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Expression of an Equine Herpesvirus 1 ICP22/ICP27 Hybrid Protein Encoded by Defective Interfering Particles Associated with Persistent Infection

MING CHEN, RONALD N. HARTY,† YUHE ZHAO, V. ROGER HOLDEN, AND DENNIS J. O'CALLAGHAN*

Department of Microbiology and Immunology, Louisiana State University Medical Center, Shreveport, Louisiana 71130-3932

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Defective interfering (DI) particles of equine herpesvirus type 1 (EHV-1) are capable of mediating persistent infection (S. A. Dauenhauer, R. A. Robinson, and D. J. O'Callaghan, J. Gen. Virol. 60:1–14, 1982; R. A. Robinson, R. B. Vance, and D. J. O'Callaghan, J. Virol. 36:204–219, 1980). Sequence analysis of cloned DI particle DNA revealed that portions of two regulatory genes, ICP22 (IR4) and ICP27 (UL3), are linked in frame to form a unique hybrid open reading frame (ORF). This hybrid ORF, designated as the IR4/UL3 gene, encodes the amino-terminal 196 amino acids of the IR4 protein (ICP22 homolog) and the carboxy-terminal 68 amino acids of the UL3 protein (ICP27 homolog). Portions of DNA sequences encoding these two regulatory proteins, separated by more than 115 kbp in the standard virus genome, were linked presumably by a homologous recombination event between two identical 8-bp sequences. Reverse transcriptase-PCR and S1 nuclease analyses revealed that this unique ORF is transcribed by utilizing the transcription initiation site of ICP22 and the polyadenylation signal of ICP27 in DI particle-enriched infection. Immunoprecipitation and Western blot (immunoblot) analyses with antisera to the ICP22 and ICP27 proteins demonstrated that a 31-kDa hybrid protein was synthesized in the DI particle-enriched infection but not in standard virus infection. This 31-kDa hybrid protein was expressed at the same time as the ICP22 protein in DI particle-enriched infection and migrated at the same location on polyacrylamide gel electrophoresis as the protein expressed from a cloned IR4/UL3 expression vector. These observations suggested that the unique IR4/UL3 hybrid gene is expressed from the DI particle genome and may play a role in DI particle-mediated persistent infection.

Equine herpesvirus type 1 (EHV-1), a member of the *Herpesviridae* family and *Alphaherpesvirinae* subfamily, causes spontaneous abortion, respiratory infection, and neural disorders in horses (35, 39, 40, 42). The KyA (Kentucky A) strain of EHV-1 has been extensively studied in our laboratory as a useful model for herpesvirus gene regulation and persistent infection (34, 36–38, 40, 41, 49). The EHV-1 genome is a 144-kb double-stranded DNA molecule composed of a unique long (UL [110 kb]) region attached to a short region (34 kb). The short region contains a unique segment bracketed by two identical inverted repeats (IRs) (25). EHV-1 can establish a lytic infection in several cell types, and its 77 genes are expressed in an immediate-early early, and late cascade fashion (16).

EHV-1 defective interfering (DI) particles have been isolated both from in vivo infection of Syrian hamsters (8) and from infected cells in culture (23–25, 34, 36, 38). Virus titers of serial, undiluted passages of EHV-1 exhibited a definite cyclic pattern, and the presence of DI particles was demonstrated by interference assay (23). Electron microscopic studies indicated that capsid production in DI particle-enriched infections was altered compared with that in standard virus (STD) infection (24). Infection of permissive hamster embryo cells with EHV-1 enriched with DI particles resulted in persistent infection and transformation (10, 11, 52). Ten persistently infected cell lines, designated DI-1 to DI-10, were independently established and shown to exhibit biological properties (immortality, increased saturation density, growth in soft agar, tumorigenicity in hamsters, etc.) inherent to transformed cells; 2 to 20% of these cells were shown to release virus constantly as judged by electron microscopic studies and infectious center assays (10, 11, 52). Both viral DNA and proteins were detected in the DI particle-enriched virus-infected cells (38, 50, 52), and these persistently infected cells continued to release infectious virus and DI particles after several years in culture.

Restriction endonuclease digestion, Southern blot analyses, and DNA sequence analyses of cloned DI particle genomic DNA (4) indicated that DNA sequences conserved in the DI particle genomes are derived from three regions of the STD genome: (i) the left terminus of the UL region that contributes a portion of the CPS (cleavage and packaging signal), intact and functional UL1 and UL2 genes, and a portion of the ICP27 sequence; (ii) the middle region of the IR that contributes a functional Ori S sequence and a portion of the ICP22 sequence; and (iii) the terminal region of the IR that contributes a portion of the CPS sequence (Fig. 1). These three regions are joined together and repeated tandemly and are packaged as DI particle DNA that has a size similar to that of the standard virus genome (3–6, 10, 51, 63, 64). A large portion of the total viral transcripts in DI particle-enriched infection was also found to be expressed from these regions (17). DNA sequence analyses revealed that sequences at the left terminus and middle IR regions were joined by homologous recombination involving an identical 8-bp sequence present in both of these sequences. Interestingly, this recombination linked the 5′

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Louisiana State University Medical Center, 1501 Kings Highway, P.O. Box 33932, Shreveport, LA 71130-3932. Phone: (318) 675-5750. Fax: (318) 675-5764.

† Present address: Department of Microbiology, Mount Sinai School of Medicine, New York, NY 10029-6574.

