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Accumulation of Herpes Simplex Virus Type 1 RNAs of Different Kinetic Classes in the Cytoplasm of Infected Cells

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We have analyzed the accumulation of herpes simplex virus type 1 RNA of the immediate early (IE; infected cell polypeptide types 4 and 0 [ICP-4 and ICP-0]), early (thymidine kinase), and early late (ICP-5) kinetic classes in the cytoplasm of infected cells in the presence of anisomycin, canavanine, or phosphonoacetic acid and in the course of a normal infection. IE RNAs were overproduced and were the only class of transcript detected in anisomycin-blocked cells. Phosphonoacetic acid treatment resulted in overaccumulation of early RNAs and underaccumulation of early late RNAs. Although low-stringency canavanine treatment resulted in accumulation of RNA from all kinetic classes, high-stringency conditions restricted accumulation of herpes simplex virus type 1 RNAs to the IE class. More importantly, the IE RNAs for ICP-4 and ICP-0 accumulated to a lesser extent under high-stringency canavanine conditions compared with their accumulation in anisomycin-treated cells. Therefore, the absence of newly synthesized viral proteins (anisomysin treatment) and the presence of analog proteins (stringent canavanine treatment) have different consequences with regard to the accumulation of these two IE RNAs. The kinetics of cytoplasmic accumulation for these RNAs was different for each class of RNA. The IE RNAs were detectable at 1 h postinfection and reached a maximum accumulation at ca. 3 h postinfection. The IE RNAs for both ICP-4 and ICP-0 persisted at late times of infection; however, they differed in that the RNA for ICP-4 remained at relatively low levels and the RNA for ICP-0 remained at relatively high levels as compared with their peak levels of accumulation. The 1.4-kilobase RNA for the herpes simplex virus type 1 thymidine kinase was detected by 2 h, with maximum accumulation occurring at ca. 5 h postinfection. After the peak of accumulation, the amount of thymidine kinase RNA declined rapidly from 8 to 14 h postinfection. The early late RNA for ICP-5 was detected between 2 and 3 h, after which accumulation increased to a peak between 8 and 10 h postinfection. The level of ICP-5 RNA remained at close to the peak level until 14 h postinfection. We also compared the accumulation of viral mRNAs in the cytoplasm with the rates of synthesis of their respective polypeptides. Our results suggest that translational controls may be involved in the regulation of IE genes but not early or late genes.

The regulation of herpes simplex virus type 1 (HSV-1) genes is a complex process, resulting in a coordinate cascade of viral gene expression. The virus lytic cycle can be temporally divided into immediate early (IE), early (E), and late (L) stages. Each stage is characterized by the maximum synthesis of IE or α, E or β, and L or γ proteins (12, 13).

Inhibitors of protein and DNA synthesis as well as amino acid analogs have been used to operationally define the different gene classes and to elucidate their regulatory functions and synthetic requirements (13, 14, 23, 24). For example, when protein synthesis is inhibited by cycloheximide, viral transcription is limited to the IE genes (36), and thus only IE proteins are synthesized soon after the reversal of the block (13). IE protein synthesis is sustained and a subset of E proteins is synthesized when 2.8 mM canavanine is added at the onset of infection (14, 24). Inhibitors of viral DNA synthesis have been used to differentiate between E and L proteins and between different subsets of L proteins. A block in DNA synthesis imposed either by inhibitors such as phosphonoacetic acid (PAA), adenine arabinoside, or cytosine arabinoside or by DNA-negative temperature-sensitive mutants results in (i) the delayed shutoff of E protein synthesis, (ii) a reduction in the synthesis of the leaky late (EL) proteins which are a subset of polypeptides in the late class, and (iii) the absence of detectable synthesis of true L proteins (4, 13, 23, 25).

Viral proteins are the mediators of the regulatory events, and a variety of studies suggests that the proteins act at the level of transcription (9, 11, 16). Differential rates of transcription in the nucleus, the presence of RNA sequences in the cytoplasm, and the accumulation of functional RNA in the cytoplasm have been observed by filter hybridization of pulse-labeled RNA to restriction fragments of viral DNA, by solution hybridization of RNA to labeled DNA, and by in vitro translation of infected cell RNA, respectively (3, 12, 17, 31, 36). Given the complex and compact arrangement of genes within the HSV-1 genome (33), it has become apparent that a more specific approach is required for further characterization of HSV-1 gene expression. With some exception (29), little is known about the fate of individual transcripts, either in drug-treated cells or in the course of a normal infection, or to what extent the accumulation of viral RNAs correlates with the synthesis of their respective polypeptides. As a result, it has been difficult to evaluate the contribution of translational control to viral gene expression. Therefore, we first determined the effects of drug treatments on viral protein synthesis as well as the kinetics of viral protein synthesis in a normal infection. Using the same conditions of infection, we next determined the pattern of accumulation of the 4.7-kilobase (kb) infected cell polypeptide 4 (ICP-4) and 2.7-kb ICP-0 IE mRNAs, the 1.4-kb E thymidine kinase (TK) mRNA, and the 6.0-kb EL ICP-5 mRNA in both drug-treated infected cells and in the course of a normal infection.

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Our goals were as follows: (i) to establish the pattern of accumulation of HSV-1 RNAs which encode proteins of known kinetic classes and (ii) to assess the possible contribution of posttranscriptional, particularly translational control, on the regulated expression of these genes.

MATERIALS AND METHODS

Cells and virus. Plaque-purified HSV-1 (F) was passaged once in CV-1 cells before the inoculation of CV-1 cells at 0.01 PFU per cell for stock preparations. Virus was isolated from both the culture medium and cell lysates, and titers were subsequently determined on Vero cells. Vero cells were grown at 37°C in minimal essential medium containing 5 to 10% bovine calf serum, 100 μg of neomycin-sulfate per ml, and 2 mM glutamine.

