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Gene Expression of Herpes Simplex Virus

I. Analysis of Cytoplasmic RNAs in Infected Xeroderma Pigmentosum Cells

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RNAs which are synthesized and accumulate in the cytoplasm of uninfected and herpes simplex virus type 1 (HSV-1)-infected xeroderma pigmentosum (XP) cells in the presence of cycloheximide (early RNAs) or absence of drugs (late RNAs) were analyzed by electrophoresis through denaturing polyacrylamide gradient slab gels. HSV RNAs were selected by hybridization to HSV DNA covalently bound to cellulose. No HSV-specific low-molecular-weight (4S to 10S) RNAs were detected. However, several changes were observed in the electrophoretic pattern of the host low-molecular-weight RNAs during HSV infection. Five HSV RNAs ranging in size from 16S to 28S accumulated in the cytoplasm of infected XP cells in the presence of cycloheximide. These are of the size range predicted to encode the major early viral polypeptides. The cytoplasmic and polyadenylated early RNAs from HSV-infected XP cells were translated in vitro to produce proteins whose electrophoretic pattern resembled that of the early viral proteins synthesized in vivo.

Studies on herpes simplex virus type 1 (HSV-1) RNAs have been hampered by relatively slow turn-off of host cell transcription (26), the large number of RNA species which must be produced to synthesize at least 48 viral polypeptides (6), and the poor resolution of techniques used to separate RNAs. It has been shown that the appearance of HSV-1 transcripts in the cytoplasm of infected cells is temporally regulated. In the cytoplasm of cells infected with HSV in the presence of cycloheximide, immediate early transcripts which hybridize to about 10% of the viral DNA were detected by RNA-driven liquid hybridization (9). Under these conditions, the number of viral polypeptides synthesized upon removal of cycloheximide is reportedly limited to two to six polypeptides (7, 13, 19). Late in infection when many viral polypeptides are being synthesized, transcripts in the cytoplasm were found to be complementary to about 50% of the viral DNA by liquid hybridization (9, 25). These results obtained by liquid hybridization have been extended by using hybridization to restriction fragments. The immediate early cytoplasmic HSV RNA synthesized in the presence of cycloheximide hybridizes to restricted portions of the HSV genome, whereas late viral cytoplasmic RNA hybridizes to all of the HSV DNA fragments (4, 8).

Little is known about the events leading to the production of HSV mRNA and about the size and structure of individual HSV mRNA's. At least the initial phase of viral transcription is carried out by the host polymerases since the naked viral DNA is infectious (20). Evidence has been presented suggesting that viral mRNA is generated by transcription of a high-molecular-weight precursor RNA which is processed into lower-molecular-weight viral mRNA (23, 27). Results of UV transcriptional mapping have confirmed that most of the primary transcripts are larger than necessary to encode the viral polypeptides (Millette and Klaiber, submitted for publication).

Studies on the structure of HSV mRNA have revealed that a large portion of the mRNA contains polyadenylic acid [poly(A)] (21, 23) and that cytoplasmic HSV RNA has a 5'-terminal "cap" structure and internal basemethylated nucleosides (1, 14). The size distribution of the viral mRNA early and late in infection has been determined by isolating the viral RNA with DNA excess hybridization in solution, recovering the hybrids by hydroxyapatite chromatography, and centrifuging the RNA in sucrose gradients containing formaldehyde (23). The results of these experiments showed a very broad size distribution of the viral RNA both early and late in infection. This method gives some details of viral mRNA size but lacks the resolution required for

biochemical analyses of individual HSV mRNA's.

To effectively study individual HSV RNAs several problems must be overcome. Host cell RNA must be eliminated from the RNA samples, the number of viral RNA species to be studied must be reduced to a workable number, and a separation system capable of resolving the viral RNA species must be found. In the work presented here, we have made progress toward solving these problems and have analyzed the cytoplasmic RNAs in HSV type 1 (HSV-1)-infected xeroderma pigmentosum (XP) cells. We have compared RNA species which accumulate in the cytoplasm of uninfected and infected cells in the presence of cycloheximide (early RNA) or absence of drug (late RNA) by gel electrophoresis and have identified viral specific RNAs by hybridization to HSV DNA covalently coupled to cellulose. This paper reports the characterization of HSV-induced changes in low-molecular-weight RNAs, the identification of the presumptive early HSV mRNA's, and the in vitro translation of early RNA into polypeptides whose electrophoretic pattern resembles that of early viral polypeptides synthesized in vivo.

