Virology 283, 273–284 (2001)

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Herpes Simplex Virus 1 ICP27 Is Required for Transcription of Two Viral Late (γ2) Genes in Infected Cells

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Received January 18, 2001; accepted March 1, 2001

The herpes simplex virus infected cell protein 27 (ICP27) is required for the expression of certain early viral proteins and for many late proteins during productive infection. Expression of at least one late (γ2) gene, that encoding glycoprotein C, is severely restricted in the absence of functional ICP27. The exact mode of action by which ICP27 induces late gene expression is not known, but the effect is apparent at the mRNA level as demonstrated by Northern blot analysis. To determine whether ICP27 activates late genes via transcriptional or posttranscriptional mechanisms, we initially used nuclear run-on assays to measure transcription of viral genes in Vero cells infected with wild-type (WT) virus or an ICP27 nonsense mutant virus, *n*504. We observed a 4-fold reduction in the nuclear run-on signal from the coding strand of the *gC* gene for *n*504-infected cells compared to that of WT-infected cells. However, interpretation of the results was complicated by the observation of a significant signal from the noncoding strand in these experiments. To obviate the problem of symmetrical transcription, we utilized *in vivo* RNA pulse-labeling to measure the amount of transcription of viral genes in cells infected with either WT virus or *n*504 virus. We found a 5- to 10-fold reduction in the transcription of the *gC* and U_L47 genes, two late genes, in cells infected with *n*504 compared to that in cells infected with WT virus. In contrast, transcription of the ICP8 gene, an early gene, was similar in WT and *n*504 virus-infected cells. We also examined the stability of the *gC* and U_L47 gene transcripts in *n*504-infected cells, and we found it to be comparable to that in WT virus-infected cells, further supporting an effect on transcription. Transcription of the *gC* and U_L47 genes by *n*504 was normal in a cell line that expresses WT ICP27. From these results we conclude that ICP27 is required for transcription of the late *gC* and U_L47 genes during productive infection. © 2001 Academic Press

INTRODUCTION

The cascade pattern of viral gene expression during productive infection by herpes simplex virus 1 is well documented (Clements *et al.,* 1977; Honess and Roizman, 1974, 1975; Jones and Roizman, 1979; Wagner, 1985). Soon after penetration of the host cell by the virus and transport of the capsid to the nucleus, the 152-kbp double-stranded DNA genome circularizes and is transcribed in the nucleus by the host RNA polymerase (pol) II (Costanzo *et al.,* 1977). Three broad classes of viral genes are expressed in a temporally coordinated fashion: α or immediate-early (IE), β or early, and γ or late. The α genes do not require *de novo* protein synthesis for expression, but transcription of these genes is stimulated by the VP16 virion protein and is maximal between 2 and 4 h postinfection. The α proteins regulate later classes of viral genes. Of the five IE proteins, two are essential for viral growth: infected cell polypeptide (ICP) 4 (Dixon and Schaffer, 1980; Knipe *et al.,* 1978; Watson and Clements, 1980) and ICP27 (Sacks *et al.,* 1985). The second group of genes that are expressed is the β genes. Expression of all β genes requires ICP4 (Knipe *et al.,* 1978; Preston, 1979) and expression of some β genes also requires ICP27 (McGregor *et al.,* 1996; Samaniego *et al.,* 1995; Uprichard and Knipe, 1996). β Proteins are synthesized maximally between 4 and 6 h postinfection and are involved mainly in viral DNA replication and metabolism. The last group of genes that are expressed is the γ genes, the protein expression of which peaks after 10 h postinfection. The γ proteins are involved in virus assembly, maturation, and egress. They are further subdivided into two subgroups, γ1 and γ2. The γ1 genes are expressed at significant levels in the absence of viral DNA replication but are further increased by viral DNA synthesis, whereas γ2 proteins are produced only if viral DNA synthesis takes place. The mechanisms involved in the transition from early to late gene expression is not well understood, but the expression of late genes requires one *cis*-acting factor, viral DNA replication, and at least three *trans*-acting factors: ICP4 (Dixon and Schaffer, 1980; Knipe *et al.,* 1978; Watson and Clements, 1980), ICP8 (Chen and Knipe, 1996; Gao and Knipe, 1991), and ICP27 (McCarthy *et al.,* 1989; Rice *et al.,* 1989; Uprichard and Knipe, 1996).

The IE protein ICP27 is a nuclear phosphoprotein

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(Ackermann *et al.,* 1984; Hay and Hay, 1980; Wilcox *et al.,* 1980) that plays an important role in the expression of β and γ genes and during productive infection. Studies of viruses with temperature sensitive (ts) and deletion mutations in the ICP27 gene demonstrated that ICP27 is required for viral DNA synthesis and γ gene expression (McCarthy *et al.,* 1989; Rice *et al.,* 1989; Sacks *et al.,* 1985). The effect of ICP27 on viral DNA synthesis is in large part the result of stimulation of early gene expression (McGregor *et al.,* 1996; Samaniego *et al.,* 1995; Uprichard and Knipe, 1996), in particular of those genes encoding the less abundant viral DNA replication proteins (Uprichard and Knipe, 1996). A number of other functions or effects of ICP27 have been reported, including inhibition of host cell RNA splicing (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994), rearrangement of host cell snRNPs (Phelan *et al.,* 1993), binding to RNA (Brown *et al.,* 1995; Ingram *et al.,* 1996; Mears and Rice, 1996), shuttling from the nucleus to the cytoplasm (Phelan and Clements, 1997; Soliman *et al.,* 1997), regulation of differential polyadenylation (Hann *et al.,* 1998; McGregor *et al.,* 1996; McLauchlan *et al.,* 1992), interaction with ICP4, the major viral transcriptional activator (Panagiotidis *et al.,* 1997), and localization to replication compartments, the site of late transcription (de Bruyn Kops *et al.,* 1998).

The ability of ICP27 to promote late viral gene expression is likely to be a function distinct from its ability to stimulate viral DNA replication because of the phenotype of a mutant that we isolated, *n*504, which contains a nonsense mutation in the ICP27 gene and encodes only the amino-terminal 504 residues of ICP27. Like the ts mutant viruses, *n*504 is defective for late gene expression, while maintaining normal levels of viral DNA replication (Rice and Knipe, 1990). The *n*504 mutant virus has a phenotype that clearly separates ICP27's role in promoting viral DNA replication from its role in late gene regulation. Further analysis of the *n*504 virus revealed that ICP27 possesses two transactivating functions, one that stimulates γ1 gene expression and another that induces γ2 genes (Rice and Knipe, 1990).

