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Characterization of the herpes simplex virus type 1 glycoprotein D mRNA and expression of this protein in *Xenopus* oocytes

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ABSTRACT

We have identified and characterized a 3.0 kilobase (kb) mRNA containing coding sequences of the herpes simplex virus type 1 (HSV-1) glycoprotein D (gD) gene. The synthesis of this 3.0 kb mRNA was unaffected by the presence of cytosine arabinoside, but was made in greatly reduced amounts in cells infected with HSV-1 in the presence of cycloheximide: it was, therefore, classified as an early mRNA. By nuclease protection experiments, it was found that the 3.0 kb mRNA is unspliced and, further, that it is 3' co-terminal with a smaller 1.6 kb early mRNA which is transcribed from a DNA sequence 3' to the gD coding sequence. We describe the use of the Xenopus laevis oocyte system to produce HSV-1 gD in vitro. Oocytes injected with mRNA isolated from HSV-1-infected Vero cells synthesized gD, which was identified by immunoprecipitation. Injection of a plasmid clone containing the HSV-1 BamHI J fragment (0.89 to 0.93 map units) into the nuclei of Xenopus oocytes also resulted in synthesis of gD.

INTRODUCTION

During its replicative cycle, herpes simplex virus type 1 (HSV-1) codes for at least four glycoproteins, designated gA/gB, gC, gD and gE (1,4). The functions of these virus glycoproteins have not been fully described: however, glycoprotein gB is required for penetration of the virus into the cell (2), while gC is implicated in virus-induced cell-fusion (3), and gE has immunoglobin IgG Fc-binding capacity (4). The role of gD in virus replication remains unknown. However, in common with antisera directed against these other HSV-1 glycoproteins, anti-gD serum neutralizes the infectivity of the homologous virus (5). Moreover, unlike anti-gC and -gE sera, anti-gD serum also neutralizes infectivity of the related HSV type 2 (6,7,8,9).

Glycoprotein gD has been reported to be an early protein of HSV-1 (10): as such, its synthesis occurs within 2 hours post-infection (p.i.) and reaches a maximal rate between 4 and 6 hours p.i. (11). The protein is made as a 50,000 molecular weight (mw) precursor (pgD) and is processed to the

mature glycosylated form of approximately 59,000 mw (10,12) through a number of discrete stages (13). The mature product, gD, accumulates in the outer cell membrane and is incorporated into newly-formed virions (1).

A number of studies have been undertaken to localize the gD gene and coding sequences within the HSV-1 DNA genome. By analysing intertypic recombinants between HSV-1 and HSV-2, Ruyechan and co-workers positioned the coding sequence of gD within 0.90 to 0.945 genome map units (3); that is, within the rightward half of the short unique segment (U_S) of the genome (14). Other workers also positioned the gD coding sequence within U_S (15,16). Recently, using *in vitro* translation of hybridization-selected mRNA's, Lee and co-workers further localized the gD gene to a 2.4 kilobase pair (kbp) HindIII/SacI DNA fragment (17), mapping within those co-ordinates established by Ruyechan *et al.* (3). By DNA sequence analysis and expression of an HSV-1 DNA fragment using a bacterial plasmid vector, we located precisely the gD coding sequences within this 2.4 kbp HindIII/SacI DNA fragment (18). In this report, we identify and characterize a 3.0 kilobase (kb) mRNA that includes this DNA fragment and which presumably encodes gD. Furthermore, we report that injection, into the nuclei of *Xenopus laevis* oocytes, of a plasmid (pRWF6) that contains a 6.4 kbp BamHI DNA fragment which includes the gD structural gene, resulted in synthesis of the gD polypeptide.

MATERIALS AND METHODS

Cells and Viruses

African green monkey kidney (Vero) cells were grown to confluency in Modified Eagles Medium (MEM) supplemented with 5% fetal calf serum. Stocks of HSV-1 (Patton strain) were grown from thrice plaque purified virus using low multiplicities of infection as described elsewhere (19).

RNA Isolation

Cytoplasmic RNA was extracted from Vero cells 8 hours after infection with 20 PFU of HSV-1 per cell as described previously (20). Poly(A)-containing mRNA was selected by retention on an oligo deoxythymidine (dT) cellulose column (P-L Biochemicals, type 7), followed by elution with sterile water (20). Total poly(A)-containing mRNA was ethanol precipitated, resuspended in water and then used in RNA microinjection of oocytes. When indicated, metabolic inhibitors were added to cells at the time of infection at concentrations of 200 µg/ml (cycloheximide) or 50 µg/ml (cytosine arabinoside) and cells were maintained in media containing these

concentrations of drugs throughout infection.

