene deleted from HSV-1(F)Δ305. The results (Fig. 5) indicate the following. The genomic *Eco*RI fragment from L3038 cells comigrated with the γ_2-TK chimeric gene cloned in pRB3038, indicating that the cells contained nonrearranged γ_2-TK chimeric genes. The L3038 cells contained ca. 10 copies of the gene per cell, and the copy number did not increase after infection. The *Eco*RI digests of L3048 cell DNA yielded bands which comigrated with the *Eco*RI fragment cloned in pRB3048 and bands that migrated slower than the pRB3048 *Eco*RI DNA fragment. Both the band which comigrated with the pRB3048 *Eco*RI fragment and the slower migrating bands increased in intensity at 12 h postinfection. No further appreciable increase in copy number was seen at 18 h postinfection. The copy number of the nonrearranged band increased from ca. 1 per cell in the mock-infected cells at the beginning of the experiment to ca. 10 per cell at 12 h postinfection. The increase in the intensity of slower migrating bands indicates that the TK sequences that had undergone rearrangements were still linked to the HSV origin of DNA replication.

These experiments indicate that the resident TK genes in L3038 and L3048 cannot be differentiated from the natural TK gene with respect to (i) a requirement for functional α4 protein and (ii) lack of a requirement for viral DNA synthesis for their expression.

Induction of TK activity in L cells selected for resistance to G418. To control for the possibility that transfected cells selected for the TK^+ phenotype express a bias with respect to the regulation of the resident TK gene, Ltk^- cells were transfected with an artificial mixture of pSVneo and pRB3038, pRB3048, pRB3037, or pRB3047 DNA and then selected for resistance to G418. The regulation of the chimeric TK genes resident in these cell lines was determined by infection with HSV-1(F)Δ305 in the presence or absence of PAA. TK expression was induced by HSV-1(F)Δ305 in G418-resistant cell lines containing pRB3038 and pRB3048 but not in cell lines containing pRB3037 or pRB3047 (Fig. 6). In this instance, the induction of L3048 cells was slightly better in the absence of PAA than in its presence (Fig. 6A), whereas the induction of L3038 cells was unaffected by PAA (Fig. 6B). The induction of TK activity in these cell lines could not be differentiated from that observed after infection of a cell line derived by cotransfection of Ltk^- cells with pSV2neo and pRB103 carrying the natural β-TK gene.

It is of interest that, as in the case of cells selected for the TK^+ phenotype, the induced level of TK activity in cells selected for G418 resistance and carrying the β-TK gene was higher than the activities obtained in corresponding cells cotransfected with pRB3038 or with pRB3048.

The putative γ_2-TK construct is regulated as a γ_2 gene in cells infected with a recombinant virus carrying the construct. In this series of experiments, rabbit skin cells were cotransfected with intact HSV-1(F)Δ305 and pRB3112 DNAs (Fig. 2), and the progeny was selected for the TK^+ phenotype in $143tk^-$ cells plated in HAT medium. The resulting recombinant virus, R3112, contained the *Bam*HI-D′ DNA fragment inserted into the *Bgl*II site of *Bam*HI-Q with its γ_2 promoter in the correct transcriptional orientation relative to the structural sequence of the TK gene. The R3112 DNA yielded a 4,500-bp *Bam*HI fragment absent from the digests of HSV-1(F)Δ305 DNA but which comigrated with the HSV-1 *Bam*HI fragment of pRB3112 DNA (Fig. 7). The presence of the *Bam*HI D′-TK gene fusion fragment was verified by transfer of the digest to nitrocellulose strips and hybridization of the 4,500-bp fragment with pRB142 and pRB408 containing *Bam*HI-D′ and the *Bgl*II-*Sac*I fragment from *Bam*HI-Q, respectively (data not shown).

$143tk^-$ cells were infected in the presence or absence of PAA with R3112 or HSV-1(F). The infected cells were harvested at 4, 8, and 12 h postinfection and assayed for TK activity. The results showed that the TK activity induced by R3112 appeared later than that induced by HSV-1(F) (Fig. 8). Moreover, PAA inhibited the accumulation of TK induced by R3112 but not the TK activity induced by HSV-1(F). Unlike the regulation of γ_2-TK in transformed cell lines, the expression of γ_2-TK within the viral genome was stringently dependent on viral DNA replication.

