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Differential Dependence of Herpes Simplex Virus Immediate-Early Gene Expression on De Novo-Infected Cell Protein Synthesis†

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The time course of accumulation of herpes simplex virus immediate-early (IE) mRNA and the requirement for infected cell protein synthesis for mRNA transcription and accumulation were compared. Measurements of transcription in nuclear run-on assays, accumulation of cytoplasmic mRNA by Northern (RNA) blot hybridization, and rates of infected cell protein synthesis by pulse-labeling did not indicate differences among the five IE genes, consistent with previous studies. However, as a result of varying the amount of de novo protein synthesis after infection, at least three patterns of maximal expression of the IE genes were revealed. Addition of the protein synthesis inhibitor anisomycin to cells coincident with infection resulted in maximal rates of transcription and accumulation of functional ICP0 mRNA, while 0.5 h of infected cell protein synthesis prior to addition of the drug was required for maximal expression of ICP22/47 and ICP27 mRNAs. Maximal expression of ICP4 mRNA occurred only when 1 h of de novo protein synthesis occurred prior to the addition of the drug. These results are discussed in the context of alternative mechanisms for regulating IE gene expression.

Expression of the herpes simplex virus (HSV) genome is temporally regulated at the transcriptional level. Early studies defined the time of appearance and thus the kinetic classification of infected cell proteins on the basis of requirements for either de novo protein or DNA synthesis (29, 30). Analysis of infected cell transcription patterns revealed that no protein synthesis was required for the accumulation of functional immediate-early (IE) mRNA, while 2 to 3 h of infected cell protein synthesis was required for accumulation of delayed-early (DE) mRNAs. The late (L) genes could be subclassified into those (gamma-1) requiring 3 to 5 h of protein synthesis and others (gamma-2) requiring in addition the onset of viral DNA replication (9, 27, 31, 64, 66). During productive infection, viral gene expression is initiated by interaction of a structural component of the virion, variously termed VP16, ICP25, Vmw65, or α-TIF (4, 7, 43), with the cellular transcription factor Oct-1 (23, 34, 38, 46, 47, 55, 65) and at least one additional cellular protein (23, 32, 35, 68) resulting in interaction with cognate binding sites (the TAA TGARAT sequence), present one or more times in IE promoters (5, 18, 33, 40, 41). This regulatory network, however, is dispensable at least for growth in tissue culture since viral DNA is infectious and a mutant carrying a 12-bp insertion in the structural gene for VP16 is viable (1). Though efficiency of replication of the mutant is severely impaired at low multiplicity of infection (>10 particles per cell [1]), it is capable of establishing and maintaining a latent infection and efficiently reactivating from a mouse eye latency model (63).

The products of two IE genes play critical roles in the subsequent expression of DE and L genes. A large number of mutational analyses of infected cell protein 4 (ICP4) as well as cotransfection assays have demonstrated that this gene product is essential for DE and L gene transcription and is required in a functional form throughout the replication cycle (11, 24, 37, 54, 66). Similarly, ICP27 is a multifunctional protein which stimulates expression of gamma-1 genes and is essential for gamma-2 gene expression (57, 60, 62).

Genetic studies have indicated a role for a third IE protein, ICP0 (2, 30, 51, 52), in efficient reactivation from latency (8, 26, 36, 59, 71). In addition, viral DNA from ICP0 mutant viruses is less efficient at initiating foci of infection than is wild-type DNA when transfected into permissive cells (6). Finally, ICP0 has been shown to transactivate viral IE, DE, and L promoters alone or in combination with other HSV transactivating proteins in transfected cells (13, 14, 19, 20, 45, 48, 49, 56, 62). Taken together, these findings suggest an important role for ICP0 in very early events of viral gene expression, particularly in the absence of VP16. At least one study has reported the absence of Oct-1 (but not other members of the POU domain regulatory protein family) from adult mammalian sensory ganglia or spinal cord (28). On the assumption that temporally regulated viral gene expression occurs during reactivation from latency, it could be argued that an alternative pathway, underlying and independent of VP16–Oct-1 interactions, exists for activating IE gene expression.

We initiated comparative studies of IE mRNA metabolism in order to gather information on rates of transcription, accumulation of functional mRNA, and requirements for maximal expression. Our results support a model for efficient expression of IE genes which relies, at least in part, on the expression of one or more IE proteins.