The mechanism(s) by which ICP27 promotes late gene expression in infected cells remains to be elucidated despite the plethora of activities and effects that have been associated with it. Several studies have addressed the question of the stage of late gene expression that is affected directly or indirectly by ICP27. McCarthy *et al.* (1989) measured transcription rates of viral genes in infected cells, using nuclear run-on assays. Although they found a marked reduction in transcription for the late *gC* gene, this effect could have resulted from the lack of viral DNA replication in cells infected with the deletion mutant used. Smith *et al.* (1992) measured transcription of the *gC* gene in cells infected with *ts*LG4, a virus containing a ts mutation mapping in the ICP27 gene, and found that transcription was reduced by about 10-fold at the nonpermissive temperature. However, when the infection was conducted at the permissive temperature and later shifted to the nonpermissive temperature, transcription of the *gC* gene in the ts mutant-infected cells was similar to that in the wild type (WT)-infected cells. The authors concluded that ICP27 regulates these genes at the posttranscriptional level. However, the *ts*LB4 ICP27 may not be thermolabile once properly folded at the permissive temperature and thus not inactivated by temperature shift-up. The lack of an effect upon temperature shift need not indicate a posttranscriptional effect. Recently, some studies have observed a correlation between the ability of ICP27 to shuttle from the nucleus to the cytoplasm and the onset of late viral gene expression, suggesting that ICP27 stimulates late gene expression by shuttling late transcripts from the nucleus to the cytoplasm (Soliman *et al.,* 1997).

To attempt to reconcile these results and to determine if ICP27 exerts an effect that stimulates late gene expression via a transcriptional or posttranscriptional mechanism, we have used the method of *in vivo* labeling of RNA to measure transcription rates of viral genes in infected Vero cells. Our results show that ICP27 promotes transcription of two γ2 genes, *gC* and U_L47, in infected Vero cells.

RESULTS

Measurement of transcription of γ2 genes

We had shown previously that ICP27 is required for γ gene expression and that the effect is apparent at the mRNA level (Rice and Knipe, 1990). In this study we wished to determine whether ICP27 affects transcriptional or posttranscriptional processes to stimulate γ gene expression. Thus, we initially used a nuclear run-on assay, which we had used previously (Godowski and Knipe, 1986) to measure levels of transcription in infected cells. To eliminate any potential effects of ICP27 stimulation of viral DNA synthesis on late gene expression, we utilized the *n*504 ICP27 nonsense mutant virus. This mutant virus replicates viral DNA to nearly WT levels, but does not induce γ2 gene expression (Rice and Knipe, 1990). Thus, through the use of this mutant, any observed effects of ICP27 on γ gene expression could not be explained by the absence of viral DNA replication but by a more direct effect of ICP27 on γ gene expression. When we compared nuclear run-on transcription levels of the *gC* gene in nuclei from cells infected with *n*504 or WT virus, we observed that the levels of coding strand (detected by the complementary, or "c" probe) transcription from the *gC* (Figure 1), U_L47, or *ICP5* (results not shown) genes were reduced by about fourfold in Vero cells infected with *n*504 virus compared to those of WT virus. However, significant transcription from the noncoding strand (detected by the anti-complementary, or "a"

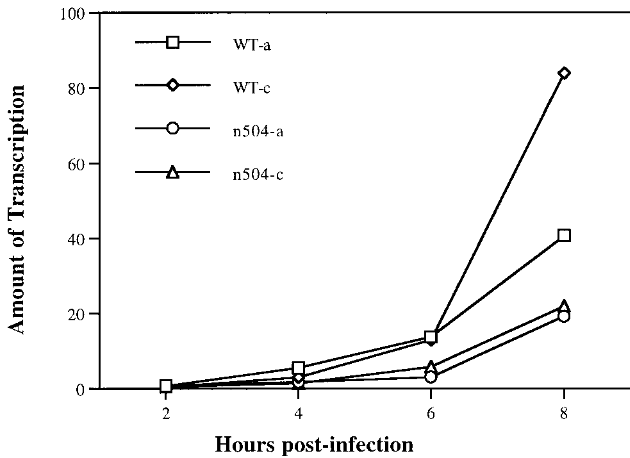
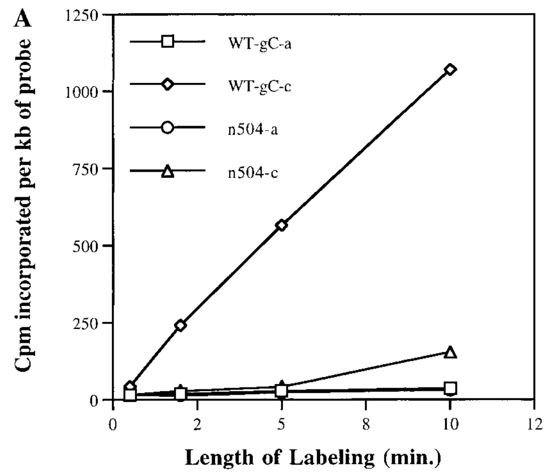
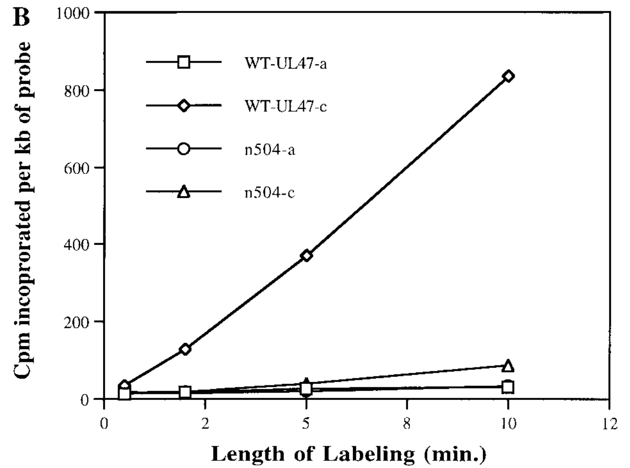


FIG. 1. Transcription of the gC gene in WT- and *n*504-infected Vero cells as measured by nuclear run-on assays. Vero cells were infected with the KOS1.1 strain of WT HSV-1 or the *n*504 mutant virus. After 2, 4, 6, and 8 h of infection, nuclei were isolated for nuclear run-on assays. Transcripts from the *gC* gene were detected by hybridization of ^{32}P-labeled RNA with single-stranded DNA probes blotted onto nylon membranes. ◇, RNA from WT-infected cells hybridized to complementary strand; □, RNA from WT-infected cells hybridized to anti-complementary strand; △, *n*504-infected cell RNA hybridized to complementary strand; ○, *n*504-infected cell RNA hybridized to anti-complementary strand.

probe) was observed (Fig. 1 and results not shown for *ICP5* or U_L47), and, in fact, nearly equivalent levels of transcription from the two strands were observed for the *n*504 mutant virus-infected cell nuclei. The "symmetrical" transcription cannot be explained by the transcription of any known gene encoded on the opposite DNA strand from that encoding the gC mRNA. This effect has been observed in several other studies utilizing nuclear run-on analyses, particularly at late times postinfection (Godowski and Knipe, 1986; McCarthy *et al.,* 1989; Smith *et al.,* 1992). We were concerned that the symmetrical transcription observed might represent artifacts that could obscure true levels of transcription of the *gC* gene.

