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Identification of a Herpes Simplex Virus Function That Represses Late Gene Expression from Parental Viral Genomes

PAUL J. GODOWSKI AND DAVID M. KNIPE*

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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The expression of herpes simplex virus γ_2 (late) genes is inhibited before the onset of viral DNA replication. We report that the block in the expression of certain γ_2 genes is relieved, at least in part, by defects in the β ICP8 protein. We have examined the expression of the γ_2 gene encoding glycoprotein C (gC) in cells infected with a temperature-sensitive ICP8 mutant. Under conditions in which viral DNA replication is inhibited, cells infected with the ICP8 mutant overproduce the gC family of mRNAs relative to the level observed in cells infected with a wild-type virus. The gC mRNA synthesized in cells infected with the ICP8 mutant virus is correctly initiated and spliced and is translated with the same relative efficiency as in cells infected with a replicating wild-type virus. These results suggest that ICP8 is involved in the negative regulation of γ_2 genes expressed from parental viral genomes. The level of gC expression was greatest in cells infected with a replicating wild-type virus. These data suggest that DNA replication and genome amplification are not absolute requirements for γ_2 gene expression but may facilitate full-level expression of these genes.

A common theme illustrated in the productive infections of eucaryotic cells by DNA viruses is the tightly controlled, developmentally regulated expression of viral genes. Certain viral gene regulatory circuits provide appropriate model systems for the study of cellular gene expression. Although several viral- or cellular-positive regulatory factors have been identified, relatively few well-defined negative regulatory functions have been characterized. In general, viral late genes are expressed at very low levels before viral DNA replication. By an as yet undetermined mechanism, DNA replication or genome amplification results in a burst of late gene activity. The factors that restrict late gene expression before DNA replication and the mechanism by which viral DNA replication increases late gene expression are poorly understood. Elucidation of the mechanism by which these viral genes are regulated may prove useful in understanding the regulation of cellular genes whose expression is linked to DNA replication.

Herpes simplex virus (HSV) is a large DNA virus that replicates in the cell nucleus (40, 44, 47). The infection of permissive cells results in the induction of more than 50 polypeptides, many of which have been shown to be encoded by the virus. The genes have been divided into three general classes, α, β, and γ, based on differences in their order of expression and the viral gene products necessary for their synthesis. The γ class has been further subdivided into the γ_1 and γ_2 classes. In the initial stages of a lytic infection, the viral α genes are expressed from the parental genomes. α gene products activate the expression of viral β genes from unreplicated genomes. The α and β genes are required for the efficient expression of viral γ_1 and γ_2 genes. Unlike α or β genes, after DNA replication, the expression of γ_1 and γ_2 genes greatly increases. γ_1 and γ_2 genes are distinguished by the observation that γ_2 gene expression is barely detectable in a normal lytic infection before viral DNA replication, whereas γ_1 gene expression is readily detected (40).

We have shown that HSV temperature-sensitive mutants encoding a defective β gene product, ICP8, overproduce certain α, β, and γ_1 proteins and mRNAs at the nonpermissive temperature (14). These results suggested an involvement of ICP8 in the regulation of HSV gene expression. ICP8 binds single- and double-stranded DNA (24, 29, 36) and is required for viral DNA replication (8, 49). These mutants are defective for viral DNA replication at the nonpermissive temperature (8, 18, 49). It was of interest to determine the effect of these defects on the expression of γ_2 genes. In this study, we show that the tight block to γ_2 gene expression in the absence of DNA replication is relieved, at least in part, by defects in ICP8. The expression of γ_2 genes is greatest in cells infected by a replicating wild-type virus. These studies suggest that, before DNA replication, γ_2 gene expression may be regulated, directly or indirectly, by ICP8.

MATERIALS AND METHODS

Cells, viruses, and inhibitors. The procedures used for propagating and titrating viral stocks have been previously described (14, 26). HSV-1 strains KOS1.1 and KOS1.1 *ts*18 (18, 21) were provided by R. Sandri-Goldin and M. Levine, University of Michigan, Ann Arbor. Sodium phosphonoacetate (PAA) was a gift of Abbott Laboratories, North Chicago, Ill. PAA was used at a concentration of 400 μg/ml. PAA was added 2 h before infection or at the time of infection and maintained throughout infection. The expression of γ_2 genes was similar whether PAA was added before or at the time of infection (data not shown). Thymine-1-β-D-arabinofuranoside (AraT; Sigma Chemical Co., St. Louis, Mo.) was used at a concentration of 3.8×10^{-4} M. Cells were pretreated for 1 h with 1.9×10^{-4} M AraT in Dulbecco modified Eagle medium–10% serum before infection. To construct KOS1.1 18*Rs*1, Vero cells were transfected (25) with 0.5 μg each of intact *ts*18 DNA and plasmid pSG18-B51 (18) which had been digested with *Bam*HI to release the viral DNA insert. After 4 days at 33°C, the cells were harvested, and virus was titrated at 33 and 39.5°C. The ts^+ recombinants were plaque purified four times at 39.5°C.

Northern blot analysis of viral mRNAs. Confluent cultures of Vero cells were infected at a multiplicity of 20 PFU per cell. At 8 h postinfection, the cells were harvested, and cytoplasmic nucleic acids were isolated after Nonidet P-40 lysis as previously described (14). Ten-microgram portions

* Corresponding author.

of total cytoplasmic RNA were denatured, fractionated in 1% neutral agarose gels containing formaldehyde, blotted onto nitrocellulose, and hybridized with nick-translated probes as previously described (14). Autoradiography was performed with intensifying screens at −70°C.

