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Biochemistry

Regulation of herpesvirus macromolecular synthesis: Transcription-initiation sites and domains of α genes*

(herpes simplex virus/5′ mRNA termini/vaccinia guanylyltransferase/DNA strand separation/*in vitro* translation)

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ABSTRACT **Herpes simplex virus 1 (HSV-1) specifies in the infected cell a set of polypeptides (α) whose mRNAs are made in the absence of protein synthesis. The individual mRNAs specifying α polypeptides 0, 4, 22, and 27 were purified by hybridization to strand-separated or total HSV-1 DNA fragments cloned in pBR322 plasmids, translated *in vitro* to verify their specificity, then mapped by hybridization to separated strands and digests of cloned DNA fragments. To map the transcription initiation sites, chemically decapped individual mRNAs were recapped with [α-^{32}P]GTP and vaccinia virus guanylyltransferase and hybridized to digests of the cloned DNA fragments. Each of the labeled 5′ termini hybridized to a specific site, two on one strand, and two on the other. The 5′ ends of the transcripts do not share sequence homology, suggesting that they are transcribed from independent promoters.**

The infected-cell polypeptides (ICPs) specified by herpes simplex virus 1 (HSV-1) form at least three groups—α, β, and γ—whose synthesis is coordinately regulated and sequentially ordered (1). The differential feature of α ICPs is that they are made immediately after infection and that their mRNA accumulates in cells infected and maintained in the absence of protein synthesis. To date, the genes specifying α ICPs 0, 4, 22, and 27 have been located by several techniques (2–6). Thus, α ICPs 4 and 22 map within the inverted repeats and the unique sequences of the S component, respectively, whereas α ICPs 0 and 27 map within the inverted repeats and unique sequences of the L component, respectively (Fig. 1). Central to the understanding of the regulation of α gene expression are the precise locations of transcription-initiation sites and the direction of transcription of these genes. Previous studies (6, 10) determined the direction of transcription of ICP 0, ICP 4, and ICP 22 mRNA by hybridization of DNA transcribed from the mRNAs by reverse transcriptase (RNA-dependent DNA polymerase) to electrophoretically separated, immobilized HSV-1 DNA fragments.

This paper reports the location of the sequences homologous to the 5′ termini of α mRNAs on the presumption that they are at or near the transcription-initiation sites, the domains, and the direction of transcription of the genes specifying ICPs 0, 4, 22, and 27. These features were determined by hybridization of purified mRNAs, labeled randomly or by replacement of the endogenous cap with a labeled cap, to digests of cloned DNA fragments and to separated DNA strands. The latter procedures involved the use of vaccinia guanylyltransferase to label only the authentic 5′ terminus of RNAs. This enzyme has been previously used to label the 5′ termini of highly abundant RNAs (11–13). The results described in this paper show that this technique can also be applied to mRNAs composing a small fraction of the total cellular RNA.

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MATERIALS AND METHODS

Preparation of Viral and Plasmid DNAs. The purification of HSV-1 strain F DNA from infected cells (14) and the properties and purification of cloned *Bam*HI fragments of HSV-1 DNA (9) have been described.

Preparation of Viral RNA. Vero cells were preincubated for 1 hr with cycloheximide at 50 μg/ml, then infected with 40–50 plaque-forming units per cell and maintained for 6 hr at 34°C in the presence of the drug. Cytoplasmic RNA was extracted as described (15).

Chemical Removal of Cap Structures. One milligram of total cytoplasmic RNA was oxidized with 0.5 mM $NaIO_4$ (1 ml) and subjected to β-elimination with 0.3 M aniline (1 ml) as described (16).