Zhang *et al.* (1987) had reported that *in vivo* RNA pulse-labeling showed hybridization specifically to the coding strands, even using RNA isolated at late times after herpes simplex virus (HSV) infection. Therefore, we attempted to circumvent the problem of the apparent symmetrical transcription by *in vivo* pulse labeling RNA in infected cells with [^{3}H]uridine. Vero cells were infected with WT virus for 6 h at a multiplicity of infection (m.o.i.) of 20 plaque-forming units (PFU) per cell. The infected cells were labeled for varying lengths of time from 30 s to 10 min at 37°C. We observed that labeled RNA hybridized specifically to the coding strand of the *gC* gene (Fig. 2A), the U_L47 gene (Fig. 2B), and the *ICP8* gene (Fig. 2C), indicating that the problem of symmetrical transcription from nuclear run-on assays had been avoided using this approach. Furthermore, incorporation of label into specific RNAs was linear for labeling periods through 10 min





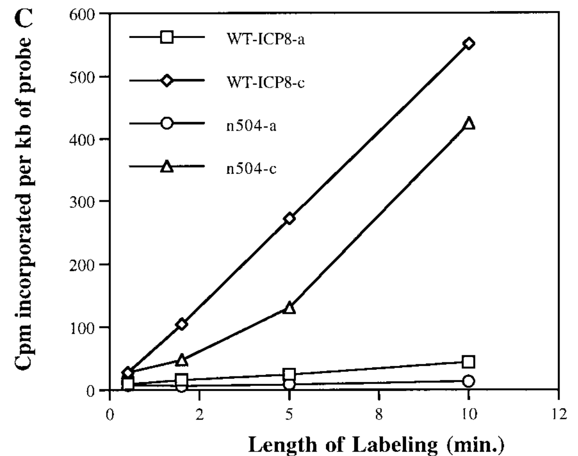


FIG. 2. Transcription of viral genes as measured by *in vivo* RNA pulse labeling. Vero cells were infected with either WT or *n*504 virus. At 6 h postinfection, the infected cells were labeled with [^{3}H]uridine for 0.5, 2, 5, and 10 min at 37°C. mRNA transcripts specific for gC (A), U_L47 (B), and ICP8 (C) were detected by hybridization to single-stranded DNA probes blotted onto nylon membranes. The amount of labeled RNA hybridized to the filters was determined by scintillation counting. ◇, WT RNA, complementary probe; □, WT RNA, anti-complementary probe; △, *n*504 RNA, complementary probe; ○, *n*504 RNA, anti-complementary probe.

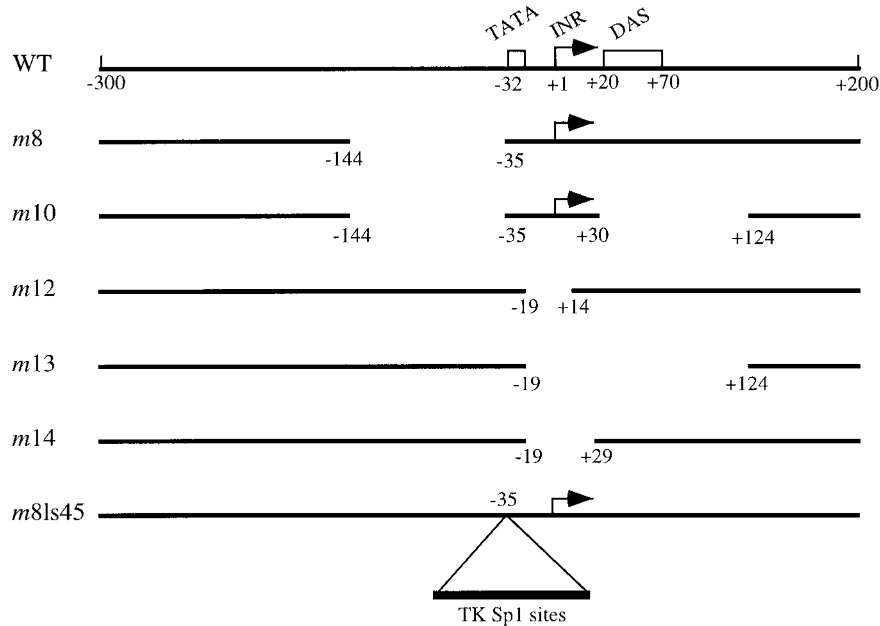


FIG. 3. Promoter structure of wild-type *gC* gene and mutant viruses. WT promoter labels: TATA, −32 indicates the position of the first T of the TATA box sequence (TATAAA) from the start site of transcription (+1). INR, initiator-like element. The start site of transcription is based on Homa *et al.* (1986), and this places the 5′ terminus of the gC mRNA 5 nucleotides 3′ of that mapped by Frink *et al.* (1983). The nucleotide sequence of the initiator element of *gC* gene: CTACCCTCACTACC, where the reported transcription start sites of *gC* gene are underlined. The A at the position 3 and the doublet, TC, at positions 7 and 8 are the start sites of transcription determined by Frink *et al.* (1983) and Homa *et al.* (1986), respectively. DAS, downstream activating sequence.

in this experiment so that the slopes of the plots in Fig. 2 were a good measure of the amount of transcription of each gene.

To determine if the pulse-labeling technique could measure quantitative differences in transcription, we measured the levels of transcription from *gC* genes with altered promoters. For this experiment we utilized viruses with the *gC* promoter mutations isolated by Homa *et al.* (1988) (Fig. 3). gC mRNA levels were measured by Northern blot hybridization, and transcription levels were measured by pulse-labeling of RNA for 10 min at 6 h postinfection (h.p.i.) and hybridization to the gC probes. These data showed that mRNA levels correlated very well with transcription levels (Table 1). For example, the promoter mutation in *m*13 reduced RNA levels by 8-fold relative to WT virus, and a 7-fold reduction in transcription was observed. In contrast, the *m*81s45 mutation increased RNA levels by 2.9-fold, and transcription was increased by 2.1-fold. Therefore, the uridine labeling technique was capable of measuring quantitative differences in transcription induced by different forms of the gC promoter.