MATERIALS AND METHODS

Cells and virus. XP cells (Jay-Tim line, ATCC CRL 1223) were grown and HSV-1 (F strain) was propagated as previously described (Millette and Klaiber, submitted for publication). Before infection, confluent monolayers of cells in 75-cm^2 plastic tissue culture flasks were incubated for 12 to 16 h in phosphate-free Dulbecco-modified Eagle medium containing 1% inactivated calf serum that had been dialyzed against water (DME-1% IDCS). Cells were infected for 1 h at 37°C with HSV-1 (F strain, obtained from Bernard Roizman) at a multiplicity of infection of 50 PFU/cell in 3 ml of phosphate-free DME containing no serum.

Labeling of RNA and cell fractionation. After removal of the virus, 75-cm^2 monolayers were overlayed with 5 ml of either phosphate-free DME-1% IDCS (for late RNA) or phosphate-free DME-1% IDCS containing 50 μg of cycloheximide per ml (for early RNA) and were incubated at 37°C for 1.5 h. One millicurie of [^{32}P]orthophosphate was added, and incubation was continued for 4 h. The cells were rinsed five times with cold phosphate-buffered saline and harvested by scraping in phosphate-buffered saline and centrifuging. The cell pellets were suspended in RSB (0.15 M NaCl-1.5 mM $MgCl_2$-10 mM Tris, pH 7.5) containing 5 μl of diethyl pyrocarbonate and 25 μg of polyvinyl sulfate per ml and were lysed by addition of Nonident P-40 (Gallard-Schlesinger, Carle Place, N.Y.) to a final concentration of 0.5% (vol/vol). After 10 min on ice, the nuclei were removed by centrifugation at 800 × *g* for 10 min.

To prepare polysomes, the cytoplasmic extract was layered over 1 ml of 1 M sucrose in RSB and centrifuged at 150,000 × *g* for 70 min in a Spinco SW 41 rotor. The polysomes were suspended in RSB. The polysome-free cytosol was recovered from the supernatant fluid. The cells that had been incubated in the presence of cycloheximide were rinsed five times with media and further incubated 15 min to allow polysome formation.

Purification of RNA. The cytoplasmic fractions were made 1% in sodium dodecyl sulfate and 10 mM in EDTA and were heated at 60°C for 5 min. The RNA was isolated by three extractions with phenol-chloroform-isoamyl alcohol (24:24:1) and precipitation with ethanol at −20°C.

Isolation of HSV DNA. HSV DNA was prepared from virus propagated in CV-1 (African green monkey kidney) cells by a modification of the procedure of Pellicer et al. (18). Confluent roller bottle cultures of CV-1 cells were infected with HSV-1 at a multiplicity of infection of 2 PFU/cell. At 2 h after addition of the virus, the infecting medium was replaced with DME containing 1% calf serum. At 40 h after infection, the cells were shaken off the glass and were removed by centrifugation at 6,000 rpm for 20 min in the Sorvall GSA rotor. The virus was recovered from the supernatant medium by centrifugation at 11,000 rpm in the GSA rotor for 90 min. The virus was suspended in 10 mM NaCl-1.5 mM $MgCl_2$-10 mM Tris (pH 7.5) and digested for 1 h at 37°C with pancreatic RNase at 0.1 mg/ml. Sodium dodecyl sulfate was added to 1% and proteinase K (EM Biochemicals, Darmstadt, Germany) was added to 0.1 mg/ml, and the mixture was incubated at 37°C for 1 h. The DNA was extracted three times with phenol saturated with 0.12 M NaCl-1 mM EDTA-10 mM Tris (pH 7.4) and then two times with chloroform containing 2% (vol/vol) isoamyl alcohol and dialyzed against 0.2 M sodium borate buffer, pH 8.0. This method yields 30 to 40 μg of HSV DNA per roller bottle. Analysis of the DNA on CsCl gradients indicated that the preparation was greater than 90% HSV DNA.

