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Characterization of the Major mRNAs Transcribed from the Genes for Glycoprotein B and DNA-Binding Protein ICP8 of Herpes Simplex Virus Type 1

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The structural genes encoding the herpes simplex virus type 1 glycoprotein B and the major DNA-binding protein ICP8 have been mapped previously within the *Eco*RI-F restriction fragment (map coordinates 0.314 to 0.420) of the viral genome. In this study the mRNAs transcribed from these DNA sequences were identified by hybridization selection of ^{32}P-labeled RNA and by Northern blot analysis of polyadenylated cytoplasmic RNA. A 3.4-kilobase RNA was the major mRNA homologous to the DNA sequences between coordinates 0.343 and 0.386 in which mutations in the glycoprotein B gene have been mapped. A 4.5-kilobase RNA was the major mRNA homologous to the viral DNA sequences between coordinates 0.361 and 0.417 in which mutations in the ICP8 gene have been mapped. Hybridization-selected mRNAs were translated in vitro to determine the primary translation products encoded in each region. The glycoprotein B- and ICP8-specific polypeptides were identified by immunoprecipitation with specific antisera. The translation products encoded by the glycoprotein B gene were 103,000 and 99,000 in molecular weight. The translation products encoded by the ICP8 gene were 125,000 and 122,000 in molecular weight.

The herpes simplex virus (HSV) genome is a linear double-stranded DNA, ca. 10^8 in molecular weight (21). Infection by HSV involves a complex pattern of viral gene expression and the synthesis of a large number of polypeptides whose functions are largely unknown. The functions of viral genes are being determined through the study of mutant gene products encoded by conditional lethal temperature-sensitive (*ts*) mutants. The physical mapping on the viral genome of the genes encoding these polypeptides is being accomplished by several approaches.

Restriction and polypeptide analysis of HSV type 1 (HSV-1) and HSV type 2 (HSV-2) intertypic recombinants has localized many structural genes on the HSV genome. Another genetic approach to physical mapping of the viral genome has been marker rescue of *ts* mutants with defined restriction fragments. RNA analysis by a variety of techniques has allowed the precise localization of specific viral mRNAs on the HSV genome. In combination with these studies, in vitro translation of these characterized mRNAs has identified the primary translation products which they encode. Characterization of the in vitro-synthesized polypeptides has been possible in many instances by immunological identification. Together, these three approaches afford an understanding of the functional organization of the HSV genome.

In this study, the mRNAs and in vitro translation products encoded by the glycoprotein B (gB) and ICP8 genes were characterized. The HSV-1 gB is essential for penetration of virions into infected cells, presumably by promoting fusion of the viral membrane with the membrane of the host cell (39). The mature glycosylated gB is ca. 120,000 in molecular weight and has been shown to be derived by additional glycosylation of a precursor polypeptide, pgB (gA), which is ca. 110,000 in molecular weight (13). The function of gB was determined from studies of *ts* mutants that do not synthesize mature functional gB at the nonpermissive temperature. The mutation in one such *ts* mutant, *tsB5* of strain HFEM, has been mapped between coordinates 0.360 and 0.368 on the HSV genome (9, 18). Recently, Holland et al. (18) have isolated a mutant, KOS1.1 *marB*1.1, which was selected for resistance to neutralization by a monoclonal antibody and expresses an antigenic variant of gB. The mutation in *marB*1.1 maps between coordinates 0.350 and 0.361.

The HSV-1 major DNA-binding protein ICP8 is an early (beta) protein ca. 128,000 in molecular weight (4, 33). This protein is required for viral DNA synthesis and maximal late gene expression (8) and is involved in the regulation of expression of viral polypeptides (14). Mutations affecting ICP8 have been mapped between coordinates 0.385 and 0.413 by marker rescue of several *ts* mutants (8, 26, 47). Conley et al. (8) have previously shown that a 128,000 (128K) polypeptide is translated from mRNA selected by hybridization with viral DNA sequences from map coordinates 0.386 to 0.397. Holland et al. (16) have previously shown early mRNA species of 4.3, 3.3, and 1.5 kilobases (kb) from coordinates 0.31 to 0.45 of the HSV-1 genome.

Based on genetic analyses, both the gene encoding gB and the gene encoding ICP8 are contained within the *Eco*RI-F restriction fragment (map coordinates 0.314 to 0.420) of the HSV-1 genome. Hybridization selection was used to identify the mRNAs homologous to these genes. In vitro translation of hybridization-selected RNAs allowed the identification of the primary translation products encoded by the gB and ICP8 genes.

MATERIALS AND METHODS

Cells and viruses. Vero cell monolayer cultures were infected with HSV-1 strain KOS (originally obtained from P. Schaffer) at a multiplicity of 10 PFU per cell. Infected cells were labeled with [^{35}S]methionine as described previously (23).

Recombinant DNA and isolation of restriction fragments. All HSV-1 restriction fragments are referred to by their accepted letter designations where appropriate and their

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TABLE 1. Virus-specific polypeptides synthesized in vitro from hybridization-selected mRNA homologous to DNA sequences between 0.314 and 0.447 on the HSV-1 genome

Plasmid	Approx map coordinates[a]	Molecular weight ($\times 10^{-3}$) of polypeptides[b]
pSG18	0.314–0.420	Major: 125, 105, 103, 54, 47, 43, 35; minor: 113, 100, 91, 62, 60, 57, 41, 40, 37
pSG18:SalE	0.343–0.361	Major: 125, 105, 103, 75, 66, 43, 35; minor: 91, 85, 71, 62, 60, 57, 41, 40, 35
pSG18:SalD	0.361–0.386	Major: 125, 105, 103, 75, 65, 54, 47, 43, 35; minor: 113, 91, 85, 68, 62, 60, 57
pSG18:SalA	0.386–0.417	Major: 125, 105, 75, 65, 54, 47, 43, 35; minor: 113, 108, 100, 91, 88, 74, 68, 62, 60, 57
pSG18:SalB[c]	0.417–0.420	Major: 133, 125, 91, 75; minor: 145,
pSG17[c]	0.420–0.447	136, 115, 105, 100, 96, 68, 65, 62, 60, 58, 54, 51, 47, 43, 41, 35

[a] Map location on the prototype arrangement of the HSV-1 (strain KOS) genome (150,000 base pairs) based on the left-hand side of the *Eco*RI-F fragment as 0.314. Each 1,000 base pairs is 0.00673 map units.

[b] Molecular weights of immunoprecipitated polypeptides were determined as described in the legend to Fig. 3.

[c] Plasmids pSG18:SalB and pSG17 gave the same polypeptides.

coordinates in map units on the prototype arrangement of the HSV-1 genome (e.g., *Eco*RI-F [map coordinates 0.314 to 0.420]). All restriction fragments used are listed in Table 1.