RNA Electrophoresis

Polyadenylated cytoplasmic RNA's were separated on 1.5% agarose gels containing 6 mM methyl mercuric hydroxide as described by Bailey and Davidson (21), and were transferred by blotting to diazobenzyloxymethyl cellulose paper (22). DNA probes were ^{32}P-labeled by nick-translation and hybridized for 20 hours at 42°C to blots in a solution containing 60% formamide, 5 x SSC, 1 x Denhardt's buffer, 300 µg/ml denatured salmon sperm DNA, 25 mM potassium phosphate, pH 6.5, and 10^6 counts per minute per ml of the denatured ^{32}P-labeled DNA probe.

Nuclease Protection Analyses

The conditions for ^{32}P-labeling the 5' and 3' termini of DNA fragments have been described (23). Hybridizations were performed by incubating 0.1 µg of the ^{32}P-labeled DNA fragment with 20 µg early cytoplasmic RNA in a volume of 25 µl hybridization buffer (90% formamide, 400 mM NaCl, 1 mM EDTA, 40 mM PIPES, pH 6.4) for 10 minutes at 70°C, then overnight at 55°C. Following hybridization, the samples were digested with nuclease S1 and exonuclease VII as described previously (23). Digests were analysed by electrophoresis on alkaline 1.4% agarose gels (24).

DNA Sequencing

The procedures used for DNA sequencing by the method of Maxam and Gilbert were as described (25,26).

^{35}S-Methionine Labeled Proteins

Vero cells were infected with HSV-1 at a multiplicity of 2 plaque forming units (PFU) per cell. At seven hours after infection, cells were fed MEM containing methionine at one fifth the normal concentration and were labeled with 100 µCi/ml ^{35}S-methionine (Amersham, specific activity greater than 600 Ci/mmol) from 8 to 10 hours after infection. Labeled cells were lysed in immunoprecipitation (IP) buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5% NP40).

DNA Isolation

The recombinant plasmids used in the microinjections contained pBR322 sequences into which HSV-1 (Patton) U_S sequences have been inserted. Supercoiled, hybrid plasmids were grown and purified as described by Moore et al. (27). DNA's were dialyzed, phenol extracted, ethanol precipitated and resuspended in 10 mM Tris-HCl pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA) prior to microinjection.

Microinjection of Xenopus Oocytes

Oocytes were surgically removed from healthy Xenopus laevis females. Microinjection was performed one to three days after their removal. The oocytes were washed, separated and maintained in Barth's buffer (28) until injection. Stage 5 and 6 oocytes were selected and were used for microinjection of the mRNA (1 µg/µl) into their cytoplasm or of supercoiled DNA (1 µg/µl) into their germinal vesicles. mRNA (50 nl per oocyte, 50 oocytes per sample) and DNA (15 nl per oocyte, 100 oocytes per sample) were injected using a modified Hamilton micropipettor (29,30). Proteins synthesized in the oocytes were labeled with ^{35}S-methionine in Barth's buffer from 4 to 18 hours after injection and were extracted following homogenization of the oocytes in IP buffer.

Immunoprecipitations

Extracts from oocytes or Vero cells in IP buffer were preadsorbed with Staphylococcus Protein A-Sepharose CL4B (Pharmacia). Polyvalent anti-HSV-1 antiserum (MacIntyre, VR3, DAKO Corp.) or monoclonal anti-gD antibodies (1S or 4S, kindly provided by Dr. Martin Zweig) were added to preadsorbed extracts. The immune complexes were precipitated by addition of Protein A-Sepharose. Proteins were eluted from the complex by boiling in sample buffer.

Electrophoresis of Proteins

Immunoprecipitated proteins were separated in a 9% polyacrylamide gel as described by Laemmli (31). The buffer used during electrophoresis contained 25 mM Tris-HCl pH 8.6, 192 mM glycine and 0.1% SDS. ^{14}C-labeled high mw proteins (Bethesda Research Laboratories, Inc.) were electrophoresed on each gel as standards.

Following electrophoresis, the gels were treated with dimethylsulfoxide and 2,5-diphenyloxazole (32) and fluorographed using Kodak XAR film at -70°C for various lengths of time.