Viral infection and drug treatment. Replicate cultures of Vero cells were infected with 20 PFU of HSV-1 (F) per cell at 37°C, with the zero time point defined as the time that virus was added to the cells. After a 1-h absorption period, the inoculum was removed and replaced with fresh medium. Unless otherwise indicated, absorption and overlay media were identical to the growth medium, except reduced to 2% bovine calf serum.

Cells were infected in the presence of either 25 μM anisomycin, 10 mM PAA, 2 mM canavanine, or 2.8 mM canavanine. The 25 μM anisomycin or 2.8 mM canavanine, each in normal medium, was added at the time of infection. Cultures were pretreated for 1 or 0.5 h before infection with 2 mM canavanine or 10 mM PAA, respectively. Whereas the 2.8 mM canavanine was present in normal arginine-containing medium (low-stringency canavanine), 2 mM canavanine was added to arginine-free medium (high-stringency canavanine) supplemented with 2% dialyzed fetal calf serum. In all cases, the drugs were present in the viral inoculum and the overlay medium.

Isolation of RNA. RNA was isolated from the cytoplasm of infected cells essentially as described by Preston (26). Cell monolayers were rinsed twice with phosphate-buffered saline before either scraping or trypsinization into cold phosphate-buffered saline. The cells were then pelleted at 4°C and washed once more with phosphate-buffered saline. After the suspension of cells in cold lysis buffer (0.2 M Tris [pH 7.6 to 8.5], 0.14 M NaCl, 2 mM $MgCl_2$, 20 μg of cycloheximide per ml), Nonidet P-40 was added to a 0.5% final concentration. After no more than 5 min on ice, nuclei were pelleted at 2,000 rpm at 4°C for 3 to 5 min. The supernatant was added to 3 volumes of TSE (10 mM Tris, 1 mM EDTA, 0.5% sodium dodecyl sulfate [SDS]) and phenol-chloroform extracted with 1 volume each of TSE-saturated redistilled phenol and chloroform until the interphase was clear. After the extraction with 1 volume of chloroform, RNA was precipitated at −20°C overnight after the addition of sodium acetate to 0.3 M and 2.5 volumes of 95% ethanol.

RNA was pelleted at 10,000 × *g* for 30 min and resuspended in 200 to 300 μl of TSE for a second ethanol precipitation. The second RNA pellet was resuspended in 30 μl of TSE plus 0.5% SDS per 8×10^6 to 10×10^6 cells. RNA was quantitated by spectrophotometry at 260 nm.

Gel electrophoresis and blot transfer of RNA. Total cytoplasmic RNA (10 μg) was denatured at 65°C in 50% formamide–2.2 M formaldehyde–1× MOPS running buffer (20 mM morpholinepropanesulfonic acid, 8 mM sodium acetate, 1 mM EDTA) for 5 to 10 minutes. The RNA was then quenched on ice, and 0.25 volumes of 5% Ficoll containing bromophenol blue was added to each sample. The RNA was electrophoresed through a 1.2% agarose gel containing 2.2 M formaldehyde (19). After electrophoresis, the RNA was blot transfered onto nitrocellulose as described by Southern and Thomas, except that 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) was used for the transfer (30, 32). After 4 to 12 h of blotting, the nitrocellulose filters were soaked briefly in 4× SSC, air dried, and baked for 2 to 3 h at 80°C in vacuo. Ethidium bromide-stained 28S and 18S rRNA or ^{14}C-labeled polyadenylated vesicular stomatitis virus RNAs were used as size markers.

DNA probes. All DNA fragments were derived from either pBR322 or pBR325 recombinant plasmids containing inserts of HSV-1 fragments as listed in Table 1. The plasmids were propagated in *Escherichia coli* strains HB101, LE392, or DH-1. The *Eco*RI clones [HSV-1 (KOS) in pBR325], the *Bam*HI clones [HSV-1 (F) in pBR322], and the pTK-1 clone [*Bam*-Q of HSV-1 (CL101) in pBR322] were the kind gifts of M. Levine, B. Roizman, and L. Enquist, respectively. *Bam*-Y and *Sal*-W were isolated from larger *Eco*RI (KOS) clones and subcloned into pBR322 in our laboratory.

Specific fragments were excised from plasmids (see Table 1) by restriction enzyme digestion as specified by the suppliers. The fragments were electroeluted from agarose, phenol extracted, and ethanol precipitated. A 0.5-μg portion of

TABLE 1. Description of HSV-1 DNA probes and summary of Northern blot analyses

Properties of DNA			Properties of RNA		
Fragment	Plasmid (virus strain)	Map coordinates (size)[b]	Size (kb)	Abundance	Class
Bam-SP	pRB104 (F)	0–0.022, (0.80–0.844), 0.976–1.0 (6.6)	4.7	High	IE
			2.6	Medium	IE
			1.3	Low	E
			0.9	Low	L
Bam-Y	pBam-Y (KOS)	0.844–0.856/0.964–0.976 (1.8)	4.7	High	IE
Bam-E/*Pst*	pRB111 (F)[a]	0.022–0.052 (4.5)	2.6	High	IE
			4.3	Medium	E
Bam-Q	pTK (F)	0.290–0.312 (3.4)	6.0	Low	Unknown
			4.6	Medium	E
			3.3	Medium	L
			1.4	High	E
Sal-W	pBH133 (KOS)	0.241–0.258 (2.5)	6.0	High	EL

[a] Clone of Bam-E in pBR322.
[b] Kilobase pairs.

purified fragment was labeled with ^{32}P by nick translation in a 50-μl reaction to a specific activity of 0.5×10^8 to 2×10^8 cpm/μg of DNA. The reaction mixture contained 4 μM [^{32}P]dCTP, 10 μm each dATP, dTTP, and dGTP, 1 ng of Worthington DNase, and 9 U of Boehringer-Mannheim polymerase I (Kornberg) in 50 mM Tris–5 mM MgCl–10 mM β-mercaptoethanol. DNA was preincubated for 15 min with DNase at 15°C before the addition of polymerase. Thereafter, the complete reaction was maintained at 15°C until it was stopped by the addition of 1 mM EDTA. After ethanol precipitation of the DNA, the unincorporated triphosphates were removed by chromatography on a G-75 Sephadex column.