S1 analysis of viral mRNAs. To identify the 5′ end of the glycoprotein C (gC) mRNA, total cellular RNA was isolated after the lysis of cells with guanidine isothiocyanate as previously described (32). Fifty-microgam samples of RNA were precipitated with ethanol. The precipitate was collected by centrifugation at 15,000 × *g* for 15 min and then washed with 75% ethanol. The precipitate was dissolved in 20 μl of a solution containing 100 to 200 ng of 5′-end-labeled DNA probe, 80% formamide, 400 mM sodium chloride, 40 mM PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) [pH 6.4]), and 1 mM EDTA (4), heated at 76°C for 10 min, and then incubated at 64°C for 8 h. The samples were diluted into 200 μl of buffer (0°C) containing 280 mM sodium chloride, 50 mM sodium acetate, 4.5 mM zinc acetate, 25 μg of denatured salmon sperm DNA per ml, 5% glycerol, and 500 U of S1 nuclease (Boehringer Mannheim, Mannheim, Federal Republic of Germany) per ml. After incubation at 35°C for 1 h, the reaction was terminated by the addition of ammonium acetate to 0.5 M and EDTA to 12.5 mM. The reaction was extracted with equal volumes of phenol and chloroform, and the nucleic acids were then precipitated with 2 volumes of ethanol. The products of the S1 nuclease reaction were resolved by electrophoresis in 8% polyacrylamide gels containing 8 M urea (33). The gels were fixed in 10% acetic acid for 20 min and then dried. Autoradiography was performed with Kodak XRP-5 film at −70°C with an intensifying screen. To identify the gC mRNA splice acceptor sites, 2.5 μg of polyadenylated RNA (isolated from total cytoplasmic RNA by oligodeoxythymidylic acid-cellulose [Collaborative Research, Inc., Waltham, Mass.] chromatography) was mixed with 100 μg of *Escherichia coli* tRNA (Boehringer Mannheim) and precipitated with 2 volumes of ethanol. The precipitate was treated as described above, and the products of the S1 nuclease reaction were separated by electrophoresis in 4% polyacrylamide gels containing 8 M urea (33).

Preparation of nick-translated and end-labeled probes. Plasmid DNAs were isolated by CsCl equilibrium density gradient centrifugation of alkaline lysates from plasmid-containing cells (32). The HSV-1 or α-tubulin DNA inserts were cleaved from the vector DNA by digestion with the appropriate restriction enzyme. For nick translation, the fragments were purified by electrophoresis through neutral agarose gels and then isolated by electroelution (34). They were labeled by nick translation (39) with [α-^{32}P]dCTP (1,600 Ci/mmol; New England Nuclear Corp., Boston, Mass.) to a specific activity of 1 × 10^8 to 3 × 10^8 cpm/μg. Before end labeling, restriction endonuclease-generated DNA fragments were treated with calf intestine alkaline phosphatase (Boehringer Mannheim). DNA probes labeled at the 5′ end were prepared with polynucleotide kinase and [γ-^{32}P]ATP (ICN Chemicals, Inc.) (33).

Synthesis of viral DNA in infected cells. Confluent monolayer cultures of Vero cells were infected as described above with 20 PFU per cell of the indicated HSV strain. As indicated, cells were incubated from the time of infection until the time of harvesting in the presence of 400 μg of PAA per ml of medium. At 1 h postinfection the inoculum was removed, and the cells were overlaid with medium 199–1% inactivated calf serum containing 20 μCi of [*methyl*-^{3}H]thymidine (New England Nuclear) per ml. At 8 h postinfection, DNA was extracted from the cells as previously described (22). The DNA was banded in NaI equilibrium density gradients in a Beckman Ti50 rotor at 44,000 rpm for 48 h at 15°C. Fractions of equal volume (175 μl) were collected with a Microfractionator (Hoefer Scientific Instruments, Inc., San Francisco, Calif.). Portions (20 μl) of the fractions were spotted onto DE-81 filters (Whatman Laboratory Products, Inc., Clifton, N.J.), and radioactivity was determined as previously described (32).

Quantitation of viral DNA molecules in infected cells. Vero cells were infected with either *ts*18 or KOS1.1 in the presence or absence of PAA as described above. At 8 h postinfection, the cells were harvested and lysed with 1% Nonidet P-40 as described for the isolation of cytoplasmic RNA (14). The nuclear pellet was washed with a detergent mixture (26) and then suspended in 10 mM Tris-hydrochloride–10 mM EDTA (pH 8.0) with 0.6% sodium dodecyl sulfate. Pronase was added to 200 μg/ml, and the nuclei were incubated for 12 h at 37°C. NaCl was added to 0.3 M, and the suspension was extracted once with an equal volume of phenol and chloroform and then once with chloroform. The aqueous phase was treated with 10 μg of RNase A per ml for 30 min at 37°C, and nucleic acids were precipitated with 2 volumes of ethanol. Equal quantities of DNA were digested to completion with *Bam*HI. As an internal control for complete digestion, 1 μg of lambda DNA was added to each sample, and digestion was monitored by electrophoresis through neutral agarose gels and ethidium bromide staining. The nuclear DNA molecules were resolved by electrophoresis through a 0.8% neutral agarose gel and transferred to nitrocellulose (32). The conditions for hybridization of the probe to DNA immobilized on the nitrocellulose strips were exactly as described previously (14) for hybridization to RNA for Northern blot analysis.

Immunoprecipitation and polyacrylamide gel electrophoresis. Infected or mock-infected Vero cells were pulse-labeled for 15 min at 8 h postinfection with [^{35}S]methionine as described previously (14, 26). The cells (6 × 10^6) were harvested and pelleted by centrifugation for 35 s at 15,000 × *g*. The cell pellet was suspended in 150 μl of RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 10 mM Tris-hydrochloride [pH 7.2]) (13) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma) and 10 μM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) (Sigma) and then subjected to 15 1-s pulses with a cell disruptor (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). The extract was clarified by centrifugation at 15,000 × *g* for 15 min at 4°C. Ascites fluid containing C16 antibody (19; provided by J. C. Glorioso, University of Michigan, Ann Arbor) was added to 10% (vol/vol) and then incubated on ice for 45 min. One-half volume of a 10% solution (vol/vol) of formaldehyde-treated *Staphylococcus aureus* (IgSorb; Enzyme Center Inc.) in RIPA buffer with PMSF and TLCK was added and incubated at 0 to 4°C for 30 to 60 min. The immunoprecipitate was collected by centrifugation at 15,000 × *g* for 30 s at 4°C. The pellet was washed four times with RIPA buffer containing PMSF and TLCK. The proteins were dissolved in gel sample buffer and then resolved by electrophoresis through a 7.25% polyacrylamide gel (26). Labeled proteins were detected by fluorography (27).