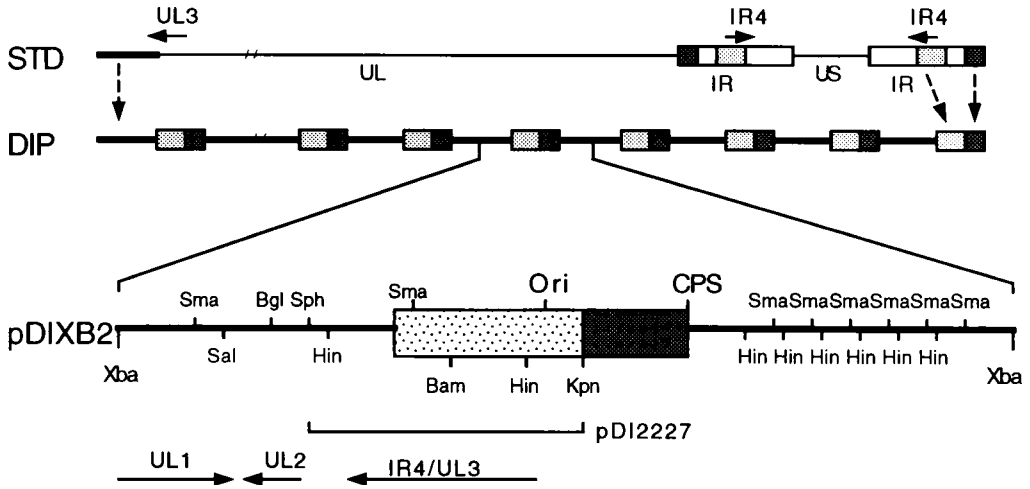

FIG. 1. Structure of the EHV-1 DI particle genome. STD, EHV-1 STD virus genome with a UL region, two IRs, and a unique short (US) region. The locations of the IR4 (ICP22) and UL3 (ICP27) genes are shown. DIP, DI particle genome with three regions (one from the left terminus of the UL and two from the IR) from the STD genome joined together and tandemly repeated until it reaches a size similar to that of the STD genome. pDIXB2, detailed map of one cloned DI particle DNA repeat unit with restriction endonuclease sites (Xba, *Xba*I; Sal, *Sal*I; Sma, *Sma*I; Bgl, *Bgl*II; Sph, *Sph*I; Hin, *Hin*dIII; Bam, *Bam*HI; Kpn, *Kpn*I), the replication origin (Ori), and the CPS. The locations of UL1, UL2, and hybrid IR4/UL3 ORFs and of the subclone pDI2227 are shown.

196 codons of the IR4 gene in frame to the 3′ 70 codons of the UL3 gene. Therefore, a hybrid open reading frame (ORF) in the genome of the DI particles was predicted (63). Since both IR4 (herpes simplex virus type 1 [HSV-1] ICP22 homolog) and UL3 (HSV-1 ICP27 homolog) are important early regulatory genes (28, 58, 67), the expression of this hybrid gene and its potential roles in DI particle-mediated persistent infection are of interest.

Here we report that this hybrid ORF, designated as the IR4/UL3 gene, is expressed at high levels in DI particle-enriched infection. Reverse transcriptase (RT)-PCR and S1 nuclease analyses demonstrated that this hybrid gene is transcriptionally active and that the IR4/UL3 mRNA appears to utilize the ICP22 promoter region and the ICP27 polyadenylation signal. Western blot (immunoblot) analysis revealed that antisera raised against either the ICP22 protein or the ICP27 protein reacted with the predicted 31-kDa hybrid protein produced in DI particle-enriched infection but not in standard virus infection. To our knowledge, this is the first demonstration that a unique hybrid ORF is expressed in an animal virus DI particle-enriched infection.

MATERIALS AND METHODS

Virus and cells. EHV-1 (Kentucky A strain) was grown in murine fibroblastic L-M cells (35, 39, 42, 43) after its adaptation from infection in hamsters (12). The EHV-1 STD was propagated at a low multiplicity of infection (MOI; 0.01 to 0.1 PFU per cell) in L-M cell suspension cultures. EHV-1 preparations enriched in DI particles were generated by serial, undiluted high-MOI passage of EHV-1 in suspension cultures of L-M cells as described previously (4, 11, 23–25, 36, 38). All virus preparations were titrated by plaque assay on L-M cell monolayer cultures (35, 39, 42). DI particle-enriched EHV-1 preparations employed in this study include DI63 (3.9×10^7 PFU/ml), DI64 (1.9×10^6 PFU/ml), DI69 (9.4×10^7 PFU/ml), DI75 (3.1×10^7 PFU/ml), and DI76 (1.6×10^6 PFU/ml), for which the numbers after DI represent the times that the DI particle-enriched virus stocks were passaged.

Cloning and sequencing. As described previously (4), EHV-1 DI particle DNA was purified and digested completely with *Xba*I. The resultant fragments were inserted into the *Xba*I site of pACYC184. Five of these cloned *Xba*I fragments were characterized. One of these clones, pDIXB2, was digested with *Kpn*I and *Sph*I, and the resultant *Kpn*I-*Sph*I fragment, which encodes the IR4/UL3 gene, was subcloned into the pGEM3z vector. The subclone, designated pDI2227, was sequenced by the dideoxy chain termination method with Sequenase (U.S. Biochemical Corp., Cleveland, Ohio). Both DNA strands were sequenced for accuracy. The IR4/UL3 ORF encoded by pDI2227 was subcloned into the expression vector pcDNAI under the control of cytomegalovirus immediate-early promoter. Since no restriction site is available near the translation initiation site, two complementary synthetic oligonucleotides (5′-AGC TTC CCA GCC ATG CCC CAC GGA CAG CCG TGC GGG GCG TGC GAC G-3′ and 3′-AG GGT CGG TAC GGG GTG CCT GTC GGC ACG CCC CGC ACG CTG CCT AG-5′) were used to create a *Hin*dIII-*Bam*HI fragment that contains the upstream portion of the coding sequence. This *Hin*dIII-*Bam*HI fragment was ligated into the *Hin*dIII-*Sph*I site of pcDNAI along with the *Bam*HI-*Sph*I fragment derived from pDI2227. The resulting IR4/UL3 expression vector, designated CMV-IR4/UL3, was transfected into L-M cells by lipofectin-mediated procedures to express the IR4/UL3 hybrid protein.

Isolation of infected cell poly(A) mRNA. L-M cells (10^8) were infected with each DI particle-enriched virus preparation or with EHV-1 STD at an MOI of 1.0 PFU per cell. Additionally, 10^8 L-M cells were mock infected as controls. At 8 h postinfection, the mock-, DI-, and STD-infected cell suspensions were harvested and washed twice with phosphate-buffered saline (PBS). The poly(A) mRNA was isolated with the Fast Track mRNA isolation kit, version 2.1 (Invitrogen Corp., San Diego, Calif.).