MATERIALS AND METHODS

Cells and virus. Vero cells were grown in Dulbecco's modified Eagle medium-H (DMEM-H) supplemented with 5% bovine calf serum. HSV-1 strain F was used throughout this study and propagated as described previously (27). All infections were performed at a multiplicity of infection of 10

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for 1 h. Times postinfection (p.i.) were calculated from the time of addition of the inoculum.

Analysis of protein synthesis. Replicate 35-mm cultures of Vero cells were infected and then treated with 25 μM anisomycin where indicated in the figure legends. Experience with emetine and cycloheximide at the outset of these studies suggested that these drugs were not ideally suited, since the former had toxic effects on cells when present for prolonged periods (5 to 8 h), and the latter did not rapidly reverse under our conditions of drug treatment. At 7 h p.i., DMEM-H containing drug was removed, and monolayers were washed once with drug-free medium and overlaid with DMEM-H containing actinomycin D (5 μg/ml). After 30 min, the medium was removed, and the monolayers were washed twice with methionine-free medium and labeled for 30 min with methionine-free medium supplemented with 5% dialyzed fetal calf serum and 30 μCi of [^{35}S]methionine per ml. Lysates were prepared by washing monolayers with phosphate-buffered saline (PBS) and scraping cells into a buffer consisting of 5 mM Tris (pH 6.8), 1.4 M 2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), 5% glycerol, and 0.0001% bromophenol blue. Protein samples were boiled for 3 min prior to storage at −70°C. Aliquots of protein lysates were electrophoresed on 9% acrylamide SDS-polyacrylamide gels cross-linked with *N*,*N*-diallyl tartardiamide as described previously (27, 61). Autoradiographs of the resulting gels were prepared by fluorography.

Analysis of steady-state RNA levels. Replicate 100-mm cultures of Vero cells were infected and harvested at indicated times between 0.5 and 14 h p.i. or infected in the presence of drugs as indicated and harvested at 8 h p.i. Cytoplasmic RNA samples were prepared, and 5-μg aliquots were fractionated on formaldehyde agarose gels and then transferred to nitrocellulose membranes as described previously (27). Double-stranded DNA probes (0.5 μg of plasmid insert) were labeled by nick translation and hybridized to replicate Northern (RNA) blots as previously described (27, 61). The following mRNA-specific hybridization probes were used: for ICP4, a *Bam*HI (0.844 to 0.856/0.964 to 0.976 map units [m.u.]) 1.8-kb fragment (Bam Y [53]); for ICP0, a *Bam*HI-*Pst*I (0.022 to 0.052 m.u.) 4.5-kb fragment from pRB111 (53); for ICP22, a *Pvu*II-*Bst*EII (0.869 to 0.874 m.u.) 0.75-kb fragment from pSG25 (25); for ICP27, a *Bam*HI-*Sal*I (0.741 to 0.748 m.u.) 1.05-kb fragment from pSG28 (25); and for ICP47, a *Bam*HI-*Bst*EII (0.952 to 0.955 m.u.) 0.45-kb fragment from pSG25 (25, 58). In the interest of space and convenience of presentation, only those portions of the autoradiographs containing signal are presented (Fig. 1 and 3).

Analysis of transcription rates. (i) Isolation of nuclei. Nuclei were isolated by the method of Marzluff (44). Briefly, 100-mm cultures of infected cells were washed with PBS, resuspended in lysis buffer [0.32 M sucrose, 3 mM $CaCl_2$, 2 mM $Mg(CH_3COO)_2$, 10 mM Tris-HCl (pH 8), 1 mM dithiothreitol (DTT), 0.1% Triton X-100] at 10×10^6 to 50×10^6 cells per ml and disrupted by Dounce homogenization. The lysate was diluted with 0.5 volume of suspension buffer [2.0 M sucrose, 5 mM $Mg(CH_3COO)_2$, 10 mM Tris-HCl (pH 8), 1 mM DTT], layered over a 2-ml cushion of suspension buffer, and centrifuged for 45 min at 25,000 × *g* at 4°C. The nuclear pellet was resuspended in storage buffer [25% glycerol, 5 mM $Mg(CH_3COO)_2$, 50 mM Tris-HCl (pH 8), 5 mM DTT] and stored in liquid N_2 for up to 1 month prior to use.