5′ Termini of γ_2-TK mRNAs from cells infected with recombinant virus R3112 and from L3038-transformed cells are identical and map in *Bam*HI fragment D′. To determine whether the γ_2-TK genes resident in L3038 cells and in R3112 virus were transcribed from the γ_2 promoter contained in the donor *Bam*HI fragment D′ or from a secondary site within the TK gene, we analyzed the mRNA extracted from mock-infected and infected L3038 cells and from cells

productively infected with R3112, by S1 nuclease mapping. Cytoplasmic RNA was extracted from the following cells: (i) R3112-infected Vero cells, (ii) HAT-selected L3038 mock-infected cells, (iii) HAT-selected L3038 cells infected with HSV-1(F)Δ305, and (iv) mock-infected Ltk$^-$ cells.

In the first series of experiments, RNA was hybridized to probe A, a 350-nucleotide *Mlu*I-*Kpn*I probe, 5′ end labeled at the *Mlu*I site (Fig. 9). The *Mlu*I site is located at nucleotide +133 within the wild-type TK gene, whereas the *Kpn*I site is located approximately at nucleotide −160 relative to the γ_2 transcription initiation site within *Bam*HI-D′. After hybridization and treatment with S1 nuclease, the protected portions of the probe were sized on an 8% polyacrylamide sequencing gel, with reference to markers generated by 5′ end-labeled, *Hpa*II-digested pBR322. The results showed that ca. 190 nucleotides of probe A were protected by RNA extracted from R3112-infected cells (Fig. 10). Identical results were obtained with RNA extracted from both infected and mock-infected L3038 cells. As expected, the quantity of probe A DNA protected by RNA extracted from infected L3038 cells was greater than that protected by the RNA extracted from mock-infected cells. Our results indicate the following. If transcription of the γ_2-TK gene were initiated within the domain of the TK gene, i.e., at the *Bgl*II site or downstream, the size of the protected probe A DNA would be 83 nucleotides or less. Since the size of the protected DNA was ca. 190 nucleotides, the results indicate that transcription initiated at the predicted position in *Bam*HI-D′, 107 nucleotides from its left terminus.

Dennis and Smiley (9) reported that in cells converted to the TK$^+$ phenotype with a γ_1-TK chimera consisting of the

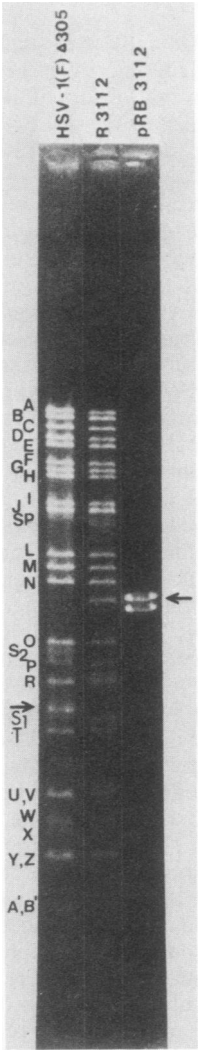

FIG. 7. Photograph of electrophoretically separated *Bam*HI digests of HSV-1(F)Δ305, R3112 viral, and plasmid DNAs. The digests were subjected to electrophoresis on a 0.8% agarose gel. In the recombinant virus R3112, a new viral *Bam*HI DNA fragment (right arrow) comigrated with the HSV-1 *Bam*HI fragment in the recombinant plasmid (4,500 bp). The parental HSV-1(F)Δ305 DNA band representing *Bam*HI fragment Q containing the TK gene carrying a deletion is also identified by an arrow (left arrow).

