Viral DNA Synthesis Is Required for the Efficient Expression of Specific Herpes Simplex Virus Type 1 mRNA Species

LOUIS E. HOLLAND,* KEVIN P. ANDERSON,* CHARLES SHIPMAN, JR.,†
AND EDWARD K. WAGNER*,1

*Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California 92717,
and †Department of Oral Biology and the Dental Research Institute, School of Dentistry,
The University of Michigan, Ann Arbor, Michigan 48109

Inhibition of HSV-1 DNA synthesis with either arabinosyladenine plus the adenosine deaminase inhibitor pentostatin, or with arabinosylthymine, showed a viral mRNA population identical to that seen prior to viral DNA replication (early) by the criteria of quantitative hybridization, specific mRNA species identifiable by Southern blot hybridization of size-fractionated RNA, and migration of polypeptides resolved by *in vitro* translation of purified viral mRNA. The amount of viral mRNA associated with infected cell polyribosomes was determined by quantitative DNA excess solution hybridization. At 2 hr postinfection (p.i.) (before viral DNA synthesis) and in drug-treated cells at 6 hr p.i., the majority of the polyadenylated RNA was cell specific with some virus-specific RNA detectable. In contrast, at 6 hr p.i., in the absence of drugs (during maximum viral DNA synthesis), nearly all the polyadenylated polyribosomal RNA was viral. Blot hybridization of size-fractionated viral RNA confirmed several specific differences between the viral mRNA species occurring before and after HSV-1 DNA synthesis, which have been reported previously from this laboratory. These differences also were reflected in the *in vitro* translation products encoded by the viral mRNAs. The mRNA species and the encoded polypeptides that were present in the absence of viral DNA synthesis are a subset of those viral mRNA species and polypeptides expressed in the presence of viral DNA synthesis. The viral mRNA species fall into several groups based on their relative abundance at various times of infection. These data suggest that, in the normal virus infection cycle, the onset of viral DNA synthesis is necessary for normal expression of later viral genes.

INTRODUCTION

Much information is accumulating on the transcriptional and translational events in herpes simplex virus type 1 (HSV-1) infected cells. However, the role of HSV-1 DNA replication in the temporal control of viral mRNA expression is not well understood. Although viral mRNA sequences expressed after viral DNA replication (late) are also detectable before replication (early) (Kozak and Roizman, 1974; Roizman et al., 1974), it is very clear that differences in abundance of viral mRNAs exist (Silverstein et al., 1973; Swanstrom and Wagner, 1974; Swanstrom et al., 1975; Stringer et al., 1977). Prior to viral DNA replication, viral

mRNA species representing approximately 25% of the HSV-1 DNA sequences are found in 10- to 20-fold higher concentrations than the mRNA species representing an additional 20% of the viral genome. By 6 hr postinfection (p.i.), when viral DNA synthesis is maximal, 40 to 45% of the viral genome is represented as abundant RNA. However, inhibitors of DNA synthesis prevent this change in abundance class ratio (Swanstrom et al., 1975).

Approximately 50 virus-specific polypeptides have been identified in HSV-1 infected cells (Honess and Roizman, 1973; Marsden et al., 1976). These have been differentiated into three groups—α, β, and γ—based on their kinetics of synthesis (Honess and Roizman, 1974). The α polypeptides are the ear-

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1 To whom requests for reprints should be addressed.

liest polypeptides made and are the only ones made immediately following removal of inhibitors of protein synthesis, suggesting that they do not require prior protein synthesis in order for their mRNA to be expressed. The α polypeptides are encoded by the α mRNAs, or immediate early mRNAs, which represent 10 to 15% of the viral DNA. These immediate early mRNAs are contained in the abundant class of viral transcripts early in infection, and map in noncontiguous regions of the viral genome (Kozak and Roizman, 1974; Clements et al., 1977; Jones et al., 1977). Following the appearance of the α polypeptides, new mRNA is required for the expression of the β polypeptides (Honess and Roizman, 1974; Preston, 1979). However, all β polypeptide synthesis is not necessarily controlled by the same α polypeptide(s) (Pereira et al., 1977; Preston, 1979). The β mRNA species presumably compose the remainder of the viral mRNA abundant prior to DNA synthesis.

Taken together, the mRNA species encoding α and β proteins can be classified as early mRNA. Stringer et al. (1978) used R-loop mapping techniques to show this early mRNA maps in noncontiguous regions of the viral genome. Jones and Roizman (1979) used HSV-1 restriction fragments hybridized in solution to an excess of RNA to similarly show that the viral mRNA abundant in the presence of the DNA synthesis inhibitor phosphonoacetic acid is transcribed from noncontiguous regions throughout the viral genome. Using Southern blot hybridization of size-fractionated polyribosomal poly(A) RNA, we have been able to specifically identify and map 16 of these early abundant HSV-1 mRNA species (Holland et al., 1979).

Although the early mRNA species are abundant prior to viral DNA synthesis, this is not in itself a sufficient condition to define the role of viral DNA replication in the normal expression of the late mRNA species coding for the synthesis of γ proteins. It is possible that the presence of the β polypeptides alone is sufficient for normal γ polypeptide expression. Thus, γ polypeptides could be detected in cells where readily detectable viral DNA synthesis was inhibited, although their levels were reduced at least two- to threefold over normal levels (Honess and Roizman, 1974; Powell et al., 1975). However, Ward and Stevens (1975) examined total infected cell polypeptides from normal cells and from cells in which DNA synthesis was inhibited, and suggested a rigorous requirement for viral DNA replication in expression of certain viral functions. Honess and Watson (1977) have similarly shown that under stringent conditions for inhibition of HSV-1 DNA synthesis the late polypeptides are reduced by at least 85%. Also, Powell et al. (1975) showed that certain late (γ) viral polypeptides were missing in cells in the absence of DNA synthesis.

Because of pleiotropic effects of DNA negative mutants (Marsden et al., 1976; Stow et al., 1978; Preston, 1979), the impossibility of rigorously excluding some viral DNA synthesis under any experimental regime, and possible nonspecific toxic effects of inhibitory drugs, it is not clear that we can completely define the role of viral DNA replication in gene expression. We have, therefore, asked a more limited question: Does the inhibition of HSV-1 DNA synthesis result in a population of viral mRNA species of greater complexity than that seen prior to viral DNA replication?