RESULTS

Expression of γ_2 mRNAs by HSV-1 mutant viruses encoding a defective ICP8. To determine the effect of ICP8 on γ_2 viral gene expression, we have examined the expression of the HSV-1 gC gene. The major transcription product of the γ_2 gC

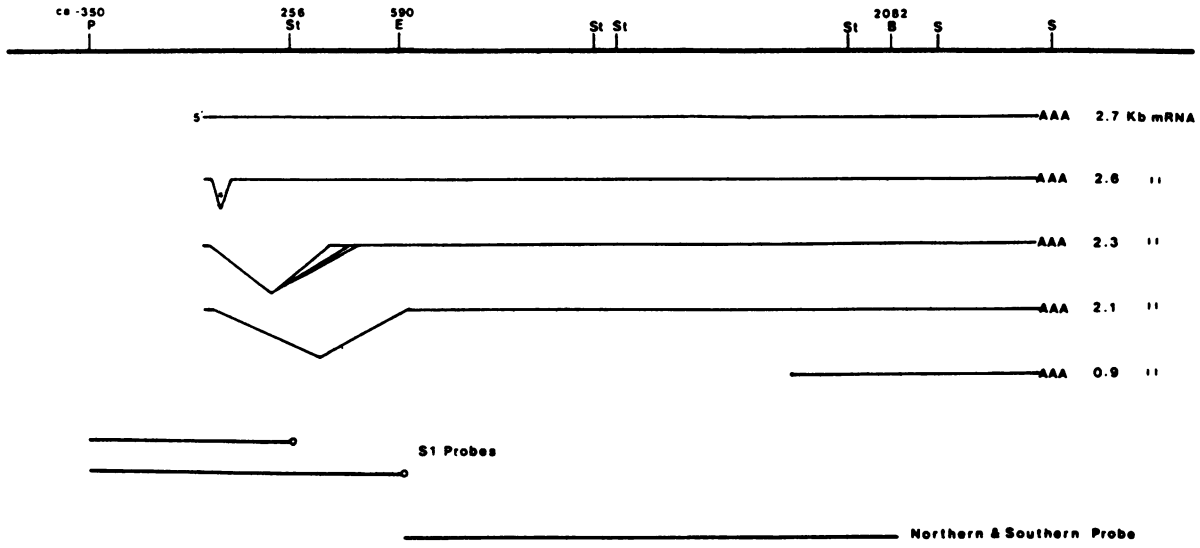

FIG. 1. Map of the HSV-1 gC gene. The sites for the restriction enzymes *Pst*I (P), *Sst*II (St), *Eco*RI (E), *Bam*HI (B), and *Sal*I (S) and their position relative to the initiation site of the major 2.7-kb gC mRNA transcript identified by Frink et al. (11) are indicated. The minor processed forms of the 2.7-kb mRNA, 2.6, 2.3, and 2.1 kb, are also shown. The 2.3-kb mRNA actually consists of at least three species that differ slightly in the splice acceptor sites. The independently initiated 0.9-kb γ_2 mRNA coterminal with the gC family of mRNAs is also shown. The fragments used in the S1 mapping experiments (*Pst*I-*Sst*II and *Pst*I-*Eco*RI) and the fragment used as a probe for the gC mRNA and to determine the gC gene copy number (*Eco*RI-*Bam*HI fragment I-I) are indicated.

gene is an unspliced 2.7-kilobase (kb) mRNA. A number of minor transcripts (2.6, 2.3, and 2.1 kb) are derived from the 2.7-kb mRNA by splicing. These mRNAs are referred to as the "gC family" of mRNAs (10, 11). The minor transcripts share a short leader sequence at the 5' end that is spliced to sequences ca. 100, 400, or 625 bases downstream from the start of the 2.7-kb mRNA (Fig. 1). Nucleotide sequence data indicate that both the 2.7- and the minor 2.6-kb mRNAs can encode the gC polypeptide. The functions of the 2.4- and the 2.1-kb mRNAs are not known. In addition, a 0.9-kb mRNA overlaps the 3' end of the 2.7-kb mRNA. This unspliced mRNA terminates at the same site as does the 2.7-kb mRNA but appears to be initiated at its own promoter. A 4.3-kb minor transcript also maps in this region but has not been fully characterized (11).

We used Northern blot analysis to characterize the effects of DNA replication on the accumulation of these mRNAs. These mRNAs were very abundant in samples of RNA isolated from cells infected with a wild-type virus (Fig. 2A, lane 6). The inhibition of viral DNA replication in cells infected by the wild-type virus resulted in a drastic decrease in the accumulation of the gC family of mRNAs (Fig. 2A, lane 4). The 0.9- and 4.3-kb mRNAs were also reduced when DNA replication was blocked. In other experiments, we used quantitative slot blots to compare the amounts of these transcripts in cells infected with ts^+ virus in the presence or absence of AraT, another inhibitor of viral DNA replication (2, 17). The amount of stable RNA transcribed from this region in the presence of AraT was only 1 to 2% of the level observed in the absence of AraT (data not shown). In agreement with the observations of Frink et al. (10, 11), these mRNAs appear to be regulated as γ_2 transcripts.

We then examined the effects of a defect in ICP8 on the expression of these mRNAs. ICP8 is an essential function for viral DNA replication. Cells infected with the temperature-sensitive ICP8 mutant KOS1.1 *ts*18 at the nonpermissive temperature (39.5°C) synthesize less than 0.1% of the amount of DNA synthesized in cells infected by the wild-type virus (18). The mutant protein is defective for binding to viral DNA in vivo (28). In cells infected with *ts*18 at 39.5°C and blocked for DNA replication by the temperature-sensitive defect or by the temperature-sensitive defect and PAA, the inhibition of gC mRNA accumulation was partially overcome (Fig. 2A, lanes 5 and 3, respectively). Under these latter conditions, the gC family of mRNAs accumulated to ca. 10% of the level found in cells infected by a replicating wild-type virus. It is noteworthy that the 0.9-kb mRNA accumulated to a level nearly equal to that found in cells infected by a replicating wild-type virus. At the permissive temperature (33°C) there was only a very low amount of the gC family and of the 0.9- and 4.3-kb mRNAs in PAA-treated cells infected with either *ts*18 or ts^+ virus (Fig. 2A, lanes 1 and 2). Thus, the regulation of these mRNAs in cells infected with the mutant appeared nearly normal at the permissive temperature.