Selection of HSV-Specific mRNA by Hybridization to Plasmid DNAs Bound to Diazobenzyloxymethyl (DBM) Paper. Plasmid DNAs (25 μg) were bound to DBM circles as described (17). Dimethyl sulfoxide was omitted when strand-separated DNA was linked to DBM paper. For selection, β-eliminated RNA (2–4 mg/ml) in hybridization buffer [50% (vol/vol) recrystallized formamide/0.8 M NaCl/0.2% $NaDodSO_4$/10 mM 1,4-piperazinediethanesulfonic acid (Pipes), pH 6.5/2 mM EDTA] was incubated for 6 hr at 57°C with DBM circles (about 60 μl per circle) that had been pretreated with tRNA at 0.5 mg/ml in hybridization buffer for 1 hr at 57°C. After hybridization, the circles were rinsed three times with 2× (0.15 M NaCl/0.015 M sodium citrate) (NaCl/Cit) containing 0.2% $NaDodSO_4$ at 25°C, washed twice for 15 min with 0.1× NaCl/Cit containing 0.2% $NaDodSO_4$ at 65°C, and RNA was eluted by three 5-min washes at 70°C with 0.4 ml of 99% recrystallized formamide/10 mM Pipes, pH 6.5. Eluted RNA and 10 μg of calf thymus DNA carrier were precipitated with ethanol. The RNA was resuspended in 25 μl of water; of this solution 1-μl portions were used for *in vitro* translations, 3 μl for random labeling, and 12–25 μl for 5′-end-labeling. Prior to all hybridizations with labeled RNA, the probes were treated with DNase I at 20 μg/ml (10 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$, at 37°C for 30 min) to remove small amounts of HSV DNA leached from DBM circles.

5′-End-Labeling of RNA. The procedure for purification of guanylyltransferase (18, 19) was modified as suggested by J. Hurwitz (personal communication). Solubilized purified vaccinia virions were chromatographed on two DEAE-cellulose columns. To remove the 3′-poly(A) polymerase activity, the flow through was then applied to a phosphocellulose column (19). Column fractions were assayed for (guanine-7-)-methyltransferase activity (20), which copurifies with guanylyl-

Abbreviations: HSV-1, herpes simplex virus 1; ICP, infected-cell polypeptide; DBM, diazobenzyloxymethyl; NaCl/Cit, 0.15 M NaCl/0.015 M sodium citrate; Pipes, 1,4-piperazinediethanesulfonic acid.

* This is paper no. 9 in a series; paper no. 8 is ref. 4.