(ii) Run-on transcription. Thawed nuclei were diluted with a reaction mix to give final concentrations of 10 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 0.3 M KCl, 2 mM ATP, CTP, and UTP, 5 mM DTT, and 100 μCi of [α-^{32}P]GTP (800 Ci/mmol, 10 mCi/ml). Reaction mixes were incubated for 30 min at 25°C. Control reactions for RNA polymerase II were performed in the presence of 100 μg of α-amanitin per ml; under these conditions, no hybridizable counts per minute were detectable (data not shown). Reactions were stopped by addition of 1.5 volumes of HSB (0.5 M $NaCl_2$, 50 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Tris-HCl [pH 7.4]) containing 50 μg of RNAse-free DNAse I per ml and further incubation at 30°C for 5 min.

(iii) Preparation of RNA, hybridization conditions, and quantitation. Nuclei were digested by the addition of 0.2 volume of 5% SDS–0.5 M Tris-HCl (pH 7.4)–0.125 M EDTA and proteinase K (200 μg/ml) and incubation at 42°C for 30 min. RNA was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated on ice for 30 min in 1 volume of 20% trichloroacetic acid and 100 μg of tRNA per ml. The RNA was pelleted, washed four times with 5% trichloroacetic acid resuspended in 200 μl of [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] (TES) buffer (10 mM TES [pH 7.4], 10 mM EDTA, 0.2% SDS) and precipitated by addition of 2 volumes of 95% ethanol. RNA was pelleted, resuspended in 1 ml of TES buffer, and, following limited hydrolysis of the RNA in 1 N NaOH, diluted to 5 ml by addition of TES buffer–0.6 M NaCl. Labeled run-on RNA was hybridized for 36 h at 65°C to single-stranded phage M13 probes immobilized on nitrocellulose filters, specific for ICP4, ICP0, ICP22, ICP27, ICP8, ICP5, and gC mRNAs. DNA inserts complementary to mRNA encoding ICP4, ICP8, ICP5, and gC (24) and ICP22 and ICP27 (37) have been previously described. The DNA insert complementary to ICP0 mRNA is a 658-bp *Xma*I-*Sal*I fragment near the 3′ end of the gene (from W. Sacks and P. Schaffer). Prior to use in run-on transcription analyses, the specificity of each clone was verified by Northern blot hybridization against RNA preparations isolated from cells infected in the presence of anisomycin or in the absence of drug at various times p.i. Filters were washed twice for 1 h in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) at 22°C, once for 30 min at 37°C in 2× SSC containing 10 μg of RNase A per ml, and once for 1 h in 2× SSC at 37°C. Filters were air dried, and hybridization was quantitated with an AMBIS radioanalytic beta scanner.

RESULTS

Kinetics of appearance and metabolic requirements for individual IE gene expression. We previously reported experiments which defined in Vero cells the kinetics of appearance of representative IE (ICP4 and ICP0), DE (thymidine kinase), and L (ICP5) mRNAs and the metabolic requirements for their accumulation; in addition, we presented a time course of appearance of infected cell proteins in which the corresponding protein products could be identified (27). Figure 1 presents a comprehensive Northern blot analysis of all five IE genes, based on identical RNA samples purified from replicate Vero cell cultures. At the indicated times, cytoplasmic RNA was isolated and fractionated by formaldehyde gel electrophoresis, transferred to nitrocellulose, and hybridized with labeled DNA probes as described in Materials and Methods. Where indicated, cells were treated with 25 μM anisomycin or 2.0 mM canavanine in arginine-free medium or 10 mM phosphonoacetate (PAA). In this analysis, all of the IE mRNAs accumulated as early as 1 h p.i., peaked in accumulation at 3 to 5 h p.i., and thereafter declined in relative abundance (Fig. 1A). The additional transcript detected with the ICP47 probe and displaying late

