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Transcriptional and Translational Analyses of the UL2 Gene of Equine Herpesvirus 1: a Homolog of UL55 of Herpes Simplex Virus Type 1 That Is Maintained in the Genome of Defective Interfering Particles

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Defective interfering particles (DIPs) of equine herpesvirus 1 (EHV-1; Kentucky A strain) mediate persistent infection. DNA sequences at the L terminus, which contain the UL2 gene (homolog of UL55 of herpes simplex virus type 1 and open reading frame 3 of varicella-zoster virus) of standard EHV-1, have been shown to be highly conserved in all clones of the EHV-1 DIP genome. The UL2 mRNA was characterized by S1 nuclease analyses, which mapped the 5′ and 3′ termini of the 0.9-kb early UL2 mRNA to approximately 26 and 16 nucleotides downstream of a TTTAAA box and polyadenylation signal, respectively. The UL2 open reading frame, present within both the EHV-1 standard and DIP genomes, was inserted into the transcription expression vector pGEM-3Z to yield constructs pGEML2 and pDIL2, respectively. After in vitro transcription and translation, both constructs yielded a comigrating 23-kDa protein, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Polyclonal antiserum was raised against the UL2 protein by injecting rabbits with a TrpE/UL2 fusion protein expressed from plasmid pATH23L2 in *Escherichia coli*. The UL2-specific antiserum reacted in Western immunoblot and immunoprecipitation analyses with a 23-kDa polypeptide synthesized in cells infected with standard EHV-1 or DIP-enriched virus. These data also indicated that the UL2 polypeptide was more abundant in DIP-infected cells than in standard EHV-1-infected cells. Results from time course and pulse-chase analyses suggested that the UL2 polypeptide has a rapid turnover rate in DIP-infected cells.

Equine herpesvirus 1 (EHV-1; Kentucky A strain) serves as a model system for investigations into the molecular aspects of persistent herpesvirus infections (for a review, see reference 34). Defective interfering particles (DIPs) of EHV-1 have been shown to mediate the coestablishment of persistent infection and oncogenic transformation in permissive, primary hamster embryo fibroblasts (11, 35, 40, 41). Numerous cell lines that are persistently infected with EHV-1 have been generated and shown to release continuously both standard EHV-1 and EHV-1 DIPs (11, 35, 40). Thus, the identification and function of the genes conserved in the genome of EHV-1 DIPs are of particular interest, since they may play an important role in the establishment and/or maintenance of EHV-1 persistence.

To date, the DIP genome has been shown to be composed of DNA sequences from three distinct regions of the standard EHV-1 genome: region 1, the terminus of the unique long (UL) region; region 2, the central portion of the inverted repeat (IR); and region 3, a small portion of the IR at the UL-IR junction (2–4). DNA sequencing at the L terminus (nucleotides [nt] 1 to 3000) of the Kentucky A strain of EHV-1 revealed the presence of two major open reading frames (ORFs; UL1 and UL2) whose sequences are conserved in the DIP genome (2, 3, 24, 25, 52). The UL1 gene exhibits homology to the ORF2 gene of varicella-zoster virus (VZV; 12) and the *circ* gene of bovine herpesvirus 1 (43). No homolog of UL1 has yet been identified in herpes simplex virus type 1 (HSV-1). Recent transcriptional analyses have identified a UL1-specific 1.2-kb early mRNA possessing a major ORF of 258 amino acids (25). In vitro transcription and translation analyses revealed that the UL1 ORF directs the synthesis of a 30-kDa protein (24). In addition, UL1-specific antiserum has been used to identify and characterize the UL1 polypeptide in cells infected with standard EHV-1 or EHV-1 DIPs (18).

The joining of DNA sequences at the L terminus (region 1) with sequences within the IR (region 2) to generate the DIP genome was shown to occur via a homologous recombination event (51) which resulted in the formation of a unique hybrid ORF (264 amino acids) present only in the DIP genome. The N-terminal 196 codons of the IR4 ORF (homolog of ICP22 of HSV-1) present within region 2 (28) were joined to the C-terminal 68 codons of the UL3 ORF (homolog of ICP27 of HSV-1) present within region 1 (53). Preliminary studies have indicated that this unique hybrid gene is expressed in cells infected with EHV-1 DIPs but is not expressed in cells infected with standard virus alone (19). In addition to the N-terminal portion of the IR4 gene, region 2 also contributes a perfectly conserved and functional origin of DNA replication (5, 50).

Last, DNA sequencing analyses of cloned DIP DNA revealed that region 3 (the UL-IR junction) is joined to the ORI sequences within region 2 (19). The IR sequences at the IR-UL junction, downstream of the immediate-early gene, contain highly repetitive sequences and have been shown to hybridize to a 2.2-kb RNA species present in cells persistently infected with EHV-1. To date, this 2.2-kb RNA

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species has not been detected in cells cytolytically infected with EHV-1 (22).

This report is the first to identify and characterize the mRNA and polypeptide synthesized from the UL2 gene (homolog of UL55 of HSV-1 [33], ORF3 of VZV [12], and ORF4 of EHV-1 Ab4 strain [47]) mapping at the L terminus (49, 52). S1 nuclease analyses were utilized to fine map the UL2 mRNA, whereas the UL2 polypeptide was characterized in cells infected with either standard EHV-1 or DIP-enriched virus, using polyclonal antiserum raised against a TrpE/UL2 fusion protein synthesized in *Escherichia coli*. These data revealed that the UL2-specific mRNA and polypeptide are expressed more abundantly in EHV-1 DIP-infected cells than in standard EHV-1-infected cells. These initial studies to identify and characterize one of the gene products of sequences highly conserved in the DIP genome may lead to a better understanding of the role of DIPs in EHV-1 persistence.

MATERIALS AND METHODS

Virus and Cells. EHV-1 (Kentucky A strain) was grown in murine LM fibroblasts and assayed by plaque titration as described previously (36, 37). EHV-1 virions were purified by polyethylene glycol precipitation and several cycles of rate-velocity banding in dextran-10 or potassium tartrate gradients (37). EHV-1 preparations enriched for DIPs were generated by serial, high-multiplicity passage of EHV-1 in suspension cultures of LM cells, as described previously (11, 26, 35). DIP-enriched virus preparations (passages 63 [3.9×10^7 PFU per ml], 64 [1.9×10^6 PFU per ml], and 69 [9.4×10^7 PFU per ml]) were utilized in these experiments.

Viral DNA clones. Plasmid pGEML2 was constructed by digesting the *Xba*I G fragment of EHV-1 with *Hin*dIII and *Sal*I and ligating this 1,161-bp *Hin*dIII-*Sal*I fragment into a *Hin*dIII-*Sal*I-digested pGEM-3Z vector (Promega Corp., Madison, Wis.). Plasmid pGEML2 contains viral sequences from bp 1796 to 2957 from the L terminus and thus contains the entire UL2 ORF (bp 2825 [5′] to 2223 [3′] (52). Plasmid pDIL2 was constructed by digesting the EHV-1 DIP DNA clone pDIXB2 (3) with *Hin*dIII and *Sal*I and ligating the 1,161-bp fragment into pGEM-3Z (Promega Corp.). Thus, pDIL2 contains the UL2 ORF derived from the DIP genome, whereas pGEML2 contains the UL2 ORF derived from the standard EHV-1 genome. Plasmid pATH23L2 was constructed by digesting pGEML2 with *Sph*I and *Sal*I and ligating the resulting 794-bp fragment containing the C-terminal 122 codons of the UL2 ORF into the pATH23 vector (American Type Culture Collection) in frame with a truncated TrpE ORF (encoding the amino-terminal portion of anthranilate synthase) present in the pATH23 vector (30). All clones were generated by established protocols (32) and were screened by restriction endonuclease analyses and/or Southern blot hybridization (46). Plasmids pGEML2 and pDIL2 were maintained in *E. coli* XL1-Blue (*recA1* Lac$^-$ *endA1 gyrA96 thi hsdR17 supE44 relA1* [F′ *proAB lacI*^{q} *lacZ* ΔM15 Tn*10*]; Stratagene, La Jolla, Calif.). Plasmid pATH23L2 was maintained in *E. coli* TB1 (30). Plasmid DNA minipreparations were isolated by the method of Birnboim and Doly (6), whereas large DNA preparations were prepared by using the Qiagen plasmid isolation kit (Qiagen Inc., Chatsworth, Calif.).