When we used the pulse-labeling technique to compare transcription in WT and *n*504 mutant virus-infected cells, we observed that the *n*504 mutant virus-infected cells showed approximately 9-fold less transcription of the *gC* gene than WT virus-infected cells (Fig. 2A). In several experiments, the reduction in transcription of the *gC* gene by the *n*504 virus ranged from 5- to 24-fold relative to that of WT virus. Transcription of the U_L47 gene, another γ2 gene (McLean *et al.,* 1990), was reduced in *n*504 virus-infected cells to a similar extent (Fig. 2B). In contrast, transcription of the β *ICP8* gene was similar in WT and *n*504 virus-infected cells (Fig. 2C). Parallel Northern blot assays of total gC and U_L47 RNA showed 11- to 12-fold reductions in mRNA levels in *n*504 mutant virus-infected cells compared to RNA from WT virus-infected cells (Jean and Knipe, results not shown). These results argued that the increase in transcription

TABLE 1

Correlation between the Levels of Transcription and *gC* Gene Transcripts in Infected Vero Cells

Virus	Relative gC transcription level[a]	Relative gC mRNA level[b]
*m*8	0.71	0.87
*m*10	1.2	1.1
*m*12	0.24	0.18
*m*13	0.14	0.12
m14	0.58	0.47
*m*81s45	2.1	2.9
WT	1.0	1.0

[a] The amount of transcription was determined by a 10-min pulse-label with [^{3}H]uridine at 6 h.p.i. The values represent the amount of *gC* gene transcription for that virus normalized to WT virus.

[b] These levels of mRNAs were based on Northern analysis followed by quantitation with a phosphorimager. The values represent the amount of gC mRNA expressed by that virus normalized to WT virus.

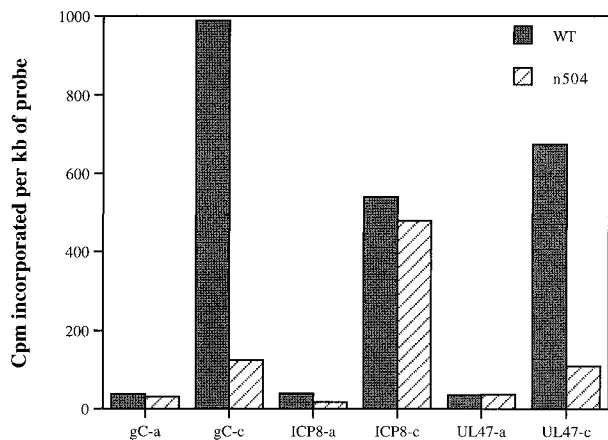


FIG. 4. Incorporation of [^{3}H]uridine into nuclear RNA in Vero cells infected with WT or *n*504 virus during a 10-min pulse-label. Vero cells were infected for 6 h, after which they were labeled for 10 min with [^{3}H]uridine at 37°C. Nuclear RNA was prepared and hybridized to ssDNA probes blotted onto nylon membranes. The amounts of specific gC, U_L47, and ICP8 RNA transcripts hybridized to the filters were determined by scintillation counting.

was a major determinant of gC and U_L47 mRNA accumulation in HSV-infected Vero cells.

We then reasoned that, if the effect of ICP27 on late gene expression was exerted at the transcriptional level, the difference should be observed in nuclear RNA as well as in total RNA from infected cells. Thus, we pulse labeled Vero cells for 10 min, isolated nuclear RNA, and used the RNA in a hybridization reaction. Indeed, the pulse-labeled nuclear transcript levels for gC and U_L47 were reduced by 9-fold in the *n*504-infected cells compared to those for WT virus-infected cells (Fig. 4) just as for total cell RNA. Very low levels of hybridization to the anti-complementary probe were observed (Fig. 4). As above, transcription of the *ICP8* gene was similar in WT and mutant virus-infected Vero cells (Fig. 4). Moreover, while the total amount of nuclear RNA isolated was low (10% of total RNA, as expected), the total amount of [^{3}H]uridine incorporated into nuclear RNA was comparable to that obtained from total RNA isolation. This demonstrated an effect of ICP27 on nuclear RNA levels in infected cells and confirmed the results with whole cell RNA.

In vivo labeling of *gC* and *U_L47* gene transcripts in WT and *n*504 virus-infected Vero cells during varied labeling periods

The amount of incorporation of [^{3}H]uridine into mRNA is a direct measure of the activity of RNA polymerase II. The shorter the labeling period, the more the measurement reflects the activity of the polymerase enzyme. However, if the RNA is unstable (for example, with a half-life less than 10 min), posttranscriptional processes that lead to changes in stability could still affect the net amount of labeled RNA detected in the hybridization reaction, even during short labeling periods. We first addressed the question of RNA stability by examining the accumulation of labeled RNA during labeling periods of different lengths. If transcriptional regulation was the sole or main mechanism by which ICP27 affects the expression of late genes, one would expect the ratio of [^{3}H]uridine incorporation into specific γ viral RNAs between WT and *n*504 virus-infected cells to remain unchanged during long labeling periods. However, effects on other processes such as RNA stability would cause the ratio of the level of uridine incorporation in *n*504 versus WT virus-infected cells to increase accordingly. By the latter scenario, the net effect would then become more pronounced as the cells were labeled for longer times. Thus, we measured gC RNA levels in cells labeled for 10 min or 3 h starting at 6 h.p.i. Total RNA was isolated, DNase-treated, and then used in a hybridization reaction with single stranded DNA probes for the *gC, ICP8,* and *U_L47* genes, as described above. We observed that the ratio of gC transcripts for WT to *n*504 was similar for 10 min or 3 h of labeling (Table 2), providing additional evidence that the differences in RNA levels were due to differences in synthesis and not differences in processing or stability.

Relative stabilities of gC and U_L47 mRNAs during infection with WT and *n*504 viruses

The previous experiment indicated that the stability of the gC transcript was not altered in cells infected with the ICP27 mutant *n*504 virus. To look more directly at the stabilities of gC and U_L47 mRNAs, we conducted a pulse-chase experiment. Although it is difficult to obtain reliable absolute measurements of RNA half-lives, the procedure was suitable for comparing relative mRNA stabilities in cells infected with different viruses. Cells were infected for 6 h and then pulse-labeled for 1 h with [^{3}H]uridine. The cells were then washed with PBS and incubated for 0, 1, 2, and 3 h with medium containing 100 μg/ml unlabeled uridine and 35 mM glucosamine at 37°C, uridine chase conditions used by Pilder *et al.* (1986). Total RNA was isolated and then subjected to single-stranded hybridization to (ss)DNA probes for gC,

TABLE 2

Incorporation of [^{3}H]Uridine into gC mRNA during Varied Labeling Times

Labeling time (min)	Ratio of gC transcription by WT virus to gC transcription by *n*504 virus			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
10	4.9	8.5	24	5.7
180	4.7	7.8	13	6.3

Note. These numbers were obtained by dividing the net amount of incorporation of [^{3}H]uridine into gC mRNA in WT virus-infected Vero cells by that in *n*504 virus-infected cells.