To control for the possibility that mRNA samples were degraded or lost, the same blot was probed with a nick-translated α-tubulin cDNA clone (30). The amount of the 1.6-kb α-tubulin mRNA varied somewhat but did not correlate with the amount of gC mRNAs (Fig. 3).

The conditional, lethal temperature-sensitive phenotype of *ts*18 is the result of a mutation in the ICP8 gene (18, 28). However, it is formally possible that a second, nonlethal temperature-sensitive mutation is responsible for the altered regulation of these γ_2 mRNAs. We constructed a ts^+ derivative of *ts*18 by marker rescue with the cloned HSV-1 *Bam*HI V DNA fragment (HSV-1 map coordinates 0.397 to 0.411). This fragment is contained almost entirely within the gene boundaries of ICP8 (18, 38; L. Su and D. Knipe, unpublished results). We then determined whether this ts^+ derivative (KOS1.1 18*Rs*1) regulated γ_2 gene expression normally. In cells infected with 18*Rs*1 at 39.5°C, the accumulation of gC mRNAs was strongly inhibited by the treatment of cells with PAA (Fig. 2B, lanes 2 and 3). We also selected a spontaneous ts^+ revertant of *ts*18. This revertant also regulated γ_2 mRNAs normally (E. Richards, P. Godowski, and D. Knipe,

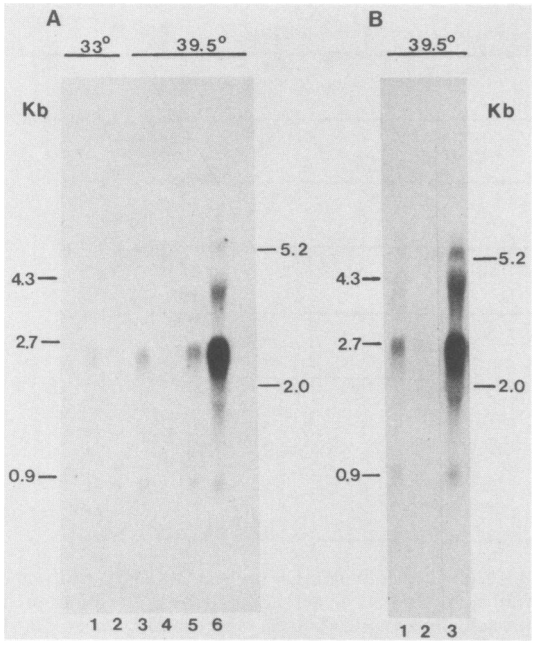

FIG. 2. gC mRNA levels in cells infected with wild-type or mutant virus. Ten-microgram samples of total cytoplasmic RNA from PAA-treated or untreated cells were subjected to electrophoresis in formaldehyde-agarose gels and transferred to nitrocellulose as described in the text. The blot was probed with the HSV-1 *Eco*RI-*Bam*HI fragment I-I (see the legend to Fig. 1). (A) RNA samples were from cells infected with either *ts*18 or ts^+ virus, in the presence (+PAA) or absence (−PAA) of PAA, at the indicated temperature. Lane 1, *ts*18, +PAA, 33°C; lane 2, ts^+, +PAA, 33°C; lane 3, *ts*18, +PAA, 39.5°C; lane 4, ts^+, +PAA, 39.5°C; lane 5, *ts*18, −PAA, 39.5°C; and lane 6, ts^+, −PAA, 39.5°C. (B) RNA isolated from cells infected as follows: lane 1, *ts*18, +PAA, 39.5°C; lane 2, KOS1.1 18*Rs*1, +PAA, 39.5°C; and lane 3, KOS1.1 18*Rs*1, −PAA, 39.5°C. The sizes of the major RNA species detected with the probe are indicated in kilobases on the left of each panel. These sizes were determined relative to migration of 28S (5.2 kb) and 18S (2.0 kb) rRNA markers, indicated on the right of each panel. Autoradiography was performed at −70°C with intensifying screens.

unpublished data). These results indicated that the altered regulation of γ_2 mRNAs in cells infected with *ts*18 at 39.5°C was related to the defect in ICP8.

Viral DNA replication and DNA copy number in mutant-infected cells. We determined the extent of the block of DNA replication in cells treated with PAA and infected with either *ts*18 or the ts^+ virus. Infected or mock-infected cultures were labeled with [^{3}H]thymidine from 1 to 8 h postinfection. The amount of labeled DNA that sedimented in NaI gradients with the density of viral DNA was determined, and the results are shown in Fig. 4. The amount of labeled DNA sedimenting with the density of viral DNA from PAA-treated cells infected with either *ts*18 or ts^+ virus was similar to the amount found in the mock-infected culture. Thus, PAA inhibited viral DNA replication very efficiently under these conditions. The efficiency of plaque formation at 33°C of the mutant *ts*18, the wild-type virus, and the rescued virus 18*Rs*1 in the presence of various concentrations of PAA was identical (data not shown). Therefore, differences in sensitivity to PAA did not account for the altered regulation of these γ_2 mRNAs.

In these experiments, cells were infected with 20 PFU per cell of either the *ts* or ts^+ virus. Conceivably, the temperature-sensitive defect could affect the viral particle/PFU ratio. This might result in a difference in the number of parental viral genomes that enter the nucleus and express γ_2 genes. We compared the gC gene copy number in the nuclei of cells infected with the *ts* or ts^+ virus. Total nuclear DNA was prepared from infected cells, digested with *Bam*HI, fractionated by agarose gel electrophoresis, and then transferred to nitrocellulose. The blot was incubated with the same labeled DNA fragment used as probe to determine the level of the gC mRNAs. The gC gene copy number was very similar in cells treated with PAA and infected at 39.5°C with *ts*18 or the ts^+ virus (Fig. 5, lanes 1 and 2). As expected, there was a huge increase in the gC gene copy number in the nuclei of cells in which viral DNA replication was occurring (Fig. 5, lane 3). Taking into consideration that 50-fold less DNA from this sample was loaded onto the gel, we calculate that there were, on the average, 200- to 250-fold more copies of the gC gene in the nuclei of cells in which viral DNA replication was occurring. Obviously, this analysis does not indicate the fraction of these DNAs that serve as templates for transcription. We conclude that the difference in the expression of γ_2 genes from parental genomes in cells infected with *ts*18 or ts^+ virus at 39.5°C was not due to differences in the number of viral DNA molecules that enter the nucleus.

