JOURNAL OF VIROLOGY, Aug. 1979, p. 299-314
0022-538X/79/08-0299/16$02.00/0

Vol. 31, No. 2

Regulation of Herpesvirus Macromolecular Synthesis

VIII. The Transcription Program Consists of Three Phases During Which Both Extent of Transcription and Accumulation of RNA in the Cytoplasm Are Regulated

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Received for publication 2 February 1979

This report concerns the stable viral RNA sequences that accumulate in HEp-2 cells infected with herpes simplex virus type 1. By hybridizing labeled total DNA and restriction endonuclease DNA fragments with excess unlabeled total nuclear and cytoplasmic RNA, we determined the genetic complexity of the RNA and we mapped the regions on the physical map of herpes simplex virus type 1 DNA that are homologous to the RNA. Our results show the following. (i) The viral RNAs accumulating in the nucleus and cytoplasm of cells infected and maintained in the presence of inhibitory concentrations of either cycloheximide or emetine were homologous to 33 and 12% of viral DNA, respectively. All of the fragments tested contained sequences homologous to nuclear RNA. However, only the fragments mapping between 0.00 and 0.18, and 0.53 and 1.00 map units contained sequences homologous to cytoplasmic RNA. (ii) The viral RNAs that accumulate in the nucleus and cytoplasm of cells infected and maintained in the presence of inhibitory concentrations of phosphonoacetic acid were homologous to 39 and 26% of viral DNA, respectively. In this instance all of the fragments except those mapping between 0.42 and 0.53 map units contained sequences homologous to cytoplasmic RNA. (iii) The viral RNAs that accumulate in the nucleus and cytoplasm 8 h after infection were homologous to greater than 50 and 41%, respectively. All of the fragments tested contained sequences homologous to cytoplasmic RNA. (iv) The viral RNAs that accumulate in the nucleus and cytoplasm of cells infected and maintained in the presence of canavanine are homologous to 33 and 19% of viral DNA, respectively. All of the fragments tested contained sequences homologous to both nuclear and cytoplasmic RNAs. Our results indicate the following. First, there are at least three phases of transcription of viral DNA. Phase 1 does not require the synthesis of host cell or viral proteins. Phase 2 requires the synthesis of viral proteins made before the initiation of viral DNA synthesis. Phase 3 appears to be related to the initiation of viral DNA synthesis. Second, both the extent of transcription and the accumulation of viral RNA in the cytoplasm are tightly regulated. The genetic complexity of total RNA accumulating in infected cells increased in each successive phase. Moreover, the genetic complexity of nuclear RNA was invariably higher than that of cytoplasmic RNA in each phase. Lastly, the results of the studies on viral RNA accumulating in canavanine-treated cells reinforce the hypothesis made previously that more than one polypeptide in each of the α and β polypeptide groups is involved in the transcription preceding the transitions from α to β and β to γ polypeptide synthesis, respectively, and that canavanine selectively inactivated subsets of these polypeptides permitting only partial transitions from α to β and β to γ to occur.

This report deals with the transcriptional program of herpes simplex virus type 1 (human herpesvirus 1, HSV-1) based on analyses of viral RNA accumulating in the nuclei and cytoplasm of infected cells by hybridization with individual restriction endonuclease fragments of HSV-1 DNA. The major emphasis of the report is on evidence indicating that both transcription and translocation of viral RNA are regulated. Pertinent to this report are the following.

(i) Evidence that gene expression is regulated emerged from the earlier analyses of viral poly-

peptide synthesis in HSV-infected cells. Specifically, approximately 50 infected cell polypeptides were identified as virus specific (20, 35, 38). Based on analyses of the kinetics and the requirements for their synthesis, these polypeptides comprise at least three groups designated as α, β, and γ whose synthesis is coordinately regulated and sequentially ordered (21, 44).

(ii) Several reports (2, 11, 12, 25, 26, 30, 31, 44, 47, 48, 51–53, 56–58) have focused on the transcription of viral DNA late in the reproductive cycle, i.e., during the synthesis of γ polypeptides. These studies have shown that the transcripts accumulating in the cytoplasm late in infection include also the transcripts made earlier in infection and are homologous to approximately 43% of viral DNA (30). That the cytoplasmic transcripts arise from transcription of noncomplementary DNA sequences is suggested by the observation that the genetic complexity of the RNA available for hybridization to viral DNA does not decrease significantly after preincubation of the RNA under conditions optimal for maximum RNA-RNA renaturation (31). In contrast, the viral RNA transcripts that accumulate in the nucleus late in infection are homologous to >50% of the DNA (11, 12, 30). That these transcripts arise in part by transcription of complementary DNA sequences is indicated by the observation that there is a significant decrease in the genetic complexity of the RNA after self-annealing (2, 25, 26, 31). Moreover, isolated double-stranded viral RNA was shown to be homologous to at least 60% of viral DNA (25).

(iii) Less well defined is the genetic complexity of the viral transcripts accumulating before the onset of viral DNA synthesis when the infected cells are making predominantly α and β polypeptides. Specifically, in analyzing the genetic complexity of viral RNA accumulating in cells at 2 h postinfection, Kozak and Roizman (30) observed that the complexity varies depending on the multiplicity of infection and that in cells infected at an input multiplicity of 40 PFU/cell it attains values as high as 40% of viral DNA. Wagner and his colleagues in initial studies (52, 53, 56, 58) observed no multiplicity-dependent fluctuation in the genetic complexity and reported that at 2 h after infection or in the presence of inhibitors of DNA synthesis, the vast majority of transcripts accumulating in infected cells are homologous to 20% of total viral DNA. However, more recently Stringer et al. (51) mapped the regions of the HSV genome homologous to polyribosomal RNA isolated from cells 3 h after infection and reported homology extending over most of the total viral DNA length.

(iv) Discordant results have also been reported on the genetic complexity of the RNA accumulating in the nuclei and cytoplasm of cells infected and maintained in the presence of inhibitors of protein synthesis. Kozak and Roizman (30) had shown that the nuclear and cytoplasmic RNAs accumulating in the presence of cycloheximide are homologous to 45 to 50 and about 10% of total viral DNA, respectively. The viral RNA accumulating in the cytoplasm in the presence of cycloheximide specifies α polypeptides: cells infected and maintained in the presence of cycloheximide synthesize only α viral polypeptides immediately upon withdrawal of the drug (21). Similarly, they are the only polypeptides made in infected cells which were physically (10) or chemically (10, 21) enucleated before the withdrawal of the drug. Furthermore, from the results of the hybridization of cytoplasmic RNA to restriction endonuclease fragments of viral DNA, Jones et al. (27) concluded that αRNA arises from defined noncontiguous regions on the viral genome. Clements et al. (6) confirmed the results of Jones et al. (27) but concluded that both the nuclear and the cytoplasmic RNAs have the same genetic complexity. Wagner et al. (52, 53, 56, 58) likewise concluded that early in infection HSV transcription is restricted and found no evidence of nuclear retention of any specific class of viral RNA at any time during viral replication.

This report focuses on the complexity and site of homology on the DNA of RNA accumulating in the cytoplasm and nucleus under four conditions: (i) in the absence of infected cell protein synthesis, i.e., in cells treated with inhibitory concentrations of cycloheximide and emetine from the time of infection; (ii) in the absence of the normal transition from α to β and γ viral protein synthesis as observed in cells infected in the presence of effective concentrations of the arginine analog L-canavanine; (iii) in the absence of viral DNA synthesis, i.e., in cells treated with concentrations of phosphonoacetic acid (PAA) previously shown to completely block viral DNA synthesis (R. J. Jacob, L. S. Morse, and B. Roizman, manuscript in preparation); and (iv) in untreated cells late in infection, i.e., after the appearance of infectious progeny.

MATERIALS AND METHODS

Cells and viruses. All RNA preparations were made in human epidermoid carcinoma no. 2 (HEp-2) cells infected with strain HSV-1(F). HSV-1(F), originally isolated from a recurrent facial lesion, has been passaged a maximum of four times at low multiplicity in HEp-2 cells (9) and has served as a prototype for biological studies of HSV-1 in this laboratory. All

individual restriction endonuclease fragments of HSV-1 DNA were made from HSV-1(MP) DNA. A distinguishing feature of HSV-1(MP) DNA is that it lacks the *Hsu*I restriction endonuclease cleavage site present in HSV-1(F) DNA between fragments O and I (16); as a consequence, the fragments present in the *Hsu*I digests can be readily resolved electrophoretically. The procedure for the propagation of HSV-1 in HEp-2 cell roller bottle cultures and the pertinent properties of strains HSV-1(F) and HSV-1(MP) were described elsewhere (9, 16, 19, 24, 29, 45, 50).