Northern (RNA) blot hybridization. RNA was analyzed by formaldehyde agarose gel electrophoresis and blotted onto GeneScreen Plus filters (New England Nuclear, Boston, Mass.) by the method of Southern (46) as described previously (20, 21, 23). Size markers included 28S (4.9-kb) and 18S (1.9-kb) calf liver rRNA and 23S (2.9-kb) and 16S (1.5-kb) *E. coli* rRNA (Pharmacia, Uppsala, Sweden). Probes were radiolabeled with [^{32}P]dGTP and [^{32}P]dCTP (New England Nuclear) by the nick-translation method of Rigby et al. (38).

Southern blot hybridization. DNA clones were analyzed on nondenaturing agarose gels and blotted onto GeneScreen Plus filters (New England Nuclear) by the method of Southern (46). Probes were radiolabeled with [^{32}P]dGTP and [^{32}P]dCTP (New England Nuclear) by the nick-translation method of Rigby et al. (38).

S1 nuclease analyses. The protocol for S1 nuclease analysis used by this laboratory has been described in detail elsewhere (20, 23, 28). Molecular weight markers included a *Hae*III digest of φX174 DNA and molecular standards VIII purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind.

Isolation of poly(A) RNA. Isolation of poly(A) RNA was accomplished with the Fast Track mRNA isolation kit (Invitrogen Corp., San Diego, Calif.) as described previously (23). To isolate mRNA under early conditions, LM cells were preincubated with 100 μg of phosphonoacetic acid (PAA; Abbott Laboratories, Chicago, Ill.) per ml 1 h before infection. Cells were maintained in the presence of PAA, and mRNA was isolated at 6 h postinfection (p.i.). To isolate mRNA under late conditions, LM cells were lysed and the mRNA was harvested at 8 h p.i. in the absence of metabolic inhibitors.

Isolation of the TrpE/UL2 fusion protein. The pATH23L2 construct was transformed into *E. coli* TB1, and the TrpE/UL2 fusion protein was isolated by the protocol of Koerner et al. (30). Briefly, the pATH23L2-transformed cells were grown overnight at 37°C in the presence of ampicillin (100 μg/ml; Sigma Chemical Co., St. Louis, Mo.) and tryptophan (20 μg/ml; Sigma Chemical Co.). The overnight culture was used to inoculate a 10-ml culture of modified M9 medium containing tryptophan. The culture was grown for 2 to 4 h (midlogarithmic phase) with shaking, and expression of the TrpE/UL2 fusion protein was induced by diluting the culture 10-fold in modified M9 medium lacking tryptophan. After 2 h at 37°C, 3-β-indoleacrylic acid (10 μg/ml; Sigma Chemical Co.), an analog of tryptophan, was added. After incubation for an additional 4 h, the induced cells were harvested at $3,300 \times g$ for 5 min and washed in 10 mM Tris-HCl, pH 7.5. After a second centrifugation step, the cells were resuspended in 50 mM Tris-HCl (pH 7.5)–5.0 mM EDTA–3.0 mg of lysozyme per ml and incubated at 0°C for 2 h. After the addition of 5.0 M NaCl (final concentration of 0.3 M), the cells were lysed completely with Nonidet P-40. The DNA was sheared by sonicating the samples for 30 s at 350 W. The cell lysate was then fractionated into soluble and insoluble fractions by centrifugation at $9,000 \times g$ for 10 min. The insoluble pellet was washed once with 10 mM Tris-HCl (pH 7.5)–1.0 M NaCl and once with 10 mM Tris-HCl, pH 7.5, and then was suspended in 1.0 ml of 10 mM Tris-HCl, pH 7.5. After the addition of 2× Laemmli sample buffer, the insoluble proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% acrylamide). The TrpE/UL2 fusion protein was visualized by staining the gel with 0.25% Coomassie brilliant blue. The gel was then destained with deionized water, and the fusion protein was excised from the gel.

Preparation of TrpE/UL2 antiserum. The gel slice containing the TrpE/UL2 fusion protein was crushed in phosphate-buffered saline, and an equal volume of Freund's complete

adjuvant (Sigma Chemical Co.) was added. Prior to injection of antigen, the rabbits were bled to obtain preimmune serum. Two milliliters of the TrpE/UL2 slurry was injected (0.5 ml per leg) into two male New Zealand White rabbits. Booster injections were administered at approximately 2-week intervals. The TrpE/UL2 antiserum was isolated and prepared as described by Harlow and Lane (17).

Isolation of infected cell extracts. At 8 h p.i., LM cell monolayers (2×10^6 cells) were washed twice with cold phosphate-buffered saline and lysed with 0.5 ml of radioimmunoprecipitation assay–1.0% SDS buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 1.0% SDS, 0.5% deoxycholate, and 1.0% Nonidet P-40) with protease inhibitors (aprotinin, 50 μg/ml; leupeptin, 50 μg/ml; and phenylmethylsulfonyl fluoride, 300 μg/ml). Cells radiolabeled with [^{35}S]methionine were preincubated for 1 h in methionine-free medium and labeled at the appropriate time with [^{35}S]methionine (50 μCi/ml; New England Nuclear). Infected cell extracts were stored at −70°C until use.

Immunoprecipitation and SDS-PAGE. One hundred microliters (4×10^5 cell equivalents) of [^{35}S]methionine-labeled cell extracts was incubated with 15 to 20 μl of preimmune antiserum at 4°C overnight on a rocker platform. Six volumes (120 μl) of protein A-coupled beads (Repligen Corp., Cambridge, Mass.) was added to the samples, and the samples were incubated an additional 1 to 2 h at 4°C. The protein A beads were pelleted, and the cleared supernatant was removed to a new tube. The supernatant was then incubated with 15 to 20 μl of UL2-specific antiserum at 4°C overnight on a rocker platform. After treatment with the protein A beads as described above, the beads were washed four times with 1.0 ml of cold radioimmunoprecipitation assay buffer. The antigen was then eluted from the beads by adding 40 μl of 1× Laemmli sample buffer (62.5 mM Tris-HCl [pH 6.8], 2.0% SDS, 10.0% glycerol, 5.0% 2-mercaptoethanol, and 0.1% bromophenol blue) and boiled for 5 min. The beads were pelleted, and the supernatant was fractionated by SDS-PAGE. SDS-PAGE was performed as described previously (23, 24). The samples were fractionated through a 5% stacking gel and a 10% resolving gel. ^{14}C-labeled molecular weight standards were purchased from Bethesda Research Laboratories, Gaithersburg, Md.

Western immunoblot analysis. Immunoblot analyses were performed essentially as described previously (44). After SDS-PAGE analysis, proteins were electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, N.H.) at 30 V overnight by using a transblot cell (BioRad Laboratories, Richmond, Calif.) containing approximately 2.5 liters of transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). The following day, the filters were rinsed in TBST buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) for 5 min and then rinsed in blocking solution (0.5% gelatin) for 30 min. The filters were then transferred to a solution of TBST containing the appropriate dilution of UL2 antiserum (1:10,000 to 1:15,000) and incubated for 30 min while rocking. The filters were washed extensively in several changes of TBST and then added to a solution of TBST containing a 1:7,500 dilution of the secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G; Promega). After a 30-min incubation, the filters were again washed extensively in TBST buffer. The reactive proteins were visualized by submerging the filter in AP buffer (0.1 M Tris-HCl [pH 9.5], 0.1 M NaCl, 5.0 mM $MgCl_2$) containing nitroblue tetrazolium (0.33 mg/ml; Bethesda Research Laboratories) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 0.165 mg/ml; Bethesda Research Laboratories).