We have utilized our previously developed viral mRNA mapping procedures to examine the viral mRNA abundant in the absence of DNA synthesis. In order to inhibit viral DNA synthesis without inducing undue toxic effects on the cells, we have used two nucleoside analogs of recent therapeutic interest, 9-β-D-arabinofuranosyladenine (ara-A) and 1-β-D-arabinofuranosylthymine (ara-T). The value of these drugs is based on the observation that, at therapeutic levels, they efficiently inhibit HSV-1 DNA synthesis while being relatively nontoxic to the host cells (Shipman et al., 1976; Aswell et al., 1977).

In addition to mapping the viral mRNA species abundant when viral DNA replication is inhibited, we have used HSV-1 DNA bound to cellulose as a reagent for the isolation of HSV-1-specific mRNA (Anderson et al., 1979; Holland et al., 1979). Such purified viral mRNA is shown to be an efficient template for synthesis of viral polypeptides

in a reticulocyte lysate system. We find that viral polypeptides encoded by earlier mRNA in this system are the same as those seen using viral mRNA from the drug-treated cells and both are essentially a subset of those encoded by late viral mRNA. Taken together, these data confirm that viral DNA replication is necessary for the normal expression of late viral genes.

MATERIALS AND METHODS

Drugs. Ara-A was provided through the courtesy of Dr. Harold E. Machamer of Parke, Davis and Company, Detroit, Michigan. Pentostatin, a known adenosine deaminase inhibitor (Woo *et al.*, 1974), was also the gift of Dr. Machamer. This drug was used along with ara-A to insure that ara-A was not deaminated to the less effective arabanosylhypoxanthine in the cells (Schwartz *et al.*, 1976). Ara-T was kindly provided by Dr. G. A. Gentry, University of Mississippi Medical Center, Jackson, Mississippi.

Cells and virus. Monolayer cultures of HeLa cells were grown in Eagle's minimum essential medium with Earle's salts, 10% calf serum, and no antibiotics. Growth conditions and assay for mycoplasmal contamination were as previously described (Sutherland *et al.*, 1975). Stocks of the KOS strain of HSV-1 were grown in HeLa cells at a multiplicity of infection of 0.1 PFU/cell and prepared as previously described (Wagner *et al.*, 1976).

Infection and labeling of cells. Infection at a multiplicity of 10 PFU/cell and labeling conditions for [^{32}P]orthophosphate (New England Nuclear Corp.) and [^{3}H]uridine (28 Ci/mmol, Schwarz/Mann) were as previously described (Holland *et al.*, 1979). Cells were maintained on drug-containing or control medium for 1 hr before infection (or mock infection), and maintained continuously on drug-containing or control medium after infection (or mock infection) until extraction.

Isolation of RNA. Polyribosome-associated RNA was isolated from cells lysed with Triton X-100 by the Mg^{2+} precipitation method of Palmiter (1974), followed by proteinase K (Merck and Co., Inc.) digestion and phenol–chloroform extraction (Stringer

et al., 1977; Holland *et al.*, 1979). Polyadenylated (poly(A)) RNA was purified by use of oligodeoxythymidylic acid-cellulose (oligo(dT)-cellulose, Collaborative Research, Inc.).

Isolation of HSV-1 DNA. Supernatant and cytoplasmic virions purified from cells at 20 hr p.i. by differential centrifugation were digested with proteinase K in the presence of 2% Sarkosyl and 0.5% sodium dodecyl sulfate (Holland *et al.*, 1979). The viral DNA then was purified by a single cycle of isopycnic centrifugation (Wagner *et al.*, 1974).

Analysis of cellular and viral DNA synthesis. Cells grown in 25-cm^2 T-25 flasks (Corning Glass Works) were rinsed twice with cold 0.15 M NaCl, then lysed in the flask by the addition of 100 mM NaCl–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–20 mM EDTA–2% Sarkosyl (pH 7.8). After digestion with 200 µg proteinase K/ml for at least 8 hr at 37°, samples were extracted with phenol and chloroform and ethanol precipitated. The nucleic acid pellet was dissolved in 0.1 × SSC (SSC is 0.15 M NaCl–0.015 M Na$_3$ citrate), and its absorbance at 260 nm was determined. Two A_{260} units (~100 µg) of each sample were adjusted to a final density of 1.700 g/ml by the addition of CsCl (6.551 g CsCl to 5 ml of sample). The gradients were layered with mineral oil, then spun for 44 hr at 44,000 rpm at 17° in a Beckman 65 rotor. Gradients were collected dropwise from the bottom into 0.175-ml fractions and refractive indexes were read to determine density. The TCA-insoluble material was collected on Whatman GF/C filters and the radioactivity was determined using toluene scintillation fluid.

Viral RNA quantitation by solution hybridization. Samples for RNA quantitation were digested with 100 µg electrophoretically purified DNase I (Sigma Chemical Co.) per milliliter in 10 mM NaCl–10 mM Tris–4.5 mM MgCl$_2$ (pH 7.4) for 20 min at 37°. Samples were adjusted to contain 100 mM NaCl–5 mM EDTA, then were phenol–chloroform extracted and ethanol precipitated. The RNA was stored in 100 mM NaCl–10 mM Tris–1 mM EDTA (pH 7.4) at 20° until hybridization.

The proportion of HSV-1 specific RNA in RNA preparations was determined as by Rice et al. (1979). A small amount of radioactive RNA (0.2–1.5 μg), containing an unknown amount of viral RNA, was hybridized to an excess of HSV-1 DNA. Each hybridization had a total DNA concentration (HSV-1 or Escherichia coli) of 100 μg/ml, and the total RNA concentration was adjusted to 1 mg/ml with uninfected HeLa cell RNA carrier. Hybridizations were carried out under mineral oil at 57° in 0.05 ml of 400 mM Na^{+}–100 mM HEPES–5 mM EDTA (pH 8.0) (hybridization buffer) containing 80% recrystallized formamide. These conditions allow HSV-1 DNA:RNA reassociation in the absence of any DNA:DNA reassociation (Casey and Davidson, 1977; Holland et al., 1979). Hybridizations were carried out to a DNA C_0t of 16–20, sufficient to drive the maximum amount of radioactivity into hybrid. Hybridized RNA was defined as the TCA-precipitable radioactivity remaining after digestion with 50 μg pancreatic RNase (Sigma Chemical Co.) and 10 units RNase T$_1$ (Calbiochem–Behring Corp.) per milliliter for 45 min at 37° in 2 × SSC. The proportion of sample RNA that was viral was then calculated from the efficiency of hybridization of a pure viral RNA sample included in each hybridization as a control (Rice et al., 1979).