Fine structure analysis of γ mRNAs produced in the presence or absence of viral DNA replication. The process of viral DNA replication influences the primary sites of initiation for the mRNAs transcribed from the simian virus 40 early transcription unit (5, 12, 16) and from the gene encoding the adenovirus 72,000-molecular-weight protein (72K protein)

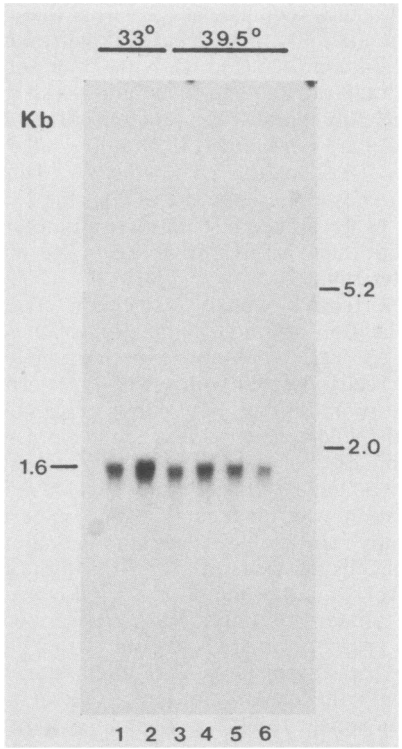

FIG. 3. Control for mRNA recovery from cells infected with wild-type or mutant virus. The Northern blot shown in Fig. 2A was rehybridized with an α-tubulin cDNA probe (30). The origins of the RNA samples are the same as described in the legend to Fig. 2A. The size of the major transcript detected with this probe is indicated in kilobases on the left.

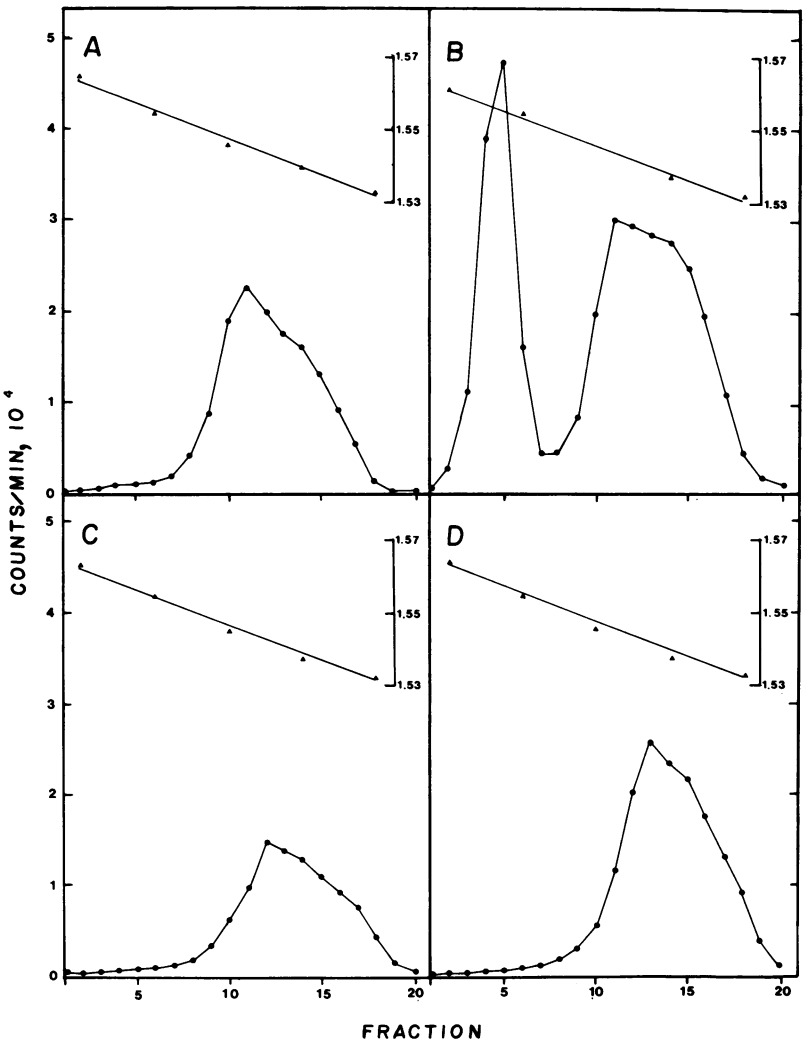

FIG. 4. DNA replication in PAA-treated (+PAA) or untreated (−PAA) cells. The distribution in NaI isopycnic density gradients of [^{3}H]thymidine-labeled DNA (●) isolated from infected or mock-infected cells incubated at 39.5°C was determined as described in the text. Panels: (A) mock-infected, +PAA; (B) ts^+, −PAA; (C) ts^+, +PAA; and (D) *ts*18, +PAA. The density of gradient fractions (in grams per cubic centimeters) as calculated from the refractive index (31) is also shown (▲).