TABLE 3

Incorporation of [3H]Uridine into RNA in Infected Vero Cells during Pulse-Chase Labeling Conditions

Length of chase (min)	Total cpm incorporated in RNA from cells infected with [a]	
	WT	*n* 504
0	2.3×10^7	3.6×10^7
60	1.3×10^7	3.2×10^7
120	0.93×10^7	3.1×10^7
180	0.22×10^7	3.1×10^7

[a] Cells were pulse-labeled with [3H]uridine for 1 h as described under Materials and Methods and then chased for various lengths of time in media containing unlabeled uridine and glucosamine. The data are presented as the total amount of radioactivity in RNA preparations.

ICP8, and U_L47. The total amount of [3H]uridine incorporation into the RNAs of the infected cells did not increase during the chase (Table 3), consistent with chase conditions being imposed. We found no difference in the stabilities of gC and U_L47 (or ICP8) transcripts between WT and *n*504 virus-infected cells (Figs. 5A–5C). Thus, the ICP27 function that is lost in the *n*504 virus did not affect the stability of late mRNA sequences, as measured by the ability to hybridize to the ssDNA probes.

Complementation of the transcriptional defect in cells expressing WT ICP27

The results described above demonstrated that a virus expressing a mutant ICP27 shows reduced transcription of γ2 genes. To ensure that the phenotype of the *n*504 virus was due to the ICP27 defect and not to other mutations, we measured transcription of viral genes in V27 cells, which express ICP27 upon HSV-1 infection (Rice and Knipe, 1990). We infected V27 cells with the *n*504 nonsense mutant, the *d*27 null mutant, and WT viruses and then labeled the cells with [3H]uridine for 10 min at 37°C. Total RNA was extracted and hybridized to single-stranded DNA probes as before. As a control, parallel experiments were conducted in Vero cells. We found that both *d*27 and *n*504 viruses transcribed the gC and U_L47 genes in V27 cells at levels similar to those of WT virus (Fig. 6). Thus, ICP27 was specifically required for transcription of the two γ2 genes, gC and U_L47.

ICP27 stimulation of expression of mutant *gC* genes

To attempt to determine the sequences in the *gC* gene promoter region that were required for transcriptional stimulation by ICP27, we isolated double mutant viruses containing the *n*504 mutation in the *ICP27* gene and each of the *gC* gene promoter mutations described in Fig. 3. gC mRNA levels were determined in Vero cells and in V27 cells infected with each of the mutant viruses. To compensate for differences in recovery, the gC RNA

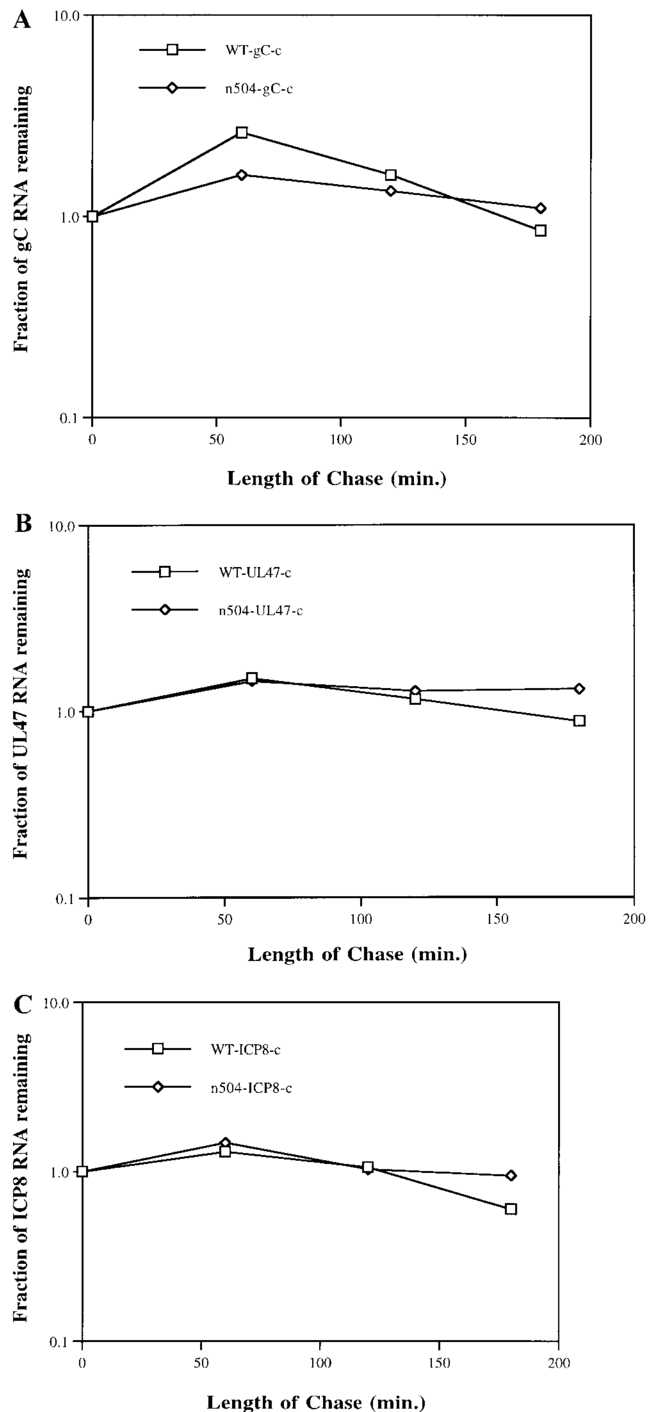


FIG. 5. Pulse-chase analysis of viral RNAs in Vero cells infected with either WT (□) or *n*504 (◇) virus. Cells were infected for 6 h and subsequently labeled with [3H]uridine for 1 h. The cells were then washed with cold phosphate-buffered saline and refed with medium containing 100 μg/ml unlabeled uridine and 35 mM glucosamine. The cells were incubated at 37°C for 0, 1, 2, or 3 h, at which time total RNA was extracted. Specific labeled viral RNAs for gC (A), U_L47 (B), or ICP8 (C) were detected by hybridization to single-stranded probes blotted onto nylon filters. The level of radioactivity on each piece of filter was determined by scintillation counting. The fraction of mRNA remaining was calculated as a ratio of mRNA at a given time point relative to the amount of the start of the chase period.