Infection of cells. We prepared five different RNA preparations from HEp-2 cells infected with HSV-1(F). Throughout exposure to the virus and subsequent incubation at 37°C, the cells were maintained in a medium consisting of mixture 199 supplemented with 1% heat-inactivated calf serum.

Untreated infected cell RNA was prepared from cells grown in roller bottle cultures (~2×10^8 cells per bottle) and exposed to 10 PFU/cell. After 2 h of incubation, the inoculum was replaced with 65 ml of maintenance medium, and the cultures were reincubated at 37°C. The cells were harvested at 8 h postinfection. Throughout these studies, time after infection was calculated from the time of exposure of the cell culture to the virus.

Canavanine RNA was prepared from cells grown in roller bottle cultures infected with 20 PFU/cell as described above except that 2.8 mM L-canavanine (Sigma Chemical Co., St. Louis, Mo.) was present in the medium during both exposure of cells to virus and subsequent incubation at 37°C until 16 h postinfection.

Cycloheximide and emetine RNAs were prepared from cells grown in 32-ounce (ca. 0.946-liter) bottles (~4×10^7 cells per bottle) and infected with 10 PFU/cell. Cycloheximide at 50 μg/ml (Acti-Dione, Calbiochem, San Diego, Calif.) or emetine at 5×10^{-6} M (Sigma Chemical Co.) were present in the medium throughout the exposure of cells to virus and subsequent incubation until 7 h postinfection. In contrast to the effects of cycloheximide the effects of emetine on protein synthesis are not reversible (13–15).

PAA RNA was prepared from 32-ounce-bottle cultures infected with 20 PFU/cell. Disodium phosphonoacetate hydrate, a gift of Abbott Laboratories, North Chicago, Ill., was present in the medium at 300 μg/ml throughout exposure of virus to cells and subsequent incubation until 24 h postinfection.

Cell fractionation. The infected cells in the monolayer cultures were rinsed and scraped into ice-cold phosphate-buffered saline (8), collected by centrifugation, and washed once more with ice-cold phosphate-buffered saline. The nuclear and cytoplasmic fractions were prepared after detergent lysis of the cells in 1% (vol/vol) Nonidet P-40 (Gallard-Schlesinger, Carle Place, N. Y.) as previously described (27).

RNA extraction. To minimize the loss of polyadenylic acid-containing RNA species, the cytoplasmic RNA was extracted by the method of Brawerman et al. (4) as previously detailed (27, 30, 31). For the extraction of total nuclear RNA, the washed nuclei were resuspended in 5 volumes of buffer (5 mM Tris [pH 6.9] and 5 mM $MgCl_2$), lysed by the addition of sodium deoxycholate to a final concentration of 2%, digested with pancreatic DNase I (RNase-free, Worthington Biochemical Corp., Freehold, N. J.) at 50 μg/ml for 45 min at 37°C, diluted with two volumes of 0.1 M Tris (pH 9.0) and sodium dodecyl sulfate to a final concentration of 0.5%, and extracted with an equal volume of water-saturated redistilled phenol at 56°C (41). The interphase and phenol phase together were reextracted with 0.1 M Tris, pH 9. The aqueous phases from both extractions were combined and extracted twice with equal volumes of water-saturated, redistilled phenol at room temperature; each time the interphase and phenol phase were reextracted with 0.1 M Tris, pH 9.0 (41). The aqueous phase was then extracted at least three times with equal volumes of chloroform containing 2% (vol/vol) isoamyl alcohol, and the RNA was precipitated in the presence of 0.1 M KCl with 2.5 volumes of absolute ethanol at −20°C. The precipitate was collected, resuspended, and dialyzed against 5 mM Tris (pH 6.9), made 5 mM $MgCl_2$, treated with pancreatic DNase I at 50 μg/ml for 30 min at 37°C, and then diluted with two volumes of 0.15 M Tris, pH 9.0. The subsequent two cycles of phenol/chloroform-isoamyl alcohol extraction of the nuclear RNA were identical to those described for the extraction of cytoplasmic RNA. After the final ethanol precipitation, the RNA was resuspended in sterile water at a concentration of more than 20 mg/ml and exhaustively dialyzed against 0.04 M sodium phosphate buffer pH 6.8. Under these conditions, the DNA was completely depolymerized. This was shown in reconstruction experiments in which two cycles of treatment with pancreatic DNase I at 50 μg/ml at 37°C for 30 min in the presence of 5 mM Tris (pH 6.9), 5 mM $MgCl_2$, and exogenous unlabeled calf thymus DNA at 3 mg/ml resulted in the complete hydrolysis of 15,400 cpm of ^{3}H-labeled HSV-1 DNA as measured by trichloroacetic acid precipitation.

Purification of viral DNA. HSV-1(MP) and HSV-1(F) DNAs were prepared from purified nucleocapsids disrupted with 1% sodium dodecyl sulfate and 2% Sarkosyl (ICN, K and K Laboratories, Inc., Cleveland, Ohio) and sedimented in neutral sucrose gradients as described by Kieff et al. (29). HSV-1(F) DNA was further purified as previously described (27) and was used for preparation of total in vitro-labeled DNA probe.

Restriction endonuclease digestion. The preparation of the restriction endonucleases and the conditions for the limit digestion of HSV-1(MP) DNA with the individual restriction endonucleases *Hsu*I, *Bgl*II, and *Eco*RI were as detailed elsewhere (37). The digestion of HSV-1(MP) DNA with both *Hsu*I and *Bgl*II simultaneously was done in 20 mM Tris (pH 7.6), 20 mM $MgCl_2$, and 30 mM NaCl.

Agarose gel electrophoresis. Electrophoresis was done in columns (1 by 30 cm) of agarose (18) gel (Seakem ME grade; Marine Colloids, Inc., Rockland, Maine) of either 0.35% or 0.45%, the latter necessary for optimal resolution of the *Eco*RI fragments and of those from the *Hsu*I/*Bgl*II double digest. The DNA fragments were subjected to electrophoresis for 36 to 40 h at 1.6 V/cm at 4°C in a buffer system of 36 mM Tris (pH 8.0), 30 mM NaH_2PO_4, and 1 mM EDTA (17).

Isolation of restriction endonuclease fragments of HSV-1 DNA. The purification of restriction endonuclease fragments of HSV-1 DNA has been previously described (27). Figure 1 shows the location of the restriction endonuclease fragments used in this study to map the regions of HSV-1 DNA homologous to cytoplasmic and nuclear RNA accumulating in infected cells.

In vitro labeling of DNA and DNA restriction enzyme fragments. HSV-1(F) DNA and individual, purified HSV-1(MP) DNA restriction endonuclease fragments were labeled in vitro to a high specific activity by a micromodification (33) of the method described by Rigby et al. (43) by using the nick translation reaction of *Escherichia coli* DNA polymerase I (28). The nick translation reaction as described below was performed in 4-μl volumes. A 30-ng amount of DNA at a concentration of ≥2 μg/ml, 0.1 nmol of [^{3}H]TTP ([*methyl*-^{3}H]thymidine 5'-triphosphate tetrasodium salt; specific activity, ≥50 Ci/mmol; New