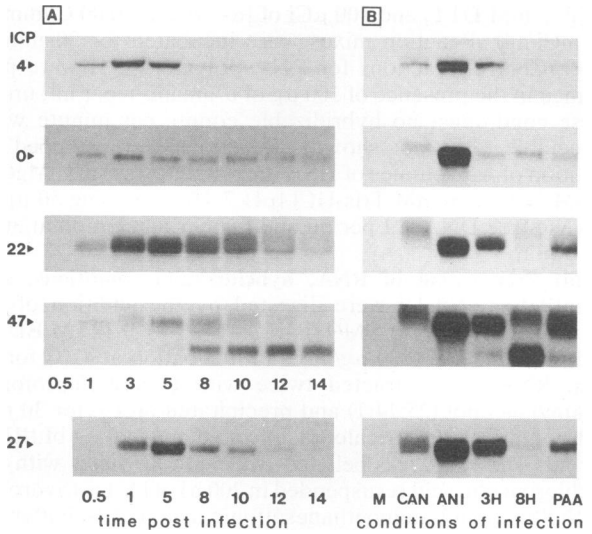

FIG. 1. Kinetics and metabolic requirements for accumulation of IE mRNAs. Northern blot analysis of cytoplasmic RNA isolated from infected cells at indicated times (hours) p.i. (A) or under various conditions of infection (B) are presented. IE mRNA-specific probes and conditions of drug treatment are described in Materials and Methods. M, mock-infected cells; CAN, canavanine; ANI, anisomycin; 3H, 3-h-p.i. no-drug control; 8H, 8-h-p.i. no-drug control. Mock and drug-treated RNA samples were from cells harvested at 8 h p.i. The ICP designations at the left refer to the IE protein-coding potential of the corresponding mRNAs; an alternative nomenclature refers to these mRNAs as IE1, IE3, IE4, IE5, and IE2, respectively. The additional transcript detected by the ICP47 probe beginning at 5 h p.i. (A) and in the 8-h sample (B, lane 8H) is the 1.5-kb L mRNA of the US11 gene (57).

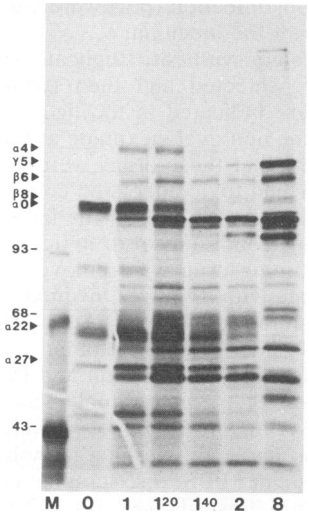

FIG. 2. Effect of the time of anisomycin addition on the subsequent pattern of infected cell protein synthesis after drug reversal. Samples of [^{35}S]methionine-labeled proteins from infected cells initially treated with anisomycin between 0 and 2 h p.i., as indicated below the lanes, and labeled from 7.5 to 8 h p.i. were separated by SDS-PAGE as described in Materials and Methods. Lane 8 contains control 8-h-infected cell proteins. The mobilities of various infected cell proteins (alpha [IE] 4, 0, 22, and 27, beta [DE] 6 and 8, and gamma [L] 5) are indicated. Other numbers refer to molecular sizes (in kilodaltons) of marker proteins (lane M).

gene kinetics is US11 mRNA (57). Importantly, the absence of requirements for de novo protein synthesis (anisomycin) and viral DNA replication (PAA) for at least minimal accumulation were shared by all IE species (Fig. 5B).

Earlier reports demonstrated that the normal transition from IE to DE and L gene expression required functional IE proteins, since treatment of cells from the onset of infection with the arginine analog canavanine resulted in IE gene expression but no DE or L gene expression (30, 51). Treatment with the arginine analog canavanine (Fig. 1B) demonstrated that normal metabolism of IE mRNA requires the presence of functional infected cell proteins. While levels of ICP22, ICP27, and ICP47 mRNAs were reduced to approximately 20% of control (lane 3H) levels and to approximately 10% of the levels attained in the absence of protein synthesis (lane ANI), they were higher than the levels normally seen in control cells infected for an equivalent time in the absence of drug (lane 8H). Similar results were obtained for ICP4 and ICP0 mRNA levels when the effects of canavanine and anisomycin were compared, though canavanine-treated samples and the 8-h control levels were comparable. The latter result probably reflects the persistence of ICP4 and ICP0 mRNAs at late times p.i. compared with ICP22, ICP27, and ICP47 (Fig. 1A and reference 27). For all of the IE mRNAs, the difference between the levels of mRNA obtained in the absence of protein synthesis (Fig. 1B, lane ANI) and the presence of defective proteins (lane CAN) is striking. The overaccumulation of IE mRNAs in the presence of protein synthesis inhibitors as well as genetic analyses (11, 48, 50) have indicated that ICP4 plays a role in the down regulation of IE gene expression (but see reference 17), though it is unclear how repression is mediated for each of the individual IE genes. Reduced levels of IE mRNA in the presence of canavanine suggest that one or more infected cell proteins play a positive regulatory role in IE mRNA synthesis or accumulation (see Discussion).