Isolation of HSV RNA. HSV DNA cellulose was prepared by the method of Noyes and Stark (17). The usual preparation contained 13 μg of viral DNA per mg of cellulose. Cytoplasmic RNA from 9 × 10^6 cells was dissolved in 0.2 ml of hybridization buffer (80% [vol/vol] formamide-0.4 M NaCl-1 mM EDTA-10 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid [HEPES], pH 7.0). Formamide (Mallinckrodt, St. Louis, Mo.) was deionized with a mixed bed resin [AG 501-X8(D), Bio-Rad Laboratories, Richmond, Calif.). The RNA solution was added to packed cellulose corresponding to 60 μg of HSV DNA. The hybridization mixture was heated to 80°C for 2 min and was placed in a shaking water bath at 57°C for 6 h. The DNA cellulose was washed successively with hybridization buffer, 2 × SSC (1× SSC, 0.15 M NaCl-0.015 M sodium citrate) and 99% formamide containing 0.1% sodium dodecyl sulfate at 0°C. The HSV RNA was eluted with 99% formamide-0.1% sodium dodecyl sulfate at 60°C. The formamide was diluted to 20%, sodium chloride was added to 0.1 M, and the RNA was precipitated by addition of two volumes of ethanol.

Isolation of polyadenylated RNA. Polyadenylated RNA was separated from nonadenylated RNA by

affinity chromatography on columns of polyuridylic acid immobilized on glass fiber filters by the method of Silverstein et al. (21).

Electrophoresis. Electrophoresis of RNA was carried out by a modification of the system described by Spradling et al. (22). The RNA was electrophoresed through polyacrylamide slab gels (0.15 by 14 by 17 cm) which were made with gradients of either 2 to 4% or 4 to 15% acrylamide (ratio of acrylamide to *N,N'*-methylene-bis-acrylamide, 30:0.8). The 2 to 4% gels were run at 4°C for 16 h at 180 V, and the 4 to 15% gels were run at room temperature for 16 h at 100 V. The gels were wrapped in Saran Wrap, and the RNA bands were located by autoradiography with Kodak X-Omat RP film with intensifying screens (Dupont, Wilmington, Del.)

Proteins were electrophoresed as previously described (Millette and Klaiber, submitted for publication) through polyacrylamide slab gels made with 8.5% acrylamide and 0.37% *N,N'*-diallyltartardiamide with the discontinuous buffer system of Laemmli (10). The proteins used for molecular weight calibration were β', β, σ, and α subunits of *Escherichia coli* RNA polymerase, heavy and light chains of immunoglobulin, bovine serum albumin, ovalbumin, chymotrypsin, and papain with molecular weights of 163,000, 155,000, 90,000, 40,000, 50,000, 23,500, 65,400, 43,000, 25,700, and 20,700, respectively.

In vitro translation. A nuclease-treated reticulocyte lysate translation kit (New England Nuclear Corp., Boston, Mass.) was used for in vitro translation. Each 25-μl assay contained 43 μCi of [^{35}S]methionine, 1 mM magnesium acetate, 80 mM potassium acetate, 2 μl of translation cocktail, 10 μl of rabbit reticulocyte lysate, and 2 μl of RNA. This RNA was prepared as described except that diethyl pyrocarbonate and polyvinyl sulfate were omitted. The assay mixtures were incubated at 37°C for 1 h. The proteins were electrophoresed as described above and visualized by fluorography by the procedure of Bonner and Lasky (3).

RESULTS

XP cells were used for these RNA studies to correlate the results with those of UV mapping studies of HSV-1 gene expression in infected XP cells (Millette and Klaiber, submitted for publication). We have analyzed the kinetics of HSV-1 infection in XP cells and, as compared with infected HEp-2 cells, XP cells exhibit a better shutoff of host protein synthesis and more rapid kinetics of viral DNA and protein synthesis (Pedersen et al., manuscript in preparation).

Cells were mock infected or infected with HSV-1 for 1 h. After removal of the infecting medium, the cells were incubated in medium containing cycloheximide to prepare samples of early RNA or containing no drug to prepare samples of late RNA. Cytoplasmic RNA was isolated from uninfected and early and late infected cells and examined on polyacrylamide gradient slab gels containing 7 M urea. Gradient gels made with 2 to 4% acrylamide were used to study RNAs larger than 1,000 nucleotides in length. Gradient gels made with 4 to 15% acrylamide were used to study the RNAs smaller than 1,000 nucleotides. RNA from the same number of cells was applied to the gel rather than the same amount of radioactivity. This avoids problems due to unequal decline in synthesis of various host RNA species which occurs during HSV infection (26) and enables us to compare the amount of an RNA component in one sample with the amount of that component in the other samples.