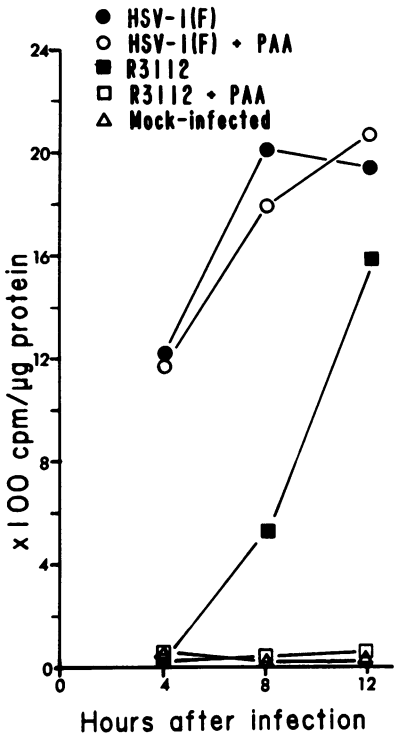

FIG. 8. TK activity in 143tk$^-$ cells infected with HSV-1(F) or R3112 in the presence or absence of PAA. Cells were infected with 5 PFU per cell.

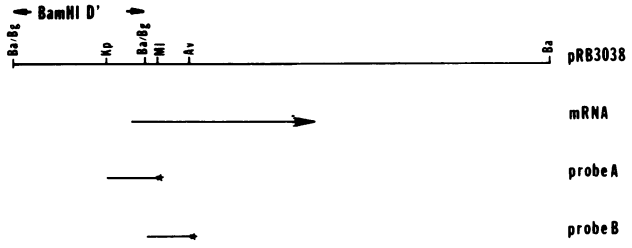

FIG. 9. Location of predicted γ_2 mRNA and probes used for S1 nuclease mapping. Probe A, derived from pRB3038, was 5′ end labeled at the *Mlu*I site within TK and extended to the *Kpn*I site within *Bam*HI-D′. Probe A was used in the experiments shown in Fig. 10. Probe B, derived from pRB408, was 5′ end labeled at the *Ava*I site within TK and extends to the *Bgl*II site of TK. Probe B was used in experiments shown in Fig. 11. The star at the end of the lines representing the probes identifies the labeled 5′ terminus.