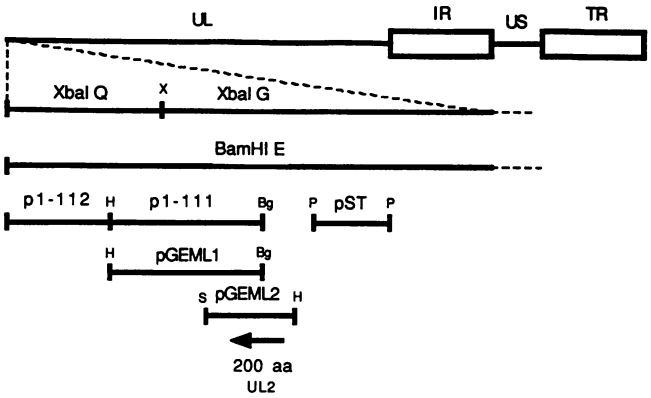

FIG. 1. Diagram of the EHV-1 genome (top), with the UL2 gene region expanded below. Several EHV-1 clones mapping at the L terminus are shown, including *Xba*I Q, *Xba*I G, *Bam*HI E, p1-112, p1-111, pST, pGEML1, and pGEML2. The UL2 ORF (nt 2825 to 2225) of 200 amino acids (arrow) lies within the pGEML2 clone. Abbreviations: UL, unique long; IR, inverted repeat; US, unique short; TR, terminal repeat; Bg, *Bgl*II (nt 2513); H, *Hin*dIII (nt 1044 and 2957); P, *Pst*I (nt 3115 and 3808); S, *Sal*I (nt 1796); and X, *Xba*I (nt 1436).

Pulse-chase analysis. Monolayers of LM cells (2×10^6 cells) were infected with DIP passage 64 (multiplicity of infection, 2.0 PFU per cell), and the virus was allowed to attach for 2 h at 37°C. At 4 h p.i., the medium was removed and 5.0 ml of L-methionine-free medium with 2.0% newborn calf serum was added to the monolayers. At 5 h p.i., the monolayers were pulse-labeled for 30 min with [^{35}S]methionine (50 μCi/ml; New England Nuclear). At 5.5 h p.i., the monolayers were washed two times with chase medium containing 2.0% serum and excess cold L-methionine (15 mg/liter; Sigma Chemical Co.). The infection was allowed to proceed (in chase medium), and the monolayers were harvested at the indicated times. The cell extracts were immunoprecipitated and analyzed by SDS-PAGE as described above.

In vitro transcription and translation. The protocols used for in vitro transcription and translation have been described previously (23, 24, 44).

Hybrid-arrest translation. The protocol of Haeuptle et al. (16) was utilized as described previously (24). Oligonucleotide L2 (Synthetic Genetics, San Diego, Calif.) is a 30-mer with the sequence 5′-GCTGTAACACCCGCCGTGGTTCC TCCCGTG-3′ corresponding to nt 2736 (5′) to 2765 (3′) from the L terminus of the genome. Oligonucleotides 4 and 5 (Synthetic Genetics) are both 20-mers derived from sequences located within the immediate-early (IE) gene of EHV-1 (bp 1030 to 1049 and 468 to 449, respectively) (15).

RESULTS

S1 nuclease analyses to map the termini of the UL2 mRNA. Previous Northern blot analyses of mRNA synthesized from the UL2 gene region (Fig. 1) suggested that UL2 encodes an approximately 0.9-kb polyadenylated mRNA in the presence of PAA (25). To characterize further the UL2-specific mRNA, S1 nuclease analyses were performed to map the 5′ and 3′ termini of the UL2 transcript. To position the 5′ terminus, mRNA isolated from mock-infected or EHV-1-

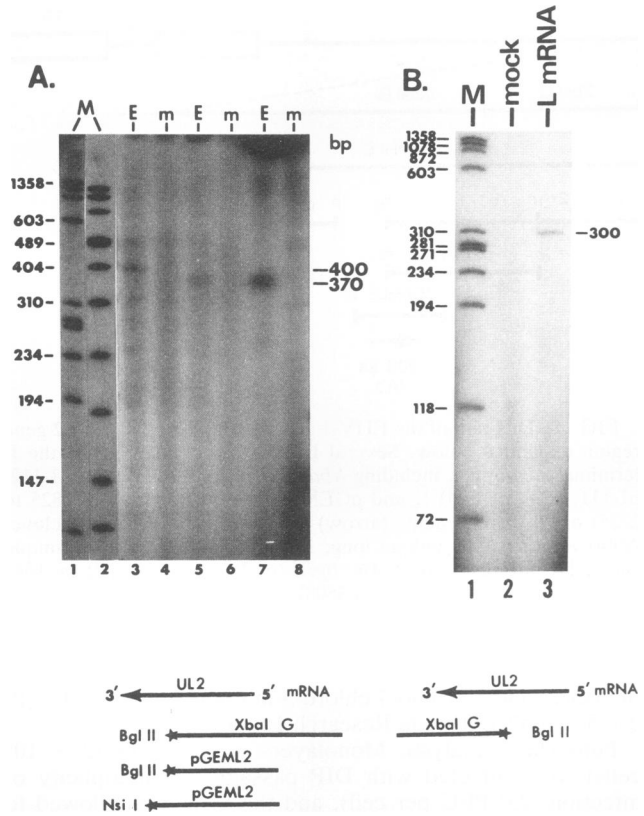

FIG. 2. S1 nuclease analyses at the 5′ and 3′ termini of the UL2 mRNA. Diagrams at the bottom (not to scale) represent the probes used to map the 5′ and 3′ termini (*, [γ-^{32}P]ATP radiolabel). (A) Plasmid pGEML2 was 5′ end labeled at either the *Nsi*I site (nt 2486) (lanes 3 and 4) or the *Bgl*II site (nt 2513) (lanes 5 and 6) and hybridized to early (lanes E; 6 h p.i. in the presence of PAA) or mock (lanes m) mRNA at 60°C. Plasmid *Xba*I G, 5′ end labeled at the *Bgl*II site, was also used (lanes 7 and 8). S1 nuclease-resistant fragments were analyzed on a 6% polyacrylamide–urea gel. Molecular weight standards (M) included DNA fragments from a *Hae*III digest of φX174 (lane 1) and molecular standards VIII (lane 2) purchased from Bethesda Research Laboratories. The gel was overdeveloped to visualize the S1-resistant fragments. (B) Plasmid *Xba*I G was 3′ end labeled at the *Bgl*II site (nt 2513) with [^{32}P]dGTP, [^{32}P]dCTP, and the Klenow fragment. After hybridization of the 3′ end-labeled probe to mock (lane 2) or late (lane 3; 8 h p.i. with no inhibitors) mRNA and digestion with S1 nuclease, the S1-resistant fragments were fractionated on a 6% polyacrylamide–urea gel. Molecular weight (M) standards (*Hae*III digest of φX174) are shown in lane 1.

infected (6 h p.i. in the presence of PAA) cells was hybridized to one of three different probes: plasmid pGEML2 5′ end labeled at the *Nsi*I site (nt 2486; Fig. 2A, lanes 3 and 4), plasmid pGEML2 5′ end labeled at the *Bgl*II site (nt 2513; Fig. 2A, lanes 5 and 6), and plasmid *Xba*I G 5′ end labeled at the *Bgl*II site (nt 2513; Fig. 2A, lanes 7 and 8). After digestion with S1 nuclease, a protected fragment of approximately 400 bp was observed with early mRNA hybridized to the probe 5′ end labeled at the *Nsi*I site (Fig. 2A, lane 3), while a fragment of approximately 370 bp was observed with early mRNA hybridized to either pGEML2 5′ end labeled at the *Bgl*II site (Fig. 2A, lane 5) or *Xba*I G 5′ end labeled at the *Bgl*II site (Fig. 2A, lane 7). Fragments corresponding to these sizes were not observed in the adjacent lanes containing mock mRNA (Fig. 2A, lanes 4, 6, and 8). These results positioned the transcription initiation site of the UL2 mRNA between nt 2883 and 2886 from the L terminus. The UL2 transcription initiation site mapped 26 to 29 bp downstream of a potential TATA box (nt 2917 to 2912), with the sequence TTTAAA identified by DNA sequencing analysis (52). The initiation codon for the UL2 ORF lies at nt 2825, and thus the untranslated leader region at the 5′ terminus of the UL2 mRNA is 58 to 61 nt in length.