Small cytoplasmic RNAs in HSV-infected XP cells. Figure 1 shows a polyacrylamide gel electrophorogram of the small RNAs which are synthesized and accumulate in the polysomes and in the polysome-free cytosol of uninfected and HSV-infected XP cells both early and late in infection. Several changes in the RNA patterns of the low-molecular-weight RNAs are apparent during infection with HSV. As shown in

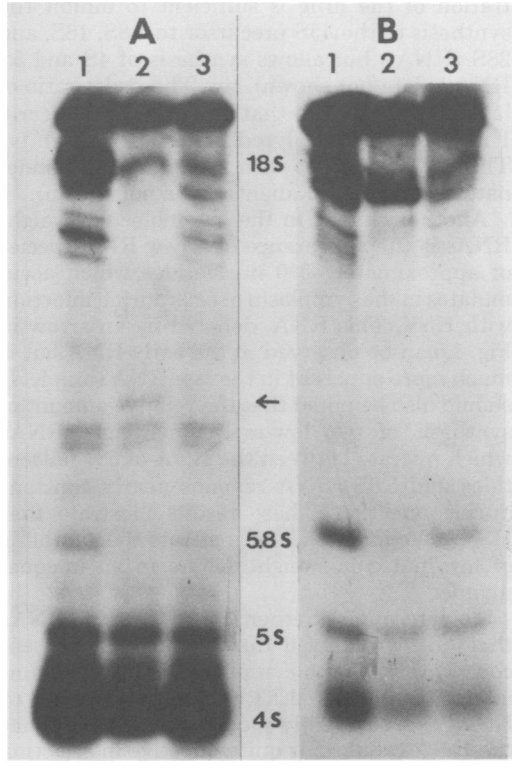

FIG. 1. *Electrophorogram of small cytoplasmic RNAs from uninfected and HSV-infected XP cells. RNA was isolated from polysomes and polysome-free cytosol and separated by electrophoresis through denaturing gels made with gradients of 4 to 15% acrylamide. (A) Polysomal RNA; (B) RNA from polysome-free cytosol; lane 1, uninfected RNA; lane 2, late RNA; and lane 3, early RNA.*

Fig. 1 and observed by others (26), the accumulation of labeled 4S RNA decreases during HSV infection but is still being synthesized late in infection. The synthesis and accumulation of 5S rRNA also decline somewhat, but to a lesser extent relative to the other changes observed. One of the most striking changes in the low-molecular-weight RNAs in HSV-infected cells is the apparent shutoff of the synthesis of an RNA which migrates with an apparent length of 180 nucleotides. The amount of incorporation into this RNA is decreased in the early RNA samples (Fig. 1, lanes A3 and B3) and is not observed in the late RNA samples (Fig. 1, lanes A2 and B2). This RNA is probably the 5.8S rRNA (28S-associated RNA) for the following reasons. (i) The size of this RNA is close to that of 158 nucleotides reported for 5.8S rRNA (15). (ii) It is enriched in the polysomes (Fig. 1, lane B1 compared with lane A1). (iii) It is not synthesized in uninfected XP cells in the presence of 0.08 μg of actinomycin D per ml. This concentration of the drug is sufficient to inhibit the synthesis of the 45S precursor to 5.8S, 18S, and 28S rRNA's but allows synthesis of 4S and 5S RNAs (data not shown). (iv) The molar ratio of label in this RNA to that in 28S rRNA is nearly 1 in all samples analyzed (data not shown). (v) This RNA migrates with 28S rRNA on nondenaturing sucrose gradients (data not shown).

Another change in the low-molecular-weight RNAs is the appearance of a new RNA species of approximately 380 nucleotides which accumulates in the cytoplasm of cells during infection with HSV. This RNA, denoted by an arrow in Fig. 1, can be observed in the early RNA but is much more apparent in the late RNA sample. It should also be noted that the relative amount of synthesis of two low-molecular-weight RNAs which migrate between the RNA of 380 nulceotides and 5.8S rRNA remains nearly constant during infection. These results illustrate that HSV infection of XP cells affects the synthesis of low-molecular-weight RNAs in an unequal manner.

To determine whether any of the small RNAs that are synthesized during infection are encoded by HSV, the cytoplasmic RNA from uninfected and infected XP cells was hybridized to HSV DNA covalently coupled to cellulose. That the DNA cellulose is quite effective in selecting HSV RNA is indicated by the following percentages of the radioactivity that were recovered by hybridization: 7.5% of the early RNA sample, 8.3% of the late RNA sample, but only 0.2% of the uninfected RNA sample.