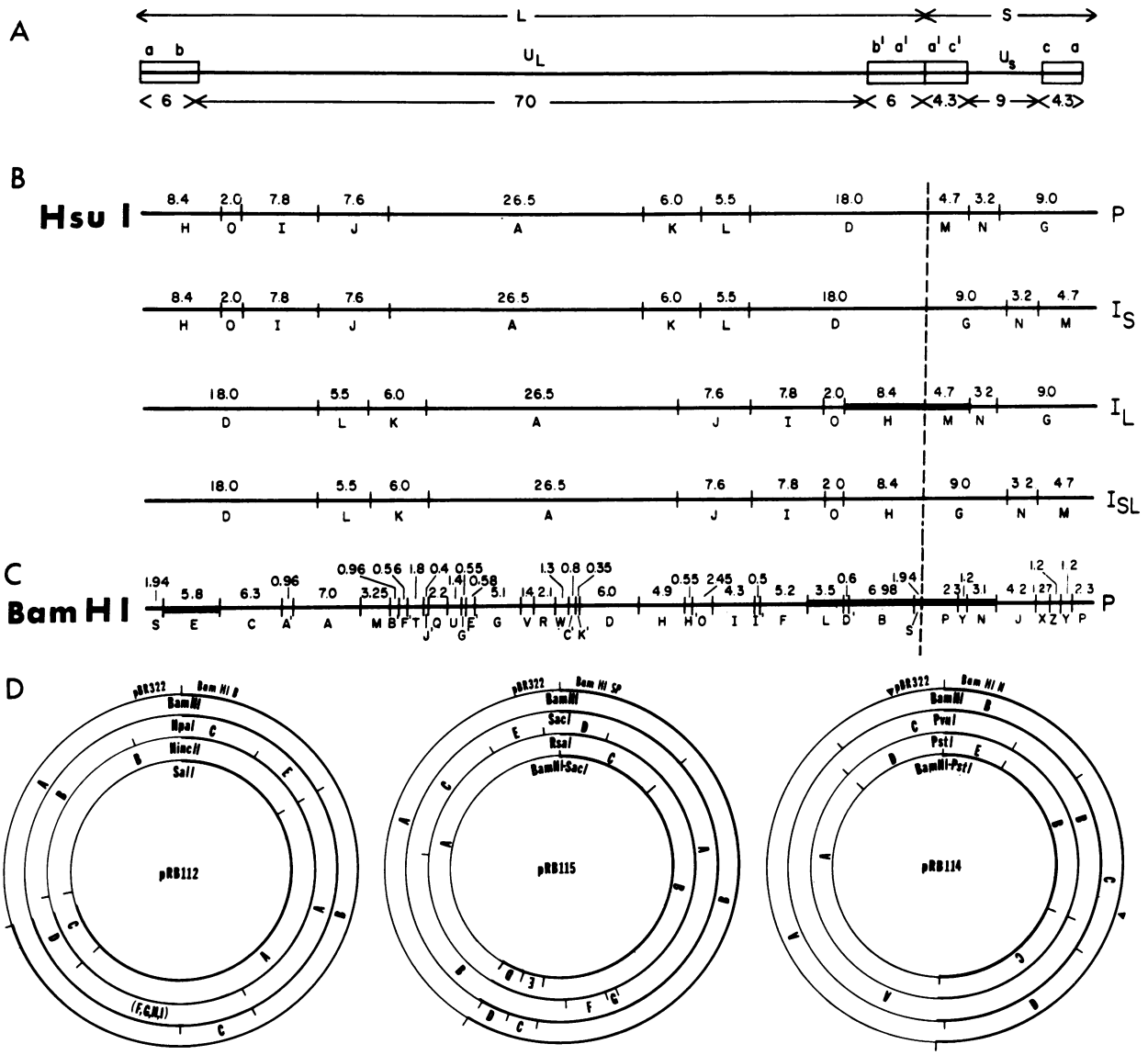

FIG. 1. Maps of HSV-1 DNA. (*A*) Diagram of the HSV-1 genome. The genome consists of two covalently linked components, L and S, which can invert relative to each other (7). Each component consists of unique sequences flanked by inverted repeats. The inverted repeats *ab* and *b'a'* each contain 6% of the DNA, whereas the inverted repeats *a'c'* and *ca* each contain 4.3% of DNA. (*B*) *Hsu* I (*Hin*dIII) map in the prototype (P) and inverted arrangements, illustrating the different fusion fragments generated at the L–S junction by inversion of L (I_L), S (I_S), or L and S (I_{SL}) components. The vertical broken line indicates the L–S component junctions. All numbers are molecular weight × 10^{-6}. (*C*) *Bam*HI map of the P arrangement. The heavy lines identify the cloned fragments used in this study. (*D*) Circular representations of the maps of the plasmids pRB112, pRB114, and pRB115 carrying the HSV-1 *Bam*HI B, N, and SP fragments, respectively. The heavy lines designate HSV-1 DNA and the thin lines, pBR322 DNA. The triangles indicate *Hsu* I cleavage sites. The maps for pRB115, pRB114, and pRB112 plasmids were either reported in part elsewhere or were obtained by double digestions with other enzymes whose cleavage sites were known (8, 9). The sizes of the fragments generated by the various enzymes are given in molecular weights × 10^{-6} as follows. *Hin*cII digest of pRB112: A, 2.5; B, 2.17; C, 1.4; D, 0.88; E, 0.73; F, 0.73; G, 0.45; H, 0.4; I, 0.33. *Pvu* I digest of pRB114: A, 2.25; B, 1.62; C, 1.1; D, 0.95. *Pst* I digest of pRB114: A, 3.3; B, 1.35; C, 1.25. *Sac* I digest of pRB115: A, 3.91; B, 2.74; C, 0.22; D, 0.22. *Rsa* I digest of pRB115: A, 2.7; B, 1.94; C, 1.04; D, 0.53; E, 0.45; F, 0.35; G, 0.09.