To map the 3′ terminus of the UL2 mRNA, mock or late viral mRNA was hybridized to the *Xba*I G clone 3′ end labeled at the *Bgl*II site (Fig. 1). After S1 nuclease digestion, a protected fragment of approximately 300 bp was observed with late viral mRNA (Fig. 2B, lane 3) but not with mock mRNA (Fig. 2B, lane 2). These results mapped the transcription termination site to lie at approximately nt 2213 from the L terminus. Thus, the transcription termination site for the UL2 mRNA mapped approximately 16 nt downstream of a consensus polyadenylation signal sequence (AATAAA; nt 2234 to 2229). Therefore, on the basis of these S1 nuclease data, the size of the UL2 mRNA (0.9 kb) is in good agreement with that reported with Northern blot analysis (25).

Cloning and expression of the UL2 ORF. The predicted molecular size of the UL2 polypeptide based on sequencing analyses is 22,380 Da. To determine whether a protein of this size is synthesized from the UL2 ORF, the UL2 ORF was cloned into the transcription expression vector pGEM-3Z (Fig. 1 and 3A). The *Hin*dIII-*Sal*I fragment containing the entire UL2 ORF from the standard genome was inserted downstream of the Sp6 bacteriophage promoter to generate the 3.9-kb construct pGEML2 (Fig. 1 and 3A). The identity of the insert in pGEML2 was confirmed by restriction endonuclease analyses with several different restriction endonucleases (Fig. 3B, lanes 2 to 5). To express a UL2-specific RNA in vitro, pGEML2 was first digested with *Sal*I and the linearized template was added to an in vitro transcription reaction containing the Sp6 polymerase. As expected, a 1.2-kb UL2-specific RNA was transcribed from pGEML2 (Fig. 3C). Northern blot analyses revealed that the 1.2-kb RNA hybridized to probes pGEML1 (Fig. 3C, lane 1) and p1-111 (Fig. 3C, lane 2) but not to probe p1-112 (Fig. 3C, lane 3) or the pGEM-3Z vector (Fig. 3C, lane 4). Thus, the pGEML2-derived RNA contained the UL2 ORF from the standard virus genome and could be used in in vitro translation experiments to identify the UL2 protein.

After in vitro translation of the pGEML2-derived RNA in a rabbit reticulocyte lysate containing [^{35}S]methionine, the resultant polypeptides were analyzed by SDS-PAGE (Fig. 4). In vitro translation of pGEML2-derived RNA yielded a protein of 23 kDa (Fig. 4, lanes 4 and 7) which was not present in translation reactions lacking pGEML2-derived RNA (lane 3). The size of the UL2 polypeptide translated from pGEML2-derived RNA was in excellent agreement with that predicted by DNA sequencing analyses. Hybrid-arrest translation was performed to confirm that the 23-kDa protein was indeed translated from the UL2 ORF. pGEML2-derived RNA was first hybridized to 2 or 3 μg of oligonucleotide L2 (Materials and Methods), which is antisense and complementary to sequences within the UL2 ORF, and then translated in vitro. Synthesis of the 23-kDa protein was dramatically reduced after hybridization to either 2 or 3 μg of oligonucleotide L2 (Fig. 4, lanes 5 and 6, respectively). In contrast, synthesis of the 23-kDa protein was not reduced significantly after hybridization to 3 μg each of two oligonucleotides that were not complementary to UL2 sequences

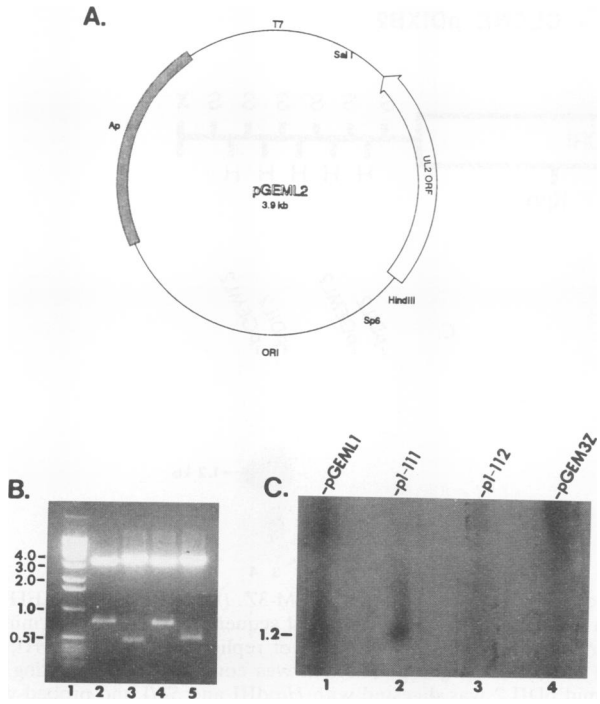

FIG. 3. Cloning of the UL2 ORF into the transcription expression vector pGEM-3Z. (A) Diagram of the pGEML2 construct (3.9 kb), showing the 1.2-kb *Hin*dIII-*Sal*I fragment (nt 2957 to 1796) containing the UL2 ORF cloned behind the bacteriophage Sp6 promoter. Abbreviations: T7, bacteriophage T7 promoter; ORI, origin of replication; and Ap, ampicillin resistance gene. (B) Restriction endonuclease analyses of the pGEML2 construct. Lanes: 1, molecular weight standards (Bethesda Research Laboratories); 2, pGEML2 digested with *Bgl*II (nt 2513) and *Sal*I (nt 1796); 3, pGEML2 digested with *Hin*dIII (nt 2957) and *Bgl*II (nt 2513); 4, pGEML2 digested with *Nsi*I (nt 2486) and *Sal*I (nt 1796); and 5, pGEML2 digested with *Nsi*I (nt 2486) and *Hin*dIII (nt 2957). Molecular standards are in kilobase pairs. (C) Northern hybridization of UL2-specific RNA transcribed in vitro from a *Sal*I-digested pGEML2, using the Sp6 polymerase. Probes included pGEML1, p1-111, p1-112, and pGEM-3Z.