RESULTS

Map location and characterization of the gD mRNA: The location of the 2.4 kbp HindIII/SacI fragment of HSV-1 DNA reported to encode the gD mRNA (17) is indicated in Fig. 1. Previously, we reported that the gD coding sequence initiated 72 base pairs (bp) to the right of this HindIII site and extended a further 1182 bp in the rightward direction (18). To identify and characterize the gD mRNA, we analysed the cytoplasmic RNA species of HSV-1-infected cells which map within the genome co-ordinates established

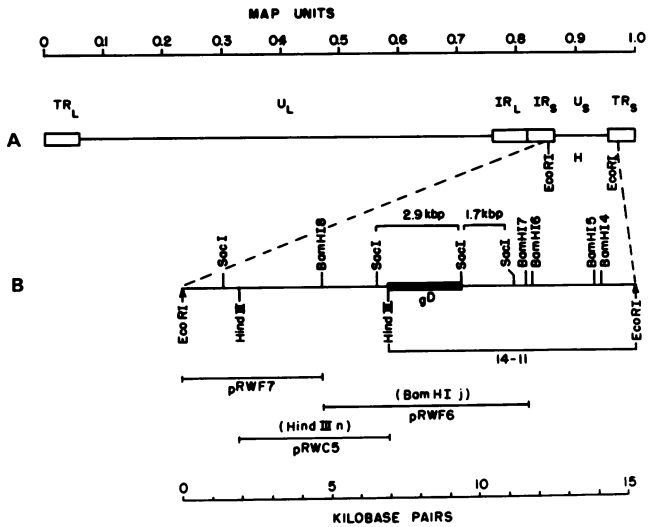

Fig. 1 (A) Diagrammatic representation of the HSV-1 DNA structure. The inverted repeat sequences (TR_L, IR_L, IR_S and TR_S) which bound the unique DNA sequences (U_L and U_S) are represented by open boxes. (B) Restriction endonuclease cleavage map of the EcoRI H DNA fragment for the enzymes SacI, HindIII, and BamHI. The HindIII/SacI DNA fragment within which the coding sequences of gD were localized by Lee et al. (17) is denoted by the thicker line. The HSV-1 DNA fragments contained by plasmids pRWF7, pRWC5 and pRWF6 and by bacteriophage lambda 14-11 are indicated.

above. Cells were infected with HSV-1 in the presence or absence of metabolic inhibitors to correlate the appearance of the mRNA and the reported kinetics of synthesis of gD (10).

Polyadenylated RNA species were isolated from cells infected with HSV-1 for 8 hours under three separate regimens; (1) in the presence of cycloheximide (immediate-early, IE mRNA), (2) in the presence of cytosine arabinoside (early mRNA), and (3) in the absence of metabolic inhibitors (late mRNA). These mRNA's were resolved by electrophoresis on a methyl mercury agarose gel and were transferred by blotting to diazobenzyloxymethyl-cellulose paper. A number of different, ^{32}P-labeled DNA probes were then hybridized to the blotted mRNA's. The results of these analyses are shown in Fig. 2. Hydridization with the HindIII/EcoRI HG fragment (Fig. 1) contained by bacteriophage lambda clone 14-11 (33) revealed that four major early mRNA species mapped to the right of the HindIII site at 0.90 map units. These early mRNA's had estimated sizes of 3.0, 2.8, 1.6 and 1.3 kb. The three larger early mRNA's were present late after infection, but

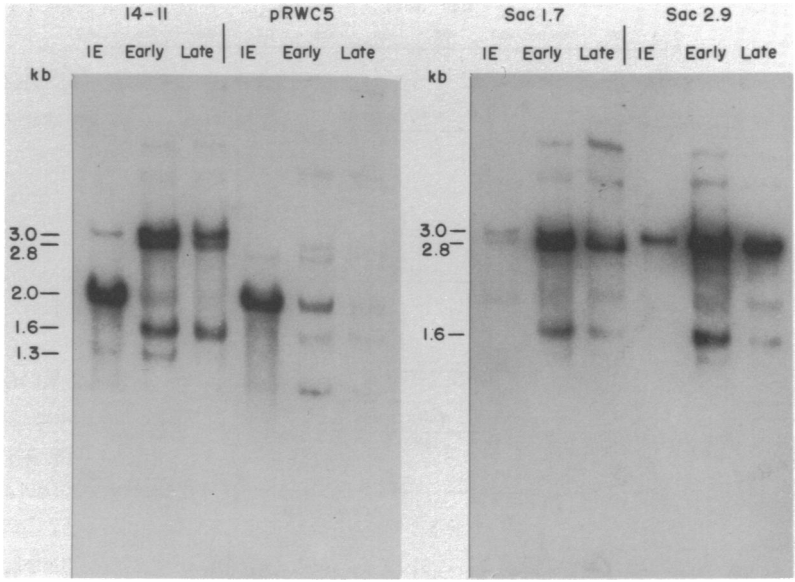

Fig. 2 Blot analysis of HSV-1 mRNA's. Polyadenylated immediate-early (IE), early and late RNA's were resolved by electrophoresis on methyl mercury agarose gels and blotted onto DBM-paper. The RNA blots were hybridized with ^{32}P-labeled nick-translated DNA probes (lambda 14-11, pRWC5, and the 1.7 kbp and 2.9 kbp SacI subfragments of pRWF6) and autoradiographed. The estimated sizes of these mRNAs (in kilobases) are indicated to the side of the autoradiographs.

the 1.3 kb species was virtually absent. The 3.0 kb early mRNA was made in reduced amounts in the presence of cycloheximide. The 2.0 kb IE mRNA's (IE mRNA-4 and IE mRNA-5, reference 23) were found to be virtually absent early and late after infection. The presence of minor amounts of three larger mRNA species was noted also in the early and late mRNA preparations using the lambda 14-11 DNA probe.