Hybridization and autoradiography. Nitrocellulose filters were pretreated for 4 to 12 h at 42°C in a solution containing 50% formamide, 5× SSC, 5× Denhardt solution (1× Denhardt solution is 0.02% Ficoll, 0.02% polyvinylpyrolidone, 0.02% bovine albumin), 50 μg of sonicated denatured calf thymus DNA per ml, and 0.1 to 0.2% SDS. The filters were then hybridized with 0.4×10^6 to 1×10^6 cpm of ^{32}P-labeled DNA per ml in a solution containing 50% formamide, 5× SSC, 1× Denhardt, 100 μg of calf thymus DNA per ml, and 0.1 to 0.2% SDS for 36 to 48 h at 42°C. Filters were then washed twice at room temperature in 2× SSC–0.1% SDS for 15 to 30 min and twice at 52°C in 0.1× SSC–0.1% SDS for 30 min. After air drying, they were placed against Kodak XAR or Cronex film for autoradiography; Cronex intensifying screens were used when necessary.

Analysis of viral proteins. Infected cell proteins were pulse-labeled for 30 min with 20 to 30 μCi of [^{35}S]methionine per ml in methionine-free medium supplemented with 2% dialyzed fetal calf serum. For labeling anisomycin-treated cells, anisomycin was removed 30 min before labeling and was replaced with medium containing 5 to 10 μg of actinomycin-D per ml. After the labeling period, the cells were rinsed in phosphate-buffered saline and lysed in sample buffer containing 60 mM Tris (pH 6.8), 10% glycerol, 0.001% bromophenol blue, 2.7% SDS, and 5% β-mercaptoethanol.

The lysates were sonicated, boiled, and then analyzed by electrophoresis through *N,N'*-diallyltartardiamide cross-linked 7.5% polyacrylamide gels (10, 18). Gels were fluorographed, dried, and exposed to Kodak XAR film at −70°C (2).

Densitometry scanning and quantitation of probed RNA. Autoradiographs of probed RNA blots were scanned on an LKB (model no. 2200) Ultroscan laser beam densitometer. The peaks were localized, and the areas under each peak were integrated by an Apple II computer with the Gelscan-Intensity Fit program.

RESULTS

Effect of drug treatment on the synthesis of viral polypeptides. Although the effects of protein synthesis inhibitors such as anisomycin, amino acid analogs such as canavanine, and DNA synthesis inhibitors such as PAA on viral protein synthesis have been reported, it was necessary to determine the effects of our conditions of drug treatment on viral protein synthesis (13–15, 23–25, 34). Proteins were labeled with [^{35}S]methionine at 8 h postinfection in infected cultures treated with canavanine, anisomycin, or PAA. Infection for 8 h in the presence of the protein synthesis inhibitor anisomycin and reversal in the presence of actinomycin-D restricted the synthesis of viral polypeptides to the IE or α class. The IE ICP-4, ICP-0, ICP-22, and ICP-27 (Ymw 175, 110, 68, and 63, respectively) are the only viral polypeptides synthesized (Fig. 1, lane 7).

We determined the effect of the arginine analog canavanine on HSV gene expression by using two protocols of drug treatment (24). The first protocol (2.8 mM canavanine in normal medium) was similar to that used by Pereira et al. to block the synthesis of some E and most L proteins (24). In our hands, these conditions of canavanine treatment resulted in prolonged synthesis of IE proteins, but no apparent block in the synthesis of E or L proteins. The E TK and the EL ICP-5 were synthesized as were IE ICP-4 and IE ICP-27 (Fig. 1, lane 8). The second protocol (2.0 mM canavanine in arginine-free medium) proved to be a more stringent condition in that the pattern of viral protein synthesis was restricted to the synthesis of IE proteins. This result was similar to that reported by Pereira et al. (24). The IE ICP-4, ICP-0, ICP-22, and ICP-27 were synthesized at high rates under stringent conditions of canavanine (lane 9) as compared with the 5-h control (lane 10). Moreover, we failed to detect the synthesis of E TK or EL ICP-5 under these stringent conditions of canavanine. We did, however, observe a low level of E ICP-6 synthesis.

The inhibition of DNA synthesis by PAA gave results similar to that reported for other DNA synthesis inhibitors (11, 23, 25, 34). Synthesis of IE proteins such as ICP-4 and ICP-27 was prolonged, and E proteins such as TK and ICP-8 were synthesized at a higher rate in PAA-treated cells than in untreated cells (cf. lanes 6 and 4). The rates of synthesis for the EL ICP-5 was reduced in PAA-treated cells, and the synthesis of other L proteins such as ICP-15 was dramatically depressed in PAA-treated cells compared with the untreated control.