Figure 2 shows the results of the hybridization experiments with respect to the low-molecular-weight RNAs. No HSV-specific low-molecular-weight RNAs can be observed in the hybridized samples (lanes C of early and late RNA). Of particular interest is the absence of binding to the HSV DNA cellulose of the 380-nucleotide RNA found in the infected cells. This RNA could be a host RNA which either is induced during infection or is a degradation product of a host RNA which is synthesized in uninfected cells but is degraded differently in infected cells. Alternatively, this RNA could be a viral RNA which, due to its small size, did not hybridize to the HSV DNA cellulose under the conditions used.

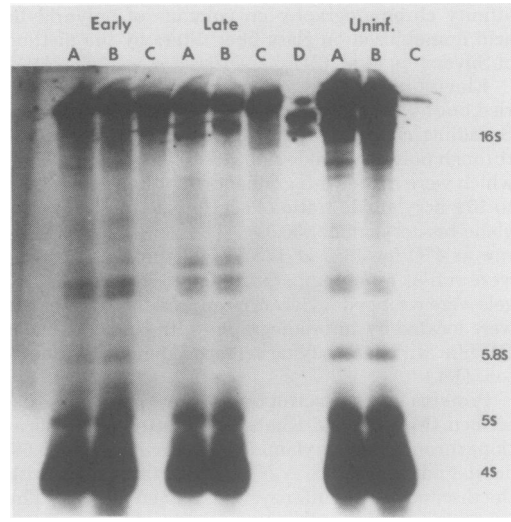

FIG. 2. *Electrophorogram of low-molecular-weight RNAs hybridized to HSV DNA cellulose. Uninfected and early and late infected cell cytoplasmic RNA was hybridized to HSV DNA covalently coupled to cellulose and electrophoresed through 4 to 15% polyacrylamide gradient gels containing 7 M urea. (A) Unfractionated cytoplasmic RNA; (B) RNA that did not hybridize to the DNA cellulose; (C) hybridized RNA; (D) E. coli rRNA.*

Large cytoplasmic RNAs of HSV-infected XP cells. The polysomal RNAs from infected and uninfected XP cells were analyzed on low-percentage acrylamide gradient gels to resolve RNAs of greater than 1,000 nucleotides in length (Fig. 3). Several RNA bands are found in the early (Fig. 3, lane A) and late (Fig. 3, lane B) RNA samples that are not visible in the polysomal RNA from uninfected cells (Fig. 3, lane C). These RNAs migrate between 16S and 28S rRNA markers and are of the size expected for HSV mRNA's (see below). When similar RNA samples were isolated with HEp-2 host cells, the electrophoretic patterns of the RNAs from infected and uninfected samples were nearly iden-

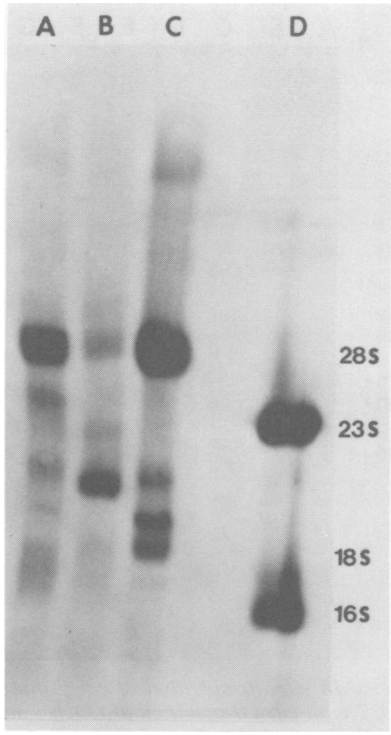

FIG. 3. *Electrophorogram of uninfected and early and late infected cell polysomal RNA. Polysomal RNA was isolated and electrophoresed through denaturing gels made with gradients of 2 to 4% acrylamide. (A) Early infected cell polysomal RNA; (B) late infected cell polysomal RNA; (C) uninfected cell polysomal RNA; (D) E. coli rRNA.*

tical (data not shown). Thus, in our hands, XP cells seem to be better host cells than HEp-2 cells in which to study HSV mRNA.

As shown in Fig. 3 and as previously reported by Wagner and Roizman (26), the synthesis and accumulation of 18 and 28S rRNAs decline during HSV infection. This is further reflected by the disappearance of labeled 5.8S rRNA (Fig. 1 and 2). The low amounts of labeled 18S rRNA compared with 28S and 4S RNAs in uninfected cycloheximide-treated cells has been observed before and is due to the degradation of newly synthesized 18S rRNA (5).