The early mRNA's mapping within the HSV-1 DNA region represented by lambda 14-11 were further localized using the 2.9 kbp and 1.7 kbp SacI DNA subfragments as probes (Fig. 2). It was found that the 2.9 kbp SacI DNA fragment, which contains the gD coding sequence, hybridized to the 3.0 kb and 1.6 kb early mRNA's, while the 1.7 kbp SacI DNA fragment hybridized predominantly to the 2.8 kb early mRNA species and to a reduced extent to the 3.0 kb and 1.6 kb species. Neither of these probes hydridized to the 1.3 kb early mRNA, which then must map to the right of the 1.7 kbp SacI DNA fragment. The 3.0 kb and 1.6 kb early mRNA species did not hybridize to

pRWC5, which contains the HindIII N fragment, and therefore must map to the right of the HindIII site at 0.90 map units.

From the data detailed above, it appeared likely that gD was encoded by either the 3.0 kb or 1.6 kb early mRNA. It is unlikely that gD is encoded by one of the other minor species of early mRNA observed in the blot analysis (Fig. 2), as these larger species would be expected to hybridize to DNA fragments adjacent to the 2.9 kbp SacI DNA fragment. Selection of gD mRNA using adjacent DNA sequences was not observed, however (17). To determine which of these two early mRNA species contain gD coding sequences, the locations of these transcripts on the HSV-1 DNA map were precisely identified. To locate the 5' and 3' termini of the 3.0 kb and 1.6 kb early mRNA's, and to determine whether these species are spliced, nuclease protection experiments were performed (23) using subfragments of the BamHI J fragment contained by pRWF6 as probes (Fig. 5). In these analyses, hybrids were formed between early cytoplasmic RNA and DNA fragments uniquely labeled with ^{32}P at either a 5' or 3' terminus. Following hybridization, samples were treated with the single-stranded-specific nucleases S1 or exonuclease VII and the products of digestion were separated by electrophoresis on an alkaline agarose gel. Control experiments, in which yeast tRNA was substituted for early RNA, were also performed to ensure that protection against nuclease digestion was specific for duplex formation with early RNA.

Hybridization to early RNA of the BstEII/BamHI 8 DNA fragment, labeled at the 5' BstEII terminus, resulted in protection of two fragments (1.73 kb and 0.365 kb) against both nuclease S1 and exonuclease VII digestion (Fig. 3). The larger fragment of 3.7 kb present in the exonuclease VII digests probably represents undigested DNA fragment probe. Hybridization of the BstEII/BamHI 8 DNA fragment, 3'-^{32}P-labeled at the BstEII terminus, to early RNA did not result in protection of DNA sequences against nuclease digestion (data not shown). These analyses indicate, therefore, that the direction of transcription of the 3.0 kb and 1.6 kb early mRNA's was from left to right (Fig. 5). As the same size DNA fragments were obtained with both S1 and exonuclease VII, these mRNA's do not appear to be spliced in the 5' regions analysed here. Therefore, the 5' termini of the 3.0 kb and 1.6 kb early mRNA's map 1.73 kbp and 0.365 kbp, respectively, to the left of the BstEII site, as indicated in Fig. 5.

A similar analysis to that above was used to map the 3' termini of the 3.0 kb and 1.6 kb early mRNA's. In this instance, the probe used in the nuclease analyses was a TthI/HpaI DNA fragment (Fig. 5), 3'-^{32}P-labeled at