Kinetics of viral protein synthesis during a normal infection. The pattern of viral protein synthesis during a normal infection is also shown in Fig. 1. Proteins were pulsed for 30 min at various times after infection as indicated in the figure

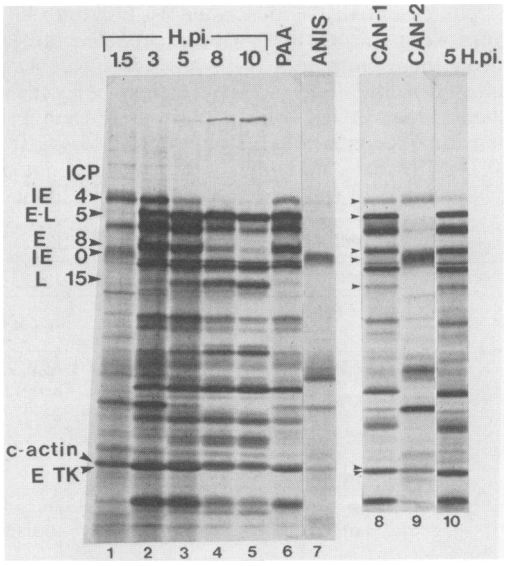

FIG. 1. Synthesis of HSV-1-infected cell polypeptides in drug-treated cells and in a normal infection. Cells were HSV-1 infected and pulsed with [^{35}S]methionine from 1 to 1.5 h postinfection (lane 1), 2.5 to 3 h postinfection (lane 2), 4.5 to 5 h postinfection (lane 3), 7.5 to 8 h postinfection (lanes 4, 6, and 7), 9.5 to 10 h postinfection (lane 5), or 5 to 5.5 h postinfection (lanes 8 to 10). Cells were treated with PAA (lane 6), anisomycin (ANIS [lane 7]), low-stringency canavanine (CAN-1 [lane 8]), high-stringency canavanine (CAN-2 [lane 9]), or no drug (lanes 1 to 5 and 10) as described in the text.

legend. We detected the synthesis of the IE ICP-4, ICP-0, ICP-22, and ICP-27 by 1 to 1.5 h (lane 1). In general, ICP-4 and other IE proteins attained peak rates of synthesis at around 3 h postinfection and declined in synthesis by 5 h postinfection. ICP-4, which was easily detected in HSV-1-infected cells, was synthesized as late as 10 h postinfection (lane 5). ICP-0, on the other hand, migrated as a diffuse band after pulse-labeling and was never very abundant in a normal infection.

The 43,000-dalton HSV-1 TK, which migrates just below cellular actin, has similar kinetics of synthesis to that of the E ICP-8 (the major DNA binding protein). TK, which was not detected by 1.5 h postinfection, was synthesized by 3 h postinfection. Synthesis of TK peaked between 3 and 5 h postinfection and declined by 8 to 10 h.

The leaky late or EL ICP-5 (the major capsid protein) was synthesized by 3 h postinfection and increased in the rate of synthesis by 5 h postinfection. ICP-5 continued to be synthesized at high rates at later times of infection. We were also able to identify viral proteins such as ICP-15 which were not synthesized until 3 to 5 h after infection. ICP-15 belongs to the set of proteins which are labeled at high rates at 8 h postinfection and are classified as L or true late proteins.

Accumulation of HSV RNA in the cytoplasm of drug-tested cells. We analyzed the accumulation of the individual viral transcripts corresponding to the polypeptides analyzed in the previous section. RNA was isolated from the cytoplasm of cells which had been infected for 8 h in the presence of either 10 mM PAA, 25 μM anisomycin, 2.0 mM canavanine, 2.8 mM canavanine or in the absence of drug as described above.

(i) IE RNA (ICP-4). *Bam*-SP and *Bam*-Y (Table 1, lines 1 and 2) were used to probe the 4.7-kb mRNA for IE ICP-4 which maps within the short repeat of the genome (7). *Bam*-SP is a junction fragment which lies totally within the repetitive sequence of the IR_S (*Bam*-P) and the IR_L (*Bam*-S) (for a review, see reference 28). The ICP-4 transcript initiates in *Bam*-N and extends leftward through *Bam*-Y into *Bam*-P (3, 20). As detected by the *Bam*-Y probe, the IE ICP-4 RNA greatly overaccumulated in infected cells when protein synthesis was inhibited (Fig. 2A). The level of ICP-4 RNA in anisomycin-treated cells was about 10-fold more than is present in control, untreated cells (Fig. 2A, lanes 1 and 4).

When cells were infected under either low- or high-stringency canavanine conditions, ICP-4 RNA accumulated to levels similar to that seen in untreated cells (Fig. 2A, lanes 2 to 4). This was quite surprising, since under conditions of 2.0 mM canavanine in arginine-free medium (high stringency), no E or L transcripts were detectable; thus, we would not have expected IE transcripts to be under any negative transcriptional control mediated by β or γ polypeptides. In contrast, transcripts of all subsequent gene classes accumulated to detectable levels in infected Vero cells treated with 2.8 mM canavanine (low stringency) (see below).

PAA inhibits the HSV-1 DNA polymerase and therefore blocks viral DNA synthesis (15). The expression of ICP-4 RNA was unaffected by preventing DNA replication, since approximately the same level of ICP-4 RNA accumulated in PAA-treated cells as in the drug-free 8-h control (Fig. 2A, lanes 4 and 5).

(ii) IE RNA (ICP-0). The mRNA for the IE protein ICP-0 maps entirely within the IR_L region of the HSV-1 genome and hybridizes to the terminal repeat sequences contained within *Bam*-E (21). Since the unique sequences of *Bam*-E hybridized to an L transcript which is similar in size to the