RT-PCR analysis. RT-PCR was performed with the GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, Conn.). The downstream primer L3136 (5′-GCT TGT GCG CGT CTA TC-3′), which hybridized to the UL3-positive strand, and the RT were added to transcribe cDNA at 37°C for 30 min. The upstream primer IR8889 (5′-CCT GCA GCT GCT GCC CG-3′), which hybridized to the IR4-negative strand, and *Taq* DNA polymerase were then added to the reaction mixtures. The amplifications were carried out for 30 thermal cycles. The amplified DNA was separated on a 1.5% agarose gel and stained with ethidium bromide. Samples without RT added were also tested to ensure that the final fragment was amplified from mRNA.

S1 nuclease protection assay. The protocol for the S1 nuclease protection assay used in this laboratory has been described elsewhere (22, 27, 66). Molecular weight markers included a radiolabeled fragment mixture from cleavage of pUCBM21 DNA with the restriction endonucleases *Hpa*I and *Dra*I-*Hin*dIII, which were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind.

Isolation of infected cell extracts. L-M cells (10^8) were infected by DI75, DI76, and EHV-1 STD, respectively, at an MOI of 10 PFU per cell. Mock-infected L-M cells (10^8) served as controls. At 8 h postinfection, the mock-, DI-, and STD-infected cell suspensions were harvested and washed twice with PBS. The infected cells were then lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 0.1% sodium dodecyl sulfate [SDS], 0.5% deoxycholate, 1% Nonidet P-40) on ice for 30 min, and the soluble supernatants were stored at −85°C for further study.

Immunoprecipitation. One milliliter of infected cell extracts (10^7 cell equivalents) was incubated with preimmune serum at 4°C overnight on a rocker platform and then incubated for 2 h with protein A-coupled beads (Repligen Corp., Cambridge, Mass.). The beads were pelleted, and the clarified supernatant was removed to a new tube. This procedure was repeated by addition of UL3-specific antiserum. The pelleted samples were washed twice with RIPA buffer, eluted from the beads by addition of Laemmli sample buffer (10% 2-mercaptoethanol, 4% SDS, 20% glycerol, 120 mM Tris-HCl [pH 6.8], 0.001% bromophenol blue), and boiled for 5 min.

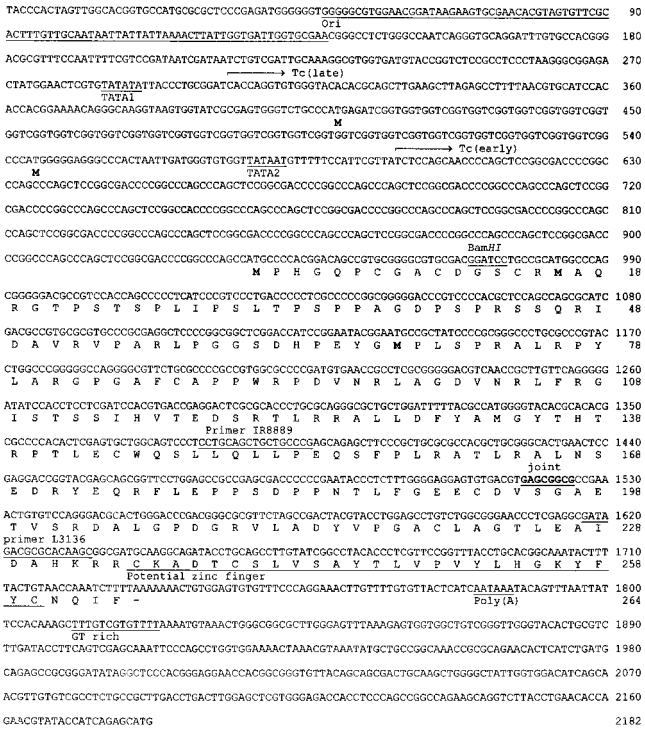

FIG. 2. Sequence analysis of the hybrid IR4/UL3 ORF present in DI DNA clone pDI2227. The 8-nt sequence (1518 to 1525) present in the 5′ portion of the IR4 gene and the 3′ portion of the UL3 gene, which is the site of recombination, is shown in boldface and is underlined. The origin of replication (Ori), two potential TATA boxes (TATA1 and TATA2), the poly(A) signal, and the GT-rich region are underlined. The predicted early and late transcription initiation sites (Tc) are noted. Five potential translation initiation sites are noted by boldface M's. The predicted amino acid sequence is indicated in the single-letter code, and the potential zinc finger domain is underlined. The two primers (IR8889 and L3136) used in RT-PCR and the *Bam*HI site used in the S1 nuclease protection assay are underlined.

Western blot analysis. Infected cell lysates or immunoprecipitated protein samples were boiled with Laemmli sample buffer for 5 min and fractionated through 5% stacking and 10% resolving SDS-polyacrylamide gel electrophoresis (PAGE) as described previously (19–21). Proteins in the gel were electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, N.H.) at 20 V overnight. The membrane was rinsed in TBST buffer (100 mM Tris-HCl [pH 7.5], 0.9% NaCl, 0.1% Tween 20) for 5 min and then incubated with 3% bovine serum albumin, specific IR4 and/or UL3 antiserum (1:1,000 to 1:10,000), and secondary antibody (1:10,000; goat anti-rabbit antibody conjugated with alkaline phosphatase). Each reaction mixture was incubated in TBST at room temperature for 30 min while rocking, followed by three washes with TBST for 5 min. The reactive proteins were visualized by submerging the filter in alkaline phosphatase 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) and 5-bromo-4-chloro-3-indolylphosphate toluidinium (0.165 mg/ml) (GIBCO BRL).