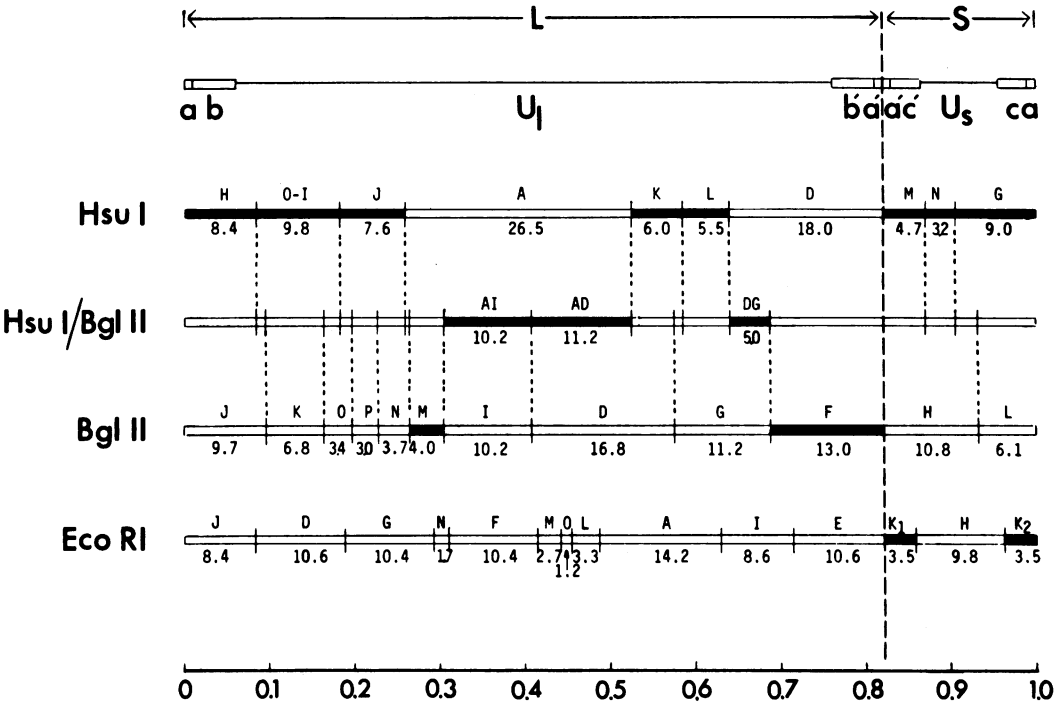

FIG. 1. *Sequence arrangement and restriction endonuclease maps of HSV-1(MP) DNA. Lines 1 and 2 are a diagrammatic representation of the arrangement of unique and reiterated sequences in HSV-1 DNA (55). HSV-1 DNA is a linear, double-stranded molecule of approximately 96 × 10^6 molecular weight (1, 29) and consists of two covalently linked components (46, 55). L refers to the large component of the DNA consisting of the unique region U$_L$ which contains 70% of the total DNA and is bound by the terminal sequence ab and its inverted repeat b'a', each containing 6% of the total DNA (55). S refers to the small component of the DNA consisting of the unique region U$_S$ which contains 9% of the total DNA and is bound by the terminal sequence ca and its inverted repeat a'c', each containing 4.3% of total DNA (55). It should be noted that the L and S components can invert relative to each other and that HSV-1 DNA extracted from virions consists of four populations of molecules differing only in the relative orientations of the L and S components (7, 17). The four arrangements have been designated as P (prototype), I$_S$ (inversion of S component), I$_L$ (inversion of L component), and I$_{SL}$ (inversion of both S and L components) (17, 37). Lines 3, 4, 5, and 6 show the size (molecular weight ×10^{-6}) and position of the DNA fragments generated by the cleavage of the P arrangement of HSV-1(MP) DNA by the restriction endonucleases HsuI, BglII, EcoRI and by codigestion with both HsuI and BglII simultaneously (16, 17, 37; G. S. Hayward, T. G. Buchman, and B. Roizman, unpublished data). The designation of the restriction endonuclease fragments follows the convention reported elsewhere (27). The fragments obtained by digestion with single enzymes are designated alphabetically in order of decreasing size. The exception is HsuI fragment O-I which is the fusion product of fragments O and I, inasmuch as HSV-1(MP) DNA lacks the HsuI cleavage site at 0.104 map units. The fragments generated by codigestion with both HsuI and BglII enzymes are identified by the designations of the two single enzyme fragments from which they are generated. Pertinent to the maps presented in this figure is that the restriction endonuclease EcoRI cleaves within the reiterated sequences of the S component and yields two electrophoretically comigrating fragments K$_1$ and K$_2$ from the I$_S$, I$_{SL}$ and P, I$_L$ arrangements of the DNA, respectively. The thick lines indicate the fragments used in the hybridization tests. Line 7 designates the fraction of HSV-1 DNA length in map units.*

England Nuclear Corp., Boston, Mass.), and 0.1 nmol of [^{3}H]dCTP (deoxy-[5-^{3}H]cytidine 5'-triphosphate ammonium salt; specific activity, ≥20 Ci/mmol; New England Nuclear Corp.) were individually evaporated to dryness in a glass tube (6 by 50 mm) in a vacuum desiccator over Drierite. To the dried DNA and ^{3}H-labeled deoxynucleoside triphosphates ([^{3}H]dNTP's) was added 3 μl of a solution containing 50 mM potassium phosphate buffer (pH 7.5), 5 mM $MgCl_2$, and 0.4 nmol each of unlabeled dATP and dGTP (Sigma Chemical Co.). A 0.5-μl of amount activated pancreatic DNase I (33) was added to the reaction mixture so that its final concentration was 30 ng/ml; the mixture was incubated for 60 s at room temperature and then immediately cooled in an ice-water bath. One unit of *E. coli* DNA polymerase I (Boehringer Mannheim Corp., Indianapolis, Ind.) was added to the nicked DNA, and the reaction mixture was incubated at 14°C for approximately 5 h. The reaction was terminated by the addition of 25 μl of 0.1 M EDTA (pH 7.0) and heated at 68°C for 10 min to inactivate the DNase and polymerase. To the inactivated reaction were added an additional 75 μl of 0.1 M EDTA (pH 7.0) and 25 μg of calf thymus DNA as carrier DNA (Sigma Chemical Co., St. Louis, Mo.). Unincorporated dNTP's were removed by Sephadex G50 (medium) gel filtration chromatography, after which the labeled DNA product was deproteinized after extraction with phenol and chloroform containing 2% isoamyl alcohol, concentrated, and then exhaustively dialyzed against 0.04 M sodium phosphate buffer, pH 6.8. The final product had a specific activity of ≥2 × 10^7 cpm/μg and sedimented at 4S to 6S in alkaline sucrose gradients. After denaturation at 109°C for 7 min followed by rapid cooling in ice water, usually less than 5% of the product was resistant to digestion with the S_1 single-strand-specific nuclease.

Solution hybridization procedure. Hybridization reactions between the in vitro ^{3}H-labeled viral DNA probe and excess unlabeled nuclear or cytoplasmic RNA were done by a modification of the procedure previously described (11, 27, 30). Specifically, 20-μl reaction mixtures containing 8 to 10 mg of RNA per ml and 1 to 2 ng of heat-denatured [^{3}H]DNA probe per ml in 0.04 M sodium phosphate (pH 6.8) and 0.23 M NaCl were sealed in 50-μl capillary pipettes and incubated at 75°C (25°C below T_m for HSV-1 DNA at 0.3 M salt concentration) for varying lengths of time. The fraction of DNA hybridized to RNA was determined from the proportion of the labeled DNA probe that was resistant to S_1 nuclease digestion. A 15-μl portion was removed from the hybridization reaction mixture and diluted to 250 μl at a final concentration of 25 mM sodium acetate (pH 4.6), 0.15 M NaCl, 1 mM $ZnSO_4$, 200 μg of native exogeneous DNA (either calf thymus DNA or salmon sperm DNA [Worthington Biochemicals Corp., Freehold, N. J.]), and 40 μg of denatured exogenous DNA per ml. One half of the sample was precipitated with 5% trichloroacetic acid, whereas the other half was digested with S_1 nuclease for 1 h at 45°C before trichloroacetic acid precipitation. The single-strand-specific S_1 nuclease was purified by a modification of the procedure of Vogt (54) from crude α amylase from *Aspergillus oryzae* (Sigma Chemical Co.). The amount of enzyme used in the assay digested at least 99% of single-stranded DNA without significantly affecting native DNA. Experiments designed to standardize the assay have shown that 2 mM sodium phosphate has no measurable effect on the activity of S_1 nuclease. For HSV-1(F) DNA and for most of the fragments tested, the amount of DNA reassociation occurring within the duration of the hybridization and in the presence of comparable concentrations of uninfected HEp-2 cellular RNA did not exceed 5%. The results of the hybridizations presented in this report were corrected for the amount of DNA reassociated in the absence of viral RNA and for the fraction of the denatured DNA probe resistant to digestion with S_1 nuclease. For the purposes of this report, the genetic complexity of viral RNA is defined as the fraction of total HSV-1 DNA homologous to the RNA.

RESULTS

Genetic complexity of RNA accumulating in nuclei and cytoplasm of HEp-2 cells infected with HSV-1. In this series of experiments, we measured the genetic complexity of RNA in cells infected and maintained in the presence of cycloheximide, emetine, canavanine, or PAA with total in vitro-labeled HSV-1 DNA as a probe. In addition, we measured the genetic complexity of the RNA at 8 h postinfection in the absence of metabolic inhibitors. The results of the hybridization tests are shown in Fig. 2. The rationale and the results of these experiments were as follows.