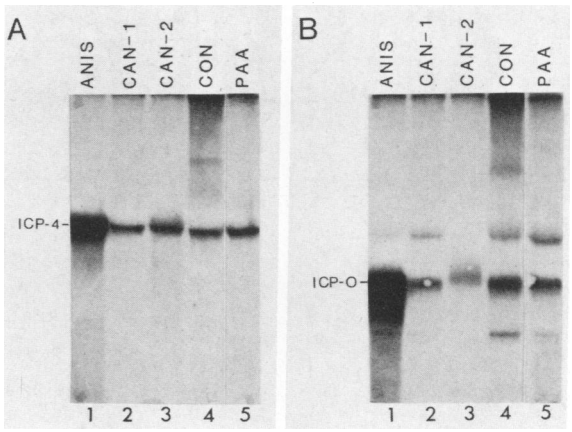

FIG. 2. Effect of drug treatment on the accumulation of IE RNA for ICP-4 (A) and ICP-0 (B) in the cytoplasm of HSV-1-infected cells. Cells were treated with anisomycin (ANIS [lane 1]), low-stringency canavanine (CAN-1 [lane 2]), high-stringency canavanine (CAN-2 [lane 3]), PAA (lane 5), or no drug (CON [lane 4]) as described in the text. Cytoplasmic RNA was isolated at 8 h postinfection and analyzed by Northern blot hybridization as described in the text. Blots were probed with *Bam*-Y (A) which hybridizes to RNA for ICP-4 and *Bam*-E/*Pst* (B) which hybridizes to RNA for ICP-0.

ICP-0 RNA (manuscript in preparation), we used a 4.8-kb subfragment of *Bam*-E which extends from the left *Bam*-HI site to a *Pst* site to probe for ICP-0 RNA (Table 1, line 3). Although this IE transcript has been estimated to be 3.0 kb in length with rRNA markers (36), we estimate the size to be 2.6 to 2.7 kb with vesicular stomatitis virus RNA size markers (data not shown).

The 2.7-kb RNA for ICP-0 was greatly overproduced in anisomycin-treated infected cells (Fig. 2B, cf. lanes 1 and 4). About 10-fold more ICP-0 RNA was detected in cells blocked in protein synthesis than in the control cells. Infection in the presence of either PAA (lane 5) or 2.8 mM canavanine (lane 2) failed to substantially alter the accumulation of the ICP-0 RNA relative to the control level (lane 4). Accumulation of this IE transcript in cells treated with 2.0 mM canavanine was not quantitatively different from the control. However, we consistently observed a diffuse, slower migrating form of the ICP-0 mRNA under these conditions of infection (lane 3). Such a shift was not apparent in infected cells treated with anisomycin, PAA, or lower stringency conditions of canavanine, but it has been observed in cytoplasmic RNA of 2.0 mM canavanine-treated cells in three independent experiments. Furthermore, the two forms of ICP-0 RNA could be distinguished when RNA from anisomycin-treated and canavanine-treated cells were mixed and electrophoresed in the same lane (data not shown). The cause for this shift in migration is not known and will be discussed later.

(iii) E RNA (TK). *Bam*-Q (Table 1, line 4) was used to probe for the 1.4-kb RNA encoding the E protein TK (22, 29, 37). The fragment contains sequences complementary to a 4.6-kb E transcript, a 3.3-kb L transcript, and several low-abundancy transcripts which migrate slower than does 28S rRNA. The 5′ terminus of the 3.3-kb L transcript has been shown to overlap the 3′ noncoding region of the TK transcript (29).

No transcripts were detected by the *Bam*-Q probe in cytoplasmic RNA of infected cells treated with anisomycin or with 2.0 mM canavanine in arginine-free medium (Fig. 3,

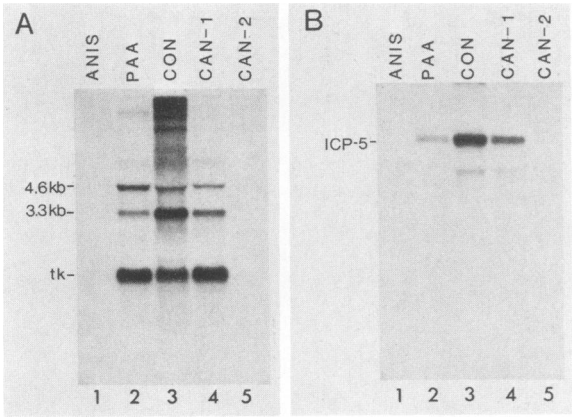

FIG. 3. Effect of drug treatment on the accumulation of E RNA for TK and the EL RNA for ICP-5. RNA was isolated from cells treated as follows: lane 1, anisomycin (ANIS); lane 2, PAA; lane 3, no drug (CON); lane 4, low-stringency canavanine (CAN-1); or lane 5, high-stringency canavanine (CAN-2); Northern blots were probed with *Bam*-Q (A) and *Sal*-W (B).

lanes 1 and 5, respectively). This is in contrast to the results obtained when cells were treated with 2.8 mM canavanine in normal medium. Under the latter conditions, the E 1.4-kb TK RNA was overrepresented, the 4.6-kb E RNA was present in approximately control amounts, and the 3.3-kb L RNA was slightly underrepresented (lanes 3 and 4). In PAA-treated infected cells, both of the E transcripts accumulated in excess of that normally found at this time of infection, whereas the L 3.3-kb RNA failed to accumulate to control levels (lane 2).

(iv) EL RNA (ICP-5). *Sal*-W (Table 1, line 5) was used to probe for the abundant 6.0-kb mRNA which maps between 0.228 and 0.267. This RNA has been shown to encode ICP-5, the major capsid protein (5, 6). The 6.0-kb RNA was not detected in cells infected in the presence of anisomycin or under high-stringency conditions of canavanine treatment (Fig. 3B, lanes 1 and 5, respectively) but accumulates to less than the control (lane 3) level in cells treated under the low-stringency canavanine conditions (lane 4). The 6.0-kb transcript was also underrepresented in infected cells which were blocked in DNA synthesis by PAA (lane 2). Although the level of ICP-5 mRNA was reduced in PAA-treated cells, the RNA was readily detectable in amounts between 20 to 30% of the control.

Accumulation of HSV-1 RNA during normal infection. To determine the kinetics of accumulation of transcripts during a normal infection, we isolated total cytoplasmic RNA at 1 to 6, 8, and 10 h postinfection. The RNA was electrophoresed and analyzed by Northern blot hybridization as described above.