To simplify the RNA analyses in the following experiments, we focused only on the early RNAs. Two types of fractionation of the early RNA were used to identify the herpes mRNA's. HSV RNA was selected by hybridization to HSV DNA covalently coupled to cellulose, and polyadenylated RNA was selected by affinity chromatography on columns made with polyuridylic acid immunobilized on glass fiber filters. In the poly(A) cytoplasmic RNA preparation, eight major RNA species are observed (Fig. 4, lane C). From their electrophoretic mobilities relative to those of XP and *E. coli* rRNA's, the apparent molecular weights of these RNAs are 1.66×10^6, 1.38×10^6, 1.09×10^6, 0.94×10^6, 0.75×10^6, 0.61×10^6, 0.38×10^6, and 0.32×10^6. The width of the RNA bands could be due to several factors such as different extent of polyadenylation of the RNA (2, 21, 23), degradation of the RNA, or the presence of several RNAs of similar sizes which are not separated on these gels.

The HSV DNA cellulose hybridization selects from the early cytoplasmic RNAs a subset of the RNAs which are observed in the polyadenylated RNA sample. Five major RNA species hybridize to the HSV DNA and are shown in Fig. 4, lane D. The molecular weights and corresponding lengths in nucleotides of these early viral RNA species are listed in Table 1. Table 2 shows the molecular weights of the major early viral polypeptides (see below and Millette and Klaiber, submitted for publication) and the number of nucleotides required to encode these polypeptides. From the size of the early HSV RNAs and from the number of nu-

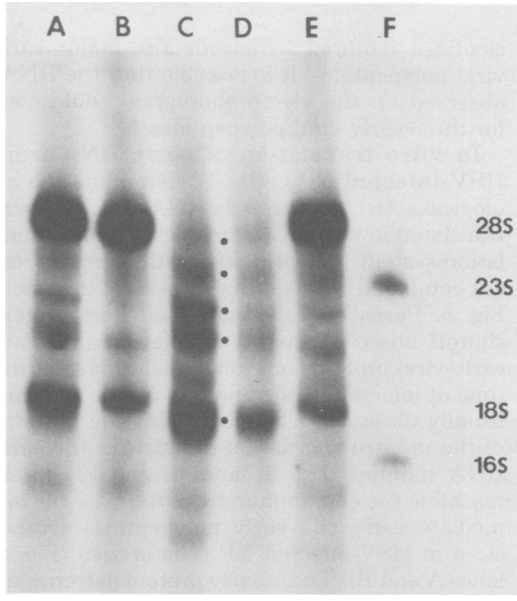

FIG. 4. *Electrophoretic analysis of HSV early RNAs. Early RNA from infected cells treated with cycloheximide was fractionated by polyuridylic acid affinity chromatography and HSV DNA cellulose hybridization and electrophoresed through 2 to 4% polyacrylamide gradient gels containing 7 M urea. (A) Early cytoplasmic RNAs; (B) nonadenylated RNAs; (C) polyadenylated RNAs; (D) RNAs that hybridized to HSV DNA cellulose; (E) RNAs that did not hybridize to HSV DNA cellulose; (F) E. coli rRNA.*

TABLE 1. *Size of early HSV RNAs*

Mol wt[a]	No. of nucleotides[b]
1.66×10^6	5,160
1.38×10^6	4,290
1.09×10^6	3,390
0.94×10^6	2,920
0.61×10^6	1,890

[a] An average of three separate experiments and calculated by electrophoretic mobility relative to that of *E. coli* and eucaryotic rRNA's with the following weights for the standards: 23S, 1.03×10^6; 16S, 0.55×10^6; 28S, 1.75×10^6; and 18S, 0.67×10^6 (11).

[b] Calculated using 322 daltons per nucleotide.

TABLE 2. *Size of major HSV early polypeptides*

Mol wt[a]	Required nucleotides in mRNA[b]
165,000	4,300
145,000	3,780
123,000	3,210
71,000	1,850
55,000	1,430

[a] From Millette and Klaiber, submitted for publication.

[b] Calculated using 115 daltons per amino acid.

cleotides required to encode the major early viral polypeptides, it is possible that the RNAs observed on the electrophorogram could code for these early viral polypeptides.