Hybridization to HSV-1 DNA-cellulose. Our modification of the Noyes and Stark (1975) procedure for the covalent coupling of DNA to cellulose has been published elsewhere (Anderson et al., 1979). In these experiments, 10–20 μg of poly(A) RNA was hybridized to 16–24 μg of HSV-1 DNA coupled to cellulose. Hybridizations were done in 0.1 ml of hybridization buffer containing 80% recrystallized formamide at 57° for 4 hr. The eluted viral RNA was adjusted to 100 mM sodium acetate and ethanol precipitated (Anderson et al., 1979).

Restriction endonuclease digestion. The restriction endonucleases, BglII, HindIII, and XbaI, were obtained from New England Biolabs. Digestion conditions were as described by the suppliers. Electrophoresis of restriction fragments was at room temperature on horizontal slabs (42 × 20

× 1.2 cm) of 0.5% agarose (Sigma) containing 0.5 μg ethidium bromide/ml for 48 hr at 70 mA (1.2 V/cm). The electrophoresis buffer (40 mM Tris–5 mM sodium acetate–2 mM EDTA (pH 7.8)) was continuously circulated.

The published restriction maps for HSV-1 (Wilkie, 1976; Morse et al., 1977; Skare and Summers, 1977) have been confirmed for our virus strain (KOS) (Stringer et al., 1978; Anderson et al., 1979; Holland et al., 1979). One HindIII restriction site is missing in our strain, resulting in the fusion of the two adjacent fragments (I and O). To create minimum confusion in nomenclature, we have designated the fragment IO, leaving all other fragment designations consistent with those shown in a recent review by Roizman (1979). The four orientations of HSV-1 DNA and the resulting generation of half-molar and quarter-molar restriction fragments were clearly described by Roizman (1979) and will not be further discussed here.

Blot hybridization of size-fractionated RNA. RNA samples were size-fractionated by electrophoresis on 1.2% agarose gels containing 10 mM methylmercury hydroxide (Alfa-Ventron) as described by Bailey and Davidson (1976). All procedures involving CH$_3$HgOH were performed in a ventilated hood, and protective gloves were worn at all times. All contaminated materials were soaked in 50 mM β-mercaptoethanol prior to washing. Following electrophoresis, gels were soaked in 50 mM β-mercaptoethanol for 20 min, then sliced in 3-mm intervals.

Each RNA gel slice was dissolved in 1.5 ml hybridization buffer containing 65% formamide by heating at 75° for 5 min. The method of Southern (1975) for transfer of DNA from agarose gels to nitrocellulose paper (BA85, Schleicher and Schuell Co.) was used to prepare DNA blots for hybridization. Dissolved RNA gel samples and strips from the nitrocellulose DNA blots were sealed in plastic bags, hybridized at 55° for 48 hr, rinsed, and autoradiographed as described (Holland et al., 1979).

In vitro translation. RNA samples for translation were precipitated twice with ethanol. The final pellet was rinsed once
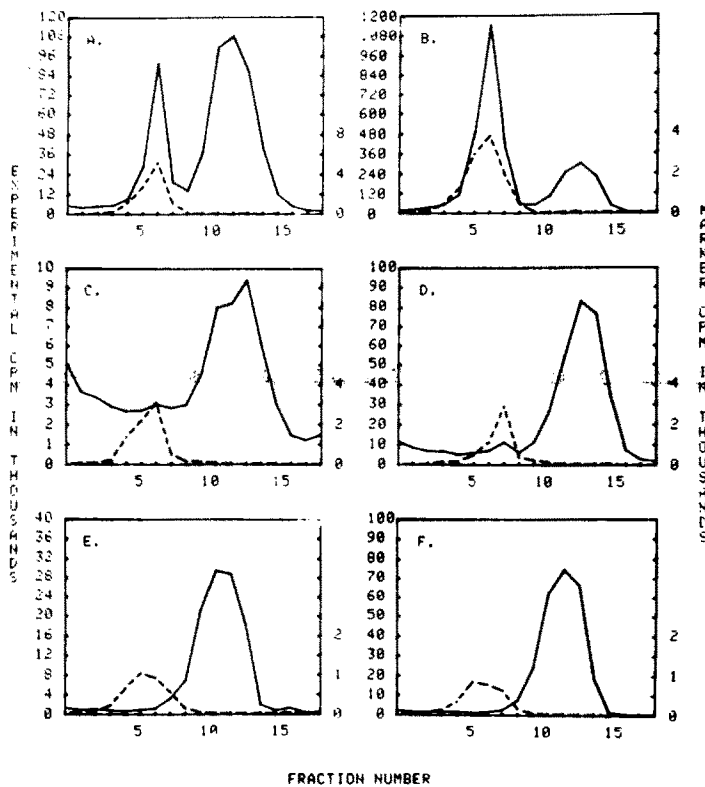
FRACTION NUMBER

FIG. 1. Effect of ara-A and ara-T on HSV-1 DNA synthesis. CsCl equilibrium density centrifugation gradients from ^{32}P-labeled infected cells, with and without drug treatment, are represented. Each gradient was fractionated into 0.175-ml fractions. The TCA-precipitable ^{32}P counts for fractions of density 1.760 to 1.670 g/ml are shown (solid line). ^{3}H-Labeled viral DNA was included in each gradient as a marker (dashed line). (A) Cells in drug-free medium labeled with 50 μCi [^{32}P]orthophosphate/ml from 0 to 6 hr p.i. (B) Same as panel A, except labeled from 0 to 22 hr p.i. (C) Cells in medium containing 1.5×10^{-4} M ara-A and 3.7×10^{-6} M pentostatin labeled with 50 μCi [^{32}P]orthophosphate/ml from 0 to 6 hr p.i. (D) Same as panel C, except labeled 0 to 22 hr p.i. (E) Cells in medium containing 1.9×10^{-4} M ara-T labeled with 50 μCi [^{32}P]orthophosphate/ml from 0 to 6 hr p.i. (F) Same as panel E, except labeled 0 to 22 hr p.i.

with cold 70% ethanol, dried under vacuum to remove all traces of ethanol, then dissolved in a small volume of sterile, glass-distilled water. A micrococcal nuclease-treated rabbit reticulocyte lysate system (New England Nuclear) was used for *in vitro* translation of RNA samples. The optimum magnesium acetate concentration for translation of HSV-1 mRNA was determined to be 0.5 mM. All other assay conditions were those recommended by the suppliers. Each 25 μl assay contained 40–50 μCi [^{35}S]methionine and 0.4–1.0 μg of RNA. Assays were incubated at 37° for 60 min and were terminated by adding 5 μl of 25 mM EDTA,

containing 100 μg pancreatic RNase and 20 units RNase T$_1$/ml, and incubated an additional 10 min at 37°.