(6). Therefore, we analyzed the site of initiation of the gC transcriptional unit in cells infected with the mutant or wild-type virus. The site of initiation of the unspliced 2.7-kb gC mRNA was analyzed in S1 mapping experiments with a DNA probe 5′ end labeled at the *Sst*II site (nucleotide 256) and extending to the *Pst*I site (nucleotide −350) as shown in Fig. 1. RNA isolated from cells infected with the replicating ts^+ virus protected DNA fragments migrating with a size of ca. 255 and 256 nucleotides (Fig. 6, lane 5). This result is in agreement with that of Frink et al. (11). DNA fragments of identical size were protected by RNA isolated from cells infected with *ts*18 and treated with AraT. Reduced but detectable amounts of these bands were observed in cells treated with AraT and infected with ts^+ virus (Fig. 6, cf. lanes 3 and 4). The amount of correctly initiated gC mRNA in cells infected with *ts*18 was ca. 10% of that accumulating in cells infected with the replicating wild-type virus. This is based on the observation that the amount of the 255- and 256-nucleotide band protected by 50 μg of RNA from *ts*18-infected cells was very similar to the amount protected by the 5 μg of RNA isolated from cells infected with a replicating ts^+ virus (Fig. 6, cf. lanes 4 and 6). Multiple bands migrating with a size greater than 255 and 256 nucleotides were also observed in this experiment. The 278-nucleotide band may be the result of S1 cutting at a sensitive site in reannealed probe, because the intensity of this band varied from experiment to experiment and because a similiar-sized band was observed in the mock-infected cell RNA on longer exposures. The 310-nucleotide band was reproducibly observed in multiple experiments with either total or polyadenylated RNA samples isolated from virus-infected cells. The amount of this band in the various samples closely paralleled that of the 255- and 256-nucleotide doublet. We suspect that the 310-nucleotide band identifies either an initiation site or splice acceptor site for a γ_2 mRNA; however, additional experiments are required to prove this point. It is worth noting the presence of a relatively AT-rich region ending 38 nucleotides upstream from the putative initiation site identified by the 310-nucleotide band (11). We conclude that the gC mRNA synthesized in cells infected by *ts*18 initiates at the same site(s) as that produced in cells infected by the replicating ts^+ virus.

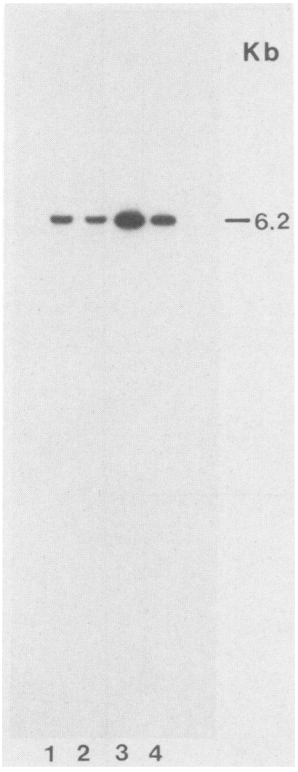

FIG. 5. Comparison of gC gene copy number in cells infected with ts^+ virus. Cells were infected at 39.5°C and incubated in the presence (+PAA) or absence (−PAA) of PAA. At 8 h postinfection, total nuclear DNA was isolated. The DNA was digested with *Bam*HI, fractionated in a 1% neutral agarose gel, and transferred to nitrocellulose. The blot was probed with the HSV-1 *Eco*RI-*Bam*HI fragment I-I (Fig. 1). Lane 1, *ts18*, +PAA; lane 2, ts^+, +PAA; lane 3, ts^+, −PAA; and lane 4, purified HSV-1 DNA digested with *Bam*HI. Lanes 1 and 2 contained 3.5 μg of total nuclear DNA, and lane 3 contained 0.07 μg of total nuclear DNA. The size of the HSV-1 *Bam*HI fragment I (in kilobases) is indicated on the right of the panel.

We determined whether the splice acceptor sites of the 2.6- and 2.3-kb mRNAs were utilized. The probe used was 5′ end labeled at the *Eco*RI site (nucleotide 560) and extended to the *Pst*I site at nucleotide −350. RNA isolated from untreated cells infected with the ts^+ virus protected a major fragment of 590 nucleotides that corresponds to the unspliced mRNA. Bands of 605, 640 (corresponding to the 278- and 310-nucleotide bands discussed above, respectively), 205, 160, and 120 nucleotides (corresponding to the multiple splice acceptor sites of the 2.3-kb mRNA [10, 11]) were also observed (Fig. 7, lanes 3 and 5). To our surprise, we did not detect a 470-nucleotide fragment corresponding to the acceptor site of the minor 2.6-kb mRNA (11). Conceivably, this reflects differences in splice acceptor utilization due to the particular HSV strain used or to different experimental conditions. These results indicate that the same acceptor sites used during a normal lytic infection by the ts^+ virus were used in infections by *ts*18. (Fig. 7, lanes 3 and 4).

γ_2 mRNAs are translated in cells infected with ICP8 mutants. We also determined whether cells infected by ICP8 mutants were competent to translate γ_2 mRNAs. Cells were infected at 39.5°C with *ts*18 or ts^+ virus in the presence or absence of PAA. After an 8-h incubation at 39.5°C, the cells were pulse-labeled with [^{35}S]methionine, extracts were prepared, and proteins were precipitated with the monoclonal antibody C16 (19). This monoclonal antibody immunoprecipitates the fully glycosolated 130,000-M_r form of gC as well as the 110,000-M_r precursor, pgC (7, 43). The precipitated proteins were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. In pulse-labeled cells infected with a replicating virus, the label was incorporated primarily into pgC (Fig. 8, lane 7). The amount of this protein that was immunoprecipitated from PAA-treated cells infected with the ts^+ virus was greatly reduced (Fig. 8, lane 3). Under these latter conditions, small amounts of this protein were reproducibly observed. The amount of pgC in PAA-treated or untreated cells infected with *ts*18 was greater than 10% of the level of pgC observed in wild-type infections (Fig. 8, cf. lanes 2 and 4 with lane 5). Thus, the block in synthesis of this γ_2 protein was partially overcome in cells infected with *ts*18. The levels