We previously noted an altered mobility of ICP0 mRNA isolated from canavanine-treated cells (27) and have observed similar results with ICP22 and ICP47 mRNAs. Whether this is related to the spliced structure of these three mRNAs is unknown. Of interest, however, is the finding that both species of mRNA in the time course show a similar decrease in relative mobility by 8 h p.i. (compare Fig. 1A, lane 8H, with Fig. 1B, lanes CAN and 8H). Finally, the increased accumulation of mRNA in the presence of PAA relative to the 8-h untreated control is consistent with the observation that failure to express DE and L proteins leads to defective shutoff of IE gene expression (10, 24, 30, 54).

Effects of de novo protein synthesis on IE gene expression. (i) SDS-PAGE and Northern blot analyses. The first indication that an additional level of regulation might exist in the metabolism of IE mRNA occurred when we determined that infected cell protein synthesis was required to achieve maximal expression of IE mRNAs. The protein synthesis inhibitor anisomycin was added to replicate infected cultures at progressively later times between 0 and 2 h p.i. The drug was removed at 8 h p.i., and proteins pulse-labeled with [^{35}S]methionine and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described in Materials and Methods. Thus, the pattern of proteins detected should reflect the ability of infected cells to accumulate the corresponding mRNAs in the presence of 0 h, 1 h, 1 h 20 min, 1 h 40 min, 2 h, and 8 h of de novo infected cell protein synthesis. Results of the analysis (Fig. 2) revealed the following: (i) the highest rate of ICP0 protein synthesis occurred in cells

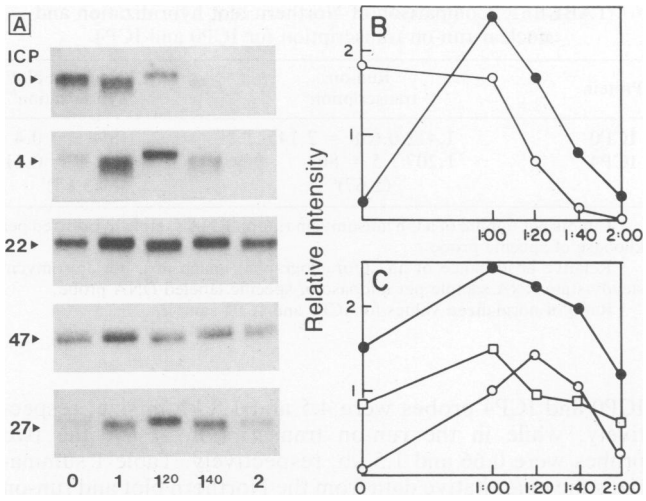

FIG. 3. Effect of the time of anisomycin addition on the accumulation of IE mRNAs. Cytoplasmic RNA from infected cells initially treated with anisomycin between 0 and 2 h p.i., as indicated below the panels, was purified from cells at 8 h p.i. and analyzed by Northern blot hybridization as described in Materials and Methods. (A) Autoradiographic results of Northern blots of IE mRNAs. Designations at the left refer to the protein-coding potential of the mRNAs as described in the legend to Fig. 1. (B and C) Relative amounts of individual IE mRNAs determined by laser densitometry of autoradiographs as described in Materials and Methods. To ensure linearity of the response, X-ray films of different lengths of exposure of each Northern blot (not necessarily those shown in panel A) were scanned. (B) ●, IE ICP4; ○, IE ICP0. (C) ○, IE ICP27; ●, IE ICP22; □, IE ICP47.