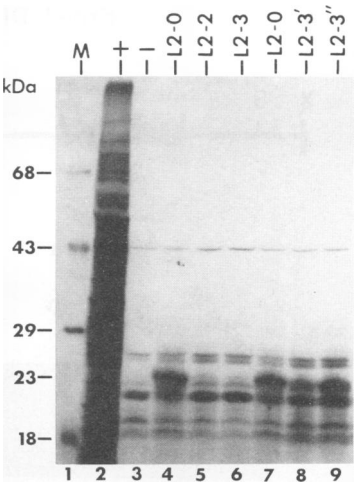

FIG. 4. In vitro translation and hybrid-arrest translation of pGEML2-derived RNA. pGEML2-derived RNA was translated in vitro for 60 min in a rabbit reticulocyte lysate (Promega) before (lanes 4 and 7; L2-0, no oligonucleotide added) or after hybridization with UL2-specific (lanes 5 [2 μg of oligonucleotide L2] and 6 [3 μg of oligonucleotide L2]) or nonspecific (L2-3′, 3 μg of oligonucleotide 4; and L2-3″, 3 μg of oligonucleotide 5) oligonucleotides. Lane 1 represents ^{14}C-labeled protein markers (Bethesda Research Laboratories). Lane 2 represents positive-control mRNA supplied with the translation kit, and lane 3 represents a negative control (no pGEML2-specific RNA added).

but were complementary to IR sequences (oligonucleotides 4 and 5, Materials and Methods; Fig. 4, lanes 8 and 9, respectively). Thus, UL2-specific RNA derived from pGEML2 was translated in vitro to yield a 23-kDa polypeptide which was shown by hybrid-arrest translation to be the product of the UL2 ORF.

Cloning of the UL2 ORF from the EHV-1 DIP genome. Previously, restriction endonuclease and Southern blot hybridization analyses have shown that the UL2 gene region is conserved in all DIP DNA clones isolated to date (3). To determine whether the UL2 gene present in the DIP genome could also direct the synthesis of a 23-kDa protein in vitro, the *Hin*dIII-*Sal*I fragment (as described above) was isolated from DIP DNA clone pDIXB2 (Fig. 5A) and inserted into the pGEM-3Z vector to generate clone pDIL2. Southern blot hybridization (Fig. 5B) confirmed that the pDIL2 construct contained UL2-specific sequences, because it hybridized to probes pGEML2 and pDIXB2 (lanes 2 and 3, respectively) but not to probe pGEM-3Z (lane 1), pST (lane 4), or p1-119 (lane 5). Last, Northern blot hybridization of RNA transcribed in vitro from either pGEML2 or pDIL2 indicated that both constructs yielded a 1.2-kb RNA species that hybridized to probe pGEML2 (Fig. 5C, lanes 3 and 4) but not to probe pGEM-3Z (Fig. 5C, lanes 1 and 2).

Generation of a TrpE/UL2 fusion protein and UL2-specific polyclonal antiserum. To identify and characterize the UL2 protein in cells infected with standard EHV-1 or EHV-1 DIPs, it was necessary to generate UL2-specific polyclonal antiserum. This was accomplished by generating a TrpE/UL2 fusion protein in *E. coli*. The pATH23 vector contains the inducible TrpE promoter and a truncated TrpE ORF capable of encoding a 37-kDa TrpE protein (30). The TrpE/UL2 fusion protein was synthesized from a hybrid ORF generated by joining the C-terminal portion (C-terminal 122 codons) of the UL2 ORF in frame with the TrpE ORF present in the pATH23 vector to yield the pATH23L2 construct (Materials and Methods). Cell extracts from TB1 cells alone (Fig. 6A, lane 3), TB1 cells transformed with pATH23 and grown under noninducing conditions (Fig. 6A, lane 2) and TB1 cells transformed with pATH23 and grown under inducing conditions (Fig. 6A, lane 1) were analyzed by SDS-PAGE, and the proteins were stained with Coomassie blue. The 37-kDa TrpE protein was observed in TB1 cells that received pATH23 and were grown under inducing conditions (Fig. 6A, lane 1) but not in TB1 cells grown under noninducing conditions (lane 2). These results demonstrate that the TrpE promoter was functional and capable of being induced. TB1 cells transformed with pATH23L2 and grown under inducing conditions clearly overexpressed the TrpE/UL2 fusion protein of approximately 45 to 47 kDa (Fig. 6B, lane 2). The TrpE/UL2 fusion protein was not observed in uninduced TB1 cells containing pATH23L2 (Fig. 6B, lane 3) nor was it observed in the control cell extracts (Fig. 6A, lanes 1, 2, and 3). The TrpE/UL2 fusion protein was subsequently used as an immunogen to generate UL2-specific polyclonal antiserum.

Immunoprecipitation of the in vitro- and in vivo-synthesized

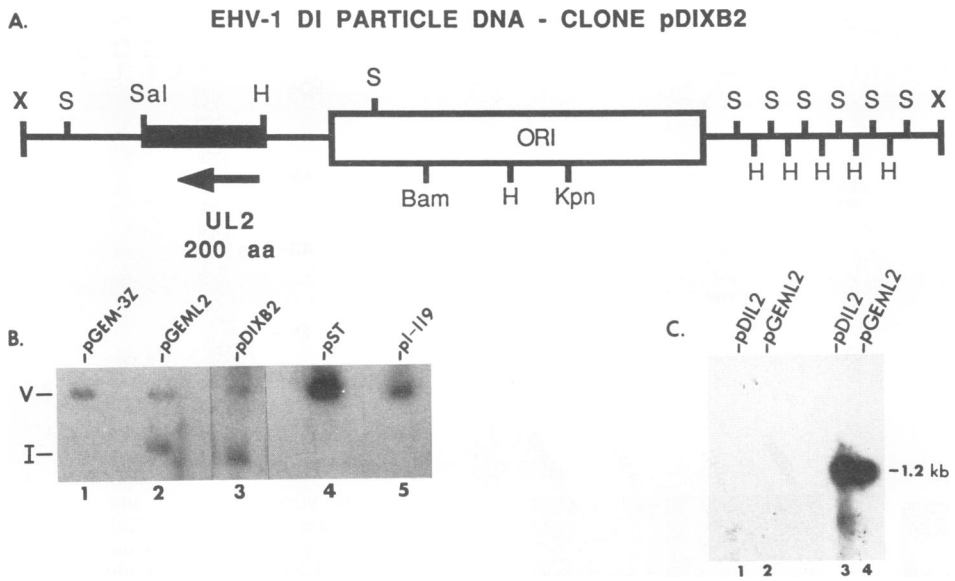

FIG. 5. Cloning of the UL2 ORF from EHV-1 DIP DNA into the transcription expression vector pGEM-3Z. (A) Diagram of the EHV-1 DIP DNA clone pDIXB2 (3), showing the position of the UL2 ORF within this clone. Solid lines represent sequences from the L terminus, while the open box represents sequences from the inverted repeat. Abbreviations: ORI, EHV-1 origin of replication; Bam, *Bam*HI; H, *Hin*dIII; Kpn, *Kpn*I; Sal, *Sal*I; S, *Sma*I; and X, *Xba*I. (B) Southern blot analyses of clone pDIL2 that was constructed by inserting the *Hin*dIII-*Sal*I fragment from pDIXB2 (shown above) into pGEM-3Z. Plasmid pDIL2 was digested with *Hin*dIII and *Sal*I and probed with pGEM-3Z, pGEML2, pDIXB2, pST, and p1-119 (p1-119 maps within the IR region). Note: Lane 3 needed to be overexposed to visualize the bands. Abbreviations: V, vector; I, insert. (C) Northern blot of RNA transcribed in vitro from either pDIL2 or pGEML2 and probed with pGEM-3Z (lanes 1 and 2) or pGEML2 (lanes 3 and 4).

UL2 polypeptide. To demonstrate the specificity of the TrpE/UL2 antiserum, cell extracts from nontransformed TB1 cells, TB1 cells transformed with pATH23 (inducing conditions), and TB1 cells transformed with pATH23L2 (inducing conditions) were immunoprecipitated with the TrpE/UL2 antiserum and analyzed by SDS-PAGE and Coomassie blue staining (Fig. 6C). As expected, the 37-kDa TrpE protein was detected by the TrpE/UL2 antiserum in extracts of TB1 cells transformed with pATH23 (Fig. 6C, lane 2) but not in extracts of untransformed TB1 cells (lane 1). Furthermore, the TrpE/UL2 fusion protein was detected in extracts of TB1 cells transformed with pATH23L2 (lane 3) but not in extracts of TB1 cells (lane 1) or TB1 cells transformed with pATH23 (lane 2). Thus, these data demonstrate the specificity of the TrpE/UL2 antiserum, since it reacted with the TrpE/UL2 fusion protein.