In vitro translation of early RNA from HSV-infected XP cells. To determine the biological activity of the early RNA, this RNA was translated in vitro in a reticulocyte lysate translation system. The proteins synthesized in vitro are compared with those synthesized in vivo in Fig. 5. Parenthetically, the good host protein shutoff observed in the sample of immediate early viral proteins (cycloheximide added at the time of infection) shown in Fig. 5, lane A, is not usually observed. The electrophoretic patterns of the in vitro translation products of the early RNA samples (Fig. 5, lanes E and F) closely resemble the electrophoretic patterns of the immediate early and early polypeptides synthesized in HSV-infected XP cells in vivo (Fig. 5, lanes A and B). These early protein patterns are very different from those of late viral proteins (Fig. 5, lane C) or uninfected cell proteins synthesized in vivo (Fig. 5, lane D) or in vitro (Fig. 5, lane H).

The in vitro translation products of early RNA samples (Fig. 5, lanes E and F) have electrophoretic mobilities very similar to the major early viral polypeptides that are synthesized in vivo (Fig. 5, lanes A and B) and are listed in Table 2. On this gel the early viral polypeptide with a

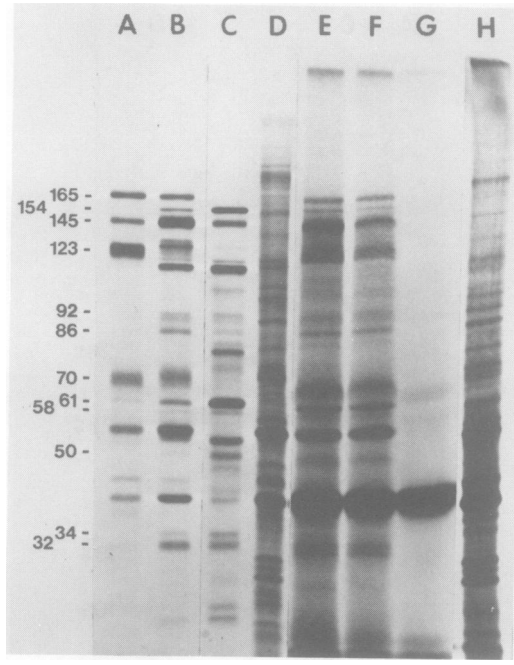

FIG. 5. *In vitro translation of early infected cell RNAs. The in vitro translation products synthesized in a reticulocyte lysate translation system are compared with viral proteins synthesized in vivo. The [^{35}S]methionine-labeled cell lysates were prepared as described elsewhere (Millette and Klaiber, submitted for publication). Briefly, XP cells were infected with 50 PFU/cell for 1 h, incubated in media with or without 50 μg of cycloheximide per ml for 6 h, and pulse-labeled for 30 min with 20 μCi of [^{35}S]methionine per ml. Lysates from uninfected and infected cells prepared with or without cycloheximide present during the infection. (A) Cycloheximide present 0 to 6 h postinfection; (B) cycloheximide present 1 to 6 h postinfection; (C) no cycloheximide present; and (D) uninfected. In vitro translation products of (E) early infected cell cytoplasmic RNA, (F) early polyadenylated RNA, (G) no added RNA, and (H) uninfected cell cytoplasmic RNA. Viral polypeptides are designated in the left margin by $M \times 10^{-3}$.*

molecular weight of 55,000 is obscured by a major host contaminant which is synthesized both in vivo and in vitro. That this viral polypeptide is synthesized in XP cells early in infection has been demonstrated (Millette and Klaiber, submitted for publication; Pedersen et al., manuscript in preparation). In addition to these major early viral polypeptides, other polypeptides, presumably viral in origin, can be detected in samples synthesized both in vitro and in vivo. These polypeptides have molecular weights of 154,000, 92,000, 86,000, 61,000, 58,000, 34,000, and 32,000. It is interesting to note that, although host RNAs are capable of being translated under

these conditions (Fig. 5, lane H), most of the polypeptides synthesized from infected cell RNA in vitro have electrophoretic mobilities similar to viral polypeptides synthesized in vivo. Furthermore, the in vitro translation products of polyadenylated RNA and whole cytoplasmic RNA are nearly identical (Fig. 5, lanes E and F).

DISCUSSION

The rationale behind our choice of adding cycloheximide at 1 h after addition of virus and studying early viral RNAs rather than adding the drug before or at the time of infection and studying immediate early viral RNAs came from three basic considerations. (i) In our hands, when pulsing cells after removal of cycloheximide, much better shutoff of host protein synthesis is observed when cycloheximide is added at 1 h after addition of the virus rather than at the time of infection. (ii) Nishioka and Silverstein (16) reported that protein synthesis is required for the degradation of host mRNA in HSV-1-infected cells. (iii) Although additional viral proteins are synthesized when cycloheximide is added 1 h after addition of virus rather than with the virus, protein synthesis is nevertheless limited to an early class of viral proteins (7). Thus, possible complications due to the accumulation of additional viral RNAs under these conditions are compensated by the lower background of host mRNA.