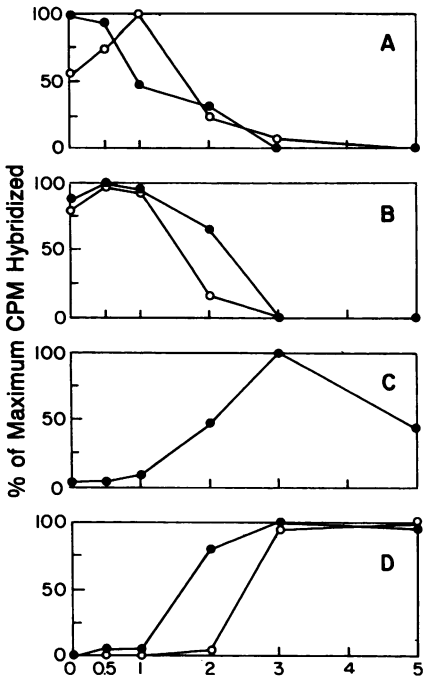

FIG. 4. Effect of the time of anisomycin addition on the transcription rates of IE, DE, and L mRNAs. Run-on transcripts were purified from nuclei of infected cells initially treated with anisomycin between 0 and 3 h p.i., as indicated below the panels, and hybridized to single-stranded mRNA-specific DNA probes, and the transcription rates of the resulting hybrids were quantitated, all as described in Materials and Methods. Results are presented as percentage of maximum counts per minute hybridized. (A) ●, IE ICP0, maximum cpm = 1,412; ○, IE ICP4, maximum cpm = 2,165. (B) ○, IE ICP22, maximum cpm = 1,510; ●, IE ICP27, maximum cpm = 1,174. (C) ●, DE ICP8, maximum cpm = 3,237. (D) ●, L ICP5, maximum cpm = 1,342; ○, L gC, maximum cpm = 1,655.

which were treated with anisomycin initially at the time of infection (0 h p.i.), and (ii) rates of ICP4, ICP22, and ICP27 were maximal when 1 h 20 min of infected cell protein synthesis was allowed prior to the anisomycin block. Two additional observations, aside from differential effects of anisomycin on maximal rates of IE protein synthesis, can be made: (i) 1 h 20 min of infection was required for the synthesis, maturation, and localization of positive regulatory factors controlling levels of DE and L gene expression (e.g., ICP8 and ICP5), and (ii) an additional 20 min (1 h 40 min) of infection was required for synthesis of negative regulatory factors controlling levels of IE expression (see also Fig. 3A).

Northern blot analysis (Fig. 3) revealed a pattern of dependence on infected cell protein synthesis for the maximal accumulation of IE mRNAs, consistent with the previous data on rates of protein synthesis. Anisomycin was added at the indicated times, and cells were harvested and RNA was extracted at 8 h p.i. The autoradiographic results of the hybridizations (Fig. 3A) indicate that the highest accumulation of ICP0 mRNA occurred in the complete absence of infected cell protein synthesis, while periods of 1 h and 1 h 20 min were required for maximal accumulation of ICP4, ICP22, and ICP47 mRNAs and ICP27 mRNA, respectively. Figures 3B and C summarize densitometric analysis of the hybridization results. Steady-state levels of ICP0 mRNA declined continuously through the course of the experiment, while levels of ICP4 mRNA initially increased to a maximum when drug addition was delayed to 1 h p.i. (Fig. 3B). Levels of ICP22, ICP27, and ICP47 increased through 1 h to 1 h 20 min before declining (Fig. 3C). It is clear that by 2 h p.i., DE or gamma-1 gene products had accumulated to sufficient levels in the infected cell to mediate the decline in levels of IE mRNAs normally seen after 5 h of infection (compare Fig. 3A with Fig. 1A).

(ii) Run-on nuclear transcription analysis. To determine whether the apparent requirement for infected cell protein synthesis was at the transcriptional or posttranscriptional level, run-on assays were performed by using nuclei from cells treated with anisomycin for increasing times between 0 and 3 h p.i. All cells were harvested and nuclei were prepared at 5 h p.i. as described in Materials and Methods. If the protein synthesis requirement were at the level of transcription, we would expect rates of RNA synthesis to mirror results from the previous protein synthesis and mRNA accumulation analyses (Fig. 2 and 3). Alternatively, if the requirement were posttranscriptional, then rates of IE transcription should be similar and the previous results would likely reflect differential mRNA stability or translational efficiency. Figure 4 summarizes results of beta emission densitometry scanning of slot blot hybridization experiments. Panels of single-stranded DNA probes specific for four of the five IE genes were hybridized with nuclear run-on RNA as described in Materials and Methods. Labeled RNA was prepared from nuclei treated for various lengths of time with anisomycin and harvested at 5 h p.i. The analysis revealed a differential pattern of IE transcription that mirrored the differences in mRNA accumulation and protein synthesis results seen earlier. Maximum transcription of