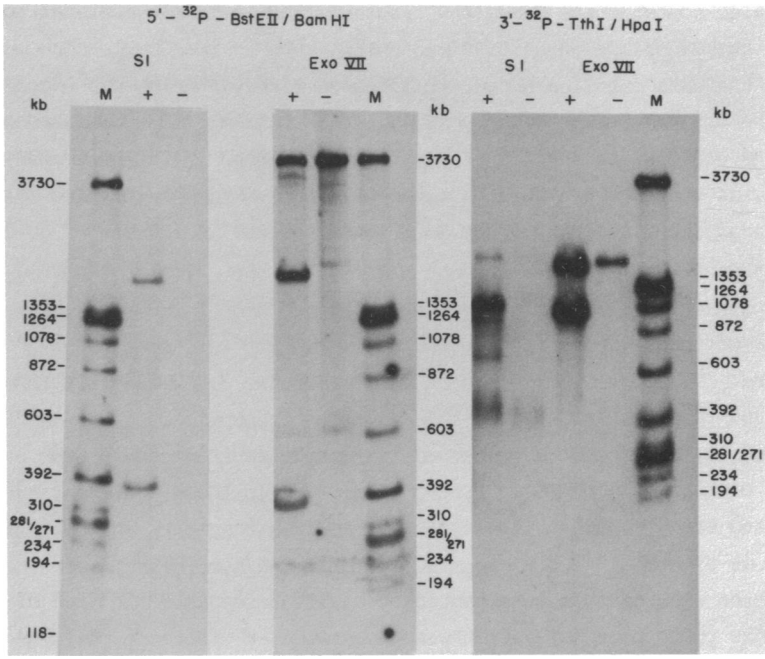

Fig. 3 Analysis of DNA fragments protected against nuclease S1 and exonuclease VII (Exo VII) digestion by electrophoresis on alkaline 1.4% agarose gels. Hybrids were formed between 5'-^{32}P-BstEII/BamHI and 3'-^{32}P-TthI/HpaI DNA fragments and cytoplasmic early RNA (+). Controls in which yeast tRNA was substituted for early RNA are denoted (-). Nuclease treated samples were run in parallel with a mixture of 5'-^{32}P-labeled HaeIII and HpaI fragments of phi X 174 replicative form DNA as markers (M). The size of the DNA markers (in base pairs) are indicated to the sides of the autoradiographs.

the TthI terminus. S1 and exonuclease VII digestion of early RNA/DNA hybrids resulted in protection of a similar 1.1 kb DNA fragment (Fig. 3). Less intense bands (0.95 and 0.73 kb) present in the S1 digest of early RNA/DNA hybrids (Fig. 3) possibly may represent splice points in a minority of the transcripts mapping in this DNA region. It is unlikely that these minor species represent alternative polyadenylation sites, as they are not present in exonuclease VII digests. It is possible also that these minor species are artifactual. These nuclease analyses indicate that the 3' termini of both the 3.0 kb and the 1.6 kb early mRNA's map 1.1 kbp to the right of the TthI site (Fig. 5), and that these mRNA's are unspliced. As the TthI site maps 0.1 kbp to the left of the BstEII site used in mapping the 5' termini, it can be calculated that the transcribed regions of these early mRNA's are 2.73 kb

and 1.365 kb in length. Considering addition of a polyadenylate tail of approximately 0.15 kb (35), these size values are in reasonable agreement with the estimates of 3.0 kb and 1.6 kb obtained by gel electrophoretic analysis. The map locations of these two early mRNA's are summarized in Fig. 5. It can be seen that only the 3.0 kb early mRNA contains the gD coding sequence. The 5' terminus of the 1.6 kb mRNA was estimated to map approximately 100 bp downstream from (3' to) the end of the gD coding sequence.

A further S1 analysis was performed to determine the DNA sequence corresponding to the 5'-terminus of the 3.0 kb early mRNA. In this analysis, an NcoI/SacI DNA fragment (Fig. 5), 5'-^{32}P-labeled at the NcoI terminus, was hybridized to cytoplasmic early RNA. The products of S1 digestion were resolved on a sequencing gel alongside the Maxam and Gilbert (25) sequencing reaction products of the NcoI/SacI DNA fragment. A series of bands was observed with the S1 digest (Fig. 4), and this may reflect, in part, terminal heterogeneity of the products of digestion commonly observed with this enzyme (36). This heterogeneity may also reflect transcription initiation at a number of sites within a defined region. It is apparent from this analysis that the 5' terminus of the 3.0 kb mRNA maps a few bases to the left of the HindIII site (Fig. 5).

Expression of gD in Xenopus laevis oocytes: To develop the gD gene as a model system to study the expression and regulation of a membrane protein, we determined if expression of this gene could be obtained in a convenient *in vitro* system, namely the *Xenopus* oocyte. These experiments involved injection of an RNA preparation containing gD mRNA (early cytoplasmic RNA) into the cytoplasm of *Xenopus* oocytes, and injection of recombinant plasmids containing various fragments of HSV-1 DNA mapping in the U_S region into the nuclei. Proteins synthesized in these oocytes were analysed by immunoprecipitation and SDS polyacrylamide gel electrophoresis.