The plasmids containing the *Eco*RI-F (pSG18) and *Eco*RI-M (pSG17) fragments of strain KOS1.1 DNA (15) were kindly provided by M. Levine, University of Michigan, Ann Arbor. Subclones of the *Eco*RI-F fragment were generated by cleaving DNA of pSG18 with *Eco*RI and *Sal*I and ligating the DNA to *Sal*I-cleaved pBR325 (D. Zantos and D. M. Knipe, unpublished data).

Plasmid DNAs were isolated from *Escherichia coli* containing each of the different plasmids as described by Clewell and Helinski (7). Briefly, the bacteria were grown in minimal medium (M9), and the plasmids were amplified by the addition of chloramphenicol (for the plasmids containing *Eco*RI restriction fragment inserts) or spectinomycin (for the plasmids containing *Sal*I restriction fragment inserts), provided by The Upjohn Co., Kalamazoo, Mich. Cleared lysates were prepared by lysozyme-Triton X-100 extraction. Plasmid DNA was purified by two cycles of cesium chloride isopycnic centrifugation.

Restriction fragments containing viral-specific DNA were used as probes for the Northern blot analysis of viral RNA. Plasmid DNAs were digested with the appropriate restriction enzyme to release the viral DNA insert from the plasmid vector. Restriction enzyme digestions were carried out as recommended by the supplier (New England Biolabs, Beverly, Mass.). The viral DNA fragments were electroeluted (29) from 0.75% agarose gels and purified by DEAE-cellulose (DE52; Whatman, Inc., Clifton, N.J.) chromatography.

Isolation of RNA. Vero cells were infected at 37°C in phosphate-buffered saline. At 1 h postinfection (pi) the inoculum was removed, and the infected cells were overlaid with medium 199–1% calf serum. For the preparation of ^{32}P-labeled RNA, Vero cells were preincubated for 4 h before infection in phosphate-free medium 199–5% fetal calf serum dialyzed against 0.15 M sodium chloride. After a 60-min adsorption in the same medium, the inoculum was removed, and the infected cells were labeled from 1 to 4 h pi with carrier-free [^{32}P]P_i (New England Nuclear Corp., Boston, Mass.) in phosphate-free medium 199–5% dialyzed fetal calf serum. For a roller bottle (ca. 2×10^8 cells), 10 mCi of carrier-free [^{32}P]P_i in 50 ml of phosphate-free medium 199–5% dialyzed fetal calf serum was used to label infected cells. For the preparation of RNA transcribed in the absence of viral DNA synthesis, the cells were infected and maintained in 400 μg of sodium phosphonoacetate (PAA) per ml provided by Abbott Laboratories, North Chicago, Ill. For the preparation of RNA transcribed in the absence of protein synthesis, the cells were preincubated for 45 min in 50 μg of cycloheximide per ml, infected, and maintained in 50 μg of cycloheximide per ml.

RNA was isolated from all infections at 4 h after the 60-min adsorption period. For RNA analysis by Northern blot hybridization, cytoplasmic RNA was isolated. The infected cells were washed twice in cold phosphate-buffered saline and removed by scraping with a rubber policeman. The cells were pelleted by low-speed centrifugation, washed twice with cold phosphate-buffered saline and resuspended in 0.15 M NaCl–20 mM Tris-hydrochloride (pH 7.6) containing 10 mM vanadium ribonucleoside complex (Bethesda Research Laboratories, Bethesda, Md.) (5) at a concentration of 2×10^7 cells per ml. The cells were disrupted with Nonidet P-40 (NP-40) (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 1% (wt/vol). The nuclei were removed by centrifugation at 3,000 rpm for 10 min at 0°C. The resulting cytoplasmic supernatant was adjusted to a final concentration of 2% sodium dodecyl sulfate (SDS) and extracted twice with an equal volume of phenol at 55°C. The RNA was precipitated by addition of NaCl and ethanol. For hybridization selection, RNA was isolated by 6 M guanidine–HCl extraction (41). Polyadenylated (poly[A]$^+$) RNA was isolated by chromatography on oligodeoxythymidylic acid-cellulose (T-3; Collaborative Research, Inc., Waltham, Mass.) (3).

RNA fractionation, transfer to nitrocellulose, and hybridization to labeled HSV-1 DNA fragments. RNA was denatured for 5 min at 60°C in 50% (vol/vol) formamide (Fluka Chemical Corp., Hauppauge, N.Y.) and 2.2 M formaldehyde (Fluka Chemical Corp.) in electrophoresis buffer (20 mM morpholinepropanesulfonic acid [pH 7.4; Sigma], 5 mM sodium acetate, 1 mM EDTA) and analyzed by electrophoresis in 1% agarose containing 2.2 M formaldehyde (27) at 50 V for 14 h with circulating buffer. The RNA was transferred to nitrocellulose (Schleicher & Schuell Co., Keene, N.H.) with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 6.80) overnight (43). The positions of the rRNA markers were determined by UV illumination. The lanes containing rRNA were removed and placed in water at 60°C for 15 min to reduce the formaldehyde concentration and facilitate staining. This portion of the gel was stained for 30 min with 5 μg of ethidium bromide per ml and destained in water for 60 min.

After transfer of the RNA to nitrocellulose, the nitrocellulose was baked at 80°C for 2 h in a vacuum oven. The filter was preincubated in 0.02% Denhardt solution (10)–50% formamide–5 × SSC–0.25% SDS-50 mM sodium phosphate (pH 7.0)–250 μg of yeast RNA per ml (Sigma) at 42°C for 48 h with 5×10^6 cpm of nick-translated restriction fragment (specific activity, 2×10^8 cpm/μg of DNA). Nick translation of purified DNA was carried out as described by Rigby et al.

(36) with *E. coli* DNA polymerase I (New England Biolabs) and [^{32}P]dCTP (800 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). The unhybridized probe was removed by two washes for 30 min at room temperature in 2× SSC–0.1% SDS, followed by a wash in 0.1× SSC–0.1% SDS for 60 min at 50°C.

Hybridization selection of viral RNA. To select RNA homologous to specific viral DNA fragments, recombinant plasmid DNA was denatured (30) and immobilized on nitrocellulose (20). Briefly, the ethanol-precipitated DNA was suspended in 20 mM Tris-hydrochloride (pH 7.6)–0.5 mM EDTA at a concentration of 500 μg/ml. The DNA was boiled for 10 min, chilled on ice, and incubated at room temperature for 20 min in 0.4 M sodium hydroxide. The DNA was then diluted to a concentration of 20 μg/ml with water. An equal volume of cold 2 M ammonium acetate was added to promote binding of the DNA to nitrocellulose. The DNA was bound in 1 M ammonium acetate–0.2 N sodium hydroxide at a final DNA concentration of 20 μg/ml. The DNA solution was spotted onto a 1-cm^2 nitrocellulose filter and allowed to adsorb. This was repeated until each filter had been loaded with 20 μg of DNA. The filters were rinsed with a drop of 1 M ammonium acetate, air dried, and rinsed with 10 ml of 6× SSC. The filters were air dried and baked at 80°C for 2 h in a vacuum oven. To remove any loosely bound DNA, the filters were boiled for 1 min in 1 mM EDTA. The wash was aspirated, and the filters were rinsed twice in 1 mM EDTA at room temperature.