The genetic complexity of the viral RNA accumulating in nuclei and cytoplasm in the presence of cycloheximide was 30 to 35 and 12%, respectively. This is in good agreement with results published from this laboratory previously (30) but does not agree with the results reported by Clements et al. (6). To test for possible fluctuation in the genetic complexity of the RNA accumulating in the nuclei, we assayed the RNA extracted from three independently prepared batches of cycloheximide-treated infected cells. As shown in Fig. 2A, there was little or no fluctuation in the genetic complexity of viral RNA accumulating in the presence of cycloheximide.

To test for the possibility that the differences in the genetic complexity of the viral transcripts accumulating in nuclei and cytoplasm of infected cells reflect selective properties of cycloheximide independent of its effects on protein synthesis, we repeated these experiments with emetine as the inhibitor of protein synthesis. Preliminary experiments summarized in Fig. 3 showed that 5 × 10^{-6} M emetine completely inhibited the incorporation of labeled amino acids into trichloroacetic acid-precipitable macromolecules. The results of hybridizations (Fig. 2B) show that the genetic complexities of the emetine nuclear and cytoplasmic RNAs are similar, if not iden-

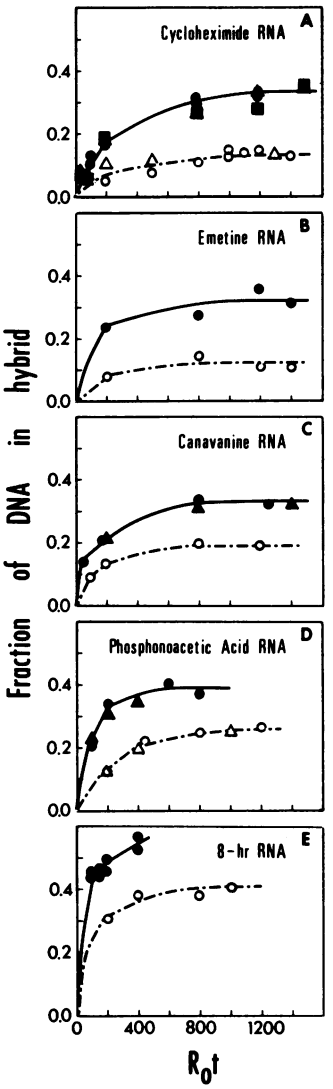

FIG. 2. *Hybridization of ^{3}H-labeled HSV-1(F) DNA with the nuclear and cytoplasmic RNA extracted from infected cells. The figures show the fraction of ^{3}H-labeled DNA probe driven into RNA-DNA hybrid by nuclear RNA (solid symbols) and by cytoplasmic RNA (open symbols) as a function of RNA concentration and time (R_0t = moles of nucleotides × second/liter). The different kinds of symbols used in cycloheximide, canavanine, and PAA RNA hybridizations represent different RNA preparations extracted from independently prepared batches of infected cells.*

tical, to those of RNAs extracted from cycloheximide-treated cells.

The experiments with canavanine were based on previous studies showing that in the presence of a 2.8 mM concentration of the amino acid analog, infected cells make α, a subset of β, and one or two γ polypeptides but that the normal transition to other β and γ polypeptide synthesis does not ensue (22). The results of the hybridizations (Fig. 2C) show that whereas the viral nuclear transcripts have the same genetic complexity as the nuclear RNA extracted from emetine or cycloheximide-treated cells, the cytoplas-

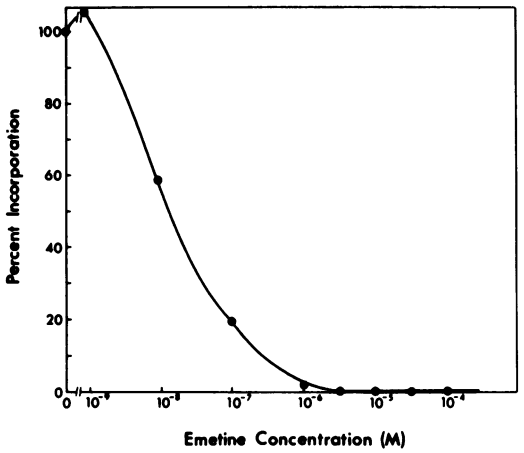

FIG. 3. *Effect of varying the concentration of emetine on protein synthesis in infected cells. Confluent monolayer cultures of HEp-2 cells grown in 32-ounce bottles (~4 × 10^7 cells per bottle) were infected with HSV-1(F) at an input multiplicity of 10 PFU/cell in 8 ml of maintenance medium consisting of mixture 199 supplemented with 1% heat-inactivated calf serum and containing emetine at the indicated final concentrations. Emetine was present in the medium both during exposure of cells to virus and during the subsequent labeling. After 2 h of gentle horizontal agitation at 37°C, the inoculum was replaced with 10 ml of labeling medium consisting of mixture 199 containing 1/10th of the normal concentration of leucine, isoleucine, and valine and supplemented with 1% dialyzed calf serum but supplemented with [^{14}C]leucine, [^{14}C]isoleucine, and [^{14}C]valine (New England Nuclear Corp., Cambridge, Mass.) each at 2.0 μCi/ml of medium. To label the polypeptides the cultures were reincubated at 37°C until 7 h postinfection. At the end of the labeling period the cells were rinsed with ice-cold phosphate-buffered saline to terminate incorporation and then harvested immediately. The labeled cells were scraped from the bottle, pelleted and denatured, and solubilized by heating for 20 min at 80°C in the presence of 2 ml of 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, and 50 mM Tris (pH 7.0). To determine the incorporation of radioactivity into trichloroacetic acid-insoluble material, 100-μl portions were precipitated with cold 5% trichloroacetic acid, collected on filters (Millipore Corp.), dried and counted in PPO–POPOP [2,5-diphenyloxazole-1,4-bis(5-phenyloxazolyl)benzene] toluene-based fluor in a liquid scintillation spectrometer. The percent incorporation shown is expressed relative to the control culture in which the infection proceeded in the absence of any drug.*

mic viral RNA was homologous to 20% of viral DNA and had therefore a higher genetic complexity than the corresponding cycloheximide or emetine RNA. This finding is consistent with the observation that canavanine-treated cells synthesize several β polypeptides not made in cells immediately after withdrawal of cycloheximide. The genetic complexity of RNA extracted from nuclei of canavanine-treated cells also exceeded that of the corresponding cytoplasmic viral RNA.

The experiments with PAA were based on reports showing that the drug is a specific inhibitor of HSV DNA polymerase (34, 39). Recent studies have shown that PAA at a concentration of 300 μg/ml totally blocks the onset of viral DNA synthesis as measured by incorporation of ^{32}P into viral DNA in cells which had been preequilibrated with ^{32}P and then infected and maintained in the presence of ^{32}P for at least 8 h postinfection (Jacob et al., manuscript in preparation). Lower concentrations of PAA reduced the rate of accumulation of viral DNA in proportion to the amount of the drug added to the medium but did not block the onset of its synthesis. The results of the hybridizations (Fig. 2D) show that the RNA accumulating in the nuclei was homologous to 39% of the viral DNA, whereas the cytoplasmic RNA from these same cells was homologous to only 26% of the DNA. These results indicate that the genetic complexity of this RNA is greater than that of the corresponding RNA accumulating in cycloheximide or emetine-treated cells or in canavanine-treated infected cells. Moreover, consistent with the other results, the genetic complexity of the nuclear viral RNA of PAA-treated cells is greater than the genetic complexity of the cytoplasmic viral RNA.

At 8 h postinfection, i.e., at the time when cells synthesize predominantly γ polypeptides, the nuclear RNA is homologous to >50% of viral DNA, whereas cytoplasmic RNA is homologous to approximately 40% of the DNA (Fig. 2E). These results are consistent with previous findings showing that viral transcripts accumulating in nuclei late in infection arise at least in part by symmetric transcription and are homologous to >60% of viral DNA (25), whereas viral RNA accumulating in the cytoplasm is homologous to no more than 43% of viral DNA (30, 48). The data are shown to emphasize that the probe DNA hybridized in a fashion consistent with previous results and that the genetic complexity of the transcripts accumulating in the nuclei and cytoplasm of cells late in infection exceeds that of transcripts present in cells treated with the various metabolic inhibitors. It should be emphasized that in this instance as well the genetic complexity of nuclear RNA exceeds that of cytoplasmic RNA.