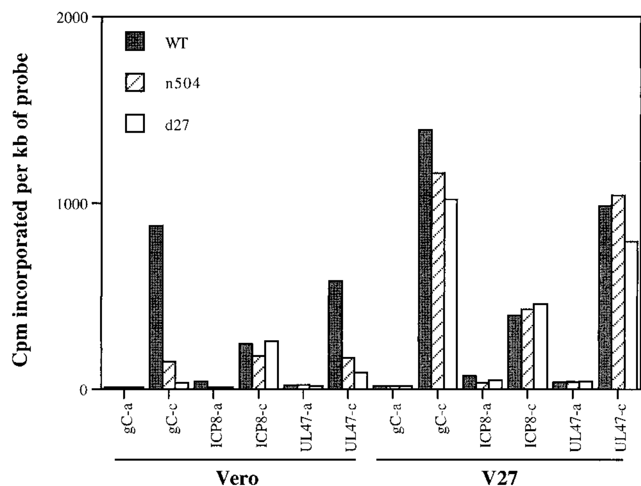


FIG. 6. Transcription of the *gC*, U_L47, and *ICP8* genes in Vero and V27 cells. Vero or V27 cells were infected with WT, *n*504, or *d*27 (open bars) virus for 6 h. The infected cells were labeled for 10 min as described in the legend to Fig. 4. Specific labeled RNA transcripts for gC, U_L47, and ICP8 were detected by hybridization to ssDNA probes blotted onto nylon membranes followed by scintillation counting.

values were normalized for ICP8 mRNA levels in the preparation. Comparison of the normalized gC mRNA levels in the presence of ICP27 (V27 cells) to the absence of WT ICP27 (Vero cells) yielded the ICP27 induction ratio for each mutant *gC* gene (Table 4). All of the mutant *gC* genes tested were induced by ICP27 to relatively similar levels, with only *dm*10 showing a slight decrease in the ICP27 induction ratio (Table 4). Thus, none of the individual sequences altered in these mutant constructs was essential for induction by ICP27.

DISCUSSION

Evidence for an effect of ICP27 on the transcription of γ genes in infected cells

It is well established that ICP27 is required for the transition from early to late gene expression during productive infection (McCarthy *et al.,* 1989; Rice and Knipe, 1988, 1990; Sacks *et al.,* 1985; Smith *et al.,* 1992). We have shown that induction of *gC* gene expression by ICP27 occurs at the mRNA level, as demonstrated by Northern analysis (Rice and Knipe, 1990; Rice *et al.,* 1989). This work provides evidence that the effect of ICP27 on expression of at least two late genes is exerted either directly or indirectly via a transcriptional mechanism. Our results show specifically that transcription of two γ2 genes, *gC* and U_L47, is decreased by approximately 10-fold in cells infected with an ICP27 nonsense mutant virus, *n*504, compared to that in cells infected with WT virus. We also showed that the stability of the *gC* and U_L47 gene transcripts is very similar in *n*504 and WT virus-infected cells. Thus, the magnitude of the transcriptional effect is sufficient to fully explain the decrease in γ2 mRNA levels in *n*504 mutant virus-infected cells. We conclude that the missing function in *n*504 ICP27 stimulates γ2 gene transcription. However, this does not rule out other possible effects of ICP27 on γ2 gene expression, such as posttranscriptional or posttranslational effects, because other functions could be performed by other portions of ICP27.

The ability of ICP27 to increase transcription of γ2 viral genes could be due to an increased rate of transcriptional initiation, an increased rate of elongation, a decreased rate of premature transcriptional termination, or a combination of these mechanisms. Our analysis of mutated *gC* gene promoters failed to identify a unique sequence needed for ICP27 induction. While this may suggest that transcriptional initiation is not affected by ICP27, this conclusion is premature because ICP27 may be able to induce transcription of the *gC* genes via multiple promoter-binding factors. We wish to emphasize that the increase in transcription could be due to (1) a direct effect of ICP27 on transcriptional processes, (2) an indirect effect of ICP27 on viral or cellular proteins by posttranslational modification of the proteins, such as phosphorylation, or (3) an indirect effect of ICP27 on expression of viral or cellular gene products involved in transcriptional processes. Possible mechanisms will be explored further below.

Our observations are consistent with two earlier reports in the literature that examined the effect of ICP27 on the transcription of late genes. Using nuclear run-on assays, McCarthy *et al.* (1989) measured transcription rates of γ genes in infected cells. They reported that transcription of the *gC* gene was reduced in cells infected with ICP27 null mutant viruses. However, this reduction could also be explained by the significantly

TABLE 4

Stimulation of Expression of the *gC* Gene in Infected Cells

	Levels of mRNA[a]				
	Vero cells (−ICP27)		V27 cells (+ICP27)		
Virus	gC	ICP8	gC	ICP8	ICP27 induction ratio[b]
*dm*8	6505	7134	84356	7957	11.6
*dm*10	16038	7716	161470	9795	7.9
*dm*12	736.1	5678	13303	6935	14.8
*dm*13	946.1	6345	15537	7091	14.7
*dm*14	4283	6138	75657	6433	16.9
*n*504	5859	8921	92462	8289	17.0

[a] Levels of gC and ICP8 mRNA were determined by Northern analysis and quantitation of a phosphor screen with a phosphorimager. The values are in arbitrary units.

[b] The induction ratio of the gC mRNA was calculated as

$$\frac{\text{gC}(+\text{ICP27})/\text{gC}(-\text{ICP27})}{\text{ICP8}(+\text{ICP27})/\text{ICP8}(-\text{ICP27})}.$$

reduced levels (about 10 to 20% of WT) of viral DNA replication in these mutant infected cells and not by a specific effect of ICP27 on *gC* gene transcription. In addition, they observed "symmetrical" hybridization that complicated interpretation of the results. Using a similar approach, Smith *et al.* (1992) observed that transcription of the *gC* gene is decreased by 5- to 10-fold in cells infected with an ICP27 ts mutant, *ts*LG4, compared to that in cells infected with WT virus. However, when the authors conducted a "temperature-shift" experiment in which cells were first infected at the permissive temperature and then later incubated at the nonpermissive temperature, they observed that transcription of the *gC* gene was comparable in WT and *ts*LG4 virus-infected cells. Because transcription was not altered following the temperature shift-up, they concluded that ICP27 stimulates late gene expression via a posttranscriptional mechanism. However, an alternative explanation for these results is that the stimulatory function of ICP27 is thermostable; thus, incubation at the nonpermissive temperature following infection at the permissive temperature did not render the ICP27 protein nonfunctional. Our results represent the first unequivocal demonstration of the transcriptional stimulation of γ genes by ICP27. This work was facilitated by the use of *in vivo* pulse-labeling of RNA and a nonsense mutant virus, *n*504, that uncouples the viral DNA replication function from the γ2 gene induction function of ICP27. This enabled us to examine the effects of ICP27 on the expression of late genes due specifically to ICP27. Thus, optimal transcription of the *gC* and U_L47 genes specifically requires ICP27, in addition to viral DNA replication and the other viral proteins, ICP4 and ICP8.