HSV-infected cell low-molecular-weight RNAs. Two main conclusions can be made from the study of the low-molecular-weight RNAs in HSV-infected XP cells. First, the results of these experiments extend the previous observation by Wagner and Roizman (26) that HSV-1 infection affects the synthesis of host RNAs in an unequal manner. Although the accumulation of labeled 4S and 5S RNAs decreased somewhat during infection, the accumulation of detectable 5.8S rRNA was completely eradicated, and a 380-nucleotide RNA, possibly of host origin, was observed in infected cells. Furthermore, the synthesis and accumulation of two other low-molecular-weight RNAs were not affected by HSV infection of XP cells. Second, no HSV-specific RNAs of 4S to 10S were detected in these experiments (Fig. 2). Additional experiments involving sucrose gradient selection of low-molecular-weight RNA of high specific activity from HSV-infected HEp-2 cells, hybridization to HSV DNA cellulose for 45 h, and subsequent gel electrophoresis have failed to detect any HSV-specific 4S to 7S RNAs (M. Pedersen, unpublished data). This is of interest because of the question regarding the existence of HSV-specific tRNA's (12, 24).

In vitro translation of early RNA. The in vitro translation studies produced several interesting findings. (i) The electrophoretic pattern of the products of the in vitro translation of early RNA closely resembled that of the early proteins synthesized in infected cells in vivo (Fig. 5). (ii) Another interesting observation is that although host RNA from uninfected cells was efficiently translated in this system (Fig. 5, lane H), the majority of the products of the in vitro translation of early RNA had electrophoretic mobilities similar to those of early viral polypeptides synthesized in vivo (Fig. 5, lanes E and F). Since purified RNA was used, this preferential translation of presumptive viral messages is not due to regulatory proteins. Furthermore, because poly(A) RNA was translated to yield a protein pattern similar to that of whole cytoplasmic RNA, other tRNA's in addition to reticulocyte tRNA's are not needed to produce this effect. Therefore, these experiments are consistent with the observation of Nishioka and Silverstein (16) that host mRNA is degraded during HSV-1 infection. (iii) The translation studies also reveal that at least some fraction of each early mRNA is polyadenylated since the in vitro translation products of the poly(A+) and whole cytoplasmic RNA were the same (Fig. 5, lanes E and F). It was previously reported that a large fraction (30 to 40%) of the polysomal HSV RNA in infected HeLa cells is not polyadenylated and that this HSV poly(A−) RNA is transcribed from the same sequences as the HSV poly(A+) RNA (23). Experiments are in progress to determine the fraction of each early mRNA that is polyadenylated in infected XP cells.

Presumptive HSV mRNA's. Five major HSV-specific RNA species were found to accumulate in the cytoplasm of infected XP cells in the presence of cycloheximide. That these RNAs could be HSV mRNA is indicated by the following. (i) RNAs of these five size classes are poly(A) (Fig. 4, lanes C and D). (ii) These RNA preparations can be translated in vitro to yield polypeptides whose electrophoretic pattern is similar to that of early viral polypeptides synthesized in vivo (Fig. 5). (iii) The size of these RNAs is approximately what would be expected to encode the major early viral polypeptides (Tables 1 and 2).

The early RNA preparations can be translated into more than five polypeptides (Fig. 5, lanes E and F), but only five viral RNA bands were detected on the gels (Fig. 4, lane D). This could be explained by the presence of more than one mRNA per gel band and may account for the band widths on the gels. Another explanation is that mRNA's for the additional proteins are in

concentrations too low to detect by our gel analyses. Studies are in progress to distinguish between these two alternatives by separating the RNAs and translating the individual fractions in vitro.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

Since submission of this paper, we have established by in vitro translation the protein coding properties of four immediate early mRNA's. The viral mRNA's of 5.2, 4.3, and 2.9 kilobases (kb) encode viral polypeptides 145, 165, and 123 ($M \times 10^{-3}$), respectively, and the RNA of 1.9 kb encodes viral proteins 71 and 55 ($M \times 10^{-3}$). These results agree in part with the data recently reported by Watson et al. (J. Virol. **31**:42-52, (1979) on three immediate early HSV-1 mRNA's.

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