RESULTS

Sequence analyses of the IR4/UL3 hybrid ORF of the genome of EHV-1 DI particles. The *Sph*I-*Kpn*I fragment of pDIXB2, a DI particle DNA clone that contains the unique ORF made up of portions of the ICP22 (IR4) and ICP27 (UL3) genes, was subcloned into a pGEM3z vector (Fig. 1). This plasmid, designated as pDI2227, was sequenced on both strands by the Sanger method (Fig. 2). The 8-nucleotide (nt) recombination site was confirmed on a pDI2227 subclone. This ORF (designated as IR4/UL3 for the hybrid of IR4 and UL3) appears to have the transcription initiation site of the IR4 gene, which gives rise to an early mRNA and a late mRNA, as described by Holden et al. (27), and the polyadenylation signal of the UL3 gene, as described by Zhao et al. (66). A comparison of the IR4 sequences in the hybrid ORF with those in the IR4 gene revealed a 28-bp deletion in the upstream noncoding region, in which 4 of the 17 seven-mer repeats were missing in pDI2227. Five in-frame ATGs were identified after the first transcription initiation site. The third ATG codon is in the best context for translation initiation according to Kozak's consensus sequence (31). Initiation of translation at the third ATG would result in a protein with a size of 264 amino acids and with a predicted molecular mass of 29 kDa. This protein would be composed of the 196 amino acids at the amino terminus of the IR4 protein and the 70 amino acids at the carboxy terminus of the UL3 protein, with 2 amino acids overlapping at the joint region. The potential zinc finger domain of the UL3 protein (66) is also conserved in the hybrid protein.

RT-PCR analysis detected an IR4/UL3 hybrid mRNA expressed only in the DI particle-enriched infection. RT-PCR was used to detect the expression of the hybrid IR4/UL3 mRNA. Two 17-mer oligonucleotide primers were designed for the experiment. Primer L3136 hybridizes to the UL3 region of the mRNA, while primer IR8889 hybridizes to the complementary strand of the IR4 region (Fig. 3). Only hybrid mRNAs with both IR4 and UL3 sequences would be expected to be amplified during the RT-PCR procedure. Since these two regulatory genes are more than 115 kb apart in the EHV-1 STD genome, no mRNAs containing sequences from both genes are expected to be expressed in the STD infection. However, if the hybrid IR4/UL3 ORF is expressed in the DI particle-enriched infection, a 254-nt fragment of the mRNA would be amplified by the RT-PCR procedure. The results are shown in Fig. 3A.

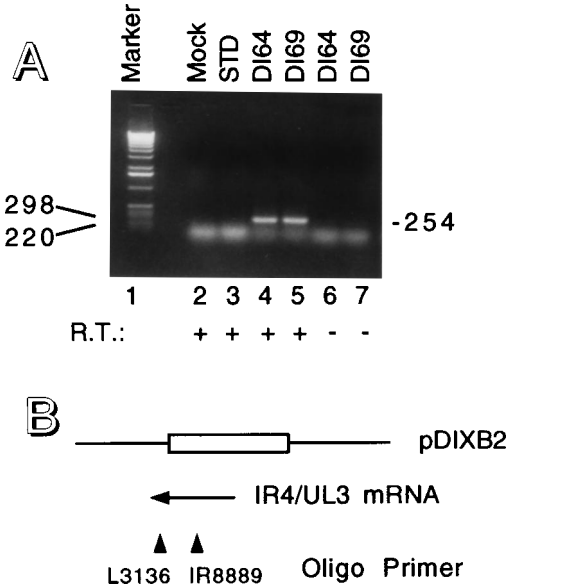

FIG. 3. RT-PCR analysis of the hybrid IR4/UL3 mRNA expressed in DI particle-enriched infection. (A) L-M cells were infected with mock, EHV-1 STD, or two DI particle-enriched virus preparations (DI64 and DI69) at an MOI of 1 PFU per cell. The polyadenylated mRNA of the infected cells was isolated at 8 h postinfection. One microgram of mRNA of each sample was reverse transcribed to cDNA with downstream primer L3136 and amplified with both L3136 and upstream primer IR8889 for 30 thermal cycles. The reaction products were analyzed on a 1.5% agarose gel with ethidium bromide stain. Lanes: 1, molecular weight markers; 2 to 5, RT-PCR analysis of infected cell mRNA; 6 and 7, same as lanes 4 and 5 but without RT added. The predicted 254-nt fragment amplified from the IR4/UL3 mRNA is marked. (B) Location of the IR4/UL3 ORF and two oligonucleotide (oligo) primers used in RT-PCR as related to the DI particle DNA clone pDIXB2.

Mouse L-M cells were mock infected or infected with EHV-1 STD or DI particle-enriched virus stocks, DI64 and DI69, which were generated by high-MOI serial undiluted passages. Total cell polyadenylated mRNAs were isolated 8 h postinfection. One microgram of mRNA of each sample was analyzed with the GeneAmp RNA PCR kit, and the products were resolved on an agarose gel and stained with ethidium bromide (Fig. 3A). The expected 254-bp band was amplified only from the mRNA isolated from DI particle-enriched infections (lanes 4 and 5), but not from mock or STD infection (lanes 2 and 3). Moreover, samples isolated from DI particle-enriched infections but amplified without addition of RT gave very light bands (lanes 6 and 7), confirming that the amplification in lanes 4 and 5 was mainly from mRNA. The same 254-bp band was also amplified with mRNA isolated from cells transfected with an IR4/UL3 expression vector or with DI DNA clone pDIXB2 (data not shown). The results of this experiment indicated that the hybrid IR4/UL3 mRNA is uniquely expressed in DI particle-enriched infections.