Possible modes of transcriptional activation by ICP27

The stimulatory effect of ICP27 on transcription of γ2 genes could occur by any of several mechanisms, either directly or indirectly, as described above. First, ICP27 could directly affect the transcriptional apparatus to promote transcription of late genes. Although ICP27 has been reported to affect posttranscriptional processes such as promoting 3′-end processing of genes containing suboptimal or weak polyadenylation signals (McGregor *et al.,* 1996; Sandri-Goldin and Mendoza, 1992), inhibition of RNA splicing (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994; Phelan *et al.,* 1993, 1996; Sandri-Goldin *et al.,* 1995; Sandri-Goldin and Mendoza, 1992), and stabilization of labile mRNAs (Brown *et al.,* 1995), these activities have been observed mostly in transfected cells and may involve gene expression processes not directly related to viral γ2 gene expression. Furthermore, ICP27 has been reported to associate with ICP4 in infected cells (Panagiotidis *et al.,* 1997) and to co-localize with ICP4 in replication compartments (de Bruyn Kops *et al.,* 1998), the likely site of γ gene transcription. Thus, at least a portion of ICP27 may be localized to the proper molecules or cellular sites, which would allow it to directly affect transcriptional processes.

Second, ICP27 could affect transcription indirectly by binding to nascent transcripts and promoting transcriptional elongation, much as has been reported for the HIV tat protein (Feinberg *et al.,* 1991). ICP27 has been reported to bind RNA directly (Brown *et al.,* 1995; Ingram *et al.,* 1996; Mears and Rice, 1996), providing a biochemical basis for ICP27 acting through RNA. Although one group reported specific binding of ICP27 to 3′ ends of certain RNAs (Brown *et al.,* 1995), others have found no specificity for RNA binding by ICP27 (Ingram *et al.,* 1996; Mears and Rice, 1996). Consistent with a possible effect of ICP27 on transcriptional elongation, we have observed that ICP27 stimulates utilization of a downstream polyadenylation site in the U_L24 gene (Hann *et al.,* 1998). Because the upstream site is a functionally weaker polyadenylation site, ICP27 is not stimulating the use of the weaker polyadenylation site but rather the use of the downstream site, possibly by stimulating transcriptional elongation or read-through of the upstream site.

Third, ICP27 could affect transcription indirectly by promoting posttranslational changes in other viral or cellular proteins. ICP4 expressed in ICP27 mutant-infected cells shows decreased electrophoretic mobility, consistent with increased phosphorylation (Rice and Knipe, 1988), and ICP4 expressed in transfected cells in the presence of ICP27 shows an increased electrophoretic mobility relative to ICP4 expressed alone (Su and Knipe, 1989). These mobility changes suggested that ICP27 causes a change in the phosphorylation of ICP4. Indeed, phosphopeptide mapping of ICP4 expressed in ICP27 mutant virus-infected cells showed altered phosphopeptides compared to ICP4 expressed in WT virus-infected cells (Xia *et al.,* 1996). These changes in phosphorylation could lead to a form of ICP4 that is more capable of binding the transcription factors involved in late gene transcription. ICP27 could also affect the modification of cellular proteins that promote late viral transcription. There is precedent for ICP27 affecting phosphorylation of cellular proteins in that ICP27 mutant viruses do not cause the normal decrease in phosphorylation of snRNP proteins (Sandri-Goldin and Hibbard, 1996) or the normal alteration in phosphorylation of pRb (Song *et al.,* 2000; Song and Knipe, unpublished results). Thus, ICP27 could increase the ability of ICP4 or cellular transcription factors to promote transcription of viral late genes by altering their phosphorylation.

Fourth, ICP27 could affect transcription indirectly by altering the expression of a cellular or viral factor needed for late gene transcription. This effect could be caused by ICP27 exerting an effect at the transcriptional, the posttranscriptional, or the translational level. For example, ICP27 is required for viral inhibition of RNA splicing, which largely affects expression of host genes because very few HSV transcripts are spliced.

Pleiotropic effects of ICP27 and other viral and cellular proteins

While we have been reporting a transcriptional effect on $\gamma 2$ gene expression by ICP27, there have been several reports showing that ICP27 can modulate various aspects of the posttranscriptional machinery, as referenced above. It is conceivable that ICP27 regulates some genes via a transcriptional mechanism while it regulates others by posttranscriptional processes. Alternatively, ICP27 could affect one or both of those processes indirectly. The HIV tat protein has been reported to have both transcriptional and posttranscriptional effects (Drysdale and Pavlakis, 1991). Similarly, the cellular helicase A binds RNA, shuttles to the cytoplasm, promotes the cytoplasmic export of RNA containing a constitutive transport element (Tang *et al.,* 1997), binds to RNA pol II holoenzyme, and mediates the association of coactivators to the pol II holoenzyme complex. Further studies are needed to determine if HSV ICP27 can function through similar mechanisms.

MATERIALS AND METHODS

Cells and viruses

Vero cells were obtained from the American Type Culture Collection (Rockville, MD). They were maintained in Dulbecco's modified Eagle medium (DMEM—high glucose) and 10% heat-inactivated fetal bovine serum, purchased from Irvine Scientific (Irvine, CA). V27 cells, described previously (Rice and Knipe, 1990), were derived from Vero cells by stable transformation with the WT ICP27 gene. These cells contain approximately one copy of the ICP27 gene per diploid genome and are maintained in medium containing 300 μg/ml G418. Following adsorption of virus in phosphate-buffered saline (PBS) containing 1% heat-inactivated calf serum and glucose, the infected cells were maintained in medium 199 (Earle's salts) supplemented with 1% heat-inactivated newborn calf serum. The WT virus used in all the experiments was the KOS1.1 strain. The HSV-1 mutants, *n*504 and *d*27, have been described previously (Rice and Knipe, 1990). The *n*504 mutant virus encodes a slightly truncated form of ICP27 containing 504 of 512 amino acid residues, whereas *d*27 fails to express ICP27 due to deletion of the promoter and almost all of the open reading frame. All infections were done at a m.o.i. of 20 PFU per cell.

Probes and plasmids

The probes used for detecting *gC* and *ICP8* gene transcripts have been described previously (Chen and Knipe, 1996; Godowski and Knipe, 1986). They are ss DNA molecules generated by cloning viral DNA fragments into M13 bacteriophage DNA. For strand-specific hybridization probes, ssDNA was isolated from phage particles. The HSV sequences in each of the probes were as follows: *gC* (U_L44) gene, a 918-bp *Eco*RI-*Xba*I fragment (nt 96,751–97,669) (Godowski and Knipe, 1986; McGeoch *et al.,* 1988); *ICP8* (U_L29) gene, a 1.4-kb *Kpn*I-*Bam*HI fragment from coordinates 0.398 to 0.407; and *U_L47* gene, a 870-bp *Pst*I fragment (nt 101,485–102,355) from plasmid pSU$_L$47, which was kindly provided by J. McKnight (University of Pittsburgh). These sequences were cloned into M13 mp18 and mp19. In all experiments, complementary (c, coding strand) and anti-complementary (a, opposite the coding strand) probes (Godowski and Knipe, 1986) were used in separate hybridization reactions. The PSX3 plasmid (called pBH-504R in Rice *et al.,* 1989) contains the *ICP27* gene with a terminator codon insertion after codon 504 of the U_L54 open reading frame.