Hybridization and elution of RNA were performed essentially as described by Ricciardi et al. (35) with minor modifications. Poly(A)$^+$ RNA was hybridized at a concentration of 160 μg/ml in 50% formamide–0.4 M NaCl–40 mM PIPES (piperazine-*N*,*N*-bis[2-ethanesulfonic acid], pH 6.4; Sigma)–1 mM EDTA–0.1% SDS. The hybridization was incubated overnight at 37°C. The reaction mixture was removed by aspiration, and the filters were washed in 1 ml of hybridization solution at 60°C. The filters were washed 10 times in 1 ml of 1× SSC–0.5% SDS at 60°C. The final three washes were in 1 ml of 5 mM EDTA (pH 7.9); the last wash was maintained at 60°C for 5 min. The hybridized RNA was eluted in 400 μl of 1 mM EDTA (ph 7.9) containing 4 μg of calf liver tRNA (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) by boiling for 2 min and quenching on ice. The reaction was divided into aliquots for four in vitro translation reactions and precipitated with salt and ethanol.

Hybridization selection of ^{32}P-labeled RNA was carried out with 20 μg of immobilized DNA and 5 × 10^5 cpm of poly(A)$^+$ RNA (specific activity, 1.7 × 10^4 cpm/μg of poly[A]$^+$ RNA) under the conditions described above. The washing of the nitrocellulose filters and elution of hybridized RNA were as described above. The hybridized RNA from each reaction was fractionated on a 0.85% agarose gel containing 2.2 M formaldehyde as described previously. The labeled rRNA species used as molecular weight markers were contained in a sample of nonpolyadenylated RNA (10^5 cpm).

In vitro translation. Before translation, the hybridization-selected RNA was pelleted at 12,000 × *g* for 15 min at 4°C, washed twice with 75% ethanol, and dried under vacuum. The in vitro translation was carried out in a reticulocyte lysate system (New England Nuclear Corp.) as described by the supplier. The radioactive tracer used for all in vitro reactions was [^{35}S]methionine (1,000 Ci/mmol; 50 μCi/25 μl reaction).

Antibodies and immunoprecipitation. The rabbit anti-HSV-1 antiserum was prepared against extracts of cells infected with HSV-1 strain KOS (H. Weiner, A. Spang, and D. Knipe, unpublished results). The rabbit antiserum directed against gB was kindly provided by R. Courtney (12). The monoclonal antibody (39S) directed against ICP8 was kindly provided by M. Zweig (40).

To prepare extracts from infected cells for immunoprecipitations, cells from cultures labeled with [^{35}S]methionine for 30 min at 4 h pi were lysed in 0.1 M NaCl–10 mM Tris-hydrochloride (pH 7.6)–1% NP-40–0.5% sodium deoxycholate–1 mM phenylmethylsulfonyl fluoride at a concentration of 4 × 10^6 cells per ml. The lysates were cleared by centrifugation at 12,000 × *g* for 30 min. An aliquot of the supernatant (2 × 10^5 cell equivalents) was incubated with an appropriate dilution of each antiserum at 4°C for 60 min. The in vitro translation reactions were diluted 10-fold into 0.1 M NaCl–10 mM Tris-hydrochloride (pH 7.6)–1% NP-40–0.5% sodium deoxycholate–1 mM phenylmethylsulfonyl fluoride. The in vitro translation reactions were clarified, and antigen-antibody complexes were formed by incubation at 4°C for 60 min with an appropriate dilution of each of the antisera. Protein A-Sepharose (Sigma) was added to 2% (wt/vol) to precipitate the antigen-antibody complexes. After an additional 60 min at 4°C, the immunoprecipitates were washed in 1 M NaCl–10 mM Tris-hydrochloride (pH 7.6)–1% NP-40–1 mM phenylmethylsulfonyl fluoride, followed by four washes in 0.1 M NaCl–10 mM Tris-hydrochloride (pH 7.6)–1% NP-40–0.5% sodium deoxycholate–1 mM phenylmethylsulfonyl fluoride. The complexes were eluted from the protein A-Sepharose by boiling for 5 min in SDS sample buffer (60 mM Tris-hydrochloride [pH 7.6], 2% SDS, 20% glycerol, 2.5% 2-mercaptoethanol) for electrophoresis. Analysis of the immunoprecipitated polypeptides was performed by electrophoresis in 9.25% polyacrylamide gels (23). Fluorography was carried out as described by Laskey and Mills (25).

RESULTS

Identification of the major RNA species homologous to the DNA sequences encoding gB and ICP8. The genes encoding gB and the major DNA-binding protein ICP8 have been mapped within the coordinates 0.314 to 0.420 on the HSV-1 genome. This region is contained within the *Eco*RI-F restriction fragment. Figure 1 shows the relative location of this region on the prototype arrangement of the HSV-1 strain KOS genome and the origins of the plasmid DNA sequences used in this study.

To identify the major RNA species transcribed from the DNA sequences encoding these two genes, plasmid DNAs containing individual viral DNA fragments were immobilized on nitrocellulose and hybridized with ^{32}P-labeled poly(A)$^+$ RNA isolated from cells infected with HSV-1 strain KOS. The selected RNA was eluted and fractionated on a denaturing formaldehyde-agarose gel (Fig. 2). The mutation in the *mar*B1.1 antigenic variant was rescued by viral DNA sequences contained in the plasmid pSG18:SalE at map coordinates 0.343 to 0.361 (18). Based on these data, the gB coding sequences are contained at least in part within this DNA fragment. Therefore, the plasmid pSG18:SalE was used for hybridization selection of homologous RNA. The major RNA species homologous to the DNA sequences between coordinates 0.343 and 0.361 was 3.4 kb in size (Fig. 2, lane 4).

The mutations in all of the HSV-1 *ts* mutants that encode a thermolabile ICP8 DNA-binding protein have been mapped within the *Sal*I DNA fragment from coordinates 0.386 to 0.417 (8, 26, 47) contained in plasmid pSG18:SalA. To identify the mRNAs transcribed from these DNA sequences,