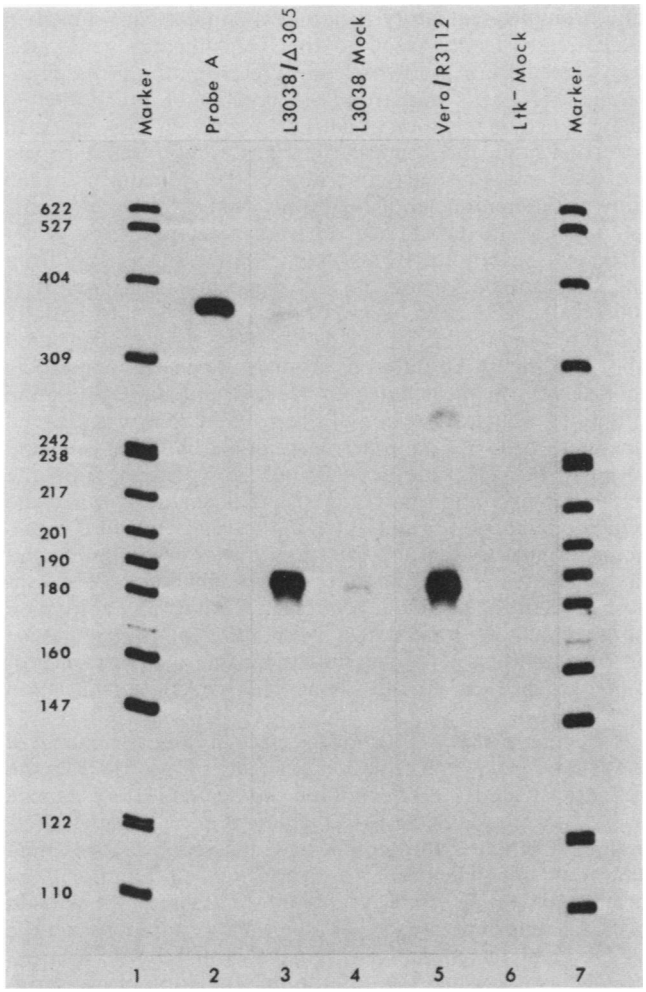

FIG. 10. Autoradiographic images or probe A DNA and of portions of probe A DNA protected from S1 nuclease digestion by RNA extracted from mock-infected and infected L3038 cells and from Vero cells infected with R3112 virus. Denatured, strand-separated probe A DNA (Fig. 9) was hybridized to cytoplasmic RNAs and then digested with S1 nuclease as described in the text. The following products were sized on an 8% polyacrylamide sequencing gel: lanes 1 and 7, 5′ end labeled, *Hpa*II-digested pBR322 to serve as size markers; lane 2, undigested Probe A; lane 3, probe A DNA protected by RNA extracted from HSV-1(F)Δ305-infected L3038 cells; lane 4, probe A DNA protected by RNA extracted from mock-infected L3038 cells; lane 5, probe A DNA protected by RNA extracted from Vero cells infected with R3112 virus; lane 6, probe A protected by RNA extracted from mock-infected Ltk$^-$ cells.

promoter-regulatory sequence of ICP5, fused to the TK gene at the *Bgl*II site (+50), the TK transcripts initiated within the domain of the TK gene at nucleotide +200 (relative to the cap site of wild-type TK mRNA), presumably by using a promoter located within the structural TK sequence. After infection of these cells with TK$^-$ virus, transcription initiated 26 bp downstream from the ICP5 promoter. Inasmuch as probe A would not detect transcripts initiated at the secondary transcription initiation site, a second series of experiments was done with probe B. This probe consisted of a 301-nucleotide *Ava*I-*Bgl*II fragment 5′ end labeled at the *Ava*I site located at position +351 within wild-type TK DNA (Fig. 9). If transcription had initiated within the TK sequence at position +200, the protected length of probe B would have been 151 nucleotides. If transcription had initiated within *Bam*HI-D′, the full-length probe (301 nucleotides) would have been protected. The entire probe was protected by RNAs extracted from infected, transformed cell lines, R3112-infected cells, and mock-infected L3038 TK$^+$ cells (Fig. 11). It should be stressed that the RNA extracted from mock-infected L3038 cells did not yield a protected probe B DNA 151 nucleotides in length. It should also be noted that although the RNA from infected L3038 cells yielded several faint bands representing fragments differing in length, the most abundant band contained the