S1 nuclease protection assays. According to the sequence analysis, the IR4/UL3 hybrid gene is expected to be transcribed by utilizing the IR4 transcription initiation site and the UL3 polyadenylation signal, which will yield an early transcript with a size of 1.2 kb and a late transcript with a size of 1.5 kb. However, DI particle-enriched virus stock also contains EHV-1 STD, and the mRNAs of IR4 and UL3 as well as mRNA transcribed from the hybrid ORF would be present in the RNA isolated from DI particle-enriched infection. Because of the similar molecular sizes of these transcripts, conventional Northern (RNA) blot analysis could not identify the IR4/UL3 transcripts definitively. To characterize the IR4 and UL3 hybrid mRNA detected by the RT-PCR, S1 nuclease analyses were performed to map the location and termini of the IR4/UL3 hybrid mRNA. To generate probes, the plasmid pDI2227 was digested at a unique *Bam*HI site and end labeled with [^{32}P]ATP at either the 5′ end or the 3′ end. mRNA isolated from mock-, EHV-1 STD-, or DI particle-enriched EHV-1-infected L-M cells (as described for RT-PCR analysis) were incubated with each probe at 68°C overnight to achieve maximum hybridization. After digestion of the reaction mixtures with S1 nuclease, the protected probes were resolved on a polyacrylamide-urea gel (Fig. 4). The 3′-labeled probe should hybridize to the normal IR4 mRNA from the labeling site to the joint site and would yield a 550-nt protected band. In addition, the IR4/UL3 mRNA, which binds to the probe from the labeling site to the polyadenylation site, should protect 825 nt of the probe. As expected, the 550-nt band was present in both STD infection and DI particle-enriched infection, while the 825-nt band was detected only in DI particle-enriched infections (Fig. 4B). Extensive expression of the IR4/UL3 mRNA is expected, since multiple copies of the hybrid ORF are present in the DI particle genome. Two smaller bands with molecular sizes of about 350 and 270 nt were also detected in the DI particle-enriched infection, indicating that different joint sites may exist in some DI particle genomes. Since the 5′-labeled probe contains a 28-nt deletion in the IR4 upstream noncoding region, the IR4 mRNA will hybridize to 436 nt from the labeling site to the deletion site and the IR4/UL3 mRNA will give rise to a 643-nt protected band if transcribed from the DI particle genome containing the 28-bp deletion. The 643-nt band was readily detected; however, the major band in the DI particle-enriched infection is 436 nt (Fig. 4A), indicating that the 28-bp deletion may not be present in most DI particle genomes. Transcripts expressed from the second transcription initiation site were not detected in either STD or DI particle-enriched infection. Overall, the S1 nuclease protection assays confirmed that the IR4/UL3 ORF is transcribed as a 1.5-kb mRNA in DI particle-enriched infection, although other transcripts may also be present.

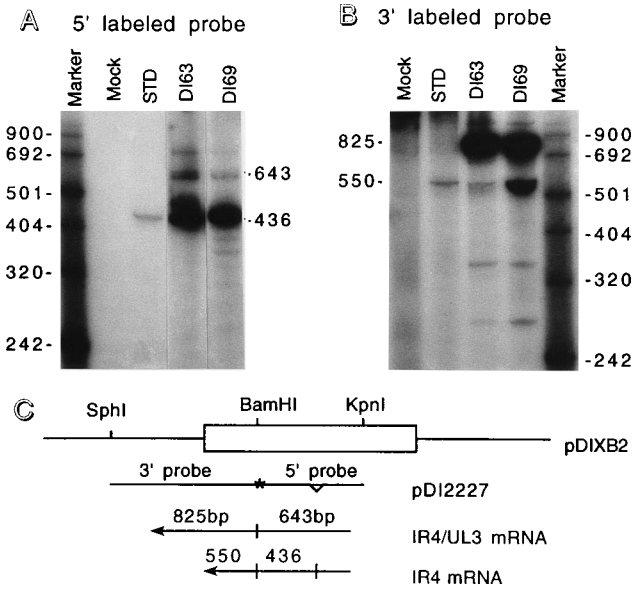

FIG. 4. S1 nuclease protection assay of the hybrid IR4/UL3 mRNA in DI particle-enriched infection. The plasmid pDI2227 was cut at the *Bam*HI site and labeled with ^{32}P at either the 5′ or 3′ terminus. Polyadenylated mRNAs from mock- STD-, and DI63- and DI69-infected cells were hybridized with either 5′-labeled probe (A) or 3′-labeled probe (B) at 68°C overnight and digested with S1 nuclease. After ethanol precipitation, the samples were analyzed on a 6% polyacrylamide–urea gel. The predicted protection sizes of 3′-end-labeled fragments (825 bp by the IR4/UL3 mRNA and 550 bp by the IR4 mRNA) and 5′-end-labeled fragments (643 bp by mRNA with the 28-bp deletion and 436 bp by mRNA without the deletion) are marked. (C) Diagram of the location of the probes, IR4/UL3 mRNA, and IR4 mRNA as related to the DI particle DNA clone pDIXB2. The location of the 28-bp deletion on the pDI2227 clone is marked with a small v.

Western blot analyses detected a 31-kDa protein encoded by the IR4/UL3 hybrid ORF. If the IR4/UL3 mRNA is translated, its protein product should contain 196 amino acids identical to the amino-terminal portion of the IR4 protein and 70 amino acids identical to the carboxy-terminal portion of the UL3 protein with 2 overlapping amino acids. Antibodies generated to these regions of the IR4 and UL3 proteins should react with the IR4/UL3 hybrid protein, which would have a predicted size of about 29 kDa (264 amino acids). Western blot analyses were performed to confirm these expectations. L-M cells were mock infected or infected with EHV-1 STD or two DI particle-enriched EHV-1 preparations at an MOI of 10 PFU per cell. At 8 h postinfection, cell lysates were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. Two specific antisera were used to detect the hybrid IR4/UL3 protein (Fig. 5E). TrpE-IR4 is a rabbit antiserum to a bacterial TrpE-IR4 fusion protein that harbors amino acids 13 to 150 of the IR4 protein (26), which are present in the IR4/UL3 hybrid protein. TrpE-UL3 antiserum was raised against a bacterial TrpE-UL3 fusion protein that harbors the carboxy-terminal portion amino acids 283 to 470 of the UL3 protein (67) and therefore contains the 70 amino acids present in the IR4/UL3 hybrid protein. The IR4 peptide antiserum was generated against a synthetic peptide presented in the carboxy-terminal region of the IR4 protein (amino acids 270 to 286). Since this peptide is not expected to be present in the IR4/UL3 hybrid protein, the anti-peptide antiserum was used as a negative control. As shown in