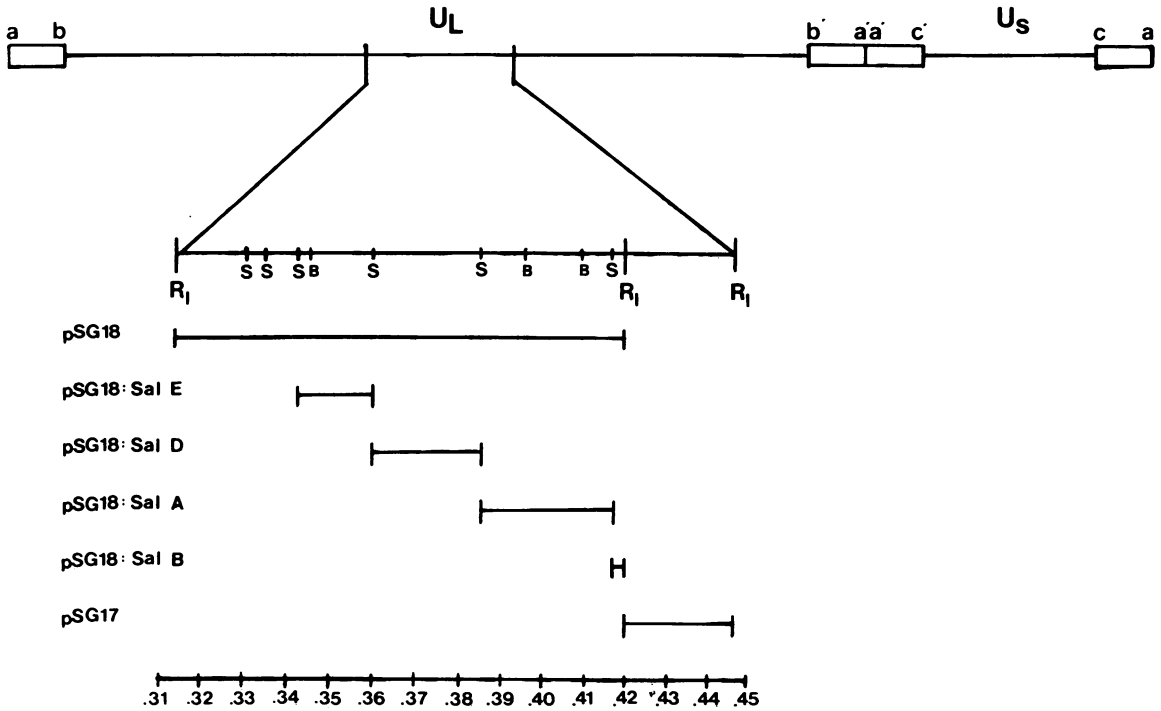

FIG. 1. Origin of HSV DNA in recombinant plasmids containing sequences between 0.314 and 0.447 on the HSV-1 (strain KOS) genome. Cleavage sites for the restriction endonucleases *Eco*RI (RI), *Sal*I (S), and *Bam*HI (B) are indicated for the region 0.314 to 0.447 on the prototype arrangement of HSV-1 DNA. These DNA sequences are contained within the *Eco*RI-F and *Eco*RI-M restriction fragments. The designations of plasmids containing restriction fragments within this region are indicated. The plasmids pSG18, containing the *Eco*RI-F fragment (map coordinates 0.314 to 0.420), and pSG17, containing the *Eco*RI-M fragment (0.420 to 0.447), were kindly provided by M. Levine. The plasmids pSG18:SalE (0.343 to 0.361), pSG18:SalD (0.361 to 0.386), pSG18:SalA (0.386 to 0.417), and pSG18:SalB (0.417 to 0.420) contain the indicated *Sal*I fragments from within the *Eco*RI-F fragment.

the plasmid pSG18:SalA was used for hybridization selection of RNA. These DNA sequences hybridized with a major RNA species of 4.5 kb (Fig. 2, lane 3).

In addition to the genes encoding gB and ICP8, the *Eco*RI-F viral DNA fragment also contains sequences encoding part of the viral DNA polymerase gene. The location of the viral DNA polymerase gene has been identified by studies of several *ts* mutants that encode thermolabile DNA polymerase activity (2). The mutations of three of these *ts* mutants, HSV-1 *tsC7*, *tsC4*, and *tsD9* of strain KOS, map within the region between the *Bam*HI V-R site and the *Kpn*I X-C site (6). This region is contained within three recombinant plasmids, pSG18:SalA (map coordinates 0.306 to 0.417), pSG18:SalB (0.417 to 0.420), and pSG17 (0.420 to 0.447). The latter two plasmids, when used for hybridization selection of RNA, detected a major 4.6-kb RNA species homologous to the DNA sequences between coordinates 0.417 and 0.447 (Fig. 2, lanes 1 and 2). The physical map locations of the genes encoding gB, the ICP8 DNA-binding protein, and the DNA polymerase correlated with the identification of major RNA species of 3.4, 4.5, and 4.6 kb, respectively. Additional minor RNAs were detected on a longer exposure which correspond to those identified in Table 2.

In vitro translation of hybridization-selected RNAs encoding gB and ICP8. To correlate the hybridization-selected RNAs with their primary translation products, selected mRNAs were identified by their ability to direct the synthesis of polypeptides in a reticulocyte cell-free translation system. The gB and ICP8 polypeptides were identified by immunoprecipitation of the in vitro-synthesized polypeptide with specific antisera.

The plasmid DNAs between coordinates 0.314 and 0.447 were bound to nitrocellulose and used to select homologous mRNAs from RNA isolated at 5 h pi from cells infected with HSV-1 KOS. The hybridized RNAs were eluted and translated in vitro. Figure 3 shows the in vitro-synthesized polypeptides immunoprecipitated with an antiserum raised against extracts from cells infected with HSV-1 KOS. Each DNA fragment selected mRNAs that directed the synthesis of a large number of viral specific polypeptides (Table 1). These polypeptides probably include correctly initiated full-length polypeptides, proteolytic cleavage products, and polypeptides initiated at internal sites within an mRNA. The antiserum did not precipitate any polypeptides synthesized from mRNA endogenous to the reticulocyte lysate or from exogenous tRNA added to the lysate (data not shown).

The gB-related polypeptides were identified by immunoprecipitation of the in vitro-synthesized polypeptides with gB-specific antiserum (Fig. 4). The pSG18:SalE and pSG18:SalD plasmids, containing sequences between 0.343 and 0.386 map units, selected mRNAs which translated in vitro to yield two polypeptides with molecular weights of 103K and 99K (Fig. 4, lanes 4 and 5). The pSG18:*Sal*-A plasmid did not select mRNA that directed the synthesis of a gB-related polypeptide (Fig. 4, lane 6). The major polypeptides immunoprecipitated from a cellular extract with a gB-specific antiserum were 103K, 100K, and 95K in molecular weight (Fig. 4, lane 3). These polypeptides may represent

under-glycosylated precursors to the mature gB (120K) because the cellular extract was prepared by labeling for 30 min with [^{35}S]methionine at 4 h pi. Alternatively, these polypeptides may represent proteolytic cleavage products of the mature forms of gB 31). Minor amounts of 115K and 112K gB-specific polypeptides, probably more extensively glycosylated forms of the protein, were also detected in the cellular extracts. The relationship of the in vitro-synthesized polypeptides to the in vivo species is discussed below.