To confirm that the TrpE/UL2 antiserum could recognize the UL2 polypeptide, in vitro-translated products synthesized from pGEML2-derived RNA and pDIL2-derived RNA were immunoprecipitated with the UL2 antiserum (Fig. 7). Indeed, the TrpE/UL2 antiserum reacted with the 23-kDa UL2 polypeptide encoded by both pGEML2-derived RNA (Fig. 7, lane 4) and pDIL2-derived RNA (lane 7). The immunoprecipitated 23-kDa polypeptide comigrated with the in vitro-translated 23-kDa protein (nonimmunoprecipitated) (compare lanes 4 and 7 with lanes 12 and 13). The TrpE/UL2 antiserum did not react with a 23-kDa protein in those samples that did not receive UL2-specific RNA (Fig. 7, lane 6). In addition, preimmune serum also did not react with the 23-kDa protein encoded by pGEML2-derived RNA (lane 5) or pDIL2-derived RNA (lane 8). As an additional negative control, antiserum raised against the IR4 protein of EHV-1 as a TrpE/IR4 fusion protein (27) was utilized and, as expected, was shown not to react with the in vitro-translated 23-kDa protein (Fig. 7, lanes 9, 10, and 11). Thus, both the standard UL2 gene and the DIP-encoded UL2 gene direct the synthesis of a 23-kDa protein that is recognized by the TrpE/UL2 antiserum.

To demonstrate that the TrpE/UL2 antiserum reacted with the UL2 polypeptide synthesized in EHV-1-infected cells, equal amounts (4×10^5 cell equivalents) of cell extracts obtained from mock-infected LM cells, EHV-1-infected cells, and cells infected with DIP-enriched virus (passage 64) were immunoprecipitated with the TrpE/UL2 antiserum (Fig. 8A). Indeed, a 23-kDa polypeptide was immunoprecipitated from cells infected with standard EHV-1 (Fig. 8B, lane 5) and from cells infected with both DIP passage 64 (Fig. 8A, lane 3) and passage 69 (data not shown). The 23-kDa protein was not observed in mock-infected cells (lane 2) or in EHV-1-infected cell extracts immunoprecipitated with preimmune serum (lane 4). Interestingly, an exposure time of approximately 1 week was required to detect the UL2 polypeptide synthesized in cells infected with standard EHV-1 (lane 5); however, the UL2 protein was visible in cells infected with either DIP 64 or 69 after an overnight exposure of the gel (lane 3). These data suggest that the UL2 protein is expressed at high levels in DIP-infected cells compared with its level of expression in standard EHV-1-infected cells.

Pulse-chase and time course analyses of the UL2 polypeptide. Western blot analyses were utilized in an attempt to detect more readily the UL2 protein from a standard EHV-1 infection and also to examine the time course of UL2 synthesis. The 23-kDa protein was detected in EHV-1-infected cell extracts isolated at 8 h p.i. (Fig. 9A, lane 2). However, to observe the UL2 protein in cells infected with standard EHV-1, it was necessary that the blot be overdeveloped and that the volume of the sample from standard

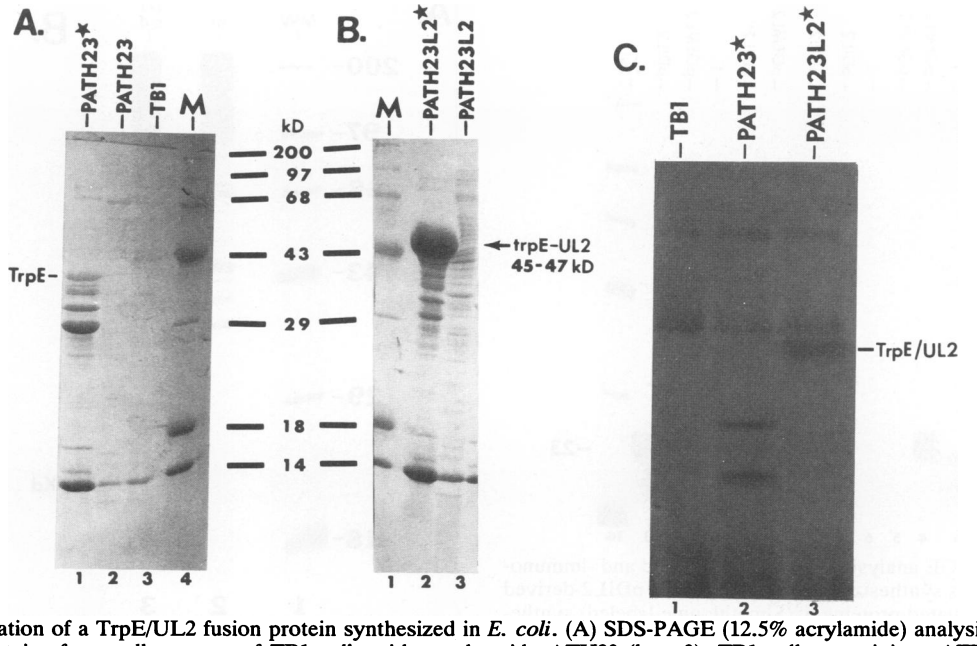

FIG. 6. Identification of a TrpE/UL2 fusion protein synthesized in *E. coli*. (A) SDS-PAGE (12.5% acrylamide) analysis and Coomassie blue staining of proteins from cell extracts of TB1 cells without plasmid pATH23 (lane 3), TB1 cells containing pATH23 grown under noninducing conditions (lane 2), and TB1 cells containing pATH23 grown under inducing (★) conditions (lane 1). Protein standards (M) are shown in lane 4. (B) SDS-PAGE (12.5% acrylamide) analysis and Coomassie blue staining of proteins from cell extracts of TB1 cells containing the pATH23L2 construct grown under inducing (★) conditions (lane 2) and TB1 cells containing the pATH23L2 construct grown under noninducing conditions (lane 3). Protein standards (M) are shown in lane 1. (C) SDS-PAGE (10.0% acrylamide) analysis of proteins immunoprecipitated with the TrpE/UL2-specific antiserum and then stained with Coomassie blue. Cell extracts immunoprecipitated with the TrpE/UL2 antiserum include TB1 cells (lane 1; without any plasmids), TB1 cells containing pATH23 grown under inducing (★) conditions (lane 2), and TB1 cells containing pATH23L2 grown under inducing (★) conditions (lane 3).

EHV-1-infected cell extracts (20 μl; Fig. 9A, lane 2) be double that of the DIP-infected cell extracts (10 μl; Fig. 9B, lanes 3 to 6), even though the protein concentration in both samples was virtually identical (2.0 and 2.3 μg/μl, respectively). Cell extracts from DIP passage 64-infected cells were isolated at 2, 4, 6, 8, 10, and 12 h p.i. and reacted with the TrpE/UL2 antiserum (Fig. 9B, lanes 1 to 6). The UL2 protein was first observed very weakly at 4 h p.i. and increased in abundance up to 12 h p.i. The UL2 protein was not observed in mock-infected cells (lane 1). These data are consistent with the placement of the UL2 gene in the early kinetic class and again demonstrate its high level of expression in cells infected with DIP-enriched virus.