Mapping of the viral RNA accumulating in the nuclei and cytoplasm of infected cells under restrictive conditions. In this series of experiments the nuclear and cytoplasmic RNAs described in the preceding section were hybridized to trace quantities of individual, labeled restriction endonuclease fragments of HSV-1 DNA. The results of these hybridizations are shown in Fig. 4 for cycloheximide RNA, in Fig. 5 for emetine RNA, in Fig. 6 for canavanine RNA, and in Fig. 7 for PAA RNA. The fractions of the DNA fragment probe and the percentage of total HSV-1 DNA within each fragment homologous to these nuclear and cytoplasmic RNA preparations were calculated and are shown in Fig. 8 and 9. It is convenient to consider the accumulation of transcripts in the cytoplasm and nucleus separately.

Figure 8 summarizes the results of the hybridizations of the labeled DNA fragment probes with the cytoplasmic RNAs. The length of the bar in each fragment position is proportional to the fraction of the total informational content of each fragment represented in cytoplasmic RNA, assuming that there is no overlap of structural genes. This assumption is in part justifiable because, as noted above, cytoplasmic RNA was shown to originate from asymmetric transcripts inasmuch as the cytoplasmic RNA does not self-anneal (31). The location of the bar within each fragment position is arbitrary; the exceptions are the bars shown within the *Hsu*I fragments M and G. In these two cases, the location has been partially determined from the results of the hybridizations of the RNAs with the *Eco*RI fragments K_1 and K_2 which map between 0.83 and 0.86 map units within the *Hsu*I fragment M and between 0.97 and 1.00 map units within the *Hsu*I fragment G. To permit comparisons with the results of hybridization of these fragments with nuclear RNA, the number on top of each bar indicates the percentage of total DNA contained in each fragment that is homologous to cytoplasmic RNAs. The salient features of the results were as follows.

(i) As could be predicted from the results reported above, the distributions of viral DNA sequences homologous to cycloheximide and emetine RNAs are virtually indistinguishable. The single, notable exception is within the region of the HSV-1 DNA bound by the *Hsu*I fragment G. The genetic complexity of the sequences within this region homologous to emetine RNA is greater than that of the sequences homologous to cycloheximide RNA. Both cycloheximide and

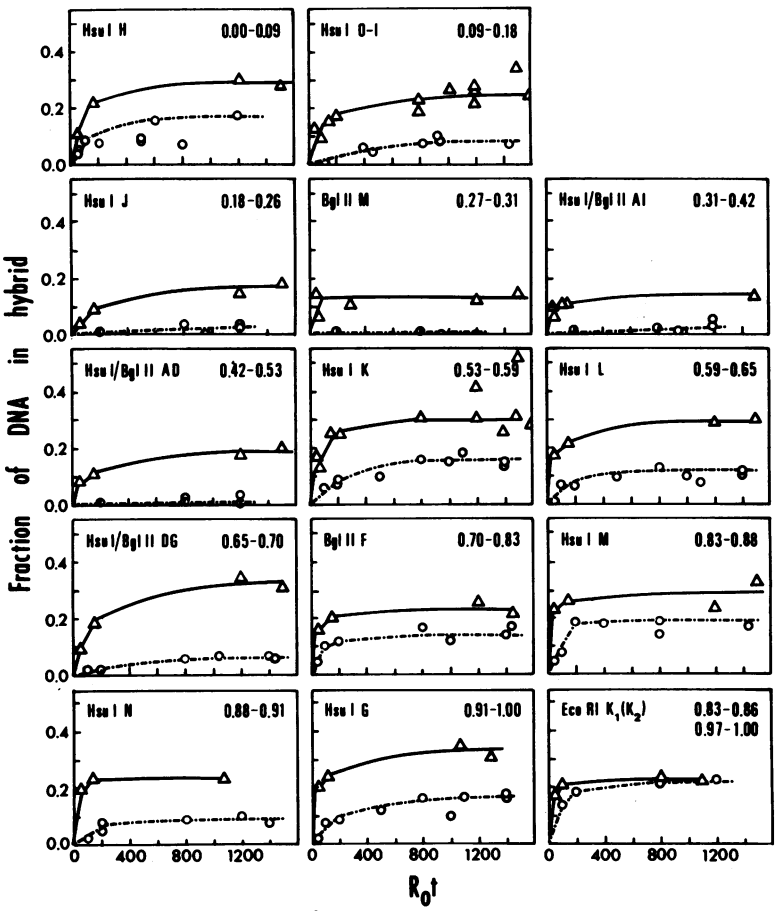

FIG. 4. *Hybridization of individual, in vitro ^{3}H-labeled restriction endonuclease fragments of HSV-1(MP) DNA with cytoplasmic and nuclear RNA extracted from cycloheximide-treated infected cells. The figures show fraction of ^{3}H-labeled DNA probe driven into RNA-DNA hybrid by nuclear RNA (Δ) and by cytoplasmic RNA (○) as a function of RNA concentration and time (R_0t = moles of nucleotides × second/liter). The numbers designated with each fragment correspond to the map units within which the fragment is positioned on the prototype arrangement of HSV-1(MP) DNA.*

emetine RNAs hybridize to viral DNA sequences situated in the inverted repeats of the L and S components and in fragments of the unique sequences of the L and S components adjacent to these repeats. Of particular interest is that region of the L component bound by 0.18 and 0.53 map units: this region shows no appreciable homology to either cytoplasmic RNA.

(ii) The PAA cytoplasmic RNA hybridized to all fragments except *HsuI/BglII* fragment AD which maps between 0.42 and 0.53 map units. The increase in the genetic complexity of PAA RNA relative to cycloheximide or emetine RNA was due to accumulation of RNA homologous to the 0.18- to 0.42-map unit region and to the accumulation of transcripts homologous to additional sequences in all fragments including those containing the inverted repeats.

(iii) Canavanine RNA hybridizes to fragments containing the inverted repeats and to several adjacent fragments to nearly the same extent as PAA RNA. It differs from PAA RNA however in two respects. First, the genetic complexity of the RNA hybridizing to *HsuI/BglII* fragment AI (0.31 to 0.42 map units) is considerably lower than that of the corresponding PAA RNA. Second, canavanine RNA contains sequences homologous to *HsuI/BglII* fragment AD (0.42 to 0.53 map units) that are absent in PAA RNA.

(iv) The 8-h RNA differs from PAA RNA in two respects. First, it hybridizes to all fragments. Second, the genetic complexity of the RNA hybridizing to fragments O-I (0.09 to 0.18), J (0.18 to 0.26), AD (0.42 to 0.53), DG (0.65 to 0.70), and

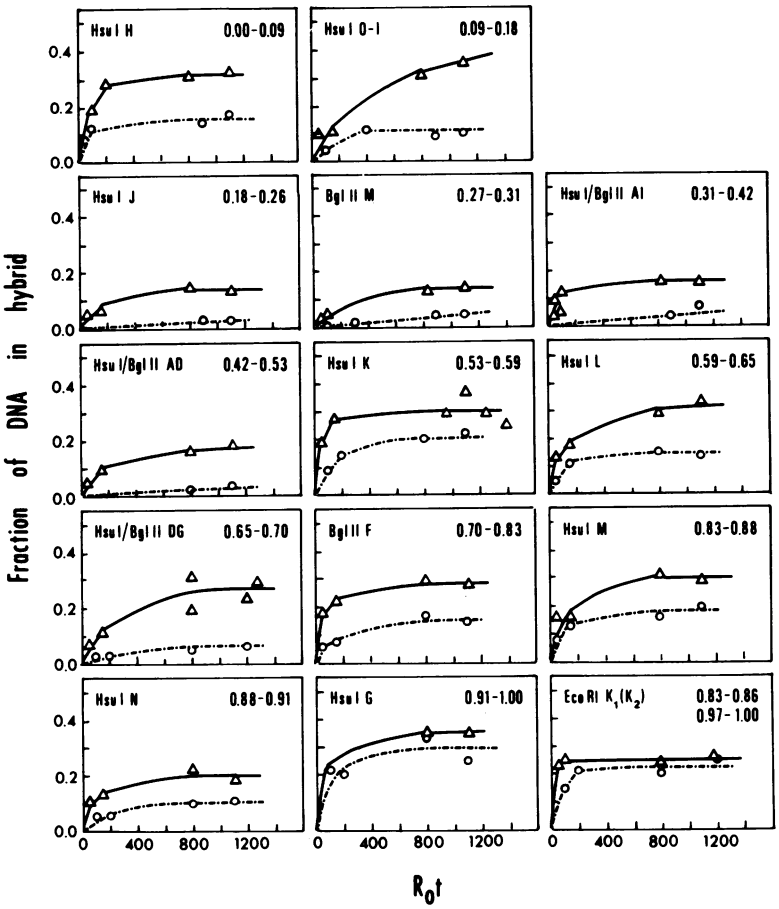

FIG. 5. *Hybridization of individual, in vitro ^{3}H-labeled restriction endonuclease fragments of HSV-1(MP) DNA with cytoplasmic and nuclear RNA extracted from emetine-treated infected cells. The figures show the fraction of ^{3}H-labeled DNA probe driven into RNA-DNA hybrid by nuclear RNA and by cytoplasmic RNA as a function of R_0t. See the legend to Fig. 4 for details.*

F (0.70 to 0.83) is considerably higher than that of the corresponding PAA RNA.