transferase. To label 5′ ends, HSV-selected RNA was resuspended in 2 μl of water and preincubated with 1 μl of 18 mM methylmercury hydroxide for 5–10 min at 25°C. The solution was brought to 20 μl containing 50 mM Tris·HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM dithiothreitol, 20 μM [α-^{32}P]GTP (New England Nuclear, 2000 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels), and 1.5 μl (10 units) of guanylyltransferase, and incubated at 37°C for 20 min. ATP was omitted to prevent generation of 5′-polyphosphate termini by contaminating kinase activity (21). The reaction mixture was processed as described (12) except that the labeled RNA was precipitated with 1.5 M NH_4OAc and 2.5 vol of ethanol.

RESULTS

Identification of the Translation Products of the Selected mRNA. mRNA extracted from the cytoplasm of cells infected and maintained in the presence of cycloheximide was hybridized to individual immobilized HSV-1 DNA fragments, eluted, and translated. The products of translation were subjected to electrophoresis in $NaDodSO_4$/polyacrylamide gels along with appropriate controls. As shown in Fig. 2, the RNA hybridizing to *Bam*HI B fragment (lane D) translated ICP 0 and ICP 27. The RNA hybridizing to *Bam*HI SP fragment translated primarily ICP 0 (lane G); a longer exposure showed that ICP 4 was also translated (lane M). RNA hybridizing to the *Bam*HI Y

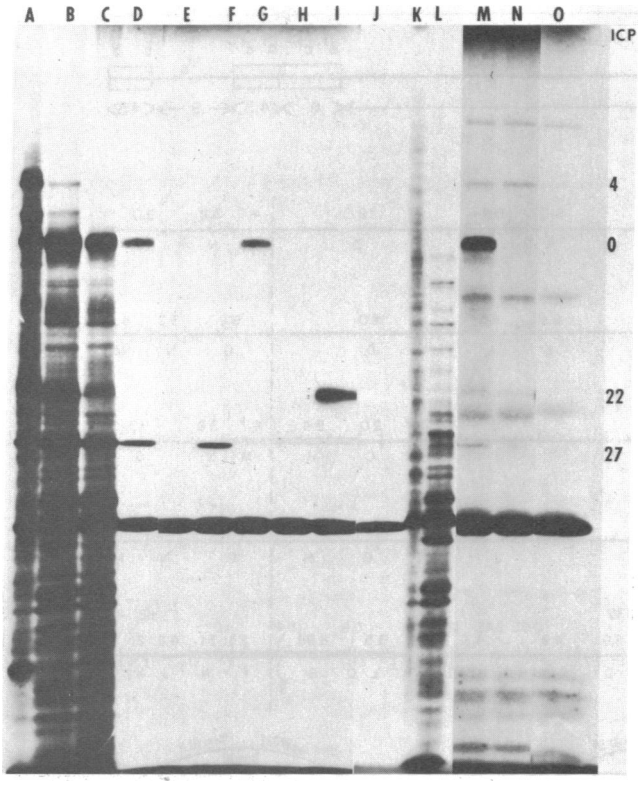

FIG. 2. Fluorogram of ^{35}S-labeled products of *in vitro* translation of mRNAs. mRNAs were translated in the reticulocyte lysate system (New England Nuclear) modified to contain 0.04 mM spermine and 0.4 mM $Mg(OAc)_2$. The products were separated on 9.25% $NaDodSO_4$/polyacrylamide gels (2). The numbers to the right identify the α polypeptides, ICPs 4, 0, 22, and 27 with molecular weights of 163,000, 128,000, 72,000, and 60,000. Lane A, polypeptides from cells infected at 10 plaque-forming units per cell, maintained in the presence of cycloheximide (50 μg/ml), and labeled with [^{35}S]methionine (100 μCi/ml) at 5 hr after infection during a 30-min chase in which cycloheximide was replaced with actinomycin D (10 μg/ml). Lanes B and C, translation products of total cytoplasmic RNA from HSV-1-infected cells before and after chemical removal of caps, respectively. Lanes D through J, translation products of RNAs selected by hybridization to the following cloned HSV-1 fragments. D, *Bam*HI B; E, slow strand of *Bam*HI B; F, fast strand of *Bam*HI B; G and M, *Bam*HI SP; H and N, *Bam*HI Y; I, *Bam*HI N; J and O, products of *in vitro* translation without added mRNA. Lane K, mock-infected cell polypeptides labeled with [^{35}S]methionine. Lane L, translation products of total cytoplasmic RNA extracted from mock-infected cells. Lanes M through O are longer exposures of lanes G, H, and J, respectively. Strand-separated DNA was prepared by phenol extraction from low-melting agarose gels of the electrophoretically separated strands of heat-denatured (22) plasmids cleaved with *Bam*HI.