The polypeptides synthesized in vitro and identified with a monoclonal antibody directed against ICP8 were 125K and 122K in molecular weight. The ICP8-specific polypeptides were synthesized predominantly from mRNA homologous to the DNA sequences between coordinates 0.361 and 0.417 (Fig. 5, lanes 6 and 7). The DNA sequences between coordinates 0.343 and 0.361 also selected mRNA which directed the synthesis of lesser amounts of the 125K ICP8 polypeptide (Fig. 5, lane 5). Very low amounts of a 125K polypeptide were synthesized by mRNA selected by pSG18:SalB (map coordinates 0.417 to 0.420) and pSG17 (0.420 to 0.447) (Fig. 5, lanes 8 and 9). The synthesis of ICP8 by mRNA homologous to map positions 0.343 to 0.361 on the HSV-1 genome is discussed further below. The major ICP8 polypeptides identified in the cellular extract by immunoprecipitation with the monoclonal antibody were 125K,

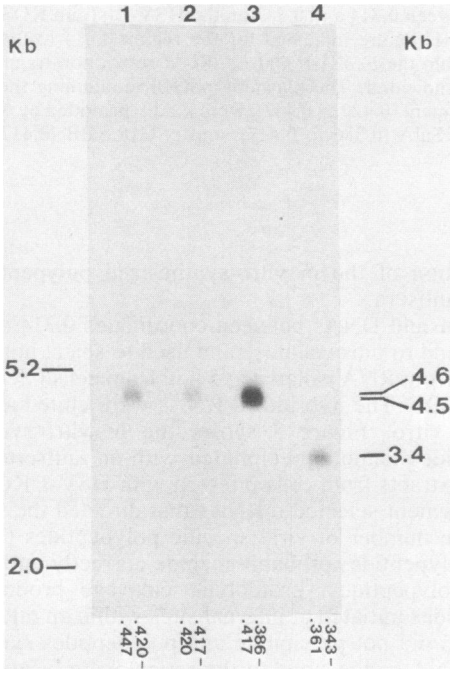

FIG. 2. Hybridization selection of ^{32}P-labeled poly(A)$^+$ RNA homologous to DNA sequences between coordinates 0.343 to 0.447 on the HSV-1 genome. Plasmid DNAs were immobilized on nitrocellulose and hybridized with poly(A)$^+$ RNA isolated at 5 h pi from HSV-1 infected cells. The hybridized RNA was eluted and fractionated in a 0.85% agarose gel containing 2.2 M formaldehyde. The gel was dried and exposed to X-ray film for 7 days. The lanes contained RNA selected by the following plasmid DNAs. Lanes: 1, pSG17 (map coordinates 0.420 to 0.447); 2, pSG18:SalB (0.412 to 0.420); 3, pSG18:SalA (0.386 to 0.417); 4, pSG18:SalE (0.343 to 0.361). The sizes of the major RNAs indicated on the right were determined relative to the migration of the 28S (5.2 kb) and 18S (2.0 kb) rRNA markers (46) indicated on the left. The additional RNAs detected on a longer exposure are listed in Table 2.

TABLE 2. RNA species homologous to DNA sequences between 0.314 and 0.447 on the HSV-1 genome determined by Northern blot analysis of RNA isolated at 5 h pi

Plasmid	Approx map coordinates[a]	Fragment size (kbp)[b]	RNA size (kb)[c]
pSG18	0.314–0.420	15.8	Major: 4.5, 3.4; minor: 10.2, 6.1, 2.3, 1.8, 1.3
pSG18:SalE	0.343–0.361	2.6	Major: 3.4; minor: 10.2, 6.1, 4.5, 2.3, 1.8, 1.3
pSG18:SalD	0.361–0.386	3.8	Major: 4.5, 3.4; minor: 10.2, 6.1
pSG18:SalA	0.386–0.417	4.6	Major: 4.5; minor: 10.2, 7.1
pSG18:SalB	0.417–0.420	0.45	4.6
pSG17	0.420–0.447	4.1	Major: 4.6; minor: 3.2, 2.6, 1.8, 1.3

[a] Map locations determined as in Table 1.
[b] The size of restriction fragments is based on comigration with *Eco*RI and *Hin*dIII digests of phage λ DNA (42). kbp, Kilobase pair.
[c] The size of RNA is based on comigration with 28S (5.2-kb) rRNA and 18S (2.0-kb) rRNA. These sizes include poly(A) tails of ca. 200 bases in length.

122K, 120K, and 88K in molecular weight (Fig. 5, lane 3). The 125K and 122K polypeptides were probably the two electrophoretic forms of ICP8 previously identified (22). The identities of the 120K and 88K polypeptides are unknown.

Virus-specific antiserum was used to identify the polypeptides encoded between 0.417 and 0.447 on the HSV-1 genome because presently there is no antiserum directed against the viral DNA polymerase. The plasmids pSG18:SalB (0.417 to 0.420) and pSG17 (0.420 to 0.477) selected mRNAs that direct the synthesis of a 133K polypeptide (Fig. 3, lanes 7 and 8). Minor amounts of a 136K and a 145K polypeptide were also detected. The 145K polypeptide may represent the 150K polypeptide previously associated with DNA polymerase activity (33). The identity of the 133K polypeptide is unknown, but it may be a proteolytic cleavage product from the 145K polypeptide.

The DNA sequences between 0.343 and 0.386 encoded polypeptides of 103K and 99K which were precipitated with gB-monospecific antiserum. This is the region of the HSV-1 genome from which the 3.4-kb RNA was transcribed. The DNA sequences between 0.363 and 0.417 encoded polypeptides of 125K and 122K which were precipitated with monoclonal antibody directed against ICP8. The major RNA transcribed from these DNA sequences was 4.5 kb. These data suggest that the 3.4-kb mRNA encodes gB, and the 4.5-kb RNA encodes the major DNA-binding protein ICP8.

Identification of the RNA species homologous to the region 0.314 to 0.447 on the HSV-1 genome. To localize further the 3.4- and 4.5-kb RNAs on the HSV-1 genome, poly(A)$^+$ cytoplasmic RNA was fractionated on a formaldehyde-agarose gel, transferred to nitrocellulose, and hybridized with nick-translated restriction fragments containing DNA sequences between coordinates 0.314 and 0.447 (Fig. 6). The RNA species homologous to this region of the genome detected at 5 h pi (early in infection) are summarized in Table 2. The 3.4-kb RNA species was transcribed from DNA sequences within the left half of *Eco*RI-F, coordinates 0.343