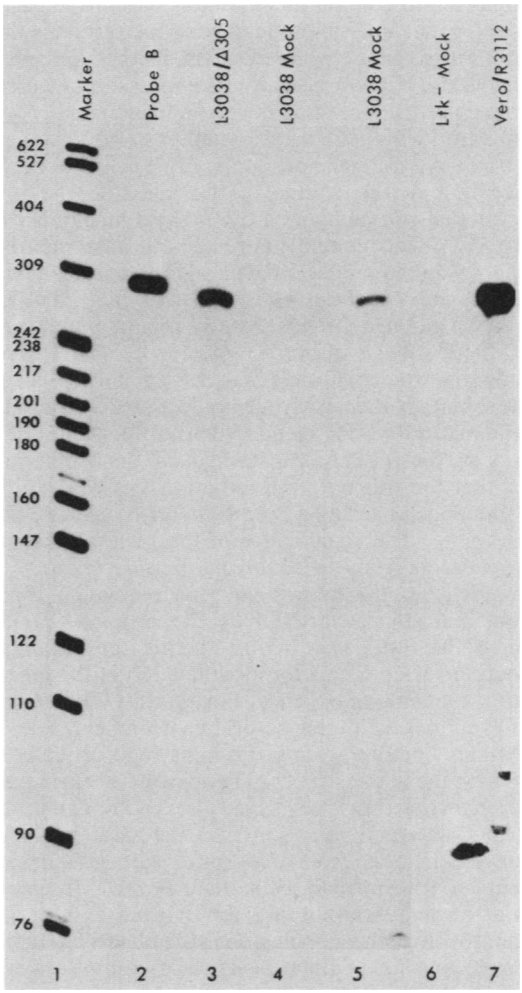

FIG. 11. Autoradiographic images of probe B DNA and of portions of probe B DNA protected from S1 digestion by RNA extracted from mock-infected and infected L3038 cells and from Vero cells infected with R3112 virus. Denatured, strand-separated probe B DNA (Fig. 9) was hybridized with cytoplasmic RNA and then digested with S1 nuclease as described in the text. The products were sized on an 8% polyacrylamide sequencing gel. The autoradiographic images shown in all lanes except lane 5 were obtained after identical exposure times. Lane 5 is the same as lane 4, but the exposure interval was doubled. Lanes: 1, size markers, as described in the legend to Fig. 9; 2, undigested probe B; 3, probe B protected by cytoplasmic RNA extracted from HSV-1(F)Δ305-infected L3038 cells; 4 and 5, probe B DNA protected by RNA extracted from mock-infected L3038 cells; 6, probe B DNA protected by RNA extracted from Ltk$^-$ cells; 7, probe B protected by RNA extracted from Vero cells infected with R3112 virus. The material at the lower right portion of the autoradiogram represents contaminating label not present in the original material.

entire probe B DNA, and all were larger than 150 nucleotides in length. The minor bands could have reflected the presence of alternate transcription initiation sites or overdigestion with S1 nuclease.

The results of the two series of experiments indicate that transcription was initiated from a transcription initiation site within *Bam*HI fragment D′ at the approximate position mapped by Hall et al. (15).

DISCUSSION

This report concerns the regulation imparted upon an indicator TK gene by an HSV-1(F) DNA sequence previously reported to contain the initiation site for the transcription of a γ_2 gene. The salient feature of the results is that the chimeric γ_2-TK gene, when resident in the viral genome, required viral DNA synthesis for its expression, whereas the chimeric gene resident in cells was induced equally well by superinfection with TK^- virus, whether or not it was amplified in *trans* by viral gene products. Moreover, there was no evidence that in the course of the infection the γ_2 gene resident in cells was amplified by the host machinery insensitive to PAA. This conclusion rests both on comparison of TK gene dosage in mock-infected and infected cells treated with PAA and on the well-established fact that HSV-1 strains lacking defects in host shutoff rapidly and effectively shut off the synthesis of host DNA (12, 13, 41). The results thus show that the chimeric gene was regulated as a γ_2 gene when resident in the virus but could not be differentiated from the natural β-TK gene when resident in the cell. Analyses of the mRNA transcribed off the chimeric gene showed that the transcription initiation site was within the donor sequence containing the promoter-regulatory domain of the γ_2 gene. The significance of the results presented in this paper stems from the following considerations.

Authenticity of the pattern of gene regulation conferred upon the indicator gene (TK) by the promoter-regulatory domain of the test gene. In introducing the technique of constructing TK chimeras for identification of the promoter-regulatory domains of α genes, Post et al. (38) first verified the α-TK chimeras in their natural environment, i.e., in the viral genome, before testing the constructs in cells transformed with these constructs. The wisdom of this approach is apparent from this study. Had the γ_2-TK construct not been tested by recombination into the viral genome, we could have concluded from analyses of the regulation of that construct in transformed cells that *Bam*HI fragment D′ carries at its left terminus a β rather than a γ_2 promoter. Consistent with such a conclusion is the observation that the γ_2-TK gene was induced in transient expression assays when cotransfected with DNA fragments carrying the α4 gene (P. Nazos, S. Silver, and B. Roizman, unpublished data). The results presented in this paper underscore the necessity of authenticating the behavior of the construct in the natural environment of the gene, i.e., within the viral genome, before proceeding with more detailed analyses in the environment of the cell genome.