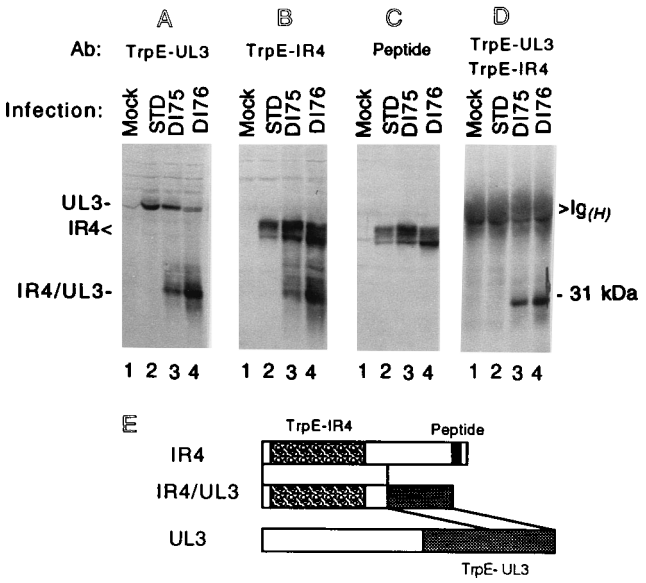

FIG. 5. Identification of the 31-kDa IR4/UL3 hybrid protein in DI particle-enriched infection. L-M cells were infected with DI75 (lane 3), DI76 (lane 4), or EHV-1 STD (lane 2) at an MOI of 10 PFU per cell or were mock infected (lane 1) as controls. At 8 h postinfection, the cells were harvested and lysed in RIPA buffer. Equal amounts of cell lysates (A, B, and C) or protein samples immunoprecipitated with TrpE-UL3 antiserum (D) were fractionated through a 5% stacking and 10% resolving polyacrylamide gel. Proteins were electrophoretically transferred to a nitrocellulose membrane and detected by Western blotting with antisera specific to TrpE-UL3 (A), TrpE-IR4 (B and D), and IR4 peptide (C). Each antiserum was generated against a specific region of the IR4 protein or the UL3 protein as shown in panel E. $Ig_{(H)}$, heavy chain of immunoglobulin G.

Fig. 5, the UL3 protein was detected as a 50-kDa band in both STD infection and DI particle-enriched infection by the TrpE-UL3 antiserum (Fig. 5A). The IR4 protein, which is present as multiple bands ranging from 42 to 47 kDa (26), was also found in both STD infection and DI particle-enriched infection by using the TrpE-IR4 and the anti-peptide antisera (Fig. 5B and C). It is likely that the multiple bands represent modified forms of IR4 protein, because recent studies revealed that this nuclear regulatory protein is phosphorylated. Unlike the STD infection, additional proteins were detected in DI particle-enriched infections (Fig. 5A and B, lanes 3 and 4). These proteins reacted with both the TrpE-IR4 and TrpE-UL3 antisera, but not with the antiserum to the IR4 peptide (Fig. 5C, lanes 3 and 4). The major band migrated at approximately 31 kDa, which is similar to the predicted molecular mass of the IR4/UL3 hybrid protein. Other minor bands may represent ORFs generated by different joint sites during the DI particle formation.

Confirmation by immunoprecipitation followed by Western blot analysis that the 31-kDa protein presented in DI particle-enriched infection is a hybrid protein reacting with both TrpE-IR4 and TrpE-UL3 antisera. It is difficult to obtain and to purify a single-genotype DI particle. Because the DI particle-enriched virus stocks used in these studies were a genotypically mixed virus population, different forms of mutated proteins may be present in the infected cells. In fact, proteins with different molecular weights were detected in the DI particle-enriched infection with either the TrpE-IR4 antiserum or the TrpE-UL3 antiserum (Fig. 5A and B). Thus, it may be argued that the 31-kDa protein detected by both the TrpE-IR4 antiserum and the TrpE-UL3 antiserum in Western blot analyses may represent two mutated proteins with a similar migration behavior. To support the conclusion that the 31-kDa protein is an IR4 and UL3 hybrid protein, cell lysates used in Western blot analyses were immunoprecipitated with UL3-specific antiserum TrpE-UL3, and the precipitated proteins were then analyzed by Western blot analysis with the IR4-specific antiserum TrpE-IR4. In this case, only a protein reacting with both the TrpE-IR4 antiserum and the TrpE-UL3 antiserum would be detected. As expected, the 31-kDa protein was detected as a single band, but neither the IR4 protein nor the UL3 protein was detected, since each protein reacts with only one of the antisera (Fig. 5D). The band present in all four lanes represents rabbit immunoglobulin heavy chain protein, which was obtained during the immunoprecipitation procedure. This experiment confirmed that the 31-kDa protein detected in DI particle-enriched infection is a hybrid of the IR4 and UL3 proteins.

Time course expression of the IR4/UL3 hybrid protein. According to sequence analyses, the IR4/UL3 ORF appears to be regulated by the upstream *cis*-regulatory elements of the IR4 gene. Therefore, the expression of IR4/UL3 protein should be regulated similarly to that of IR4 in the DI particle-enriched infection. In this experiment, L-M cells were infected with DI64 or EHV-1 STD at an MOI of 10 PFU per cell. At 2, 4, 6, 8, 10, and 12 h postinfection, cell suspensions were harvested and lysed in RIPA buffer. Equal amounts of cell lysates were resolved by SDS-PAGE, and proteins on the gel were electrophoretically transferred to a nitrocellulose membrane and subjected to Western blot analysis with the TrpE-IR4 antiserum. The results, shown in Fig. 6, revealed that both the IR4 protein and the IR4/UL3 protein were readily detected at 6 h postinfection in DI particle-enriched infection, and a very small amount of the IR4/UL3 protein could also be detected by 4 h postinfection. In STD infection, only the IR4 protein was detected at 4 h postinfection. The delay of expression of the IR4 protein in the DI particle-enriched infection may be caused by DI particle interference. This result agrees with the sequencing data that the expression of the IR4/UL3 gene is regulated by the same *cis*-acting regulatory region.