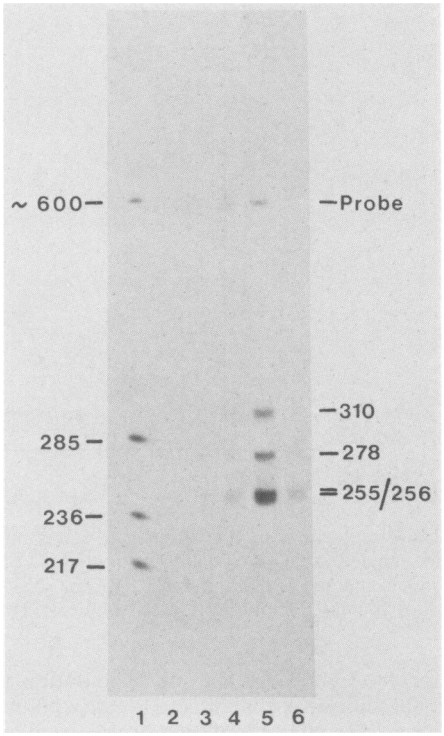

FIG. 6. S1 analysis of gC mRNA initiation sites. At 8 h postinfection, total cellular RNA was isolated from infected or mock-infected cells incubated at 39.5°C in the presence (+AraT) or absence (−AraT) of AraT. The probe used was 5′ end labeled at the *Sst*II site (nucleotide −256) and extended to the *Pst*I site (ca. nucleotide −350) (see Fig. 1). Lane 1, DNA markers; lanes 2 to 5, S1 reactions that contained 50 μg of RNA isolated from cells infected under the following conditions: lane 2, mock-infected, −AraT; lane 3, ts^+, +AraT; lane 4, *ts*18, +AraT; and lane 5, ts^+, −AraT. Lane 6 shows the products of an S1 reaction that contained 5 μg of RNA from ts^+ cells in the absence of AraT and 45 μg of RNA from mock-infected cells in the absence of AraT. The products of the reactions were separated by electrophoresis in an 8 M urea–8% polyacrylamide gel. Autoradiography was performed at −70°C with an intensifying screen. The size of the HSV marker DNA (a mixture generated by digestion of the probe with *Taq*I [nucleotide 217], *Hha*II [nucleotide 236], *Sma*I [nucleotide 285], or *Pst*I [ca. nucleotide 600]) in nucleotides is shown on the left. The size of the protected fragments along with the position of the reannelaled probe are indicated on the right.

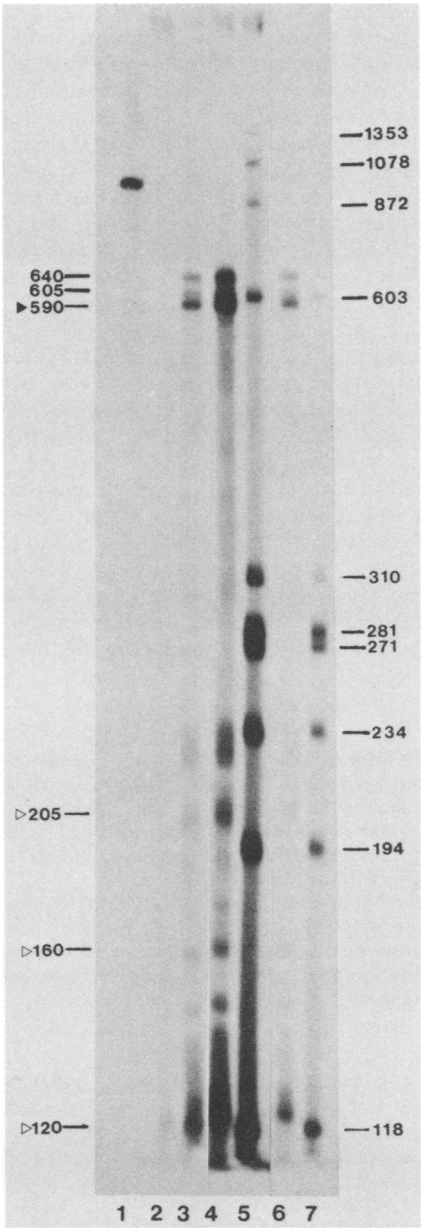

FIG. 7. S1 analysis of gC mRNA splice acceptor sites. Polyadenylated (pA^+) RNAs prepared from PAA-treated cells infected at 39.5°C with *ts*18 or from untreated cells infected with ts^+ virus was analyzed in S1 mapping experiments with a probe labeled at the *Eco*RI site (nucleotide 590) as shown in Fig. 1. Lane 1, Undigested probe; lanes 2 to 4, S1 reactions that contained the following RNAs: 100 μg of tRNA, 2.5 μg of pA^+ RNA from PAA-treated cells infected with *ts*18 and 100 μg of tRNA, and 2.5 μg of pA^+ RNA from untreated cells infected with ts^+ virus and 100 μg of tRNA, respectively. Lane 5, DNA markers. Lanes 6 and 7 show shorter exposures of lanes 4 and 5, respectively. The products of the reaction were separated by electrophoresis in an 8 M urea–4% polyacrylamide gel. The size of the markers (in nucleotides) is shown on the right. The sizes of the bands corresponding to the positions expected for the splice acceptor sites (▷) are shown on the left. Also indicated is the major initiation site (▶) identified by Frink et al. (10, 11).

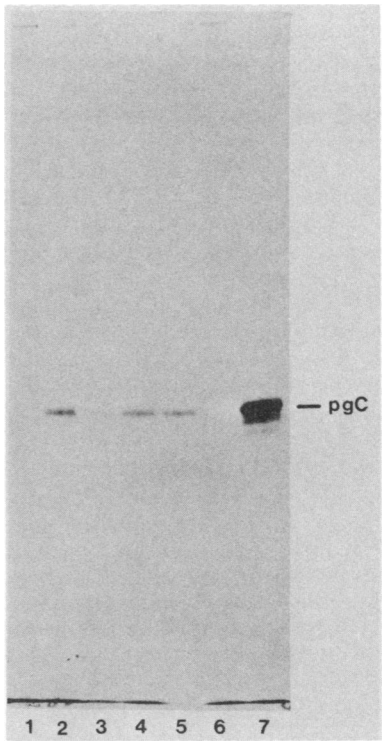

FIG. 8. Synthesis of γ_2 proteins in PAA-treated (+PAA) or untreated (−PAA) cells. At 8 h postinfection, infected or mock-infected cells were pulse-labeled with [^{35}S]methionine, and pgC was precipitated with the monoclonal antibody C16 (18). Labeled pgC was analyzed by electrophoresis and fluorography as described in the text. Lanes 1 to 7 contain proteins immunoprecipitated from extracts of cells infected as follows. Lane 1, Mock-infected, +PAA; lane 2, *ts*18, +PAA; lane 3, ts^+, +PAA; lane 4, *ts*18, −PAA; lane 5, ts^+, −PAA, 1/10 of the immunoprecipitated sample shown in lane 7; lane 6, mock-infected, −PAA; and lane 7, ts^+, −PAA.

of synthesis of this protein, as measured by immunoprecipitation, were proportional to the amounts of gC mRNAs in the cytoplasm determined by Northern hybridization (Fig. 2A).