The polypeptide products from *in vitro* translation were fractionated by electrophoresis on discontinuous polyacrylamide slab gels containing 0.1% sodium dodecyl sulfate (Laemmli, 1970). A 9% polyacrylamide separating gel (10 cm × 14 cm × 1.5 mm) was used with a 3% polyacrylamide stacking gel. Dried gels were autoradiographed using Kodak NS-2T X-ray film. The molecular weights of labeled products were determined from their migration relative to both unlabeled molecular weight markers (Phar-

TABLE 1

EFFECT OF ARABINOSYL NUCLEOSIDES ON HSV-1 DNA SYNTHESIS[a]

	cpm incorporated into DNA			
	Cellular[b]		Viral[b]	
	0–6 hr p.i.	0–22 hr p.i.	0–6 hr p.i.	0–22 hr p.i.
Mock-infected				
No drug	457,800	2,070,700	—	—
Ara-A	ND[c]	ND	—	—
Ara-T	170,800	714,600	—	—
Infected				
No drug	397,100	958,300	159,600	2,232,500
Ara-A	37,600	283,900	11,200	30,300
Ara-T	106,700	256,300	10,100	19,300

[a] Cells (2×10^6) were treated with either normal medium, medium containing $1.5 \times 10^{-4} M$ ara-A and 3.7 $\times 10^{-6} M$ pentostatin, or medium containing $1.9 \times 10^{-4} M$ ara-T for 1 hr prior to the infection, or mock infection, of the cells with 10 PFU HSV-1 per cell. Infection, or mock infection, was for 30 min, followed by overlay with 5 ml of normal, or drug-containing, medium containing 50 μCi [^{32}P]orthophosphate/ml.

[b] TCA-precipitable radioactivity incorporated during the labeling time shown. The radioactivity banding at a density of 1.685 to 1.710 g/ml was included as cellular DNA; radioactivity banding at a density of 1.715 to 1.735 g/ml, which corresponds to the position of the HSV-1 marker DNA in Fig. 1, was included as viral DNA. Each gradient contained 100 μg (2 A_{260} units) of DNA.

[c] Not done.

macia) and the labeled products from in vitro translation of the adenovirus-1 infected HeLa cell RNA supplied with the translation kit (Anderson et al., 1973; Lewis, et al., 1977).

RESULTS

Inhibition of HSV-1 DNA Synthesis by Ara-A and Ara-T

In order to confirm previous reports on the effect of arabinosyl nucleosides on HSV-1 DNA synthesis (Drach and Shipman, 1977; Aswell et al., 1977) for our cell and virus system, the following experiment was performed: Cultures of infected and mock-infected cells were grown in medium containing 50 μCi [^{32}P]orthophosphate/ml, plus either $1.5 \times 10^{-4} M$ ara-A and $3.7 \times 10^{-6} M$ pentostatin, or $1.9 \times 10^{-4} M$ ara-T, or no drug. The distribution of radioactivity from cells labeled for either 6 or 22 hr p.i. was then determined by CsCl equilibrium density centrifugation. Figure 1 shows not only the degree of separation of viral and cellular DNA obtainable by this method (A and B), but also the extent of inhibition of viral DNA synthesis due to the presence of ara-A/pentostatin (Figs. 1C and D) or ara-T (Figs. 1E and F). Gradient-purified ^{3}H-labeled HSV-1 DNA was included in each gradient as a marker.

The results of the CsCl gradient analyses are summarized in Table 1. It was evident from the absence of significant radioactivity banding at the viral DNA density that the drug concentrations used reduced viral DNA synthesis by greater than 98%, compared with that seen in infected cells without drug exposure. This is a maximum estimate of the amount of viral DNA synthesis occurring in these cells, as the amount of background radioactivity of viral density in uninfected cells was equivalent to the radioactivity of viral density in the infected, drug-treated cells (not shown). Cellular DNA synthesis, however, was only reduced to about 30% of that observed for infected cells without drug exposure. These results are entirely consistent with previous re-

TABLE 2

EFFECT OF ARABINOSYL NUCLEOSIDES ON HSV-1 RNA SYNTHESIS

| Labeling period of cells[a] | | Percentage RNase-resistant radioactivity after DNA excess hybridization[b] | | | | | | Percentage HSV-1-specific RNA in sample[c] | | |
| | | Polyribosomal RNA | | Poly(A+) RNA | | Poly(A−) RNA | | | | |
		Experimental	HSV	Experimental	HSV	Experimental	HSV	Polyribosomal RNA	Poly(A+) RNA	Poly(A−) RNA
1–2 hr p.i.	(1)	2.5	69.0	12.8	93.9	0.9	95.3	3.9	14.2	0.8
No drug	(2)	3.4	80.6	13.0	88.4	0.4	68.3			
5–6 hr p.i.	(1)	35.6	93.5	67.5	92.7	7.5	82.4	38.6	71.2	9.3
No drug	(2)	33.9	86.5	58.7	84.4	8.5	88.8			
5–6 hr p.i.	(1)	3.4	88.5	11.8	82.1	0.4	80.0	4.1	14.1	0.4
Ara-A	(2)	4.0	92.0	12.0	86.8	0.3	92.6			
5–6 hr p.i.	(1)	3.3	83.5	11.1	77.7	0.6	85.7	4.1	15.2	0.5
Ara-T	(2)	3.2	77.1	14.2	87.7	0.3	90.6			
0–2 hr p.i. No drug	(1)	ND[d]	ND	20.0	81.0	0.7	93.1	ND	24.7	0.8
0–6 hr p.i.	(1)	4.8	92.6	52.4	78.7	3.5	86.2	6.8	64.5	4.0
No drug	(2)	6.5	77.5	53.9	86.4	3.4	86.7			
0–6 hr p.i.	(1)	ND	ND	21.4	88.0	0.6	85.4	ND	24.1	0.7
Ara-A	(2)	ND	ND	21.7	90.5	0.6	83.7			
0–6 hr p.i.	(1)	1.7	89.4	20.8	81.7	0.5	88.5	1.6	26.8	0.9
Ara-T	(2)	1.1	91.3	23.4	83.1	1.0	79.3			

[a] Cells (1.2×10^7) were treated for 1 hr prior to and during infection with either normal medium, medium containing $1.5 \times 10^{-4} M$ ara-A and $3.7 \times 10^{-6} M$ pentostatin, or medium containing $1.9 \times 10^{-4} M$ ara-T. Cells were pulse-labeled for 1 hr with 15 μCi [³H]uridine/ml, or continuously labeled in the presence of 200 μCi [³²P]orthophosphate/ml at the indicated times after infection.