(i) Kinetics of accumulation of IE ICP-4 and ICP-0 RNAs. *Bam*-SP and *Bam*-E/*Pst* were used as probes for the ICP-4 and ICP-0 RNAs, respectively. Representations of the autoradiograph for each transcript are shown in Fig. 4. Both IE mRNAs were abundant by 1 h postinfection (Fig. 4A and B, lane 1) and were the only RNAs we detected at this time with our battery of probes. The amounts of these RNAs continued to increase for up to 3 to 4 h of infection. After the peak in accumulation, the level of RNA declined somewhat until 8 h postinfection for ICP-4 RNA and until 6 h postinfection

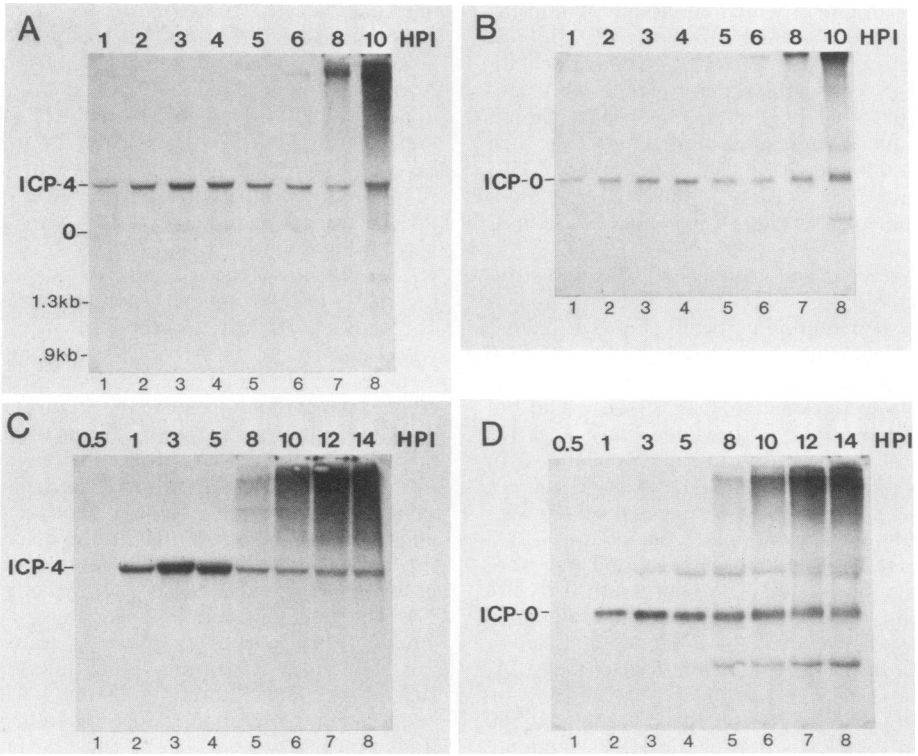

FIG. 4. Kinetics of accumulation for IE RNAs for ICP-4 and ICP-0. Cytoplasmic RNA was extracted at times indicated and analyzed as described in the text. Northern blots were probed with *Bam*-SP (A), *Bam*-E/*Pst* (B and D), and *Bam*-Y (C). The positions of the 1.3- and 0.9-kb RNAs which hybridize to *Bam*-SP on longer exposures of the blot are indicated in A.

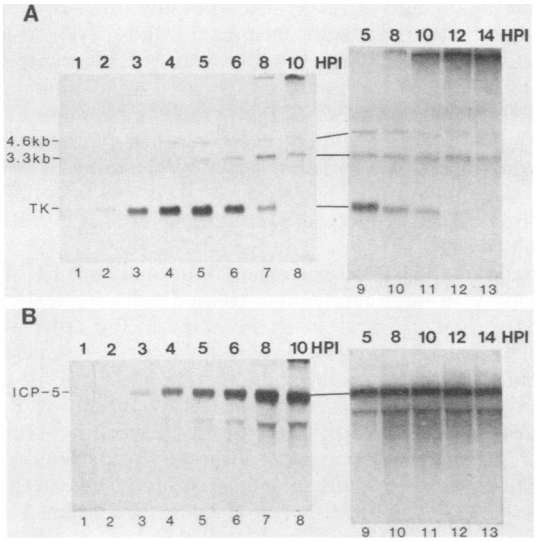

FIG. 5. Kinetics of accumulation for RNAs hybridizing to *Bam*-Q and for the EL RNA for ICP-5. Time course RNA was prepared and analyzed as described in the legend to Fig. 4, except the blots were probed with *Bam*-Q (A) and *Sal*-W (B).

for ICP-0 RNA. Subsequently, both ICP-4 and ICP-0 RNA persisted to 10 h postinfection. In Fig. 4 and 5, the hybridization near the top of lanes containing RNA isolated late in infection is presumed to represent the detection of progeny virion DNA present in the cytoplasm at these times. This hybridization was not detected when RNA was isolated at late times and polyadenylic acid selected (data not shown) or when total cytoplasmic RNA was isolated at late times from cells treated with canavanine or PAA (Fig. 2 and 3).

Although it is not apparent from the autoradiograph (Fig. 4A), we were able to detect low-abundancy 1.3- and 0.9-kb RNAs in the time course experiment with the *Bam*-SP probe. On longer exposures of this blot, the 1.3-kb transcript was detectable between 3 and 6 h of infection; the 0.9-kb RNA was only observed at 8 and 10 h postinfection. We tentatively assigned these transcripts to the E and L class, respectively.