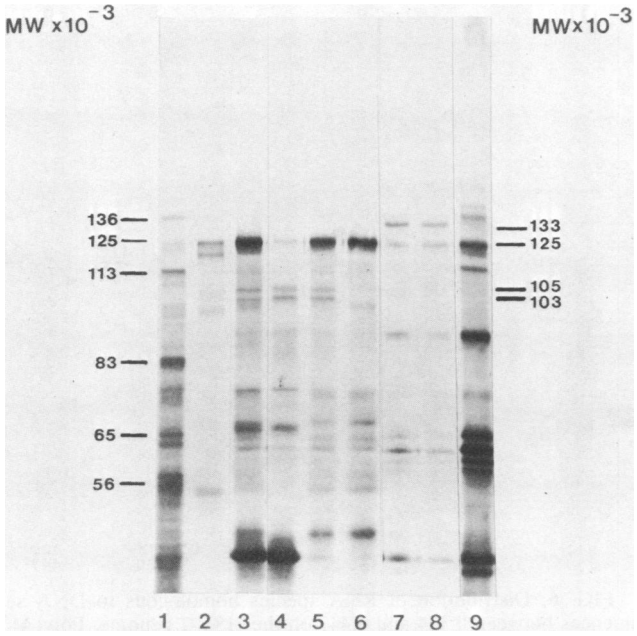

FIG. 3. Immunoprecipitation with anti-HSV-1 antiserum of polypeptides synthesized in vitro from mRNAs homologous to DNA sequences between 0.314 and 0.447 on the HSV-1 genome. Poly(A)$^+$ RNA (16 μg) isolated from infected cells (5 h pi) was hybridized with plasmid DNAs immobilized on nitrocellulose. The hybridized RNA was eluted and divided into four aliquots for translation in a commercial reticulocyte lysate with [^{35}S]methionine. The in vitro-synthesized polypeptides were immunoprecipitated with rabbit anti-HSV-1 antiserum. For the immunoprecipitation of polypeptides directed by mRNAs hybridized to pSG17 (map coordinates 0.420 to 0.447) or pSG18:SalB (0.417 to 0.420), two reactions were pooled. All other immunoprecipitations were of polypeptides synthesized in a single reaction. The immunoprecipitated polypeptides were analyzed on a 9.25% polyacrylamide gel. The dried gel was fluorographed at −80°C for 3 days (lanes 1 through 6) or 10 days (lanes 7 through 9). Translation reactions contained RNA selected by the following fragments. Lanes: 3, pSG18 (map coordinates 0.314 to 0.420); 4, pSG18:SalE (0.343 to 0.361); 5, pSG18:SalD (0.361 to 0.386); 6, pSG18:SalA (0.386 to 0.417); 7, pSG18:SalB (0.417 to 0.420); 8, pSG17 (0.420 to 0.447). Lane 9 shows polypeptides precipitated from a translation reaction containing total poly(A)$^+$RNA (200 ng). Lane 1 shows an extract from cells labeled for 30 min with [^{35}S]methionine at 4 h pi; lane 2 shows proteins immunoprecipitated from the cellular extract in lane 1. The molecular weights ($\times 10^{-3}$) on the left indicate the position of the non-immunoprecipitated polypeptides synthesized in vivo, and those on the right indicate the position of certain major immunoprecipitated polypeptides synthesized in vitro which are discussed. Molecular weights of polypeptides indicated were based on the migration of the following molecular weight standards: β-galactosidase, 117,000; phosphorylase *b*, 92,500; bovine serum albumin, 68,000; and ovalbumin, 45,000.

to 0.386 (Fig. 6, lanes 2 and 3). The 4.5-kb RNA species was transcribed from DNA sequences within the right half of *Eco*RI-F, coordinates 0.361 to 0.417 (Fig. 6, lanes 3 and 4). In addition to these major RNAs, there were several less abundant RNAs homologous to the DNA sequences within the *Eco*RI-F restriction fragment (Table 2).

The 4.5-kb RNA was the most abundant RNA species homologous to the region of the genome to which the ICP8 mutations have been previously mapped (Fig. 6, lane 4). However, there were two additional minor RNAs of 10.2 and 7.1 kb homologous to this region of the genome (map coordinates 0.386 to 0.417) (Table 2) which are large enough to encode a 125K polypeptide. A minimum of 3.4 kb pairs of coding sequences is required for a 125K polypeptide (assuming an average molecular weight of 110 per amino acid residue); therefore, either or both of the 10.2- and 7.1-kb RNAs could encode ICP8. Based on the abundance of the 4.5-kb RNA (Fig. 2 and 6) and its location within coordinates 0.361 to 0.417, which correlates with the region of the genome to which the ICP8 mutations have been mapped, the 4.5-kb RNA has been identified as the major mRNA encoding ICP8.

Similarly, the 3.4-kb RNA was the most abundant RNA species transcribed at 5 h pi from the region of the genome to which the gB mutations had been previously mapped. A minimum of 2.8 kb of coding sequences is required to encode a 103K polypeptide; therefore, the 10.2-, 6.1- or 4.5-kb RNAs are large enough to encode gB. Based on the abundance of the 3.4-kb RNA and its location to within coordinates 0.343 to 0.386, which correlates with the region of the

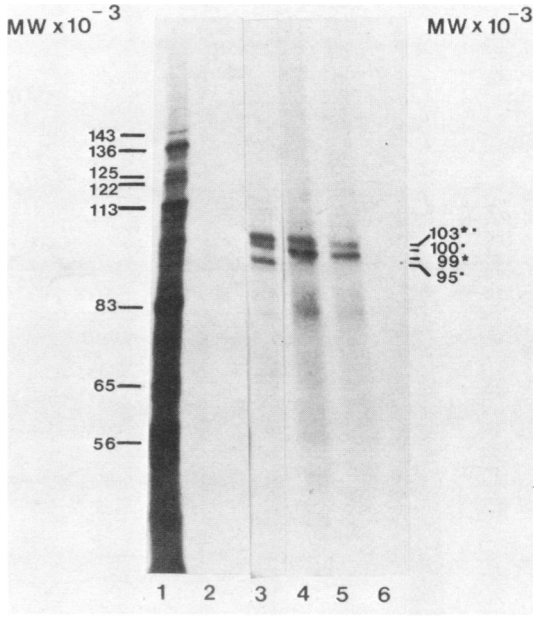

FIG. 4. Immunoprecipitation with gB-specific antiserum of the polypeptides synthesized in vitro from mRNAs homologous to DNA sequences between 0.343 and 0.417 on the HSV-1 genome. Hybridization selections of RNA and in vitro translations were carried out as described in the legend to Fig. 3. The in vitro-synthesized polypeptides were immunoprecipitated with monospecific antiserum directed against gB (kindly provided by R. Courtney). The immunoprecipitated polypeptides were analyzed on a 9.25% polyacrylamide gel. The dried gel was fluorographed at −80°C for 7 days. Translation reactions contained RNA selected by the following fragments. Lanes: 4, pSG18:SalE (map coordinates 0.343 to 0.361); 5, pSG18:SalD (0.361 to 0.386); 6, pSG18:SalA (0.386 to 0.417); 2, no mRNA added. Lane 1 contained an extract from cells labeled for 30 min with [^{35}S]methionine at 4 h pi. Lane 3 contained the polypeptides immunoprecipitated from the extract shown in lane 1. The molecular weights ($\times 10^{-3}$) on the left indicate the position of the non-immunoprecipitated polypeptides synthesized in vivo, and those on the right indicate the position of the major immunoprecipitated polypeptides synthesized in vivo (■) and in vitro (*). The molecular weights of the polypeptides were determined as described in the legend to Fig. 3.

genome to which the gB mutations have been mapped, the 3.4-kb RNA has been identified as the major mRNA encoding gB.