[b] Cytoplasmic RNA fractions were obtained as described under Materials and Methods. The hybridization mixtures contained 100 μg HSV-1 DNA/ml, 6000–8000 cpm of purified HSV-1 RNA as a standard, and either 20,000–30,000 cpm (1), or 70,000–150,000 cpm (2) of the RNA sample to be tested. Total RNase-resistant radioactivity was adjusted by subtracting a background value determined by incubation of RNA with *E. coli* DNA. The adjusted percentage of input radioactivity resistant to RNase digestion is shown for both the test RNA sample (experimental column) and the viral RNA standard (HSV column).

[c] Average of the values calculated from the ratio of the experimental to pure HSV samples.

[d] Not determined.

ports on the specificity of these drugs for inhibiting HSV-1 DNA synthesis (Drach and Shipman, 1977; Aswell *et al.*, 1977). Therefore, the drug concentrations used in this examination of DNA synthesis were routinely used in our subsequent experiments in which the role of HSV-1 DNA replication in the expression of viral mRNA was examined.

Quantitation of HSV-1 mRNA Associated with Polyribosomes

The cytoplasmic RNA population of HSV-1 infected cells was examined by using two different labeling protocols. To examine the amount of viral-specific newly synthesized RNA, infected cells with and without drug treatment were pulse-labeled with 15 μCi

[³H]uridine/ml for 1 hr prior to extraction. To examine the total amount of viral-specific RNA present, infected cells with and without drug treatment were continuously labeled from the time of infection to extraction with 200 μCi [³²P]orthophosphate/ml. Total polyribosomal-associated RNA, poly(A+) polyribosomal RNA, and poly(A−) polyribosomal RNA were hybridized in solution to an excess of HSV-1 DNA. The results of these quantitative hybridizations, expressed as the proportion of RNase-resistant radioactivity found after hybridization, are shown in Table 2. Raw data were corrected by subtracting a background RNase-resistant radioactivity determined in a parallel experiment using *E. coli* DNA instead of HSV-1 DNA. Both ³²P-labeled and ³H-labeled HSV-1 specific RNA, prepared by preparative hybridization to HSV-1 DNA coupled to cellulose, were included, respectively, in the quantitative hybridizations of the ³H pulse-labeled samples and the ³²P continuously labeled samples to standardize the efficiency of hybridization. The amount of the control viral RNA hybridized ranged from 69 to 95% (Table 2), and the data were normalized relative to 100% hybridization for the HSV-1 RNA. All samples used in quantitative hybridizations were thoroughly DNase digested prior to hybridization to remove any trace amounts of contaminating labeled DNA.

The results shown in Table 2 support our earlier finding that, at late times after infection when viral DNA synthesis is maximal, the vast majority of the labeled mRNA is virus specific (Stringer *et al.*, 1977). However, at late times of infection in the presence of drugs inhibitory for HSV-1 DNA synthesis, the proportion of the mRNA having viral origin was greatly reduced compared to that seen with the same labeling time in the absence of drugs. The amount of viral mRNA at late times of infection in the presence of ara-A or ara-T thus appeared to be very similar to that seen at very early times in the infection (prior to the start of viral DNA replication). These correlations held true for each of the RNA populations examined, as well as with both labeling protocols.

Effect of Ara-A and Ara-T on the Appearance of Specific Viral mRNA Species

We previously have shown that hybridization of size-fractionated RNA to Southern blots of restriction enzyme-digested HSV-1 DNA enables us to identify specific viral mRNA species (Anderson *et al.*, 1979; Holland *et al.*, 1979). The viral mRNA species obtained from drug-treated cells were also examined by Southern blot hybridization. The infected cells were labeled with 500 μCi of [³²P]orthophosphate/ml immediately following the adsorption period. The ³²P-labeled polyribosome-associated RNA was extracted at either 2 hr p.i. (early no-drug control) or 6 hr p.i. (drug-treated and late no-drug control). The polyadenylated RNA was isolated on oligo(dT)-cellulose, preparatively hybridized in solution to HSV-1 DNA covalently coupled to cellulose, and size-fractionated on denaturing methylmercury agarose gels. Discrete size fractions of this purified viral mRNA then were hybridized to *Hind*III/*Xba*I double-digest blots of HSV-1 DNA. The location of the restriction fragments on the HSV-1 genome is shown in Fig. 2.

The results of the Southern blot hybridizations are shown in Fig. 3. The early (Fig. 3A) and late (Fig. 3D) no-drug controls showed the same patterns of hybridization as in our previous reports (Anderson *et al.*, 1979; Holland *et al.*, 1979). The use of preparatively hybridized viral RNA in these experiments, as opposed to total poly(A) RNA in our previous reports, caused no qualitative difference in the viral RNA species observed. However, some quantitative differences were detected. These were caused by an enhancement of less abundant species by the preparative hybridization.

By comparing the viral mRNA species expressed "late," either in the presence of ara-A plus pentostatin (Fig. 3B) or in the presence of ara-T (Fig. 3C), with those seen in the two control experiments, it can be seen that the viral species present in drug-treated cells resembled very closely those species occurring in the early control and appeared to be a subset of the species observed in the late control. Several dis-
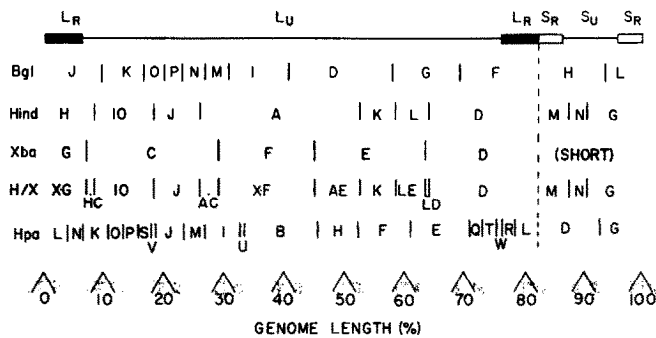
FIG. 2. Map of restriction endonuclease fragments of the KOS strain of HSV-1. The fragments generated from cleavage by restriction endonuclease BglII, HindIII, XbaI, HindIII plus XbaI, and HpaI digests are shown for the prototypical arrangement of our HSV-1 strain. The origin of these restriction maps and confirmation of our strain (KOS) have been described previously (Stringer *et al.*, 1978; Anderson *et al.*, 1979; Holland *et al.*, 1979). The structure and rearrangements of HSV-1 DNA have been reviewed recently (Roizman, 1979). Additional discussion of these restriction maps is by Holland *et al.* (1979).