Since the IE RNAs were present in the cytoplasmic RNA by 1 h and remained at 10 h after infection, we also analyzed cytoplasmic RNA at an earlier time and at later times. Total cytoplasmic RNA was extracted at 0.5, 1, 3, 5, 8, 10, 12, and 14 h postinfection and analyzed by Northern blot hybridization. Figure 4C represents the autoradiograph of a blot probed with probe *Bam*-Y for ICP-4 RNA. RNA for ICP-4 was not detected at 0.5 h postinfection (lane 1) but was readily apparent by 1 h postinfection (lane 2). Levels of ICP-4 RNA peaked between 3 and 5 h, declined by 8 h, and persisted for up to 14 h of infection. Although it is difficult to precisely measure, we estimate that 6 to 14% of the peak level of ICP-4 RNA was present at 14 h. Furthermore, pulse-labeling of proteins in infected cells suggests that the mRNA for ICP-4 is functional at 10 h postinfection (Fig. 1, lane 5).

Similar analysis of the RNA for ICP-0, as monitored by the *Bam*-E/*Pst* probe, was conducted (Fig. 4D). The early kinetics of ICP-0 RNA was similar to the ICP-4 RNA in that the RNA for ICP-0 was not detected in the cytoplasmic RNA at 0.5 h but appeared by 1 h postinfection (lanes 1 and 2). The amount of ICP-0 increased from 1 to 3 h and declined slightly from 3 to 5 h postinfection. In contrast to ICP-4 RNA, relatively high levels of ICP-0 RNA were detected at 8, 10, 12, and 14 h (lanes 5 to 8). Approximately 40% of the peak values for ICP-0 RNA persisted at 14 h of infection. Similar kinetics of accumulation for ICP-0 RNA were obtained when the 2.6-kb *Bam-Sal* fragment of the IR_L (from *Bam*-B) was used as a probe. We did not observe a slow-migrating form of ICP-0 RNA at any time of infection such as that found in cells infected under stringent canavanine conditions (Fig. 2A).

(ii) Kinetics of accumulation of E TK RNA. The autoradiogram of time course RNA probed with *Bam*-Q is shown in Fig. 5A. No RNA was detected at 1 h postinfection. The 1.4-kb TK RNA was present in the cytoplasm of infected cells by 2 h and accumulated to a maximum level at ca. 5 h of infection. The level of E TK RNA declined steadily from 6 to 10 h. (Fig. 5A, lanes 6 to 8) and from 8 to 14 h (Fig. 5A, lanes 10 to 13). Similar kinetics were obtained for the E 4.6-kb transcript which is detected by the *Bam*-Q probe, although this lower-abundance RNA was detectable at 2 h only upon longer exposures of the blot. More of this transcript persisted at late times as compared with the 1.4-kb TK RNA. Such differences may be, in part, a function of background hybridization in the region of 28S rRNA or may be in the synthesis or stability of the RNAs. The 3.3-kb *Bam*-Q transcript was present by 2 to 3 h of infection in longer exposures of the probed blot shown in Fig. 5, and it increased in abundancy between 3 and 8 h of infection. The level of this L class transcript remained high at 10 h. (Fig. 5A, lane 8) and as late as 14 h postinfection (lane 13). Based on the similarity of kinetics (see below) and drug response (Fig. 3) to the ICP-5 transcript, we assigned this RNA to the L class.

The kinetics of accumulation of the three most abundant transcripts which hybridize to *Bam*-Q in infected mouse LMTK cells have been reported by Sharp et al. (29). Our results are consistent with theirs in terms of the pattern of expression but differ with regard to the specific times. They were unable to observe the 1.4-kb TK RNA at 2 h or the 3-kb L transcript before 6 h. We attributed these differences to the different host cell lines used in the studies.

(iii) Kinetics of the EL ICP-5 RNA. *Sal*-W was used to determine the kinetics of accumulation of the RNA for ICP-5. The 6.0-kb RNA was not detected at 1 h (Fig. 5B, lane 1) but was detected at 2 h upon long exposure of the blot. ICP-5 RNA was easily seen at 3 h and accumulated almost linearly to 8 h of infection (lanes 4 to 7). High levels of ICP-5 RNA persisted in the cytoplasm at 10 h (lane 8) and for up to 14 h of infection (lanes 9 to 13).

DISCUSSION

Previous RNA studies have been useful in characterizing the overall behavior of genes within a particular class. However, information about the metabolism of individual RNAs during an infection is quite limited. Therefore, we have determined the fate of individual transcripts in both drug-treated cells and in the course of a normal infection. These studies are essential not only for ultimately understanding many aspects of regulation for a particular gene but also for comparing the expression of genes within a class and between genes of different classes.

Accumulation of HSV-1 RNA in the cytoplasm of drug-treated cells. Only IE transcripts were detectable in the cytoplasm of infected cells when anisomycin was added at the time of infection and maintained throughout. Under such conditions, the IE RNAs for ICP-4 and ICP-0 were over-

represented ca. 10-fold above the 8-h control (Fig. 2). These results correlate well with the restricted pattern of viral protein synthesis upon the reversal of the block and are consistent with the hypothesis that a protein is synthesized during the early stage of infection which "down-regulates" the transcription of IE genes.

The effect of canavanine on RNA accumulation differed, depending on the conditions of treatment. In our hands, an effective block in viral gene expression was only attained when canavanine was added to arginine-free medium. Under stringent conditions, only IE RNAs were detected, and the accumulation of TK and ICP-5 RNAs was blocked.

Consistent with the reported effects of viral DNA synthesis inhibitors on viral protein synthesis, the effects of PAA treatment on the accumulation of discrete HSV-1 RNAs were primarily quantitative. The RNA for the E TK was overrepresented at 8 h, whereas the RNA for the EL ICP-5 was underrepresented (Fig. 3). Maximum expression of L class RNAs requires viral DNA synthesis, and in the absence of sufficient L gene expression, the shutoff of E gene expression is retarded (11, 12, 23, 25, 31, 34).