Requirements for transcription of the 3.4- and 4.5-kb RNAs. The requirements for transcription of the 3.4- and 4.5-kb RNAs were determined by isolating RNA from cells infected in the presence of inhibitors of DNA or protein synthesis. The transcription of early RNAs does not require prior viral DNA synthesis (44). Therefore, poly(A)$^+$ cytoplasmic RNA was isolated at 5 h pi from cells incubated with or without PAA, an inhibitor of viral DNA synthesis. The RNA was fractionated on a formaldehyde-agarose gel, transferred to nitrocellulose, and hybridized with a nick-translated restriction fragment containing DNA sequences between coordinates 0.314 and 0.420 (Fig. 7, lanes 1 and 2). Approximately equivalent amounts of the 3.4- and 4.5-kb RNAs were detected in the presence or absence of viral DNA replication. This suggests that both RNAs encoding gB and ICP8 are expressed at early times during a productive infection.

To support the classification of the 3.4- and 4.5-kb species as early RNAs, the requirement of protein synthesis for their transcription was examined. Only immediate early (alpha) RNAs accumulate in the cytoplasm without de novo protein synthesis (24). Poly(A)$^+$ cytoplasmic RNA was isolated from cells infected and incubated with cycloheximide. Neither RNA species accumulated in the absence of protein synthesis (Fig. 7, lanes 3 and 4). However, a 4.7- and a 3.0-kb RNA species were detected in these RNA samples homologous to the DNA sequences contained within the *Bam*HI-S P restriction fragment (data not shown). These RNAs encode the immediate early (alpha) polypeptides ICP4 and ICP0, respectively (28, 45). Therefore, the 3.4- and 4.5-kb RNAs were not expressed in the absence of viral protein synthesis.

In addition to poly(A)$^+$ cytoplasmic RNA from PAA- and cycloheximide-treated cells, poly(A)$^+$ nuclear RNA was isolated and analyzed for the 3.4- and 4.5-kb transcripts (data not shown). Both RNAs accumulated in the nucleus in the absence of viral DNA synthesis, but neither species was detected in the nucleus of cells infected in the absence of protein synthesis.

Non-polyadenylated cytoplasmic and nuclear RNA isolated from PAA- and cycloheximide-treated cells were similarly analyzed for the transcription of the 3.4- and 4.5-kb RNAs (data not shown). Neither RNA species was detected in non-polyadenylated RNAs from either the cytoplasm or nucleus of treated cells. Although fivefold-greater amounts of non-polyadenylated RNAs were analyzed as compared to the poly(A)$^+$ RNA samples, neither the 3.4- nor the 4.5-kb RNA was detected. Therefore, both the 3.4- and 4.5-kb RNAs are poly(A)$^+$ RNAs expressed at early times during infection.

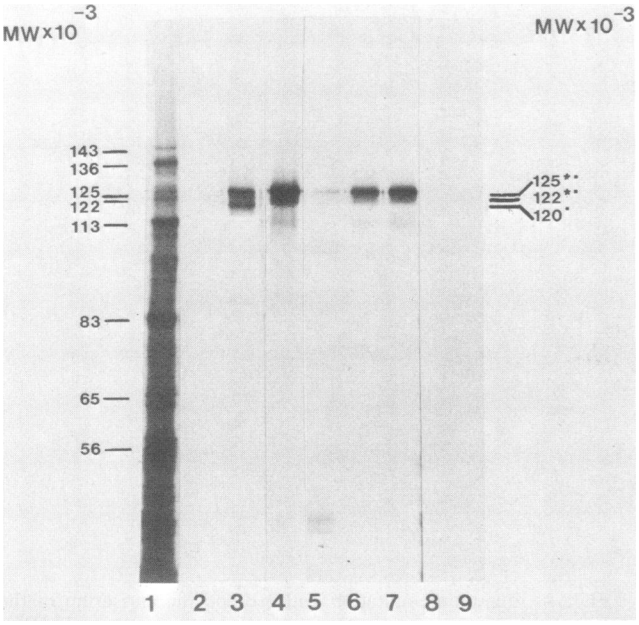

FIG. 5. Immunoprecipitation with ICP8-specific monoclonal antibody of the polypeptides synthesized in vitro from mRNAs homologous to DNA sequences between 0.314 and 0.447 on the HSV-1 genome. Hybridization selection of RNA and in vitro translations were carried out as described in the legend to Fig. 3. Aliquots of each in vitro translation reaction were immunoprecipitated with 39S monoclonal antibody directed against ICP8 (kindly provided by M. Zweig). The immunoprecipitated products were analyzed on a 9.25% polyacrylamide gel. The dried gel was fluorographed at −80°C for 7 days. Translation reactions contained RNA selected by the following fragments. Lanes: 4, pSG18 (map coordinates 0.314 to 0.420); 5, pSG18:SalE (0.343 to 0.361); 6, pSG18:SalD (0.361 to 0.386); 7, pSG18:SalA (0.386 to 0.417); 8, pSG18:SalB (0.417 to 0.420); 9, pSG18:SalA (0.386 to 0.417); 2, no mRNA added. Lane 1 contained an extract from cells labeled for 30 min at 4 h pi. Lane 3 contained the polypeptides immunoprecipitated from the extract shown in lane 1. Molecular weights indicated were determined as in the legend to Fig. 3.

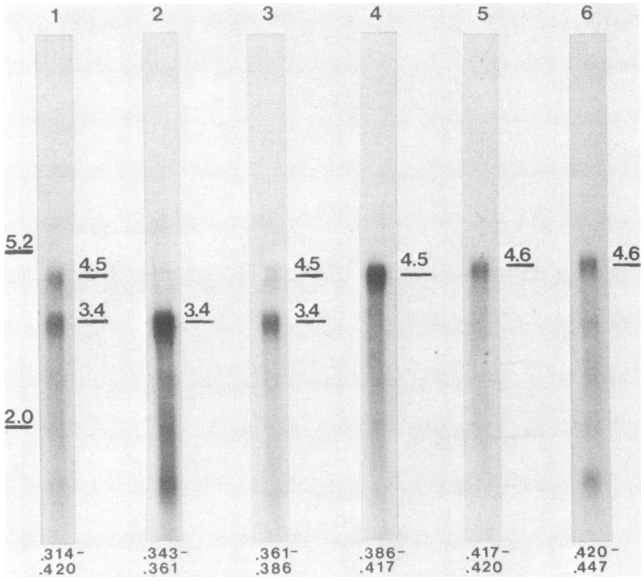

FIG. 6. Distribution of RNA species homologous to DNA sequences between 0.314 and 0.447 on the HSV-1 genome. Poly(A)$^+$ cytoplasmic RNA was isolated from infected cells at 5 h pi. The RNA (2.5 μg) was fractionated by electrophoresis in 1.0% agarose containing 2.2 M formaldehyde, transferred to nitrocellulose, and hybridized with 5×10^6 cpm of nick-translated restriction fragment. The positions of the major RNAs (3.4, 4.5, and 4.6 kb) homologous to each region are indicated on the right as determined relative to the positions of the 28S (5.2-kb) rRNA and 18S (2.0-kb) rRNA markers indicated on the left. The exposure of each lane differs to obtain the clearest representation of the major RNAs detected with each DNA probe.