DISCUSSION

We have examined the effect of a defect in the major DNA-binding protein of HSV-1 on the expression of γ_2 mRNAs. When DNA replication is blocked by drugs that inhibit the viral DNA polymerase, the accumulation of gC mRNAs is drastically reduced although not totally eliminated. The major DNA-binding protein appears to be involved in the negative regulation of γ_2 gene expression because defects of this protein resulted in an increased accumulation of γ_2 mRNAs. We did not detect an influence of viral DNA replication or ICP8 defect on the site of initiation or in the relative utilization of the multiple splice acceptor sites. Under all conditions examined in this study, the gC mRNA can be translated in vivo.

It is very unlikely that the increase in synthesis of γ_2 mRNAs in ICP8 mutant-infected cells results from an increase in residual DNA synthesis compared with PAA- or AraT-treated cells infected with the ts^+ virus. At 39.5°C, the mutant is defective in ICP8, an essential function for viral DNA replication (8, 18, 28, 49). The viral DNA polymerase appears to be equally sensitive to PAA in these strains because the efficiency of plating of *ts*18, ts^+, or 18*Rs*1 virus

at 33°C or ts^+ or 18*Rs*1 virus at 39.5°C in various concentrations of PAA are similar (data not shown). In addition, the increased expression of this γ_2 mRNA is not the result of a difference in the number of DNA molecules that enter the nucleus at 39.5°C. Thus, the increase in the accumulation of gC mRNAs appears to result from a defect in ICP8.

Control of viral late gene expression. It is well documented that the accumulation HSV γ_2 mRNAs is greatly reduced in the absence of DNA replication (10, 11, 17, 18). Preliminary experiments suggest that the rate of transcription of the gC gene is strongly inhibited in the absence of viral DNA replication (P. Godowski and D. Knipe, unpublished data). However, the results reported here suggest that DNA replication is not an absolute requirement for γ_2 gene expression. This is consistent with experiments reported by others. Several investigators have constructed cell lines containing viral or chimeric genes with HSV γ_1 (9, 41) or γ_2 (42) promoters stably integrated into the cellular genome. Although superinfection of these cell lines with HSV results in transactivation of these genes, amplification of the DNA sequences containing these genes does not appear to be necessary for promoter activity.

One model to explain the regulation of γ_2 gene expression is as follows. The transcription of γ_2 genes is dependent on the synthesis of α proteins. However, γ_2 genes are transcribed only at very low levels from unreplicated genomes. The low level of γ_2 transcription would be due to the intrinsically weak activity of these promoters and inhibition by viral and cellular *trans*-acting factors. Viral DNA replication alters the template structure, resulting in the activation of γ_2 promoters. This, along with genome amplification, results in high-level expression of γ_2 genes.

In the context of this model, ICP8 is involved, either directly or indirectly, in the repression of transcription of these genes. ICP8 can bind to either single- or double-stranded DNA (24, 29, 36), but no sequence specificity has been observed. ICP8 could repress transcription by coating double-stranded viral DNA in a nonspecific manner. Alternatively, ICP8, perhaps together with additional proteins, recognizes specific features of viral DNA not reproduced in in vitro DNA-binding assays, such as chromatin structure.

A second possibility is that ICP8 acts at a post-transcriptional level to control γ_2 mRNA accumulation. In this model, γ_2 mRNAs are transcribed from parental genomes but are rapidly degraded. DNA replication would result in genome amplification, high-level transcription of γ_2 genes, and thus, accumulation of detectable levels of γ_2 transcripts. In the context of this model, ICP8 could act directly or indirectly to affect the processing or stability of viral mRNAs. It has been reported that the functional half-life of γ mRNAs is relatively short (20, 50). ICP8 could affect the processing or stability of these transcripts, either at the nuclear matrix (37) or in a transient association with cytoplasmic ribonucleoprotein particles (M. Quinlan, Ph.D. thesis, Harvard University, Cambridge, Mass., 1984). Defects in ICP8 might result in a less rapid turnover of these messages and their subsequent accumulation. During the infection of cells by wild-type virus, ICP8 could bind to progeny DNA molecules and prevent its binding to RNA, resulting in more stable γ mRNAs.

Multifunctional proteins and their role in viral replicative cycles. The simian virus 40 T antigen, the adenovirus 72K protein, and the HSV ICP8 play multifunctional roles in their respective viral replicative cycles. These proteins are essential for viral DNA replication and function in the regulation of viral gene expression. Elegant studies have shown that simian virus 40 T antigen directly regulates early gene expression by binding to high-affinity sites near the viral origin of replication (1). Simian virus 40 T antigen increases late gene expression directly by binding to viral DNA and perhaps indirectly through genome amplification (23). The adenovirus 72K protein is essential for adenovirus DNA replication (46). This protein also appears to regulate the expression of early genes transcribed from the EIa, EIb, EII, and EIII regions apparently by affecting the stability of the mRNAs (3). This protein specifically represses the transcription of the E4 transcription unit both in vivo and in vitro (15, 35). Like the ICP8 protein, it binds to single-stranded DNA (45). To the best of our knowledge, sequence-specific binding of DNA by the adenovirus 72K protein has yet to be demonstrated. The mechanism by which ICP8 affects HSV gene expression is still unclear. Current data are consistent with ICP8 affecting the transcription of HSV early genes, but this has not been demonstrated directly (14). This study shows that ICP8 lowers the expression of HSV γ_2 genes from parental viral genomes. The results of this study are also consistent with the observations of Silver and Roizman (42) that ICP8 is not required directly for the activation of γ_2 promoters. However, ICP8 may act indirectly to increase γ_2 gene expression by promoting viral DNA replication. The mechanism by which ICP8 affects viral gene expression and the possible significance of this function in the lytic and latent life cycles of HSV are undergoing further investigation.

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