L-M cells transfected with an IR4/UL3 expression vector produce the 31-kDa hybrid protein. The IR4/UL3 ORF encoded on pDI2227 was subcloned into expression vector pcDNAI under the control of the cytomegalovirus immediate-early pro-

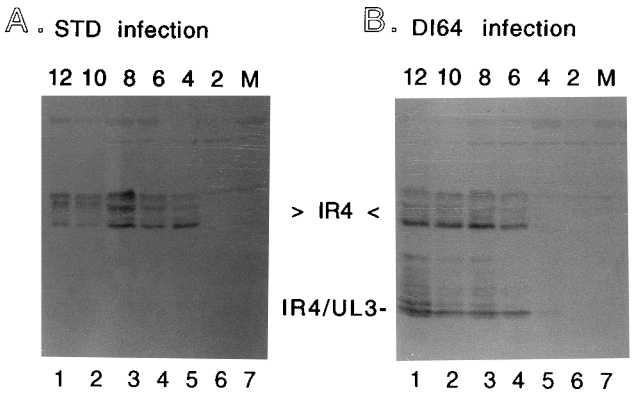

FIG. 6. Time course studies of the synthesis of the IR4/UL3 hybrid protein in DI particle-enriched infection. L-M cells were infected with DI64 (B, lanes 1 to 6) or EHV-1 STD (A, lanes 1 to 6) at an MOI of 10 PFU per cell or were mock infected (lane 7) as controls. At 2, 4, 6, 8, 10, and 12 h postinfection, cell suspensions were harvested and lysed in RIPA buffer. Equal amounts of cell lysates were resolved on SDS-PAGE. Proteins on the gel were electrophoretically transferred to a nitrocellulose membrane and detected by Western blot analysis with antiserum specific to TrpE-IR4.

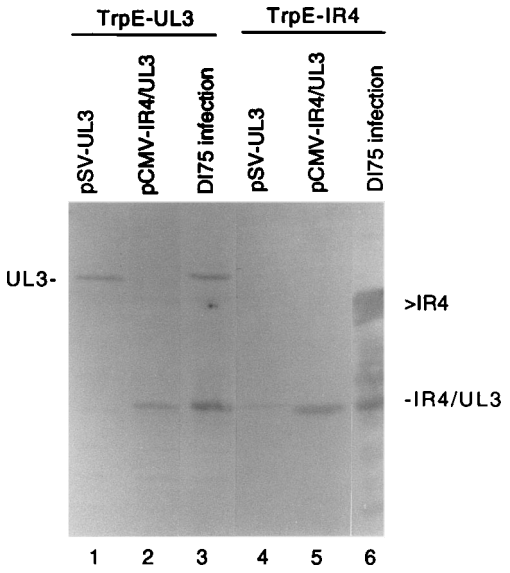

FIG. 7. Expression of the IR4/UL3 hybrid protein in pCMV-IR4/UL3-transfected cells. L-M cells were transfected with SV-UL3 (lanes 1 and 4) or CMV-IR4/UL3 (lanes 2 and 5) or were infected with DI75 (lanes 3 and 6). The proteins isolated at 72 h posttransfection or 8 h postinfection were analyzed by Western blot analysis with TrpE-UL3 (lanes 1 to 3) or TrpE-IR4 (lanes 4 to 6) antiserum. Molecular size locations of UL3, IR4, and IR4/UL3 proteins are indicated.

moter. The IR4/UL3 expression vector, designated CMV-IR4/UL3, was transfected into L-M cells by lipofectin-mediated procedures. Expression of the 31-kDa protein in the transfected cells was verified by Western blot analyses (Fig. 7). Proteins isolated from cells transfected with the UL3 expression vector (SV-UL3) and DI75-infected cells were also analyzed as controls. The 31-kDa protein expressed from the CMV-IR4/UL3 vector (lanes 2 and 5) migrated at a location similar to that observed in DI particle-enriched infection (lanes 3 and 6) and reacted with both the TrpE-IR4 antiserum and the TrpE-UL3 antiserum. As a control, cells transfected with only the SV-UL3 expression construct (lanes 1 and 4) were shown to express the 50-kDa UL3 protein that reacted only with the TrpE-UL3 antiserum (lane 1). These results support the observation that the 31-kDa protein observed in the DI particle-enriched infection is expressed from DI DNA cloned in pDI2227.

DISCUSSION

DI particles are deletion mutants that replicate and amplify their genomes at the expense of the replication of specific helper virus that encodes proteins essential for replication and structure. At least two *cis*-acting elements are required on the DI genome: a replication origin and a CPS (59). Previous studies in our laboratory demonstrated that the EHV-1 sequences upstream of the IR4 gene (map units 0.83 to 0.87) code for an origin of replication (4, 62, 64) and that sequences at the left terminus and the right terminus together make up the CPS (62). These three regions are conserved in the EHV-1 DI particle genome. Moreover, the genomes of the DI particles are usually rearranged to enhance their ability to compete for replication and encapsidation proteins with the helper virus. Kwong and Frenkel (32) demonstrated that HSV DI particles with monomeric units of less than 15 kb in size replicated faithfully, while larger amplicons developed sequence-nonspecific deletions. Therefore, recombinations and/or deletions that eliminate sequences other than the two *cis*-acting elements required for the DI particle genome to replicate properly may enhance the replicative advantage of the defective DNA molecule. In the concatemeric replicating herpesvirus genome, the left and right termini are continuous during the replication. Thus, at least two recombination events were necessary to link the origin sequence within the IR to the CPS sequences at the two termini for the generation of the genome of these EHV-1 DI particles. It is noteworthy that these IR and UL sequences are separated by more than 115 kb on the EHV-1 STD genome and are recombined at the site of the identical sequence of 8 bp. Recombination between sequences separated by considerable distances has also been observed in other herpesvirus DI particles, such as the class II defective genome of HSV (13) and DI particles of pseudorabies virus (47, 53).

The IR4/UL3 ORF characterized here was previously predicted according to the sequence analysis in our laboratory (63). Minor variations of the precise recombination site may exist. However, high-level expression of the IR4/UL3 mRNA and protein was detected in infections with several independently generated preparations of EHV-1 DI particles, indicating that generation of the IR4/UL3 hybrid ORF is not a rare event. To our knowledge, this is the first characterization of a hybrid ORF expressed in DI particle-enriched infection of an animal virus. It is of particular interest since this ORF encodes a hybrid protein composed of portions of two EHV-1 regulatory proteins. A hybrid protein encoded by DI particles of a plant virus was reported for clover yellow mosaic virus (61). Sequencing of a small group of 1.2-kb clover yellow mosaic virus DI RNAs revealed that this RNA encoded a single ORF joining the N terminus of the 191-kDa nonstructural product and the C terminus of the coat protein, and a 35-kDa fusion product was produced by in vitro translation (61).