tinguishing features are readily identifiable. For example, the 7-kb RNA hybridizing to HindIII fragment K is only detectable in the presence of DNA synthesis (Fig. 3D, compare with A, B, C). The 1.7-kb RNA hybridizing to HindIII/XBAI double-digest fragment AE and the 1.5-kb RNA hybridizing to HindIII fragment G are similarly absent without viral DNA replication. The 6-kb RNA hybridizing to HindIII fragment J, although present in small amounts in the early (Fig. 3A) and drug-treated samples (Figs. 3B, C), is much more abundant relative to other species in the late control cells (Fig. 3D). Viral DNA synthesis is required for this mRNA species to become a major abundant species. The RNA of 2.8 kb hybridizing to XbaI fragment G appeared as a more prominent species in the early control (Fig. 3A) than it did in the drug-treated (Figs. 3B, C) or late control (Fig. 3D) samples. The intensity of the spot decreased with longer labeling times. This is in contrast to the 3-kb RNA hybridizing to HindIII fragment G, which increased with the longer labeling times.

Other distinguishing features are evident in the hybridization of 3.3- and 2.8-kb RNA species shown in Fig. 4. Hybridization of 3.3-kb RNA to blots of BglII-digested HSV-1 DNA showed hybridization only to BglII fragment I for the early control (Fig. 4B),

ara-A-treated (Fig. 4C), and ara-T-treated (Fig. 4D) cells. This correlated with the 3.3-kb RNA seen hybridizing to the XbaI fragment F (see Figs. 2 and 3). However, 3.3-kb RNA from the late control cells showed additional hybridization to the BglII fragments M and N (Fig. 4E). The 2.8-kb RNA from the late control cells, which hybridized to the band containing both HindIII/XbaI double-digest fragment LE and Hind-III fragment M (Fig. 3D), is shown in Fig. 4H to hybridize to the HindIII fragment L and not HindIII fragment M. The RNA that is 2.8 kb in size from the ara-T-treated cells does not contain this mRNA species (Fig. 4G).

In Vitro Translation of Drug-Treated and Control Infected Cell RNA

We compared the translation products of purified HSV-1 mRNA from infected cells isolated at early, late, and late times in the presence of ara-A/pentostatin or ara-T with that of total poly(A) RNA from these and mock-infected cells. Our procedures for the isolation and purification of viral mRNA via use of DNA cellulose were as described above and under Materials and Methods. Samples of RNA (0.4–1.0 μg) were translated individually and the polypeptides fractionated by SDS–gel electrophoresis (Fig.
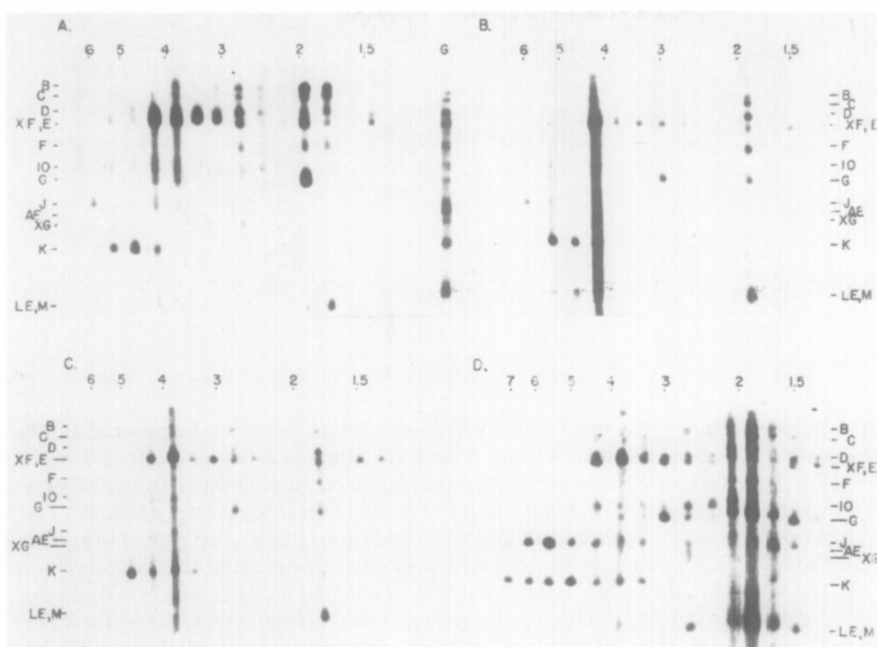
FIG. 3. Southern blot analysis of size-fractionated viral RNA from control and drug-treated cells. Polyadenylated polyribosomal RNA was preparatively hybridized to HSV-1 DNA coupled to cellulose (see Materials and Methods). The eluted viral mRNA was then size-fractionated by electrophoresis in agarose gels containing 10 m*M* methylmercury hydroxide as previously described (Anderson *et al.*, 1979; Holland *et al.*, 1979). The 3-mm gel slices were dissolved and hybridized to individual strips of *Hin*dIII/*Xba*I double-digest blots of HSV-1 DNA for 48 hr at 47° (see Materials and Methods). The top two-thirds of these blots is shown. The RNA size in kilobases is shown across the top of each panel and was determined from the position of 28 S (5.2 kb) and 18 S (2 kb) HeLa cell rRNA run in parallel gels. After hybridization, the strips were rinsed and reassembled according to RNA size. Autoradiography was for 24 hr at −70° using two intensifying screens. (A) HSV-1-specific mRNA from cells labeled from 0 to 2 hr p.i. with 500 μCi [^{32}P]orthophosphate/ml in the absence of drugs. (B) Viral mRNA from cells labeled from 0 to 6 hr p.i. with 500 μCi [^{32}P]orthophosphate/ml in the presence of 1.5×10^{-4} *M* ara-A and 3.7×10^{-6} *M* pentostatin. (C) Viral mRNA from cells labeled from 0 to 6 hr p.i. with 500 μCi [^{32}P]orthophosphate/ml in the presence of 1.9×10^{-4} *M* ara-T. (D) Viral mRNA from cells labeled from 0 to 6 hr p.i. with 500 μCi [^{32}P]orthophosphate/ml in the absence of drugs. (G) The guide strip showing the position and resolution of the fragments was made from a similar blot using ^{32}P-labeled total late poly(A) RNA.