fragment translated ICP 4, which is visible on longer exposures of the gel (lanes H and N). Finally, RNA hybridizing to the *Bam*HI N fragment yielded primarily ICP 22 (lane I). The autoradiograms revealed several other polypeptide bands present also in the autoradiogram of the electrophoretically separated translation products of RNA endogenous to the reticulocyte system.

The *Bam*HI fragments Y and N selected single RNA species corresponding to ICP 4 and ICP 22, respectively, and henceforth the RNA selected in this fashion is designated as ICP 4 and ICP 22 mRNA. To purify mRNAs specifying ICP 0 and ICP 27, we separated the strands of the *Bam*HI B fragment and selected RNA homologous to the slow (S) and fast (F) migrating DNA strands. As shown in Fig. 2, the RNA selected by the S strand translated ICP 0 (lane E), whereas that selected by the F strand translated predominantly ICP 27 (lane F). Henceforth, the RNAs selected by hybridization to the S and F strands of *Bam*HI B are designated as ICP 0 and ICP 27 mRNAs, respectively. In other experiments, the cytoplasmic RNA obtained as described above was transferred to DBM paper after electrophoretic separation on 1.5% agarose/urea gels (23) and hybridized with labeled cloned DNA probes (data not shown). These studies failed to detect RNA species homologous to *Bam*HI B, SP, Y, and N other than those identified as ICP 0, 4, 22, and 27 mRNAs. We are confident, therefore, that no additional untranslated viral RNAs were copurified by the selection procedures employed in this study.

Direction of Transcription and Domains of the DNA Sequences Hybridized to ICP 0, 27, 4, and 22 mRNAs. The purified mRNAs were fragmented with alkali, labeled by polynucleotide kinase with [γ-^{32}P]ATP, and hybridized to