The specific reagents used to identify the gD protein were the 1S and 4S monoclonal anti-gD antibodies described by Showalter *et al.* (8) Control experiments showing the specificity of the 4S monoclonal reagent are given in Fig. 6A. The monoclonal antibody precipitated two proteins of 51,000 and 49,000 mw from HSV-1-infected Vero cells but not from mock-infected Vero cells. A 42,000 mw protein was precipitated from both mock- and HSV-1-infected cultures. This protein is not HSV-1 specific and probably is actin. These results are consistent with published experiments using the 4S monoclonal antibody (8). A second control experiment is shown in Fig. 6B.

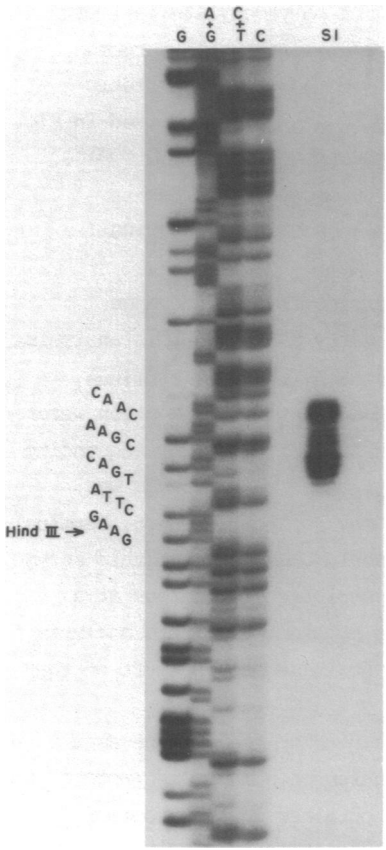

Fig. 4 Identification of the 5' termini of the 3.0 kb early mRNA. The products of S1 nuclease digestion of hybrids formed between cytoplasmic early RNA and the 5'-terminally labeled Nco/SacI DNA fragment were electrophoresed in parallel with the products of the sequencing reactions of that DNA fragment.

Here total poly(A)-containing RNA from HSV-1-infected cells was injected into Xenopus oocytes and the proteins synthesized were labeled with ^{35}S-methionine. The labeled proteins were treated with anti-HSV-1 polyvalent antiserum and the specific HSV proteins that precipitated were analysed in a SDS-polyacrylamide gel (Fig. 6B, lane 1). A number of specific HSV-1 proteins are visible demonstrating the capacity of the oocyte system to translate HSV-1 specific RNA's. A similar experiment is shown in Fig. 6B, lane 2 except that water with no RNA was injected. This control established the background of the system. The specific synthesis of HSV-1 gD in oocytes injected with HSV-1 poly(A)-containing RNA is shown in Fig. 6C. Here the labeled proteins programmed by HSV-1 RNA described in Fig. 6B were treated with the 4S monoclonal antibody and the immunoprecipitated proteins were analysed (Fig. 6C, lane 2). A diffuse band of approximately 50,000 mw was observed, demonstrating that immunoreactive gD was synthesized in Xenopus

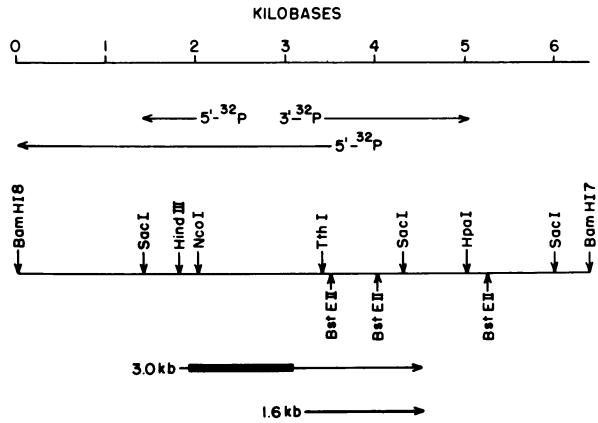

Fig. 5 Summary of the map locations of the 3.0 kb and 1.6 kb early mRNA's. Illustrated is a complete restriction map of the BamHI J fragment contained by pRWF6 for the enzymes SacI, HindIII, TthI, Bst EII and HpaI and a partial map for NcoI. The DNA fragments used in nuclease analyses of the early mRNA's are indicated above the restriction map. The directions of transcription of the 3.0 kb and 1.6 kb early RNAs are indicated. The gD coding sequence of the 3.0 kb early RNA, as determined by Watson et al. (18) is denoted by a thick line.

oocytes injected with HSV-1 RNA. No such band was observed in a similar experiment using oocytes injected with water (Fig. 6C, lane 1). The gD protein precipitated by the 4S monoclonal antibody migrated during electrophoresis as a diffuse band suggesting that processing, probably glycosylation, occurred in the oocytes.

