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An Early Gene Maps within and Is 3′ Coterminal with the Immediate-Early Gene of Equine Herpesvirus 1

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The immediate-early (IE) gene (IR1 gene) of equine herpesvirus 1 (EHV-1) encodes a single, spliced 6.0-kb mRNA during cytolytic infection. However, under early (in the presence of phosphonoacetic acid) and late (8 h postinfection; no metabolic inhibitors) conditions, in addition to the 6.0-kb IE mRNA, a 4.4-kb early (E) mRNA is transcribed from the IE gene region beginning at approximately 4 h postinfection. To map and characterize the 4.4-kb E mRNA and the protein product of this early gene (IR2 gene), Northern (RNA) blot hybridization, S1 nuclease, primer extension, and in vitro transcription and translation analyses were used. The data from RNA mapping analyses revealed that the 4.4-kb E IR2 mRNA (i) maps at nucleotides 4481 to 635 within each of the inverted repeats of the short region and thus is encoded by sequences that lie entirely within the IE gene, (ii) is transcribed in the same direction as the IE mRNA, initiating at nucleotide 4481, which lies 25 bp downstream of a putative TATA-like sequence and 1,548 bp downstream of the transcription initiation site of the IE mRNA, and (iii) is 3′ coterminal with the IE mRNA which terminates at nucleotide 635 of the inverted repeats. The IR2 open reading frame was inserted into the transcription expression vector pGEM-3Z, and the RNA transcribed from this construct (pGEM44) was shown to be a 4.2-kb transcript that contained all IR2 sequences. In vitro translation of the 4.2-kb RNA yielded a major protein of approximately 130 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. This protein corresponds to the predicted IR2 product of 1,165 amino acids that would be in frame with the major IE polypeptide (IE1 = 200 kDa; 1,487 amino acids) and thus would be a 5′-truncated form of the IE1 polypeptide. The presence and potential role of the IR2 gene embedded within the IR1 gene increase the complexity of the regulation of the IE gene region during various stages of a productive infection.

Equine herpesvirus 1 (EHV-1), an alphaherpesvirus, is a major pathogen of the equine species, causing fetal infections and abortion storms in pregnant mares, respiratory infections, and central nervous system disorders (1, 10, 47–49). EHV-1 preparations enriched for defective interfering particles mediate oncogenic transformation and persistent infection in primary hamster embryo fibroblasts, and thus EHV-1 serves as a model for studying viral gene regulation in productive and persistent infections (3, 5, 6, 20, 26, 33, 34, 48, 49, 60–62). The genome is a 144-kbp, double-stranded DNA molecule whose structure was determined by restriction endonuclease and electron microscopic analyses (4, 35, 63, 69). The EHV-1 genome of the Kentucky A cell culture strain of EHV-1 can be divided into a unique long (U_L) region (112 kbp) which is covalently attached to a short region (32 kbp). The short region contains unique sequences (U_S) (6 kbp) bracketed by two identical inverted repeats (13.2 kbp each), which allow the entire short region to invert relative to the long region, resulting in the formation of two EHV-1 genomic isomers (4, 7, 35, 48, 49, 51, 63, 69). Gene regulation of EHV-1 at the transcriptional level was first described by Huang et al. (37) and Cohen et al. (17, 18). More recently, Gray et al. (24, 25) demonstrated a temporal order of expression for EHV-1 genes at the transcriptional level, and they designated these kinetic classes immediate-early (IE), early (E), and late (L). Furthermore, Caughman et al. (12, 13) were able to show that EHV-1 protein synthesis was also coordinately regulated into IE, E, and L kinetic classes by analyzing infected cell polypeptide synthesis in the presence of specific metabolic inhibitors. These and other studies have demonstrated that EHV-1 encodes a single IE gene (map units 0.78 to 0.82 and 0.96 to 1.00) which maps within each of the two inverted repeat sequences. The EHV-1 IE gene has been (i) sequenced in its entirety (27) to reveal a major open reading frame (ORF) of 4,461 bp, (ii) characterized at the mRNA level to reveal a single 6.0-kb IE transcript (IR1 mRNA) that was spliced to remove a 372-base intron from the 5′ leader region (29, 30), (iii) characterized at the protein level to reveal that multiple IE polypeptides are synthesized from a single IE mRNA, the largest being 200 kDa (IE1) (12, 13, 59, 65), and (iv) shown to encode IE polypeptides that have transactivating functions (65). Interestingly, a second transcript of 4.4 kb was synthesized from the IE gene region during E (in the presence of phosphonoacetic acid) and L (uninhibited infection) stages of a productive infection (24, 25, 29–31). In this report, we discuss the characterization and mapping of this 4.4-kb E mRNA (IR2 mRNA) and demonstrate that it (i) maps entirely within the 6.0-kb IE mRNA, (ii) is 3′ coterminal with the 6.0-kb IE mRNA, and (iii) appears to encode a 5′-truncated form of the major IE polypeptide. Finally, the potential role of this EHV-1 E gene in viral infection is discussed.