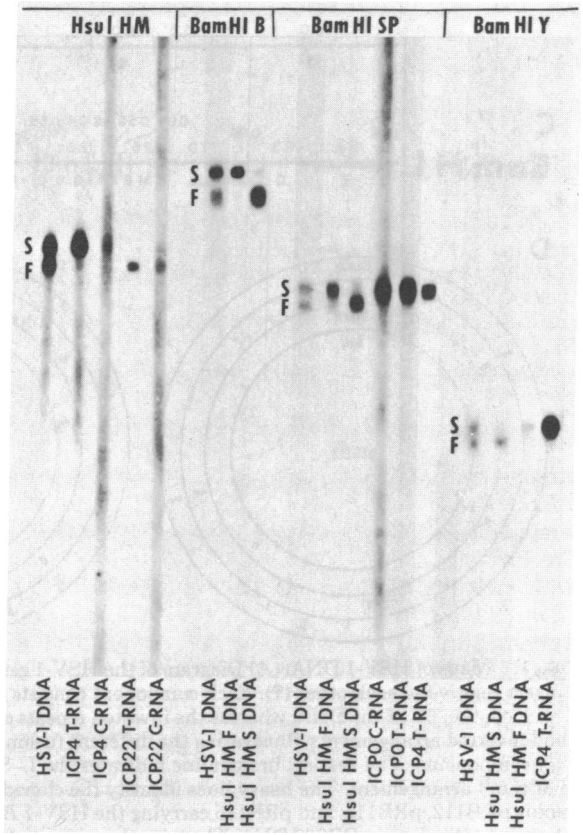

FIG. 3. Hybridization of labeled DNA and RNA to electrophoretically separated DNA strands. The cloned plasmids carrying *Hsu* I HM, *Bam*HI SP, and *Bam*HI Y DNA fragments were each cleaved with *Hsu* I, and the plasmid carrying *Bam*HI B was cleaved with *Bam*HI. The cleaved DNAs were heat denatured (22), and the strands were then electrophoretically separated on 0.7% agarose gels and immobilized on nitrocellulose strips as described (24), except that alkali treatment of gels was omitted. The labeled DNA probes used in the hybridizations are indicated on the bottom of the figure. In this and subsequent experiments, all DNA probes were fragmented and labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase by the exchange reaction (9, 25). Randomly labeled RNA (T-RNA) was prepared by fragmenting HSV-selected RNA in alkali for 30 min at 0°C as described (26) and labeling with [γ-^{32}P]ATP and T4 polynucleotide kinase by using the forward reaction conditions (27). 5′-End-labeled RNA (C-RNA) was fragmented with alkali for 30 min as described (26). In this and other experiments, all hybridizations were done at 75°C in 6× NaCl/Cit containing 0.2% $NaDodSO_4$. Denhardt's solution (0.025% bovine serum albumin/0.025% polyvinylpyrrolidone/0.025% Ficoll), was included with all DNA probes. The strips were washed in hybridization buffer at 75°C three times for 15 min each.

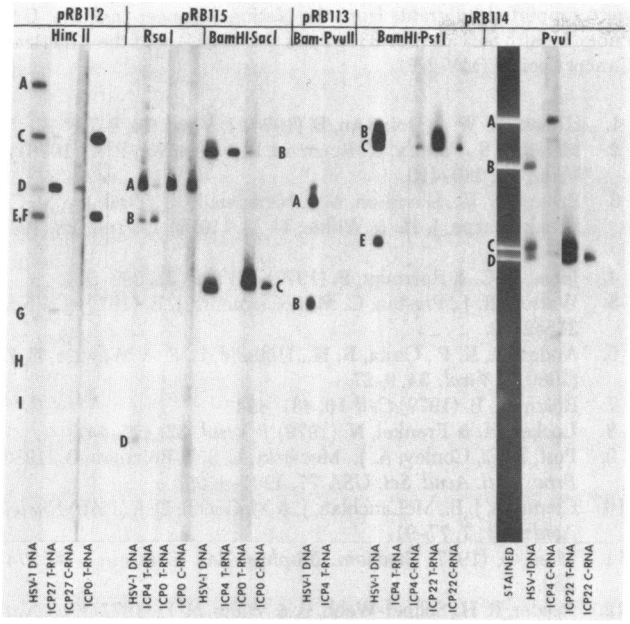

FIG. 4. Hybridization of 5′-end-labeled and randomly labeled RNAs to immobilized digests of plasmid DNAs. The DNAs were cleaved with enzymes as indicated at the top of the figure. The digests were separated on 1% agarose gels, immobilized on nitrocellulose, and hybridized to labeled probes as described in the legend to Fig. 3. The DNA fragments were designated by letter according to the maps of plasmids shown in Fig. 1. The labeled probes used in the hybridizations are listed on the bottom. The stained gel for the *Pvu* I digest of pRB114 is shown to illustrate the position of the A fragment, which does not hybridize efficiently with total HSV probe because it contains only a short stretch of HSV-1 DNA joined to pBR322 sequences (Fig. 1).

electrophoretically separated strands of *Hsu* I HM, *Bam*HI SP, and *Bam*HI Y fragments (Fig. 3), and to electrophoretically separated digests of cloned *Bam*HI DNA fragments (Fig. 4).

The *Hsu* I HM fragment contains 3.8×10^6 molecular weight DNA homologous to the L component reiterated sequences in the *Bam*HI B fragment, all of the *Bam*HI SP and Y fragments, and approximately 0.6×10^6 molecular weight DNA from the left end of the *Bam*HI N fragment (Fig. 1). Upon electrophoresis in agarose gels the denatured *Hsu* I HM DNA segregated into F and S bands. Purified labeled S band of *Hsu* I HM DNA hybridized to the F bands of *Bam*HI B, *Bam*HI SP, and *Bam*HI Y DNAs, and therefore, the S strand of *Hsu* I HM DNA contains the sequences in the S strand of *Bam*HI B, SP, and Y DNAs (Fig. 3).