ICP0 occurred in nuclei from cells treated from the time of infection (0 h) (Fig. 4A). Maximum transcription of ICP22 and ICP27 occurred in nuclei from cells treated with anisomycin at 0.5 h p.i. (Fig. 4B). While differences between the 0- and 0.5-h points for both ICP22 and ICP27 are small, they are reproducible and display a maximum rate dependent on infected cell protein synthesis. With regard to ICP22, these results are consistent with protein synthesis (Fig. 2) and mRNA accumulation (Fig. 3) data. Finally, maximal ICP4 transcription (Fig. 4A) occurred after a delay of 1 h before drug addition. ICP8, a DE gene (Fig. 4C), ICP5, an L or gamma-1 gene (Fig. 4D), and gC, an L or gamma-2 gene (Fig. 4D), displayed a pattern of delayed transcription consistent with their kinetic class designation and provided evidence that the run-on analysis faithfully reflected the regulation seen in vivo (24, 67, 69). These results argue strongly that the requirement for infected cell protein synthesis for maximum IE gene expression exists at the level of transcription.

DISCUSSION

The results of many studies describing kinetics and metabolic requirements (27, 29–31, 39), promoter structure (5, 18, 21, 22, 33, 40, 41), and rates of transcription (24, 67, 70) have supported a unified mechanism for positive regulation of the IE genes. In the experiments described here, we have attempted to determine whether any other positive regulatory mechanisms underlie the VP16-mediated effects. The impetus for these studies came from work demonstrating that the virion transactivator is not required for reactivation from latency (63) and that ICP0 appears to be important for critical early steps in the replication cycle, when virion DNA rather than infectious virus initiates infection (6). Our approach was to amplify the consequences of very early regulatory events in HSV infection. This was achieved by varying the length of time for which infected cell protein synthesis was allowed and observing the consequences on rates of transcription, accumulation, and expression of cytoplasmic mRNA. The results support the conclusion that a requirement for infected cell protein synthesis exists for maximal expression of ICP4, ICP22, and ICP27, but not ICP0, genes. We have no direct evidence for the protein synthesis requirement for maximal transcription of ICP47. From the pattern of mRNA accumulation (Fig. 3) and the fact that ICP22 and ICP47 have identical promoter sequences, it is likely that this IE gene has a similar requirement for infected cell protein synthesis for maximal expression. Accumulation of ICP27 mRNA may have an additional requirement beyond that shared with ICP4, -22, and -47 mRNAs for maximal transcription (compare results in Fig. 3B and 4B). The highest rate of synthesis under our conditions of drug treatment was coincident with that of ICP4 in the in vitro run-on assay. In an earlier analysis of transcription rates in untreated intact infected cells, the time of maximal synthesis of ICP27 lagged behind that of ICP4 (70). Though the significance of transcription rates of ICP27 compared with ICP22/47 and ICP4 is unknown, we have consistently observed delayed maximal expression of these four IE genes relative to ICP0 (data not shown). The ratio of accumulated ICP0 to ICP4 mRNAs in the absence of de novo protein synthesis was approximately 6:1 (Fig. 3), while the ratio of ICP4 to ICP0 transcription under these same conditions was approximately 2:1 (Fig. 4). This finding likely reflects differences in the size of the specific probes used in the two types of assays. For the Northern blot experiments, ICP0 and ICP4 probes were 4.5 and 1.8 kb in size, respectively, while in the run-on transcription assay, the two probes were 0.66 and 1.5 kb, respectively. Table 1 summarizes the quantitative data from the Northern blot and run-on transcription assays, presented as units of RNA detected (either relative amounts or counts per minute hybridized) per kilobase of probe for both ICP0 and ICP4 transcripts. The ratios of ICP0 to ICP4, once normalized for the method of quantitation, vary from 2.7 to 3.6.

TABLE 1. Comparison of Northern blot hybridization and nuclear run-on transcription for ICP0 and ICP4

Protein	Run-on transcription[a]	Northern blot hybridization[b]
ICP0	1,412/0.658 = 2,145	1.8/4.5 = 0.4
ICP4	1,207/1.5 = 804 (2.67)[c]	0.2/1.8 = 0.11 (3.63)[c]

[a] Counts per minute of a 0-h anisomycin run-on RNA sample hybridized per kilobase of specific probe.
[b] Relative absorbance of an autoradiographic image of a 0-h anisomycin steady-state RNA sample per kilobase of specific labeled DNA probe.
[c] Ratio of normalized values for ICP0 and ICP4 samples.