Pulse-chase analysis was performed to address the stability of the UL2 protein during EHV-1 infection. Mock-infected or DIP-infected LM cells were pulse-labeled for 30 min with [^{35}S]methionine at 5 h p.i. and then chased with excess cold methionine for up to 48 h (Fig. 9C). Extracts containing equal amounts of protein were incubated with either the TrpE/UL2 antiserum (Fig. 9C, lanes 1 to 6) or with preimmune serum (lane 7), and the immunoprecipitated proteins were analyzed by SDS-PAGE. The UL2 protein was not observed in mock-infected cells (lane 1) and was not detected in infected cell extracts immunoprecipitated with preimmune serum (lane 7). The [^{35}S]methionine-labeled UL2 protein steadily decreased in abundance as the time of chase increased (lanes 2 to 6). The data shown in Fig. 9 suggest that the UL2 protein has a rapid turnover rate. This rate of turnover of the UL2 protein is considered as rapid as the rate of turnover of the EHV-1 UL1 protein which is also produced in large quantities in persistent EHV-1 infection (18).

S1 nuclease analyses to compare the levels of UL2 mRNA in standard EHV-1 versus DIP-infected cells. Since the UL2 protein was more abundant in DIP-infected cells than in standard EHV-1-infected cells, S1 nuclease analyses were utilized to determine the levels of UL2 mRNA synthesis in DIP- and standard EHV-1-infected cells. In all reactions, 3.0 μg of the appropriate mRNA sample was used. Clone pGEML2 was 5′ end labeled at its unique *Bgl*II site and hybridized to mRNA isolated from mock-, standard EHV-1-, or EHV-1 DIP-infected cells (passages 63 and 69; Fig. 10A). As expected, after digestion with S1 nuclease, a 370-bp protected fragment was detected in EHV-1- and EHV-1 DIP-infected cells (Fig. 10A, lanes 3 to 5) but not in mock-infected cells (lane 2). Although the intensity of the signal varied between cells infected with DIP passage 64 (lane 4) and passage 69 (lane 5), both DIP passages yielded a stronger signal than that observed for cells infected with standard EHV-1 (lane 3). Some additional minor fragments were observed in the DIP-infected cells which may be derived from altered forms of the UL2 mRNA in these DIP-infected cells or from aberrant S1 nuclease digestion.

A similar result was observed with a 3′ end-labeled probe (Fig. 10B). A major S1-resistant fragment of approximately 300 bp was observed in EHV-1- and EHV-1 DIP-infected cells (Fig. 10B, lanes 3 to 5) but not in mock-infected cells (lane 2). Once again, the intensity of the signal was much greater in the DIP-infected cells than in cells infected with standard EHV-1 (compare lanes 4 and 5 with lane 3 in Fig. 10B). These results, in conjunction with the protein data, indicate that the level of expression for both the UL2 mRNA and polypeptide is much greater in cells infected with DIP-enriched preparations of EHV-1 than in cells infected with standard EHV-1. Furthermore, these results suggest

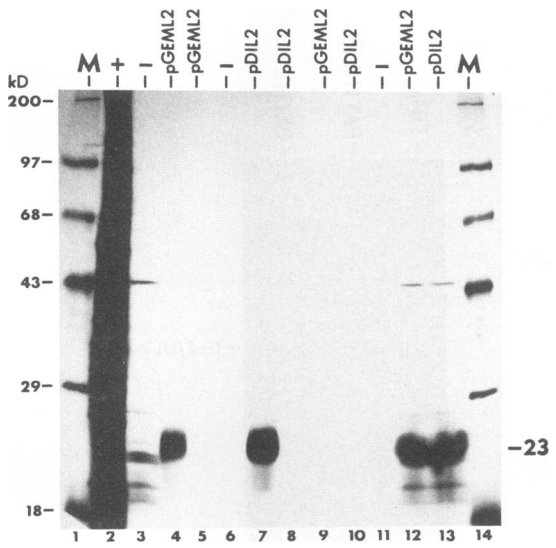

FIG. 7. SDS-PAGE analysis of in vitro-translated and -immunoprecipitated proteins synthesized from pGEML2- or pDIL2-derived RNA. In vitro-translated proteins ([^{35}S]methionine labeled) synthesized from pGEML2-derived RNA were fractionated on a 10% SDS-polyacrylamide gel (lane 12) or were first immunoprecipitated with the TrpE/UL2 antiserum (lane 4), preimmune serum (lane 5), or TrpE/IR4 antiserum (lane 9; 27) before electrophoresis. Similarly, in vitro-translated proteins synthesized from pDIL2-derived RNA were directly fractionated by electrophoresis (lane 13) or were first immunoprecipitated with the TrpE/UL2 antiserum (lane 7), preimmune serum (lane 8), or TrpE/IR4 antiserum (lane 10) before electrophoresis. In vitro translation reaction mixtures lacking any exogenous RNA were immunoprecipitated with both the TrpE/UL2 and TrpE/IR4 antisera (lanes 6 and 11, respectively). Lanes 1 and 14 contain protein standards (M; Bethesda Research Laboratories). Lane 2 represents a positive control (Adenovirus mRNA); lane 3 represents a negative control (no exogenous mRNA).

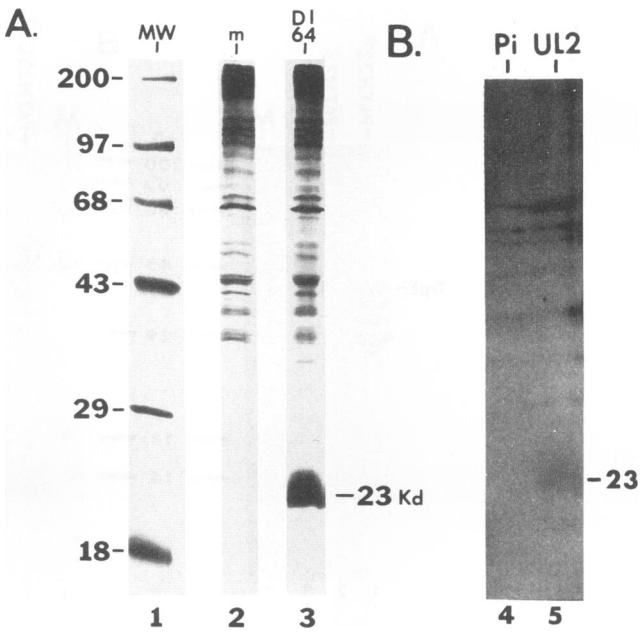

FIG. 8. Immunoprecipitation analyses of [^{35}S]methionine-labeled cell extracts. Cells were labeled from 6 to 8 h p.i. and were harvested at 8 h p.i. (A) Cell equivalents (4×10^5) from mock-infected (lane 2) or DIP passage 64-infected cells (lane 3) were incubated with the TrpE/UL2 antiserum, and the immunoprecipitated proteins were analyzed by SDS-PAGE. Lane 1 represents ^{14}C-labeled molecular weight standards. The gel was exposed for 20 h. (B) Cell equivalents (4×10^5) from standard EHV-1-infected cells (8 h p.i.) were incubated with either preimmune sera (lane 4) or TrpE/UL2 antiserum (lane 5), and the immunoprecipitated proteins were analyzed by SDS-PAGE. The gel was exposed for 1 week.

that the UL2 transcription initiation and termination sites used in a standard infection are also the major sites used in DIP-infected cells.

DISCUSSION

The ability of herpesviruses to establish persistent infection has been well documented (10, 13, 14, 31, 39, 48). Numerous studies from our laboratory have demonstrated that DIPs of EHV-1 mediate persistent infection (2, 3, 8, 11, 26, 35, 41). Indeed, a plethora of animal viruses give rise to DIPs that play a role in viral persistence (for reviews, see references 1, 29, and 42). Thus, the functions of the DNA sequences and gene products conserved in the genome of DIPs of EHV-1 are of particular interest if the molecular mechanisms governing EHV-1 persistence are to be elucidated.