The results of the hybridizations were as follows:

(*i*) Randomly labeled ICP 4 RNA hybridized to the S bands of *Hsu* I HM, *Bam*HI SP, and *Bam*HI Y DNAs (Fig. 3). The RNA also hybridized to *Rsa* I fragments A and B, to the B fragment of the *Bam*HI/*Sac* I digest of the plasmid pRB115 (Fig. 1) carrying the *Bam*HI SP DNA, and to both *Pvu* II fragments of *Bam*HI Y DNA (Fig. 4).

(*ii*) Randomly labeled ICP 0 RNA hybridized to the S band of *Bam*HI SP DNA, and therefore this RNA is transcribed from the same strand as ICP 4 mRNA (Fig. 3). The labeled ICP 0 RNA hybridized to the *Hin*cII C and E fragments of the plasmid pRB112 carrying *Bam*HI B DNA. This RNA also hybridized to the *Rsa* I fragment A and strongly to the *Sac* I/*Bam*HI fragment C and only weakly to the *Sac* I/*Bam*HI fragment B of the plasmid pRB115 carrying the *Bam*HI SP DNA (Fig. 4).

(*iii*) Randomly labeled ICP 22 RNA hybridized to the F band of *Hsu* I HM DNA (Fig. 3). Therefore, the coding sequences for ICP 22 and for ICPs 0 and 4 are on the opposite strands of the DNA. The labeled ICP 22 RNA hybridized to the *Bam*HI/*Pst* I B and C fragments and to the *Pvu* I C and D fragments of the plasmid pRB114 carrying the *Bam*HI N fragment (Fig. 4).

(*iv*) The ICP 27 RNA must be transcribed from the strand complementary to that encoding ICP 0, inasmuch as it is purified by hybridization to the F band of *Bam*HI B (Fig. 2, lane F). The randomly labeled ICP 27 RNA hybridized predominantly to the *Hin*cII D fragment and less strongly to fragment G of the plasmid pRB112 carrying the *Bam*HI B DNA. The weak hybridizations to *Hin*cII C and E are probably due to trace amounts of contaminating ICP 0 mRNA, which is apparent from the presence of small amounts of ICP 0 among the translation products of this mRNA (Fig. 2, lane F). ICP 27 RNA did not hybridize to cloned *Bam*HI fragments D′ and L (data not shown), which map to the left of the B fragment (Fig. 1*C*).

(*v*) The results of hybridizations of randomly labeled ICP 4, 0, 22, and 27 mRNAs to immobilized electrophoretically separated *Bam*HI digests of total HSV-1 DNA (data not shown) were consistent with the results obtained with cloned DNA fragments as described above.

Identification of the Sites of Initiation of Transcription of ICP 0, 27, 4, and 22 RNAs. To identify the sites of initiation of transcription, the cap structures were removed from total cytoplasmic RNA by periodate oxidation and β elimination, and the 5′-triphosphate-terminated RNA was purified by hybridization to the individual DNA fragments and then recapped with [α-^{32}P]GTP and guanylyltransferase purified from vaccinia virions. The labeled RNAs were then fragmented with alkali and hybridized to electrophoretically separated digests of individual fragments.

The procedure for the chemical removal of the cap did not adversely affect the integrity of the mRNA, inasmuch as the decapped mRNAs were readily translated *in vitro* (Fig. 2, lanes C–I). Analysis of the recapped RNAs showed that the label was present only in GpppN structures. All 5′-end-labeled RNAs were digested with appropriate enzymes and subjected to paper electrophoresis (28). The results show that tobacco acid pyrophosphatase (Bethesda Research Laboratories, Rockville, MD) released all of the label as 5′-GMP; nuclease P1 (Boehringer Mannheim) followed by bacterial alkaline phosphatase (Worthington) released all of the label as a spot comigrating with GpppG (P-L Biochemicals).