Figure 9 summarizes the genetic complexity of the RNAs accumulating in the nuclei of cells infected in the presence of cycloheximide, emetine, and canavanine. In this figure, both the bar and the number on top of each fragment position represent the percentage of the total DNA contained in each fragment that is homologous to the nuclear RNAs. The salient features of these results were as follows.

(i) The RNAs hybridized to all of the fragments tested, and these included the fragments J, M, AI, and AD to which cycloheximide and emetine cytoplasmic RNAs showed no homology.

(ii) The genetic complexity of the nuclear RNA hybridizing to each fragment was generally higher than that of the corresponding cytoplasmic RNA. The exceptions were the cycloheximide RNA homologous to *Eco*RI fragments K_1 and K_2, emetine RNA hybridizing to the *Hsu*I fragment G and the *Eco*RI fragments K_1 and K_2, and the canavanine RNA homologous to *Hsu*I fragments H, J, K, L, and G, the *Bgl*II fragment M, and the *Eco*RI fragments K_1 and K_2. In all these instances the genetic complexities of nuclear and cytoplasmic RNAs were about the same.

(iii) As noted above, the region of the L component bound by 0.18 and 0.53 map units showed no appreciable homology to either cycloheximide or emetine cytoplasmic RNA. It is of interest to note that the nuclear transcripts homologous to this region accumulate in the nuclei at relatively high abundance as judged by the initial rates of hybridization with labeled DNA fragments. This observation suggests that the

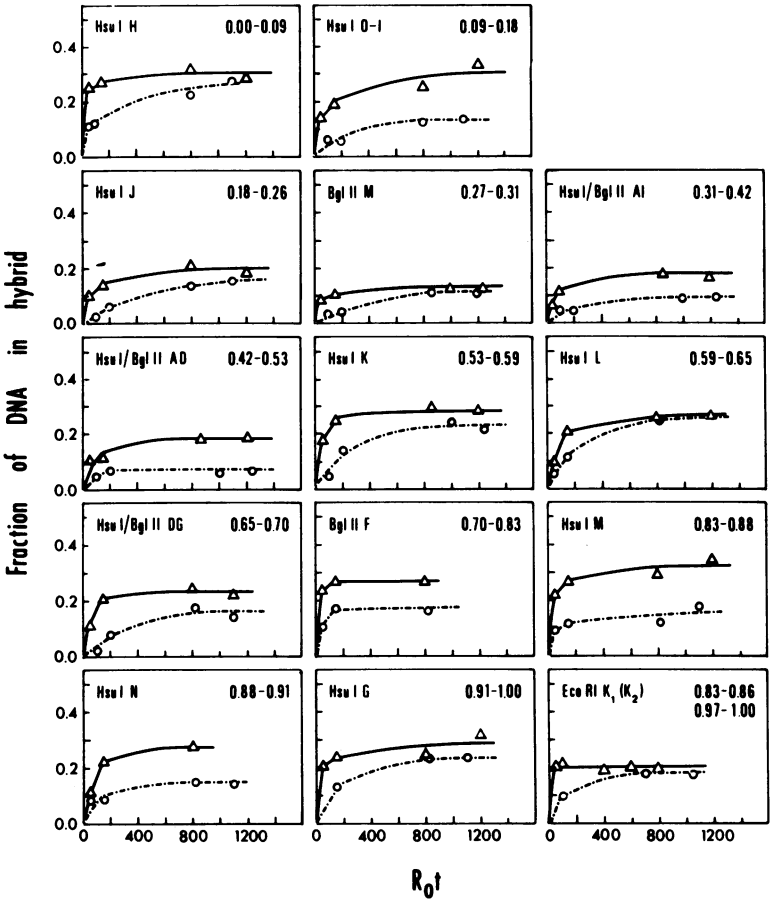

FIG. 6. *Hybridization of individual, in vitro ^{3}H-labeled restriction endonuclease fragments of HSV-1(MP) DNA with cytoplasmic and nuclear RNA extracted from canavanine-treated, infected cells. The figures show the fraction of ^{3}H-labeled DNA probe driven into RNA-DNA hybrid by nuclear RNA and by cytoplasmic RNA as a function of R_0t. See the legend to Fig. 4 for details.*

accumulation of transcripts from these regions in the cytoplasm is regulated.

DISCUSSION

In this paper we present evidence for at least three temporal phases of transcription and for a control regulating the processing and translocation of HSV-1 mRNA from the nucleus into the cytoplasm that is operative throughout the infectious cycle.

Evidence for temporal regulation of transcription. The results summarized in Table 1 show that in the absence of infected cell protein synthesis the genetic complexity of viral RNA sequences accumulating in nuclei is 33% of viral DNA. In cells synthesizing viral proteins but not viral DNA, the genetic complexity of the nuclear transcripts increases to about 39% of total viral DNA. Finally, late in infection in the absence of any restraints, the genetic complexity of the nuclear transcripts exceeds 50% of the total viral DNA. It should be noted that in earlier studies the genetic complexity of self-annealed nuclear transcripts arising from symmetric transcription late in infection exceeded 60% of viral DNA (25). It would appear from the foregoing that there are at least three phases of transcription: phase 1 in which transcription is mediated by host proteins, alone or associated with virion proteins; phase 2 in which transcription is mediated by proteins synthesized after infection but before initiation of viral DNA synthesis; and, lastly, phase 3 in which transcription is coupled to the initiation of viral DNA synthesis.

It is of interest to note that analyses of the kinetics and requirements for viral protein synthesis predicted at least three cycles of transcription yielding mRNA for the synthesis of α, β, and γ polypeptides, respectively (21, 44). Evidence for at least two of these phases of tran-

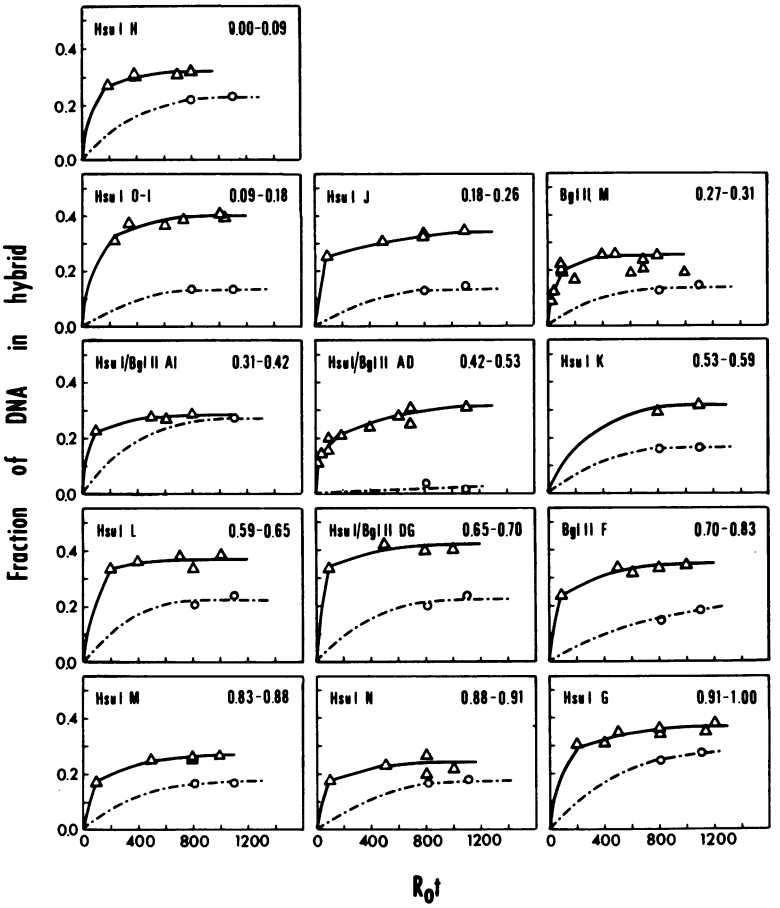

FIG. 7. *Hybridization of individual, in vitro ^{3}H-labeled restriction endonuclease fragments of HSV-1(MP) DNA with cytoplasmic RNA extracted from PAA-treated infected cells and from untreated cells 8 h postinfection. The fraction of ^{3}H-labeled DNA probe driven into RNA-DNA hybrid by 8-h cytoplasmic RNA (△) and by PAA cytoplasmic RNA (○) is shown as a function of Rot. See the legend to Fig. 4 for details.*