The UL2 gene of EHV-1 and its flanking sequences represent one of the regions (L-terminal sequences) shown to be highly conserved in the genome of EHV-1 DIPs (2, 3). The UL2 gene (52) is a homolog of UL55 of HSV-1 (33) and ORF2 of VZV (12). Since the potential mRNAs and polypeptides encoded by UL55 and ORF2 have yet to be mapped and characterized, this report represents the first to demonstrate that an HSV-1 UL55 homolog (UL2 of EHV-1) is expressed during infection and to identify and characterize its mRNA and polypeptide.

S1 nuclease analyses were utilized to position the 5′ and 3′ termini of the 0.9-kb UL2 mRNA to lie downstream of a TTTAAA box and polyadenylation signal, respectively, and to demonstrate the overabundance of the UL2 message in DIP-infected cells. S1 nuclease analyses also revealed that the UL2 transcription initiation and termination sites utilized in a standard EHV-1 infection are conserved and utilized in a DIP infection. The UL2 ORFs within the DIP genome (pDIL2) and the standard EHV-1 genome (pGEML2) were transcribed and translated in vitro to yield a comigrating 23-kDa polypeptide which was in excellent agreement with the size of the UL2 protein predicted by DNA sequencing analysis. The proteins from both ORFs were not only identical in size but also were reactive with the TrpE/UL2 fusion protein antiserum.

Immunoprecipitation and Western blot analyses of EHV-1-infected cell extracts with the TrpE/UL2 antiserum revealed the presence of a 23-kDa protein synthesized early during both standard and DIP infection (4 to 6 h). Interestingly, the 23-kDa protein appeared to be more abundant in cells infected with DIP-enriched virus than in cells infected with standard virus alone. This observation corresponds well with the similar observation of higher levels of UL2 mRNA in DIP versus standard infection. Since the DIP genome is composed of tandem repeats of its conserved regions, overabundance of the UL2 protein may be due to expression from multiple copies of the UL2 gene within the DIP genome. Indirect evidence to support this comes from the observation that expression of EHV-1 genes not conserved in the DIP genome is not significantly higher in cells

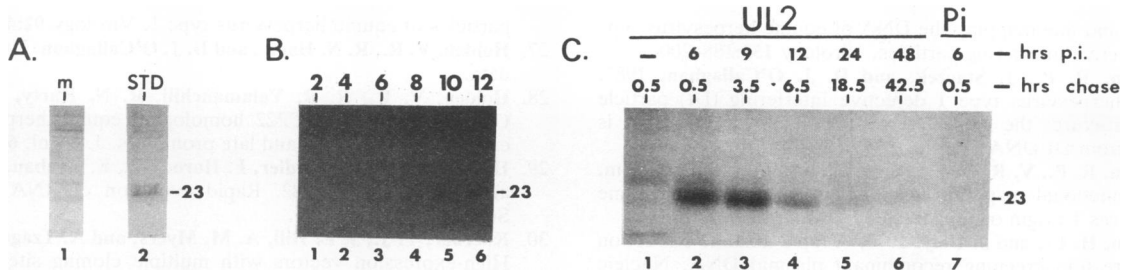

FIG. 9. Western blot and pulse-chase analyses of infected cell extracts by using the TrpE/UL2 antiserum. Protein concentrations were determined with the BCA protein assay reagent (Pierce, Rockford, Ill.). (A) Cell extracts from mock-infected LM cells (lane 1) and from cells infected with standard EHV-1 (lane 2; 8 h p.i.) were fractionated by SDS-PAGE, immunoblotted onto nitrocellulose, and reacted with the TrpE/UL2 antiserum (1:10,000 dilution). (B) Western blot analysis of proteins isolated at various times p.i. (2 to 12 h, lanes 1 to 6) from cells infected with DIP-enriched virus (passage 64). The filter was reacted with the TrpE/UL2 antiserum at a dilution of 1:10,000. (C) Pulse-chase analysis of the UL2 protein synthesized in DIP passage 64-infected cells. The hours p.i. and of chase are indicated. Cell extracts were immunoprecipitated with either the TrpE/UL2 (lanes 1 to 6) or preimmune (lane 7) antiserum. Lane 1 represents mock-infected cell extract.

infected with DIP-enriched virus versus cells infected with standard EHV-1. For example, expression of the IE polypeptide(s) from the IE gene, which is not conserved in the DIP genome, was not enhanced in cells infected with DIP passage 69 compared with cells infected with standard virus (9). It should be noted that the abundance of the UL2 protein synthesized in cells infected with standard EHV-1 at either a low (2 PFU per cell) or high (20 PFU per cell) multiplicity of infection did not match the high level of abundance of the UL2 protein synthesized in cells infected with DIP-enriched virus at either a low (2 PFU per cell) or high (20 PFU per cell) multiplicity of infection. Thus, the regulation and level of UL2 protein synthesis during infection may be influential in establishing a cytolytic versus persistent infection.

The questions concerning UL2 function in cytolytic and persistent infections and the reason for its high degree of conservation in the DIP genome remain intriguing. Whether the UL2 gene product provides a replicative advantage to the DIPs of EHV-1 remains to be determined. No function has been proposed for ORF2 of VZV, which is a homolog of UL2 of EHV-1. However, a potential function for UL55 of HSV-1 has been proposed (7). The UL55 gene and flanking sequences potentially encoding additional HSV-1 proteins may be involved in inhibition of both viral gene expression and transformation (7). Transient cotransfection experiments suggested that an intact UL55 ORF was essential for this inhibitory effect (7).

Recently, the EHV-1 UL3 gene product (homolog of ICP27 of HSV-1) has been shown by transient cotransfection assays to be required in conjunction with the EHV-1 IE protein for maximal expression of EHV-1 late promoter-reporter gene constructs (45). Initial results from transient cotransfection experiments employing a construct of the UL2 gene alone or in various combinations with other EHV-1 regulatory genes (IE and UL3) suggest that the UL2 protein does not significantly activate or inhibit expression from EHV-1 early or late promoter-reporter gene constructs over and above that observed with the IE and UL3 gene constructs (data not shown). However, it remains to be determined whether a high level of the UL2 protein, such as that detected in DIP-infected cells, can affect EHV-1 gene expression and possibly reduce the cytolytic nature of the virus.

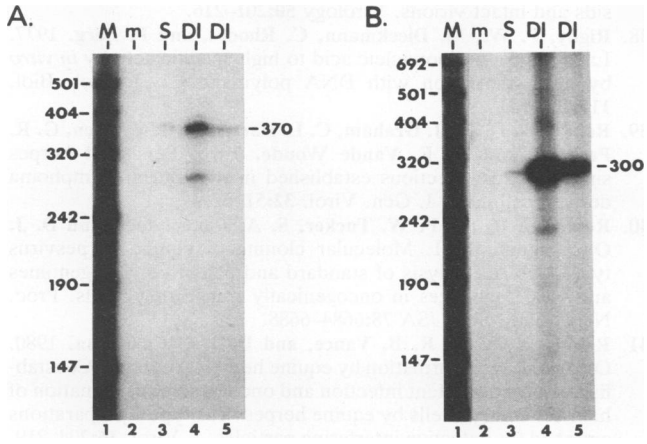

FIG. 10. S1 nuclease analyses of the 5′ and 3′ termini of the UL2 mRNA isolated from standard EHV-1- or DIP-infected cells. (A) S1 nuclease analysis of the 5′ terminus of the UL2 mRNA. Polyadenylated mRNA was isolated from mock-infected cells (lane 2), cells infected with standard virus (lane 3), and cells infected with either DIP passage 63 (lane 4) or passage 69 (lane 5). The probe used was the pGEML2 construct 5′ end labeled at the *Bgl*II site (nt 2513). Molecular weight standards (M) are shown in lane 1. (B) S1 nuclease analysis of the 3′ terminus of the UL2 mRNA. Lane assignments are the same as described in the legend to panel A. The probe used was the pGEML2 construct 3′ end labeled at the *Bgl*II site (nt 2513).

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