To determine the ability of Xenopus oocytes to synthesize gD following injection of DNA containing the gD coding sequence, three recombinant plasmids (pRWC5, pRWF6 and pRWF7) were first constructed. The HSV-1 DNA fragment inserts contained by each of these plasmids are shown in Fig. 1. The gD coding sequences are present only in pRWF6, which contains the 6.4 kbp BamHI J fragment. Plasmids pRWC5 and pRWF7 contain overlapping HSV-1 DNA fragments mapping to the left of gD (Fig. 1), and thus serve as controls. Each supercoiled plasmid DNA was purified in an isopycnic CsCl gradient and injected into the nuclei of Xenopus oocytes. Proteins synthesized in these oocytes were labeled with ^{35}S-methionine and were analysed by immunoprecipitation and electrophoresis as above. It was found that oocytes injected with pRWF6, but not those injected with either pRWF7 or pRWC5, synthesized immunoprecipitable gD of 52,000 mw (Fig. 7). These results indicate that any regulatory sequences required for expression of gD in oocytes are present within the BamHI J fragment.

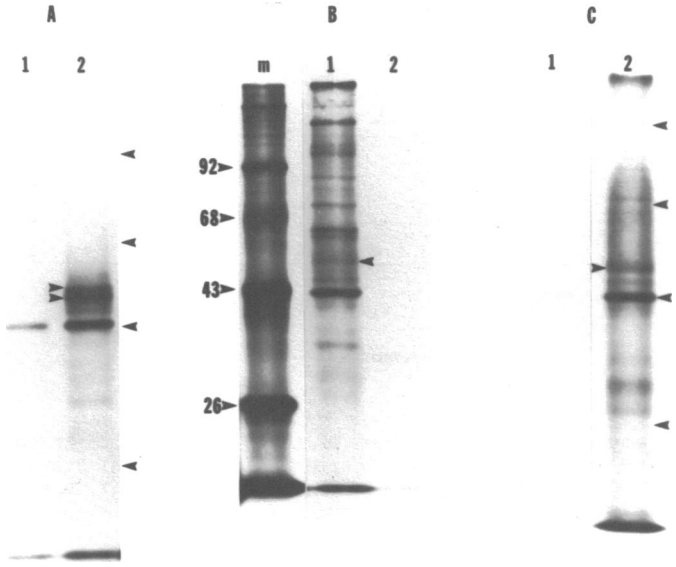

Fig. 6 Synthesis of HSV-1 gD in vitro in Xenopus oocytes.
Panel 1A: Specificity of the 4S gD monoclonal antibodies.
Lane 1 shows ^{35}S-methionine labeled proteins extracted from control cultures and immunoprecipitated by gD monoclonal antibodies (4S).
Lane 2 shows ^{35}S-methionine labeled proteins extracted from HSV-1 infected Vero cells and immunoprecipitated by gD monoclonal antibodies.
The arrows mark the migration of a 51,000 mw and a 49,000 mw polypeptide.
Panel 1B: Translation, in Xenopus oocytes, of mRNA from Vero cells infected with HSV-1.
Lane 1 shows ^{35}S-methionine labeled proteins synthesized in oocytes injected with HSV-1 mRNA. Lane 2 shows ^{35}S-methionine labeled proteins synthesized in oocytes following water injection. Lane m shows ^{14}C-labeled mw protein standards. Values shown are mw x 10^{-3}.
Panel 1C: Synthesis of gD in Xenopus oocytes.
Lane 1 shows ^{35}S-methionine labeled proteins extracted from oocytes injected with H_2O.
Lane 2 shows ^{35}S-methionine labeled proteins extracted from oocytes injected with HSV-1 mRNA. The arrow marks a 49,500 mw polypetide.
The three panels show immunoprecipitated proteins electrophoresed on separate occasions. The mw standards are the same and their positions on each gel are marked with arrow heads.

DISCUSSION

We have mapped two early mRNA's in the 2.4 kbp HindIII/SacI DNA fragment which was previously shown to contain the coding sequence of gD (17,18). These two mRNA's are unspliced and 3' co-terminal but have different 5' termini. Only the larger 3.0 kb mRNA contains gD coding sequences, while the