5A). A second experiment was carried out using only early or late HSV-1 mRNA and the polypeptides were run in a separate gel to clearly delineate the viral polypeptides seen at these times after infection (Fig. 5B). In contrast to the large number of polypeptides in the size range of 170,000 to 41,000 d encoded by uninfected HeLa cell mRNA (Fig. 5A, track 2), early viral mRNA coded for a limited number of discrete polypeptide bands (Fig. 5A, track 4; Fig. 5B). The majority of these polypeptides have no counterpart in the translation products of RNA

from mock-infected cells, demonstrating the high specificity of our method for the preparative isolation of HSV-1 mRNA. Most of the viral polypeptides seen using early viral mRNA as a template are also seen when late (6 hr p.i.) viral mRNA is used (Fig. 5A, track 9; Fig. 5B). One notable exception is the 170,000-d polypeptide, which is absent using late HSV-1 mRNA or late poly(A+) mRNA as a translation template. In addition to these polypeptides, a large number of new viral polypeptides are seen to be synthesized from late viral
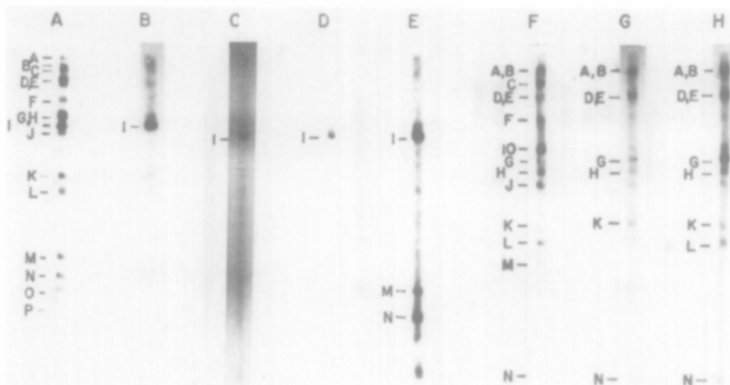
FIG. 4. Rehybridization of 3.3- and 2.8-kb RNA species to other restriction fragment blots. The RNA of these sizes from selected samples of Fig. 3 were rehybridized to either BglII or HindIII blots of HSV-1 DNA. (A) Guide strip showing position and band separation for BglII digests. (B) The 3.3-kb RNA from early control cells labeled 0 to 2 hr p.i. (Fig. 3A) hybridized to such a blot. (C) The 3.3-kb RNA from ara-A/pentostatin-treated cells labeled 0 to 6 hr p.i. (Fig. 3B) hybridized to another BglII blot. (D) The 3.3-kb RNA from ara-T-treated cells labeled 0 to 6 hr p.i. (Fig. 3C) hybridized to a BglII blot. (E) The 3.3-kb RNA from late control cells labeled 0 to 6 hr p.i. (Fig. 3D) hybridized to a BglII blot. (F) A guide strip showing position and band separation for HindIII digests. (G) The 2.8-kb RNA from ara-T-treated cells labeled 0 to 6 hr p.i. (Fig. 3C) hybridized to a HindIII blot. (H) The 2.8-kb RNA from late control cells labeled 0 to 6 hr p.i. (Fig. 3D) hybridized to a HindIII blot.

mRNA compared to early. The changes in viral polypeptides seen are entirely consistent with results of our mRNA mapping studies. We have indicated the major changes seen in Fig. 5B.

Th effect of inhibition of viral DNA synthesis using ara-A/pentostatin or ara-T on the template activity of viral mRNA is apparent from the data of Fig. 5A. The polypeptides made using total poly(A) RNA from early control (track 3), ara-A-treated (track 6), and ara-T-treated (track 7) infected cells showed a complex pattern that appeared similar to the pattern seen using poly(A) RNA from uninfected cells (track 2), with the addition of new bands of 170,000, 140,000, 125,000, 120,000, and 64,000 d easily identifiable. This result was consistent with our quantitation experiments (see Table 2), which showed large amounts of cellular poly(A) RNA remaining associated with polyribosomes under these conditions of infection. Translation of viral mRNA from these drug-treated cells, as well as in cells maintained in ara-T medium for 18 hr (not shown), resulted in the synthesis of the same polypeptides seen using early viral mRNA as a template (compare tracks 4, 5, and 8 of Fig. 5A).

A dramatic change in the proportion of viral mRNA on polyribosomes was observed by translation of total polyribosomal poly(A) RNA obtained from late control cells (Fig. 5A, track 11). In this case, virtually the same polypeptides were seen as when viral-specific mRNA was used as a template (track 9). Only one cell-specific polypeptide (of about 48,000 d), translated from the total late poly(A) RNA, was resolvable from the virus-specific polypeptides. These results also were consistent with our quantitation data, which showed the majority of the polyribosomal poly(A) RNA late after infection to be viral (Table 2).

DISCUSSION

The experiments presented here, which describe viral mRNA from cells early (2 hr p.i., prior to the onset of viral DNA synthesis) or late in infection (6 hr p.i., during maximal viral DNA synthesis), or under conditions where viral DNA synthesis has been blocked (6 hr p.i., cells treated with
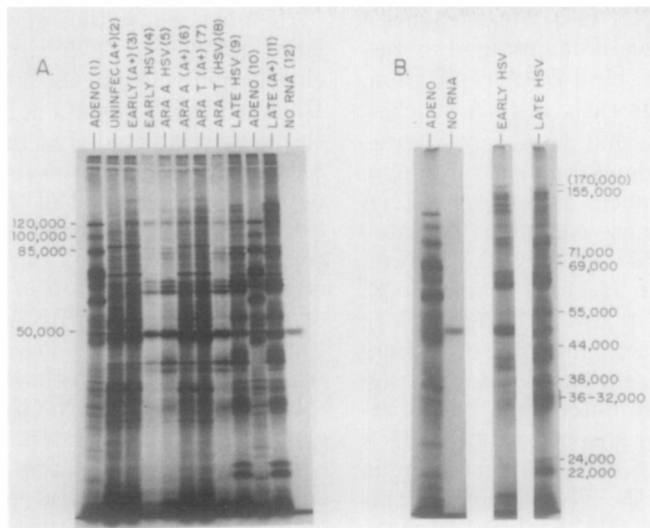
FIG. 5. Products from *in vitro* translation of viral and total polyribosome-associated polyadenylated RNA. A commercial rabbit reticulocyte lysate system was used for *in vitro* translation of both total polyribosomal polyadenylated RNA (A+ samples) and polyribosome-associated viral poly(A) RNA purified by preparative hybridization to HSV-1 DNA bound to cellulose (HSV-1 samples). Following translation, polypeptides were denatured and subjected to SDS–acrylamide gel electrophoresis as described under Materials and Methods. A sample of adenovirus infected cell mRNA (supplied with the kit) was translated and the polypeptides were run as internal size markers. The molecular weights of these standards were confirmed by their migration relative to commercial, nonradioactive size standards electrophoresed in adjacent slots and visualized by staining with Coomassie brilliant blue. (A) Samples of poly(A) or HSV-1-specific mRNA from drug-treated and control cells. Sizes of selected adenovirus polypeptides are shown on the left. The tracks are translation products of (from the left): (1) adenovirus RNA; (2) mock-infected HeLa polyribosomal poly(A+) RNA; (3) polyribosomal poly(A+) RNA from cells 2 hr p.i.; (4) HSV-1-specific mRNA from these cells; (6) polyribosomal poly(A+) RNA from such cells; (7) polyribosomal poly(A+) RNA from ara-T-treated cells; (8) HSV-1-specific mRNA from ara-T-treated cells; (9) HSV-1-specific mRNA from cells 6 hr p.i.; (10) adenovirus RNA; (11) polyribosomal poly (A+) RNA from cells 6 hr p.i.; (12) no RNA. (B) Separate samples of early (2 hr p.i.) and late (6 hr p.i.) HSV-1-specific mRNA were translated and fractionated. The polypeptide bands seen using late HSV-1 mRNA, and not present using early, are indicated along with their sizes on the right. The 170,000-d protein not seen late is indicated by the dashed line.