Accumulation of HSV-1 RNAs during normal infection. The patterns of accumulation for RNAs of IE, E, EL, and L classes are each distinct. With the exception of the IE RNAs for ICP-4 and ICP-0 (discussed below), transcripts which respond differently to a given drug treatment display different kinetics of accumulation in the course of a normal infection (manuscript in preparation). The kinetics of accumulation of ICP-4 and ICP-0 RNA were similar at early times of infection. Both IE RNAs were first detected by 1 h of infection, continued to accumulate at 3 to 4 h of infection, and were detectable at late times of infection (Fig. 4). The E 1.4-kb TK RNA was present in the cytoplasm by 2 h of infection, accumulated to maximum levels at 5 h of infection, and, in contrast to the IE RNAs, declined by 10 h (Fig. 5). The EL 6.0-kb ICP-5 RNA was barely detectable at 2 h of infection and accumulated almost linearly to a peak at 8 h. High levels of this EL were maintained for up to 14 h (Fig. 5B).

Correlation between rates of protein synthesis and the accumulation of mRNA. Our results suggest that translational controls may be involved in modulating the expression of IE genes for ICP-4 and ICP-0. We observed that the rates of ICP-4 and ICP-0 protein synthesis did not correlate well with the accumulation of the respective cytoplasmic mRNAs under several conditions of infection. The IE ICP-0 was synthesized at early times of infection (1 to 3 h postinfection) but not at late times (10 h postinfection), although up to 40% of the 3-h amount of the mRNA persisted as late as 14 h of infection. Higher rates of synthesis of ICP-4 and ICP-0 were attained in infected cells under stringent canavanine conditions as compared with the drug-free control. In contrast, neither the ICP-4 nor ICP-0 mRNA accumulated to levels significantly above the drug-free control (Fig. 2).

We speculate that the shutoff of IE protein synthesis is, in part, mediated by the inability of IE RNAs to compete effectively with E or L RNAs in protein synthesis. Differences in methylation of RNA late in infection, as reported by Bartkoski and Roizman (1), or differences in the functional stability of IE RNAs, as suggested by Read and Frenkel (27), may provide the mechanism for translational discrimination.

Comparisons of accumulated RNA and rates of protein synthesis suggested that translational controls are not important in the regulation of the TK or ICP-5 genes. The peak accumulation of TK RNA (between 4 and 5 h postinfection) corresponded well with the peak rates of TK protein synthesis, and the decline of TK synthesis at late times of infection coincided with the disappearance of the RNA from the cytoplasm. Furthermore, the high rates of TK synthesis in PAA-treated cells were matched by an overaccumulation of the mRNA. The onset of ICP-5 synthesis between at ca. 2 and 3 h postinfection and increased rates of synthesis from 3 to 8 h postinfection correlated well with the kinetics of ICP-5 RNA accumulation. The reduced synthesis of ICP-5 in PAA-treated cells reflected a reduced level of ICP-5 RNA in the cytoplasm.

Regulation of IE gene expression. Our analysis of IE RNAs in anisomycin- and canavanine-treated cells suggests that a limited set of viral proteins is involved in the down-regulation of IE gene expression. It was apparent from analyses of both mRNA and protein that accumulation of E and L transcripts can be prevented either by inhibiting protein synthesis altogether or by eliminating a normal viral function by extensive canavanine incorporation. Surprisingly, these manipulations have distinguishable consequences on IE RNA accumulation. The levels of ICP-4 and ICP-0 RNA which accumulated in anisomycin-treated cells was much greater than the levels of these RNAs which accumulated in cells under stringent conditions of canavanine. We envision three explanations which are consistent with these results. (i) The transcriptional apparatus of the cell is altered under stringent canavanine treatment. This general effect combined with the synthesis of aberrant viral proteins results in the reduced accumulation of IE RNAs and the block in E gene expression. This may not be a trivial explanation, since we observed altered migration of ICP-0 RNA (Fig. 2, lane 3) and other IE RNAs (manuscript in preparation) in canavanine-treated cells. (ii) IE genes are under negative regulation in canavanine-treated cells. This negative regulation could be mediated by one or more arginine-poor proteins which are synthesized under stringent canavanine conditions. These proteins include E polypeptides of the ICP-6 class (since E ICP-6, and not the E TK, was synthesized under stringent conditions of canavanine) and all members of the IE class, i.e., ICP-4, ICP-0, ICP-22, ICP-27, and ICP-47. Such an autoregulatory property has been proposed for ICP-4 (35). It is also possible that an IE protein, other than ICP-4 or together with ICP-4, regulates the expression of IE transcription. (iii) IE RNA is less stable in canavanine-treated cells than in anisomycin-treated cells. Fenwick and Clark (8) have suggested that ICP-0 or some "pre-α" function is important in maintaining the functional stability of IE RNAs in HSV-2-infected cells. It is proposed that this function is an HSV-1, but not HSV-2, virion component. It is possible that canavanine-substituted IE proteins (in particular ICP-0) compete or interfere with the function of the virion polypeptide consequently reduce the stability and therefore the accumulation of IE RNAs. Alternatively, IE RNAs are more stable when protein synthesis is blocked than when translation is allowed to proceed.

Our results also suggest that some aspects of regulation differ within genes of the IE class. Although the pattern of accumulation of ICP-0 and ICP-4 RNAs were similar at early times of infection, the RNA for ICP-4 persisted at low levels and the RNA for ICP-0 persisted at fairly high levels as compared with their peak levels of accumulation. Differences in the expression of ICP-4 and ICP-0 have been noted in HSV-2-infected cells (8). These differences may indicate different mechanisms for the regulation of these two genes. Similar analyses of other IE genes should reveal whether IE genes can be divided into two or more subsets of differentially expressed genes.

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