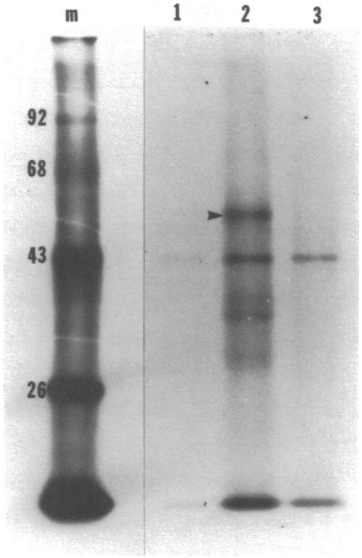

Fig. 7 Production of gD in oocytes injected with cloned HSV-1 DNA.
Lane 1 shows ^{35}S-methionine labeled proteins from oocytes injected with pRWC5 and immunoprecipitated by gD monoclonal antibodies (1S).
Lane 2 shows ^{35}S-methionine labeled proteins from oocytes injected with pRWF6 and immunoprecipitated by gD monoclonal antibodies (1S). The arrow points to a 52,000 mw polypeptide.
Lane 3 shows ^{35}S-methionine labeled proteins synthesized in oocytes injected with pRWF7 and immunoprecipitated by gD monoclonal antibodies (1S).
Lane m shows 92,000, 68,000, and 26,000 mw protein standards.
Values are presented as mw x 10^{-3}.

smaller 1.6 kb mRNA contains sequences which presumably are untranslated from the putative gD mRNA. The gD coding sequence (394 codons) terminates approximately 100 bp upstream of the 1.6 kb early mRNA 5' terminus. The presence of families of 3' co-terminal mRNA's has been previously described for other regions of the HSV-1 genome (37,38,39). In some instances, the smaller members of the family have been shown to specify proteins from coding sequences present in the 3' untranslated region of the larger members (37,40). Thus, it may be anticipated that the 1.6 kb early mRNA contains an open reading frame also present in but not translated from, the 3.0 kb early mRNA. Of relevance to this point, it has been reported that an mRNA encoding a 55,000 mw protein was selected by hybridization with the 2.9 kbp SacI fragment within which the 1.6 kb early mRNA maps (17).

Utilizing in vitro translation of selected HSV-1 mRNA, Lee and coworkers

(17) suggested that the structural gene of gD was located entirely within a HindIII/SacI subfragment of BamHI J (illustrated in Fig. 1). Our results show that the gD mRNA extends beyond the coordinates found by Lee and coworkers (17). Analysis of these mRNA's established that the gD mRNA and the 1.6 kb mRNA extend some 200 bp into the 1.7 kbp SacI fragment. This fragment, however, did not select for gD mRNA (17) suggesting that the small amount of homology was insufficient to select for mRNA's by this procedure.

The nuclease S1 analysis to map the 5' terminus of the 3.0 kb gD mRNA, revealed considerable heterogeneity of the DNA fragments protected by hybridization to this mRNA (Fig. 4). Although some of this observed heterogeneity probably reflects the mode of action of the S1 enzyme (36), the number of bands observed, and the fact that they fell into two distinct size ranges, indicates that multiple mRNA cap sites may be used. It may be relevant to note that the gD mRNA transcribed sequence is proceded by two upstream AT-rich sequences (TATAACAAA and TTTAAAAA; 41,42) that are located 7 bp apart. From the nuclease S1 analysis (Fig. 4) it was predicted that the mRNA cap sites are located in the sequence TTG TTC GGT CAT (the nucleotides corresponding to the most intense bands obtained in this analysis are underlined). It may be noted that the most probable, underlined cap sites are spaced 6 to 8 bp apart. It is therefore conceivable that two TATA boxes are utilized in transcription of gD mRNA, and that these specify cap sites at these different but closely juxtaposed positions.

Although gD is a structural component of the HSV-1 virion, it has been classified on the basis of its kinetics of synthesis as an early or β-polypeptide (10,43). Consistent with this classification, we find that synthesis of the 3.0 kb gD mRNA is unaffected by the presence of cytosine arabinoside, an inhibitor of DNA replication. In a manner similar to that of other HSV-1 early proteins, it would be expected that the gD gene is subject during lytic infection to regulation by the products of immediate early genes (11,44). In spite of such regulation, it was possible to express the gD gene in oocytes. Two other HSV-1 early proteins, thymidine kinase and alkaline exonuclease, were previously reported to be expressed in oocytes (45,46). Our studies differ from these previous studies in that we present evidence from a non-enzymatic assay for the *in vitro* synthesis of an HSV-1 structural protein. This production of gD *in vitro* establishes that the coding sequence as well as any regulatory sequences required for gD expression in oocytes are present within the BamHI J fragment. These findings do not exclude, however, the existence of additional regulatory components necessary for gD expression

during lytic infection of cells by herpesvirus.

During the course of these studies, additional mRNA's were found which map within U_S sequences. One such mRNA, a 2.8 kb mRNA, maps to the right of gD. Lee and coworkers (17) have mapped gE to that region and it seems likely that the 2.8 kb mRNA could specify that protein.

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