Authenticity of transcription initiation of the chimeric gene in the environment of the viral and cellular genomes. Several laboratories have reported that the HSV-1 TK gene has additional transcription and translation initiation sites located 3′ to the dominant sites (1, 9, 16, 39). We expected that in the infected and mock-infected transformed cells, the resident chimeric-TK gene would be transcribed from a downstream site to account for its regulation as a β gene, particularly because of two previous reports. Thus, Dennis and Smiley (9) reported that a chimeric TK gene consisting of a promoter-regulatory region of the major capsid protein (ICP5), a γ_1 gene, fused to the TK indicator gene, was transcribed from a downstream TK promoter in mock-infected TK^+ cells and from the domain of the donor γ_1 sequence upon infection of the TK^+ cells with TK^- virus. In a second case, the downstream transcription initiation site was used in a construct consisting of the indicator TK gene (downstream at nucleotide +50) inserted inside a retrovirus genome (1). In the studies reported here, mRNA was derived by transcription from the γ_2 initiation site and not from the downstream, secondary TK gene initiation site. It is unlikely that the TK gene of HSV-1(F) is substantially different from the HSV-1 TK genes used in the studies cited above. Rather, the differences might be in the constructs themselves. In the studies by Dennis and Smiley (9), the promoter-regulatory region of the γ_1 ICP5 gene was cleaved upstream from its natural transcription initiation site and fused to the TK gene at +50, i.e., downstream from the natural transcription initiation site. Conceivably, when the regulatory domain is not activated in *trans* by viral *trans*-acting regulatory factors, the transcription initiation occurs at the nearest bona fide, properly punctuated site downstream from the promoter sequence. Conversely, activation of the regulatory domain by a *trans*-acting factor may induce an increased rate of transcription by the downstream promoter region even without a bona fide transcription initiation sequence.

Induction of the γ_2-TK chimeric gene in the environment of the cellular genome: a reflection of chance or necessity? In the cells transfected and selected for neomycin G418 resistance or TK^+ expression, the γ_2-TK chimera could not be differentiated from the natural β-TK gene inasmuch as its expression was dependent on the availability of functional α4 protein and not on viral DNA synthesis. Because the virus in parental cells expresses its own genes appropriately and the γ_2-TK chimera recombined into the viral genome was expressed as a γ_2 gene, the differential regulation of transcription cannot be attributed to the primary structure of the DNA or regulation at the translational level. A possible explanation is that the secondary and tertiary structures of the chimeric genes in the environment of replicating viral and cellular genes are not identical. For example, it has been reported that HSV-1 DNA sequences resident in transformed cells are contained within nucleosomes (5, 46), whereas HSV-1 DNA in productively infected cells is not (27, 34). Although this explanation provides a basis for additional studies, it raises the question of whether α4 protein differentiates between genes located in the environments of cellular and viral genomes.

Relevant to the regulatory functions of the α4 protein are two observations. First, there is unambiguous evidence that functional α4 protein is required for the expression of HSV-1 β genes (e.g., natural TK genes) resident in transformed cells (38). Second, recent studies indicate that the pseudorabies virus gene corresponding in size and regulatory properties to the HSV-1 α4 gene induces a variety of nonviral genes introduced into the cell by transfection (14, 20). Induction is not indiscriminant, however, since neither the HSV-1 α4 protein nor the corresponding pseudorabies gene product induces α-TK chimeras residing in host cells and introduced by transfection (2, 38).

The ability of α4 and possibly that of other α genes to induce both viral and nonviral genes contained within the nucleosomal environment of the host genome may reflect an essential function rather than a mere coincidence. This hypothesis is based on two considerations. First, herpes-

viruses specify many of the enzymes required for the replication of their DNAs, and they undoubtedly utilize a large number of host factors. It is likely that the lytically infected cell of the natural host does not resemble the tissue culture cell with respect to metabolic rate and availability of the host enzymes necessary for virus replication; if this hypothesis is correct, a function of α genes would be to stimulate the transcription of specific sets of host genes. Second, current evidence suggests that in the course of latent infection, HSV DNA very likely resides in sensory neurons (22, 44). Inasmuch as HSV can remain latent for the lifetime of the host, it is likely, by analogy with the state of other herpesviruses, that HSV DNA is contained in nucleosomal structures. The mechanism of induction of expression of HSV genes is unknown; whatever the mechanism, the initial products of the expression of latent HSV, presumed to be α proteins, may have to induce not only host genes but also viral genes contained in nucleosomal structures.

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