scription arose from previous studies on the accumulation of transcripts in cells infected in the presence of cycloheximide (27, 30) and in untreated cells late in infection (11, 12, 27, 30). A possible role for viral DNA synthesis in the regulation of viral transcription was suggested from previous studies on viral protein synthesis. Several reports (3, 21, 23, 42, 59) indicated that under conditions of reduced accumulation of viral progeny DNA, the transitions from α to β and from β to γ protein synthesis were unaffected but that the amount of γ polypeptides made was grossly reduced. Recent studies (Jacob et al., manuscript in preparation) on the effects of PAA on DNA synthesis showed that the concentration of this drug used in previous studies (100 μg of PAA per ml of medium) greatly reduced but did not inhibit the incorporation of deoxynucleotides into viral DNA and that complete inhibition of viral DNA synthesis required much higher concentrations. Therefore, the effects of the lower concentration of PAA on protein synthesis and, by extension, on transcription reflected the reduction of viral progeny DNA and not the total absence of DNA synthesis.

Evidence for regulation of processing and translocation of RNA. The evidence that the accumulation of viral RNA in the cytoplasm is regulated emerged from the results (Fig. 2 and Table 1) showing that in each phase the genetic complexity of the transcripts accumulating in the cytoplasm was lower than that of the nuclear transcripts. These conclusions are reinforced by the results presented in Fig. 8 and 9. Specifically, although transcripts homologous to the region of the DNA between 0.18 and 0.53 map units were present in the nuclei of cells treated with cycloheximide or emetine, RNA sequences homologous to this region were not detected in the

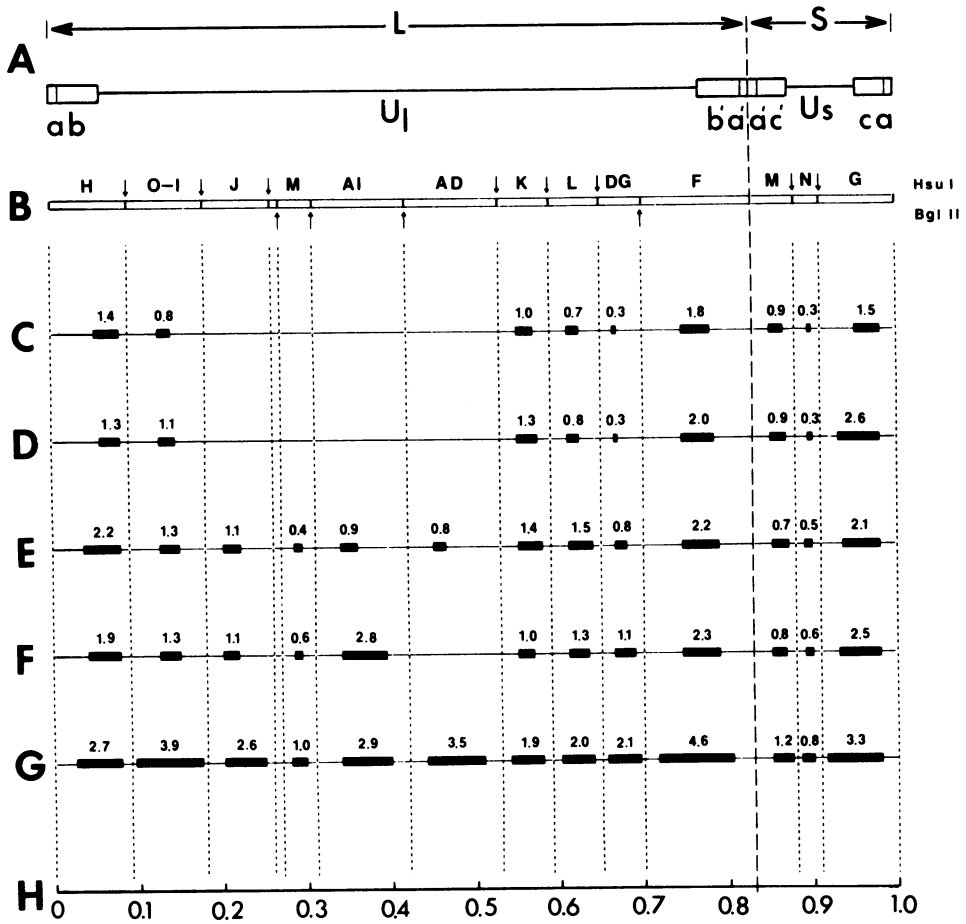

FIG. 8. *Distribution of DNA sequences homologous to cytoplasmic RNAs on the physical map of HSV-1 DNA. (A) Diagrammatic representation of the arrangement of unique and reiterated sequences in HSV-1 DNA. (B) Composite map of the restriction endonuclease fragments of HSV-1(MP) DNA selected for the hybridization tests (thick lines of Fig. 1). The arrows indicate the location of the site at which the restriction endonuclease cleaves the DNA to generate the specific fragment (↓ indicates the HsuI cleavage site and ↑ the BglII cleavage site). (C, D, E, F, and G) Distribution of viral DNA sequences homologous to RNA accumulating in the cytoplasm of cells infected in the presence of cycloheximide (C), emetine (D), canavanine (E), PAA (F) and in the cytoplasm of cells 8 h after infection (G), as determined from the results presented in Fig. 4 through 7 and discussed in the text. (H) Length of HSV-1 DNA in fractional map units.*

cytoplasm. Similarly, in cells infected in the presence of PAA, there was selective retention of the transcripts arising from the region of the DNA between 0.42 and 0.53 map units; i.e., RNA sequences homologous to this region accumulated in nuclei but not in the cytoplasm. Lastly, although only the fragments mapping within 0.00 to 0.18, 0.31 to 0.65, and 0.83 to 1.00 map units were hybridized with nuclear RNA extracted from cells 8 h postinfection (data not shown), in each instance, as could be expected from the data presented in Fig. 2 and Table 1, the genetic complexity of nuclear RNA exceeded that of cytoplasmic RNA.

Regulation of the cytoplasmic accumulation of viral transcripts was first shown in earlier studies in which total HSV DNA was hybridized to RNA extracted from cells late in infection and from cells infected in the presence of cycloheximide (11, 12, 30). These experiments showed that the genetic complexity of cycloheximide nuclear RNA and of late nuclear RNA was greater than that of the corresponding cytoplasmic RNAs, and our results are consistent with these studies. Clements et al. (5, 6), however, cite results that indicate no difference between the genetic complexity of nuclear and cytoplasmic RNAs synthesized in cycloheximide-treated infected cells. In hybridizations of ^{32}P-labeled RNA extracted from cycloheximide-treated cells