MATERIALS AND METHODS

Virus and cell culture. The Kentucky A strain of EHV-1 has been passaged in murine L-M fibroblasts suspension cultures and assayed by plaque titration as described previously (50, 54). Rabbit kidney cells (RK-13 ATCC CCL 37) were cultivated in Eagle's minimum essential medium sup-

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plemented with penicillin (100 U/ml), streptomycin (100 μg/ml), nonessential amino acids, and 5% newborn calf serum.

Virus infection and use of metabolic inhibitors. To isolate EHV-1 IE mRNA, L-M suspension cultures (10^8 cells) were preincubated with 100 μg of cycloheximide (CH); (Sigma Chemical Co., St. Louis, Mo.) per ml for 1 h at 37°C. The cells were then infected with EHV-1 at multiplicity of infection of 15 to 20 PFU per cell in the presence of CH. Following attachment for 1 h, the cells were diluted to 2×10^6/ml, and the infection proceeded in the presence of CH for a total of 4 h. To isolate mRNA under L conditions, no metabolic inhibitors were used, and the mRNA was isolated at 8 h postinfection (p.i.).

Isolation of mRNA. Total cellular RNA was isolated by the guanidinium-cesium chloride centrifugation method of Chirgwin et al. (16) as modified by Maniatis et al. (44). This protocol has been described previously (29). Poly(A)$^+$ mRNA was isolated by using the Fast Track mRNA isolation kit (Invitrogen Corp., San Diego, Calif.). Briefly, 10^8 mock-infected or EHV-1-infected cells were pelleted, washed once, and lysed with sodium dodecyl sulfate (SDS) lysis buffer containing RNase protein degrader. The cell lysate was placed in a 45°C water bath for 2 h. After incubation, 0.95 ml of 5 M NaCl was added for each 15 ml of lysate, and the DNA was sheared by passing the lysate through a syringe. The lysate was then added to preequilibrated oligo(dT)-cellulose and rocked gently at room temperature for 1 h. After a wash in binding buffer, the mRNA was eluted from the oligo(dT)-cellulose by two washes in elution buffer. The mRNA was suspended in a final volume of 0.4 ml of elution buffer and ethanol precipitated at −70°C overnight.

Northern (RNA) and Southern blot hybridization. For Northern hybridization, poly(A)$^+$ or total RNA was fractionated on 1.2% formaldehyde agarose gels as described by Maniatis et al. (44). The RNA was transferred onto GeneScreen Plus membrane filters (NEN, Boston, Mass.) by the method of Southern (66), using the protocol of the supplier. Cloned EHV-1 DNA fragments were radiolabeled with α-^{32}P-labeled deoxynucleoside triphosphates, using the nick translation method of Rigby et al. (56) as described previously (29). Oligodeoxynucleotides were 5′ end labeled with [γ-^{32}P]ATP as discussed below. Molecular weight markers used in Northern blot experiments were 28S (4.9 kb) and 18S (1.9 kb) calf liver rRNA and 23S (2.9 kb) and 16S (1.5 kb) *Escherichia coli* rRNA (Pharmacia, Uppsala, Sweden). Southern blot hybridization was performed as described above, using the blotting method of Southern (66) and GeneScreen Plus membrane filters.