Consistent with the results reported above, the 5′-labeled ICP 4 and ICP 0 RNAs hybridized to slow strands of *Hsu* I HM and *Bam*HI SP DNAs, whereas the 5′-labeled ICP 22 RNA hybridized to the fast strands of *Hsu* I HM DNA (Fig. 3). The hybridization of 5′-end-labeled RNAs to the electrophoretically separated fragments of plasmids carrying the *Bam*HI B, SP, Y, and N showed the following: (*i*) The 5′ end of ICP 4 RNA hybridized to the *Pvu* I A fragment and the *Bam*HI/*Pst* I C fragment of plasmid pRB114 carrying the *Bam*HI N DNA (Fig. 4). The RNA did not hybridize to DNA sequences contained in the *Bam*HI Y and SP fragments (data not shown). (*ii*) The 5′ end of ICP 0 RNA hybridized to the *Rsa* I A fragment and the *Bam*HI/*Sac* I C fragment of the plasmid pRB115 carrying *Bam*HI SP DNA (Fig. 4). The RNA did not hybridize to DNA sequences contained in the *Bam*HI N fragment (data not shown). (*iii*) The 5′ end of ICP 27 RNA hybridized to the *Hin*cII D fragment of the plasmid pRB112 carrying the *Bam*HI B DNA (Fig. 4). The RNA did not hybridize to DNA sequences contained in the *Bam*HI D′ and L fragments (data not shown). (*iv*) The 5′ end of ICP 22 RNA hybridized to the *Bam*HI/*Pst* I C fragment and the *Pvu* I D fragment of the plasmid pRB114

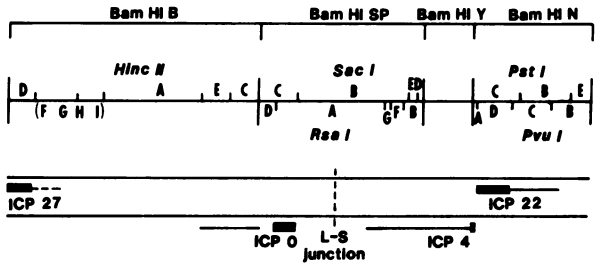

FIG. 5. Summary of the domains, direction of transcription, and sites of initiation of HSV-1 α transcripts. The restriction endonuclease fragments were designated according to the maps shown in Fig. 1. The RNAs were designated according to the ICPs they specify. The thick lines designate the maximum boundaries within which the initiation sites for transcription must be located, and the thin lines indicate the remaining DNA sequences homologous to the RNAs.

carrying the *Bam*HI N DNA (Fig. 4). The RNA also hybridized solely to the *Bam*HI N fragment on hybridization to immobilized electrophoretically separated *Bam*HI fragments of total HSV-1 DNA (data not shown).

DISCUSSION

Fig. 5 summarizes the map positions of the DNA sequences homologous to ICP 0, 27, 4, and 22 mRNAs. The directions of transcription unambiguously established from the localization of the sequence homologous to the 5′-labeled and randomly labeled mRNAs and by hybridizations of the mRNAs to separated strands are consistent with and extend previous reports (6, 10). Thus, transcription of ICP 4 and 0 mRNAs proceeds from right to left on one strand, whereas the transcription of ICP 27 and 22 mRNAs proceeds from left to right on the complementary strand of the genome as represented in Figs. 1 and 5. The significant aspect of the results concerns the identification of the sequences homologous to the 5′ termini of the mRNA, and two points should be noted. First, in the case of ICP 4 mRNA, it has been possible to localize the sequence homologous to the 5′ end of the RNA to a very small region of the reiterated sequences of the S component. Second, the failure to detect hybridization of the 5′ terminus of ICP 0 with the site of initiation of ICP 4 transcription and vice versa suggests that the leader sequences of these mRNAs have little or no homology. Similar arguments can be made for ICP 22 and 27 mRNAs. Therefore, these RNAs may be transcribed from independent promoters.

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