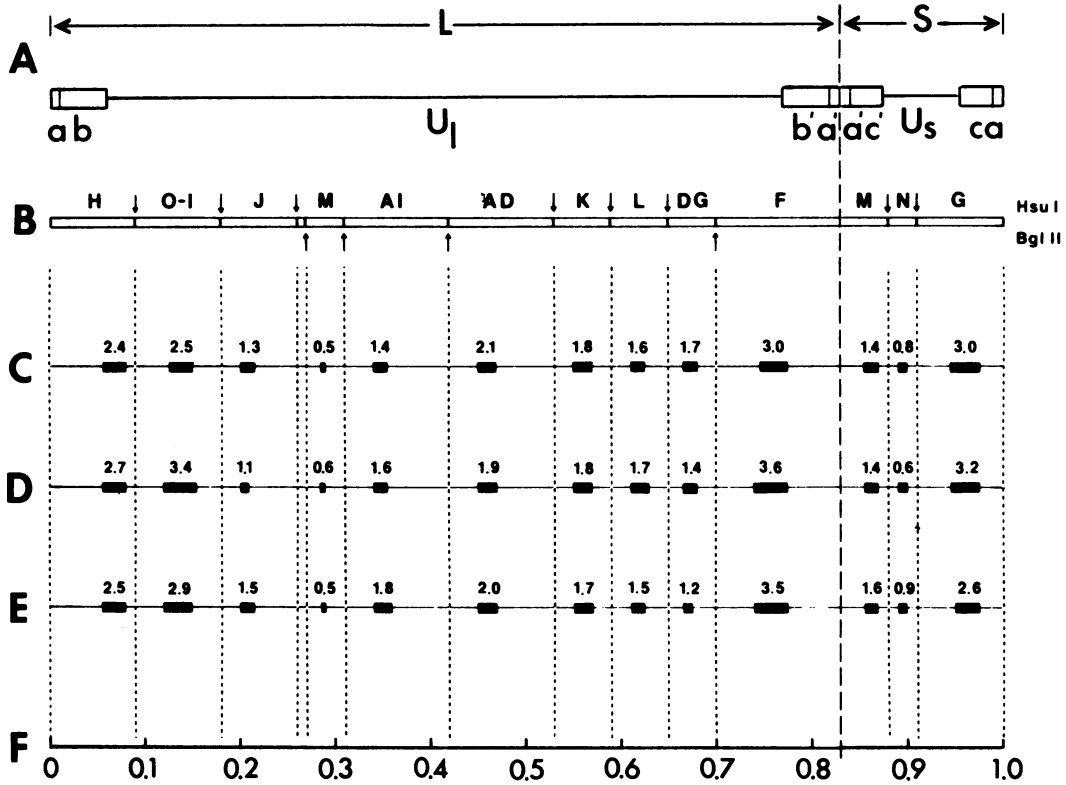

FIG. 9. *Distribution of DNA sequences homologous to nuclear RNAs on the physical map of HSV-1 DNA. A and B were defined in the legend to Fig. 8. The bars in C, D, and E diagrammatically represent the regions of the DNA homologous to RNA accumulating in the nuclei of cells infected in the presence of cycloheximide (C), emetine (D), and canavanine (E), as deduced from the data presented in Fig. 4 through 6 and discussed in detail in the text. F represents the length of HSV-1 DNA in fractional map units.*

TABLE 1. *Summary of the transcriptional program of HSV-1 in HEp-2 cells*

Phase	Genetic complexity[a] of viral sequences	
	Nuclear RNA	Cytoplasmic RNA
1. Absence of infected cell protein synthesis[b]	0.33	0.12
2. Absence of viral DNA synthesis[c]	0.39	0.26
3. Untreated infection 8 or 14 h postinfection	>0.50	0.41

[a] Fraction of viral DNA homologous to viral RNA.

[b] RNA accumulating in the presence of inhibitory concentrations of cycloheximide or emetine.

[c] RNA accumulating in the presence of inhibitory concentrations of PAA.

to restriction endonuclease fragments of viral DNA transferred to nitrocellulose blots (49), Clements et al. (6) observed that both the cytoplasmic and the nuclear RNAs hybridized to the same fragments and that these fragments map within the regions previously reported to be homologous to αRNA (27). However, the actual genetic complexities of the RNAs were not measured in their studies. Several factors could readily explain their results. First, Clements et al. (6) analyzed transcription of HSV-1 DNA in hamster BHK-21 cells, which might show a different transcriptional pattern from that occurring in human HEp-2 cells. Second, the BHK-21 cells were maintained in phosphate-free medium for 16 h before infection and in relatively low phosphate during labeling with ^{32}P. It is conceivable that in low phosphate medium there is reduction in the overall abundance of viral transcripts as well as more rapid turnover of RNA that is not processed into mRNA. Third, the ^{32}P-labeled RNA used in the hybridizations was purified by banding in Cs_2SO_4. In the absence of formaldehyde, at high salt concentrations high-molecular-weight, single-stranded RNA will aggregate and precipitate in Cs_2SO_4 solution and will band in the Cs_2SO_4 gradient at a buoyant density higher than that characteristic for single-

stranded RNA or will sediment toward the bottom of the gradient (32). As noted elsewhere (26, 57), high-molecular-weight RNA, sedimenting at >45S, constitutes a significant fraction of the stable RNA accumulating in the nuclei of HSV-1-infected cells. Therefore, the Cs_2SO_4 sedimentation in the purification of the RNA may have resulted in the selective loss of nuclear RNA sequences.

It should be noted that at this time we cannot differentiate between the failure of processing and translocation of nuclear transcripts and the rapid degradation of cytoplasmic transcripts. The observation that the abundance of nuclear transcripts is invariably higher than that of cytoplasmic transcripts does not support the latter hypothesis, but conclusive evidence on this point is lacking.

Transcriptional program of HSV-1 DNA in infected cells. The results central to the reconstruction of the transcriptional program of the virus are as follows. In phase 1, as during all three phases of transcription, the transcripts accumulating in the nucleus arise from regions in all DNA fragments tested. During the first phase, however, those transcripts that accumulate in the cytoplasm are homologous to noncontiguous regions within map units 0.00 to 0.18 and 0.42 to 1.00, but noticeably not to the region between 0.18 and 0.42 map units. Whereas during phases 2 and 3, the increased genetic complexity of nuclear RNA results from the transcription of more sequences from all regions of the DNA, the increase in genetic complexity of the cytoplasmic transcripts in phase 2 is largely the consequence of the accumulation of transcripts from the region bound by 0.18 and 0.42 map units. In phase 3 the increase in the complexity of cytoplasmic RNA is due to the accumulation of stable transcripts from the region bound by 0.42 and 0.53 map units as well as from additional sequences in other regions of the viral DNA.

Two interesting questions emerge from these results. The first is whether the transcripts accumulating in nuclei during phase 1 arise from transcription of discontinuous stretches of viral DNA. Although the number of discontinuities can be minimized by positioning the homologous sequences in adjacent fragments closer to each other, the data indicate that either there is transcription of long, continuous stretches of the DNA with rapid degradation of selected portions of transcripts or there are multiple transcriptional units including some that yield products that are absent or underrepresented in the cytoplasm. This issue remains unresolved. We should note parenthetically that there is good correspondence between the map positions of some but not all phase 1 cytoplasmic transcripts with identified α polypeptide genes (36, 38). It remains to be determined whether the virus specifies additional α polypeptides but in amounts too small to be determined.

The second question concerns the arrangement of transcriptional units in the DNA. With the exception of the region between 0.42 to 0.53 map units which could not be detected in the cytoplasmic RNA until phase 3, all other regions seem to be transcribed to a slightly greater extent in each successive phase. This observation can be reconciled by the hypothesis that the genes mapping in the region 0.42 to 0.53 map units specify late (γ) polypeptides and are not interrupted by the genes specifying β or α polypeptides present in the same or complementary strand of DNA. This may not be the case, however, for other regions of the DNA, a conclusion consistent with the apparent intermixing of β and γ genes (36, 38). It remains to be determined whether the intermixed β and γ genes each constitute an independent transcriptional unit or are a part of a large, multigene transcriptional unit in which the individual β genes are separated from each other by sequences complementary to γ genes on the complementary strand. However, the studies on the effect of canavanine are not inconsistent with the hypothesis that β and γ polypeptide genes constitute more than one transcriptional unit and that their transcription is independently regulated. Specifically, previous studies have shown that addition of canavanine to the medium several hours after infection did not affect the ongoing synthesis of viral polypeptides. However, in cells infected and maintained in the presence of the analog, only α, a subset of β (e.g., infected cell polypeptides 6 and 8) and one or two γ polypeptides (e.g., infected cell polypeptide 5) were made (22, 40). These results led to the suggestion that more than one of the α polypeptides serves to regulate subsequent transcription, that each α polypeptide enables the transcription of a different subset of β polypeptide genes, and finally that the incorporation of canavanine incapacitates some α polypeptides more effectively than it does others, thus permitting a subset of β and some γ polypeptides to be made. Concordantly, if canavanine were to incapacitate a subset of β polypeptides, only a subset of γ polypeptides would be made. It would be predicted from the foregoing that in the presence of canavanine the genetic complexity of cytoplasmic RNA would be greater than that of phase 1 cytoplasmic RNA and that some transcripts would arise from regions transcribed only late in infection. Consist-

ent with this hypothesis, Fig. 8 shows that canavanine cytoplasmic RNA contained transcripts homologous to fragments J, M, and AI (0.18 to 0.42 map units) that are absent from cycloheximide and emetine cytoplasmic RNAs and RNA sequences homologous to fragment AD (0.42 to 0.53 map units) that were absent from PAA cytoplasmic RNA. Although the hypothesis must be verified by a direct definition of the transcriptional units, the available data are consistent with a multiplicity of transcriptional units in phase 2 and 3 transcription.

ACKNOWLEDGMENTS

These studies were aided by a grant from the American Cancer Society (VC 103N) and Public Health Service grants from the the National Cancer Institute (CA 08494 and CA 19264). P. J. is a Public Health Service predoctoral trainee of the National Institute of Allergy and Infectious Diseases (AI 00238).

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