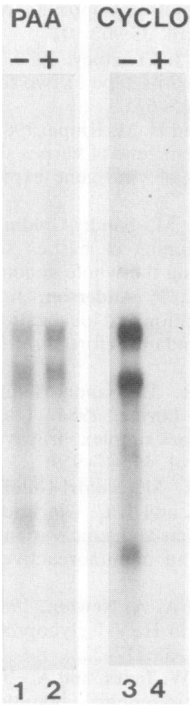

FIG. 7. Requirements for transcription of the 3.4- and 4.5-kb RNAs homologous to DNA sequences between 0.314 and 0.420 on the HSV-1 genome. Poly(A)$^+$ cytoplasmic RNA was isolated at 5 h pi from PAA- or cycloheximide-treated cells. RNA samples (2 μg) were fractionated in a 1.0% agarose gel containing 2.2 M formaldehyde, transferred to nitrocellulose, and hybridized with 5×10^6 cpm of nick-translated *Eco*RI-F restriction fragment. RNA samples were isolated from cells infected under the following conditions. Lanes: 1, in the absence (−) of PAA; 2, in the presence (+) of PAA; 3, in the absence (−) of cycloheximide; 4, in the presence (+) of cycloheximide.

DISCUSSION

In this report, RNA analysis in conjunction with in vitro translation of hybridization-selected mRNAs was used to identify and characterize the mRNAs encoding gB and the major DNA-binding protein ICP8. Hybridization selection of ^{32}P-labeled RNA was used to identify the major RNAs homologous to the regions of the genome encoding gB and ICP8 based on previous genetic analyses. In addition, hybridization selection was used to functionally define the gB- and ICP8-specific mRNAs by their ability to direct the synthesis of specific polypeptides in a cell-free system. RNA analysis by Northern blot hybridization was used to further localize the major RNAs identified by hybridization selection that were homologous to the DNA sequences between coordinates 0.314 and 0.447.

mRNA encoding gB. A 3.4-kb mRNA was identified by hybridization selection of ^{32}P-labeled RNA and by Northern blot hybridization of poly(A)$^+$ cytoplasmic RNA as the major RNA species homologous to DNA sequences between coordinates 0.343 to 0.361 on the HSV-1 genome. The mutation in the gB antigenic variant *mar*B1.1 has been mapped within this region of the genome (18). In addition, the 3.4-kb RNA contains sequences homologous to the region 0.361 to 0.386 on the HSV-1 genome. The mutation in the gB mutant *tsB5* has been mapped within this region (9, 18).

The mRNAs homologous to the DNA sequences between 0.343 and 0.386 directed the synthesis of two major gB-specific polypeptides of 103K and 99K. The 99K polypeptide may be a proteolytic cleavage product from the 103K polypeptide. Glycosylation of polypeptides in vitro does not occur in the absence of microsomal membrane preparations (37); therefore, one or both of these polypeptides are probably the primary unmodified translation products encoded by the gB gene. Previously, Inglis and Newton (19) translated cytoplasmic RNA (isolated from infected cells at 8 h pi) in a reticulocyte lysate system. An 85K polypeptide was precipitated from the in vitro translation products with gB antiserum. The gB-related polypeptides synthesized in vitro and reported in this study appear to be larger. The reason for this discrepancy is unknown. The 103K gB polypeptide is also larger than any unglycosylated species detected previously in vivo. Pizer et al. (32) have identified an 85K polypeptide as the nonglycosylated form of gB in tunicamycin-treated cells. Possibly, the 103K polypeptide does not accumulate to a substantial level in vivo because it is rapidly cleaved to the 85K non-glycosylated polypeptide. Bzik and co-workers (D. Bzik, B. Fox, N. DeLuca, and S. Person, submitted for publication) have determined the nucleotide sequence for the gB gene. The predicted molecular weight for gB is ca. 100K. Thus, the observed sizes for our in vitro translation products are consistent with the predicted size.

mRNA encoding ICP8. A 4.5-kb mRNA was identified by hybridization selection of ^{32}P-labeled RNA and by Northern blot hybridization of poly(A)$^+$ cytoplasmic RNA as the major RNA species homologous to DNA sequences between 0.386 to 0.417 on the HSV-1 genome. The 4.5-kb RNA also contains sequences homologous to the DNA sequences between 0.361 and 0.386. Holland et al. (Fig. 4 of reference 17) detected a 4.2-kb RNA transcribed from the region 0.361 to 0.417 from right to left on the prototype form of the HSV-1 genome. All of the ICP8 *ts* mutants isolated to date have mutations which map within DNA sequences between 0.386 and 0.417 (8, 26, 47). The mRNA homologous to these two regions of the genome directed the synthesis of two ICP8-specific polypeptides of 125K and 122K.

A 4.5-kb RNA was also detected as a minor species homologous to the DNA sequences between 0.343 and 0.361. Holland et al. (Fig. 4 of reference 17) have determined that a 4.4-kb RNA is transcribed from DNA sequences from within this region of the genome from left to right on the prototype arrangement on the HSV-1 genome. Therefore, the 4.5-kb RNA homologous to DNA sequences between 0.361 and 0.417, encoding ICP8, may be distinct from the 4.5-kb RNA homologous to DNA sequences between 0.343 and 0.361.

DNA sequences between coordinates 0.343 and 0.361 hybridize to mRNA which directs the synthesis of the two ICP8-specific polypeptides of 125K and 122K. A 10.2-kb RNA was detected homologous to DNA sequences throughout the region 0.343 to 0.417. Similarly, Holland et al. (Fig. 4 of reference 17) have identified a 10-kb RNA transcribed from this region from right to left on the prototype arrangement of the HSV-1 genome. Therefore, the 10.2- and 4.5-kb RNAs are transcribed from the same DNA strand. The same sequences are contained within the 4.5-kb RNA and the 5′ half of the 10.2-kb RNA. Hybridization selection of RNA with DNA sequences between 0.343 and 0.361 would select the 10.2-kb RNA which contains the ICP8 coding sequences; therefore, these sequences could direct the synthesis of the 125K and 122K ICP8 polypeptides. Anderson et al. (1) have previously identified two distinct mRNAs of 1.9 and 4 kb

which are homologous to DNA sequences between coordinates 0.55 and 0.60 and which both encode a 54K polypeptide. Multiple mRNAs generated by inefficient termination at a polyadenylation site is characteristic of HSV transcription. Two distinct mRNAs are generated having 5′ termini which are colinear. These RNAs encode the same polypeptide and translation termination signals, but the larger RNA species contains nontranslated sequences downstream from the first polyadenylation signal (1, 11).

In summary, we conclude that the major mRNA expressed from the gB gene was 3.4-kb and encoded two gB-related polypeptides of 103K and 99K. The major mRNA expressed from the ICP8 gene was a 4.5-kb mRNA. This mRNA was translated into 125K and 122K ICP8-related polypeptides.

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