ara-A or ara-T), have been directed toward elucidating the role of HSV-1 DNA replication in gene expression. The amounts of viral mRNA or polyribosomes obtained from infected cells growing either in the absence or presence of viral DNA synthesis, the distinct viral mRNA species identifiable in these mRNA populations, and the viral mRNA-specified polypeptides synthesized *in vitro*, are all consistent with a model requiring HSV-1 DNA synthesis for efficient late viral gene expression. By all these criteria, inhibition of viral DNA synthesis, over long enough time periods to allow efficient expression of late mRNA in control cells, results in a viral mRNA population typical of cells early after infection.

In addition to this conclusion, the comparison of individual mRNA species and their *in vitro* translation products confirms that several levels of viral mRNA abundance exist in HSV-1 infected cells. Viral mRNA species can be divided into groups on the basis of their relative abundance at various times after infection (see Fig. 3). Several of the mRNA species which appear as abundant species prior to viral DNA replication continue to be expressed as major species during viral DNA synthesis (i.e., the 5.3-kb mRNA in *Hind*III fragment K; the 4.3-,

3.3-, and 1.5-kb mRNAs in *Xba*I fragment
F). Their abundance is maintained or fur-
ther increased upon viral DNA replication.
We classify these as early mRNA species.

Members of a second group are charac-
terized by a dependence on viral DNA syn-
thesis for appearance as major mRNA
species. Some of these cannot be detected
at all prior to viral DNA synthesis (i.e.,
the 7-kb mRNA in *Hin*dIII fragment K;
the 2.8-kb mRNA in *Hin*dIII fragment L;
and a number of species mapping elsewhere
in the viral genome, such as in *Hin*dIII frag-
ment J). Others are detectable, but at re-
duced levels in the absence of viral DNA
synthesis (i.e., the 6-kb mRNA mapping in
*Hin*dIII fragment J). We cannot, at this
time, determine whether the differences ob-
served for these species are due to differ-
ences in steady state levels early, or to actual
differences in levels of transcription at these
times. However, it is clear that only with
HSV-1 DNA replication are any of these
viral mRNA species seen in their normal
amounts. The continued expression of some
of these mRNAs is probably responsible for
the presence of small amounts of certain γ
polypeptides *in vivo* in cells incubated for
long times in the absence of DNA replication
(Powell *et al.*, 1975). This group, as a whole,
is classified as late mRNA.

In addition to these two temporal group-
ings of viral mRNA, there are several other
patterns seen. At least one mRNA species
(the 2.8-kb mRNA in *Hin*dIII fragment G)
increases in abundance with labeling time,
even in the absence of viral DNA synthesis.
This could be an early viral mRNA with a
very long half-life, thus causing its accumu-
lation. Finally, there is a group of viral
mRNA species which decrease in relative
abundance with time of infection (4 kb in
*Hin*dIII fragment G; 2.8 kb in *Xba*I frag-
ment G). The decline of the 4-kb species
accounts for the decreased synthesis, or ab-
sence, of the 170,000 d early viral polypep-
tide at late times (Watson *et al.*, 1979; An-
derson, Holland, Costa, and Wagner, in
preparation). Further experiments will be
required to establish what factors in viral
gene expression are responsible for these
atypical patterns.

In vitro translation of viral mRNA iso-
lated under our experimental conditions
clearly confirms that the inhibition of HSV-1
DNA synthesis results in the expression of
the same restricted viral mRNA population
seen early. Further, these early viral poly-
peptides are a subset of the viral polypep-
tides encoded late during normal viral DNA
replication. Our results are, to our knowl-
edge, the first report of *in vitro* translation
of purified HSV-1 mRNA. Correlation of
the viral polypeptides identified by *in vitro*
translation here with those identified from
total infected cell mRNA by others (Cremer
et al., 1977, 1978; Preston, 1977, 1979; Wat-
son *et al.*, 1979) is quite good. However,
several additional viral polypeptides are
evident with purified viral mRNA due to
our specific elimination of cellular back-
ground. We currently are carrying out stud-
ies on the isolation and translation of in-
dividual mRNA species which will result in
the precise localization of viral mRNA
species seen at all stages of replication.

It is important to emphasize that the re-
sults presented here reflect only the viral
mRNA species of major abundance occur-
ring at the times examined. Our data sug-
gest that the sequences of low abundance
early depend upon viral DNA synthesis for
sufficient amplification to permit translation
at levels comparable to those existing for
the abundant species found early. Whether
this amplification is required to provide suf-
ficient biological activity, or is merely a con-
sequence of viral DNA replication, is not
clear. One possible mechanism for control
of this amplification is suggested by the
finding of cellular proteins specifically as-
sociated with infecting viral DNA, but not
with newly synthesized viral DNA (Rice *et
al.*, 1976). Further characterization of the
viral mRNA species and specific correlation
of the mRNAs with polypeptides and func-
tions will elucidate the significance of these
observations.

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