Nuclear run-on assay

Vero cells were infected at an m.o.i. of 20 PFU/cell and harvested at 2, 4, 6, or 8 h postinfection. Methods for the isolation of nuclei, the nuclear run-on assay, and hybridization reactions were as described previously (Godowski and Knipe, 1986). Nytran filters were prepared for hybridization by binding 10 μg of M13 ssDNA per slot in 6× SSC (0.15 M NaCl, 0.015 M Na_3Citrate), using a Schleicher and Schuell slot blot apparatus. Hybridization was performed at 68°C for 40–48 h. Filters were then washed in the following manner: twice in 2× SET (1× SET is 0.15 M NaCl, 20 mM Tris–Cl, pH 7.4, 1 mM EDTA), 0.1% sodium dodecyl sulfate (SDS) at room temperature for 30 min; twice in 0.1× SET, 0.1% SDS at 60°C for 30 min; once in 2× SET, 5 μg/ml of RNase A at 37°C for 30 min; and finally once in 0.1× SET, 0.1% SDS, at 60°C for 30 min. The filters were then exposed to either Kodak XRP films or a phosphor screen. Spots were quantitated using the phosphorimager scanning densitometer. The amount of transcription was expressed in arbitrary units, corrected for the length of the HSV-1 DNA probe used.

In vivo labeling with [^{3}H]uridine and isolation of RNA

Approximately 1 × 10^7 cells in 150-cm^2 flasks were infected with virus at an m.o.i. of 20. Virus was allowed to adsorb onto cells for about 1 h, and then the virus inoculum was replaced with 50 ml of medium 199 containing 1% newborn calf serum. The infected cells were incubated at 37°C in 5% CO_2. At 6 h postinfection, the infected cells were labeled in 5 ml of medium 199 containing 200 μCi/ml [^{3}H]uridine (35–50 Ci/mmol, New England Nuclear Corp., Boston, MA) at 37°C for the indicated length of time. The monolayers were then placed on ice and subsequently washed twice with ice-cold PBS. Total RNA was isolated by the guanidinium thiocyanate method as described by Ausubel *et al.* (1987). For nuclear RNA preparation, nuclei were isolated by resuspending the cell pellets in 4 ml of RNA lysis buffer (50

mM Tris–HCl, pH 8.0; 5 mM $MgCl_2$; 1 mM EDTA) containing 5 mM vanadyl ribonucleoside complex, followed by disruption with 6–10 strokes using a Dounce homogenizer. The nuclear pellets were washed twice with cold PBS and collected by spinning at 1000 rpm for 5 minutes. We typically isolated $3–12 \times 10^6$ cpm of total RNA (100–300 μg). For nuclear RNAs, we generally obtained 10–20 μg. The RNA was DNase-treated for 30 min at 37°C, phenol extracted, and precipitated in 2 vol of absolute ethanol. The RNA was used in a hybridization reaction with 10–20 μg of ss M13 DNA probes bound per slot onto Nytran filters.

The conditions for hybridization were a modification (Chen and Knipe, 1996) of those used by Zhang *et al.* (1987). Hybridization was performed for 40–48 h at 68°C in plastic scintillation vials containing 2 ml of 2× SSC and 5× Denhardt's solution. After the hybridization, filters were washed as for the nuclear run-on assay. To determine the amount of labeled RNA hybridized to filters, the slots were cut out and counted in a scintillation counter using Filter Count scintillation cocktail (Packard, Meriden, CT). Care was taken so that the amount of DNA probe used was always in excess. The 20 μg ss M13 DNA for each probe used contained between 11 and 20% HSV-1-specific DNA or about 2–5 μg of DNA specific for the transcript in question. The linearity of the hybridization assay was tested by showing a direct correlation between the amount of total labeled RNA added to the hybridization sample and the amount of radioactivity detected on the filter (data not shown). For each sample, a control probe of M13 ss DNA alone was included in the experiment. The net amount of HSV-specific, labeled RNA was obtained by substracting the amount of radioactivity hybridized to the control. For comparison between different genes, the final value for each probe was normalized to the length of HSV-specific DNA used in the hybridization.

Northern blots

Aliquots of 10 μg of RNA were denatured at 68°C for 10–15 min in 50% formamide, 1.1 M formaldehyde, and 1 μg/ml ethidium bromide. The samples were placed on ice for 2 min and then subjected to electrophoresis in 1% formaldehyde–agarose at 5 V/cm in 1× MOPS buffer. After electrophoresis, the gel was photographed under UV light, rinsed in glass-distilled water, and soaked in 10× SSC for 1 h. The RNA was transferred onto Hybond (Amersham, Arlington Heights, IL) paper and fixed on the membrane by baking at 80°C for 2 h or by UV crosslinking for 3 min. The blot was prehybridized in 50% formamide in 0.5% SDS, 5× SSPE, 5× Denhardt's solution, and 20 μg/ml denatured salmon sperm DNA. ^{32}P-labeled probe (10^6 cpm per milliliter) was added to the blot, and hybridization was for 20 h at 42°C. After washing, the filters were exposed to phosphor screens, and the amounts of mRNA in the specific bands were determined with a phosphoimager.

Isolation of double mutant virus strains

The construction of double mutant viruses containing the ICP27 *n*504 mutation and each of the *gC* gene promoter mutations isolated by Homa *et al.* (1988; Fig. 3) was performed in a two-step procedure. First, the *lacZ* coding sequences were recombined into the ICP27 gene to give blue plaque viruses, and then the *n*504 mutation was recombined into the *ICP27* locus to give white plaque viruses. To perform the first step, infectious DNA from each of the mutant viruses described in Fig. 3 was prepared and cotransfected with linearized p27lacZ DNA into V27 cells. The progeny viruses were plated on V27 cells in the presence of X-gal (Homa *et al.,* 1988; Rice and Knipe, 1990), and blue plaques were purified. The genomic structures were confirmed by Southern blot hybridization (Jean and Knipe, results not shown). Infectious DNA was then prepared from one of each of the desired *gC* gene mutants, and this DNA was cotransfected with linearized pPSX3 plasmid DNA. The progeny viruses were plated on V27 cells in the presence of X-gal, and white plaques were purified. The genomic structures were confirmed by Southern blot hybridization, and stocks were prepared from infected V27 cells.

ACKNOWLEDGMENTS

This work was supported by Public Health Service Grant AI20530 from NIAID. Serge Jean was supported by NIH fellowship F31 GM 13627. Kay M. LeVan was supported by NIH postdoctoral fellowship AI08385. We thank Mike Greenberg and members of the Knipe lab for their helpful comments on this work.

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