The IR4/UL3 hybrid protein may alter EHV-1 gene regulation in DI particle-enriched infection. Alterations in viral gene expression in cells infected with DI particle-enriched herpesviruses have been reported (14, 33, 48). In the case of EHV-1, these alterations include overexpression of the EHV-1 UL1 and UL2 genes that are conserved in the DI particle genome (Fig. 1). The UL1 gene, a homolog of the varicella-zoster virus ORF2 gene and the "circ" gene of bovine herpesvirus 1, encodes a 258-amino-acid virion-associated protein that is modified by myristic acid (19, 21, 22, 65). To date, no function has been proposed for UL1 or its homologs. The UL2 gene, a homolog of UL55 of HSV-1 and ORF3 of varicella-zoster virus, was shown to express a 23-kDa protein in both EHV-1 STD infection and DI particle-enriched infection (20, 65). Although the function of UL2 has not been elucidated, the homologous UL55 gene of HSV-1 may be involved in inhibition of both virus gene expression and transformation (7). Both the UL1 and UL2 transcripts and proteins are produced in large amounts in DI particle-enriched infections (19, 20), and an additional 2.2-kb transcript has been detected only EHV-1 DI particle-enriched infection (data not shown). The 2.2-kb transcript has not been characterized in detail, but preliminary data revealed that the bulk of this transcript lacks a poly(A) tail and overlaps approximately 700 nt of the 3′ end of the immediate-early mRNA (21a).

The demonstration that the IR4/UL3 hybrid ORF is expressed in infections with DI particle-enriched virus raises questions about its possible role in the establishment of persistent infection. The IR4 gene exhibits significant homology to the immediate-early gene ICP22 of HSV-1 (16% identity), ORF63 of varicella-zoster virus (24% identity), and RSp40 of pseudorabies virus (34% identity). The IR4 protein is ex-

pressed abundantly and localizes predominantly to the nucleus (26, 27), and transient expression assays demonstrate that the IR4 protein can activate a variety of EHV-1 early and late promoters in the presence of other EHV-1 *trans*-activators, such as the immediate-early protein and the UL3 protein (28). The UL3 protein exhibits significant homology to the ICP27 immediate-early regulatory protein of HSV-1 (32% identity) and to the ORF4 protein of varicella-zoster virus (13% identity) (66). Studies with ICP27 temperature-sensitive and deletion mutants have demonstrated that HSV-1 ICP27 possesses the ability to down-regulate the immediate-early gene and some early genes and to up-regulate late gene expression (44–46, 56, 57, 60). ICP27 plays an essential role in virus growth, but it does not appear to be absolutely essential for DNA synthesis (18, 54). ICP27 alone has little or no effect on gene regulation but functions significantly in the presence of ICP4 and ICP0 (44, 54, 55). ICP27 has also been suggested to regulate gene expression at a posttranscriptional level (56). The carboxy terminus of HSV-1 ICP27 is highly conserved in the EHV-1 UL3 protein, contains *trans*-repressor and *trans*-activator domains, and harbors a zinc finger motif that is considered critical for its regulatory function (18, 54). Studies in our laboratory demonstrated that the EHV-1 UL3 protein is a nuclear regulatory protein that activates immediate-early gene expression and is important for the expression of late genes (58, 67). Although the functional domains of the IR4 and UL3 proteins are not yet known, the zinc finger motif of the UL3 protein is conserved in the IR4/UL3 hybrid protein. Since the IR4/UL3 hybrid protein has amino acid sequences derived from two regulatory proteins, it is reasonable to speculate that the hybrid protein may also exhibit certain regulatory functions or be able to alter the regulatory functions of IR4 or UL3 in DI particle-enriched infections.

The IR4/UL3 ORF may play a role in DI particle-mediated persistent infection. The ubiquity of DI particles was first clearly recognized by Huang and Baltimore (30). DI particles have been studied widely, since they often interfere strongly with virus replication and facilitate the establishment and maintenance of persistent infection (2, 29, 30, 47). Herpesvirus DI particles are known to promote establishment of persistent infections and to inhibit infected cell cytolysis (13). Studies from our laboratory have demonstrated that DI particles of EHV-1 are capable of mediating persistent infection (3–6, 8, 10, 11, 36–38, 41), although the mechanism of herpesvirus DI particle-mediated persistent infection is not understood. It has been proposed that strong promoters presented on the reiterated DI DNA compete with promoters of the helper virus DNA, leading to down-regulation of the helper virus functions involved in cytolysis (13). Mutated viral genes involved in persistent infection have been reported in many cases (9), and even host cell mutations may be involved in persistent infection (1). Recently, a hybrid protein, RAZ, in Epstein-Barr virus was reported to be able to modulate the viral reactivation mechanism (15). Through alternative splicing, RAZ is formed by replacement of the transactivation domain of Z (BZLF1 or ZEBRA) with a portion of another transactivator, R (BRLF1). RAZ retains the dimerization and DNA-binding domain of Z but loses its transactivation domain. As a result, the RAZ protein is able to form RAZ:Z heterodimers and diminish viral reactivation induced by Z. Since EHV-1 DI particles with the remarkable ability to establish persistent infection efficiently and rapidly harbor and express the IR4/UL3 ORF, investigation of the possible functions of this unique protein may give some insight into the initial events associated with the establishment of herpesvirus persistent infection. If the IR4/UL3 protein does modify virus gene regulation, these alterations may affect the cytolytic functions of the standard virus. Our preliminary results from transient transfection assays suggest that the hybrid protein, produced from the pCMV-IR4/UL3 construct, can retard expression of specific EHV-1 promoters. Further studies are in progress to define the role of the IR4/UL3 hybrid protein in altering EHV-1 gene programming and to ascertain whether expression of the hybrid protein from either EHV-1 DI particles or from an EHV-1 recombinant virus that harbors the IR4/UL3 ORF is associated with the establishment of persistent infection.

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