One of the earliest papers describing the cascade regulation of HSV gene expression (29) reported that as the onset of inhibition of protein synthesis was delayed relative to the time of infection, the subsequent rates of synthesis of IE polypeptides ICP4, -0, and -27 declined continuously after removal of the block. On the basis of this and subsequent experiments (30) on the effects of canavanine on the pattern of viral protein synthesis, the authors concluded that one or more polypeptides of the DE and L classes were required for the turn-off of the IE class. While our results are consistent with this conclusion, we have noted differences in the metabolic requirements for ICP4 and ICP27 synthesis after removal of a protein synthesis block. Some obvious differences in the experimental approach include cell type (HEp-2 versus Vero) and the use of cycloheximide rather than anisomycin. Accumulation of viral mRNA restricted to the IE class has previously been demonstrated with emetine, cycloheximide, and anisomycin over a range of 0.6 to 5.5% residual protein synthesis (39). We routinely achieved 98% inhibition of protein synthesis with 25 μM anisomycin in Vero cells and could effect complete recovery of protein synthesis within a 30-min period by washing monolayers in drug-free medium and preventing further RNA synthesis by addition of actinomycin D (unpublished data). One possible explanation for the differences in protein synthesis patterns after drug reversal is the inclusion of the 30-min recovery time. Perhaps we were able to indirectly detect differences in the levels of accumulated functional mRNAs that were not detectable by labeling immediately after drug reversal, a time when translational capacity is not fully restored. In any event, these Northern blot and run-on transcription results, which should be independent of the previous considerations, clearly indicate differential effects on transcription and IE mRNA accumulation as a function of prior protein synthesis.

It is unlikely that our results obtained by using anisomycin were due to effects of the drug distinct from inhibition of protein synthesis. First, we observed an inverse relationship between the accumulation and expression of ICP4, -22, -27, and -47 mRNAs and the length of drug treatment, arguing against an mRNA stabilization or superinduction effect (42). Second, the patterns of transcription, accumulation, and expression of ICP0 and ICP4 mRNA were consistently and

demonstrably different (this work and data not shown), arguing against some general nonspecific effect of the drug on mRNA metabolism or expression. Finally, we would argue that the de novo protein synthesis-dependent change in IE transcription is not due to alterations in the properties of the virion-associated transactivator VP16, since its binding and that of Oct-1 to consensus TAATGARAT target sites can be demonstrated in vitro in the absence of protein synthesis (55). It remains a formal possibility, however, that the formation of the DNA-protein complex merely precedes a protein synthesis-dependent step that results in promoter activation.

It is tempting to speculate that the requirement for de novo protein synthesis for maximal expression of ICP4 and perhaps ICP22/47 and ICP27 is in fact a requirement for ICP0. The transactivating properties of the ICP0 protein on IE target promoters (21, 22, 48) as well as DE and heterologous promoters (12, 19, 48, 56) has been well documented. The ICP0 gene product is a nuclear phosphoprotein, and extensive mutagenesis studies and sequence inspection (8, 14, 16, 52, 71) have revealed activation domains as well as potential sites of protein-protein interaction (15). The present results clearly indicate that the ICP0 gene has requirements for maximal expression which differ from those of ICP4 and the other IE genes. This, in turn, suggests that a partially nonoverlapping set of transcription factor-promoter sequence interactions may regulate the ICP0 gene and the other IE genes. While inspection of upstream promoter sequences of ICP4 and ICP0 reveals motifs mediating Sp1 and TATA box factor binding, two sequence motifs in the ICP0 promoter are not shared with the ICP4 promoter. First, there is a near consensus CAAT box (GCCATT) centered at position -77 relative to the transcription start site. Second, there are three copies of the sequence YCTTTGGGG, at positions -213, -151, and -51. In addition, the number and sequence organization of the TAATGARAT sites in the ICP4 and ICP0 promoters differ. The ICP4 promoter contains two TAATGARAT sites which lack overlapping Oct-1 consensus binding sites (T sites [3]) and which weakly bind the VP16/Oct-1 complex as measured by a gel mobility assay. The ICP0 promoter contains four sites, consisting of overlapping octamer and TAATGARAT sequences (O/T sites [3]), which bind the VP16/Oct-1 complex more strongly. It remains to be determined whether any of these differences in consensus factor binding sites mediate the differential activation of the ICP0 promoter. Experiments are in progress to further substantiate the role of ICP0 in the regulation of IE transcription.

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