5′ and 3′ end labeling of DNA probes. EHV-1 DNA fragments and oligodeoxynucleotides (Synthetic Genetics Inc., San Diego, Calif.) were 5′ end labeled as described previously (29), using the methods outlined by Maniatis et al. (44) as modified in reference 2. Also, EHV-1 DNA fragments were 3′ end labeled as described previously (29) by a method described in reference 2.

S1 nuclease mapping. The method of Berk and Sharp (8) as modified by Weaver and Weissman (68) was used. The protocol used for S1 nuclease mapping has been described previously (29). 5′-end-labeled markers, used in S1 nuclease and primer extension experiments, were derived from a *Hae*III digest of ϕX174 (Pharmacia).

Primer extension. The primer extension method of Inoue and Cech (39) was used as described in detail elsewhere (29). The primer was oligodeoxynucleotide 1, a 20-mer with the sequence 5′-CCATGGCGTCCCCGATGTGG-3′, derived from sequences within EHV-1 clone pS1A6 at nucleotides (nt) 2586 to 2605 (Fig. 1; 27).

Cloning of viral DNA. Clone pGEM44 was generated by inserting a viral *Bsp*E1-*Eco*RI fragment (containing sequences from nt 746 to 4765 of the EHV-1 IE gene; 27), containing the major ORF of the 4.4-kb E mRNA, into the multiple cloning site of an *Xma*I-*Eco*RI-digested pGEM-3Z (Promega Corp., Madison, Wis.) vector, using established protocols (44). Screening was performed by restriction endonuclease digestion and Southern blot analysis. Furthermore, the 5′ terminus of the in vitro-transcribed mRNA synthesized from pGEM44 was analyzed by primer extension analysis (using oligodeoxynucleotide 1; Fig. 1) to map the 5′ end of the mRNA to confirm the integrity of the insert and confirm that the resultant transcript contained the appropriate ORF. Recombinant plasmid DNA was 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.) and isolated by the method of Birnboim and Doly (9).

In vitro transcription. Capped transcripts were synthesized from pGEM44 by using an in vitro transcription kit (Promega) as described by the supplier. Briefly, 5.0 μg of pGEM44 was linearized with *Eco*RI and purified from an 0.8% agarose gel by using the GeneClean kit (Bio 101, Inc., La Jolla, Calif.) as recommended by the supplier. The purified DNA was suspended in a final volume of 100 μl containing 4.0 μl of RNAsin, 20 μl of 5× transcription buffer, 10 μl of 10 mM dithiothreitol, 5.0 μl each of 2.5 mM rATP, rTTP, and rCTP 0.5 μl of rGTP, 5.0 of μl cap analog, 1.0 μl of Sp6 polymerase, and sterile H_2O. The sample was incubated at 40°C for 1 h, 1.0 μl of Sp6 polymerase was added, and the sample was incubated for 1 h at 40°C. DNase I was then added, and the mixture was incubated at 37°C for 20 min, phenol-chloroform extracted, and chloroform extracted. The RNA was quantitated and stored as an ethanol precipitate at −20°C.

In vitro translation and SDS-PAGE. Viral mRNA was translated in a rabbit reticulocyte lysate system (NEN) in the presence of [^{35}S]methionine according to the recommendations of the supplier as modified by Robertson et al. (59). For time course experiments, reactions were terminated at the designated time intervals as described above. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on 7.5% acrylamide gels according to Laemmli (42). Samples were electrophoresed for approximately 5 h, after which the gels were fixed in a solution containing 40% methanol and 10% acetic acid for at least 1 h. Molecular weight markers (Bethesda Research Laboratories, Gaithersburg, Md.) included myosin (200 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and α-chymotrypsinogen (25.7 kDa).

Electroelution of mRNA. The protocol used to isolate and purify viral mRNA from a 1.2% nondenaturing agarose gel has been described in detail elsewhere (31). The 4.4-kb E mRNA isolated by this method was detected by Northern blot hybridization (31).

RESULTS

Northern blot analysis of the 4.4-kb E mRNA. During IE conditions of a cytolytic infection (see Materials and Methods), EHV-1 synthesizes a single, spliced 6.0-kb IE transcript (IR1 gene; map units 0.78 to 0.82 and 0.96 to 1.00) within the short region (Fig. 1; Fig. 2A, lane 3) (24, 25, 29, 30). However, EHV-1 mRNA isolated under E (6 h p.i. in

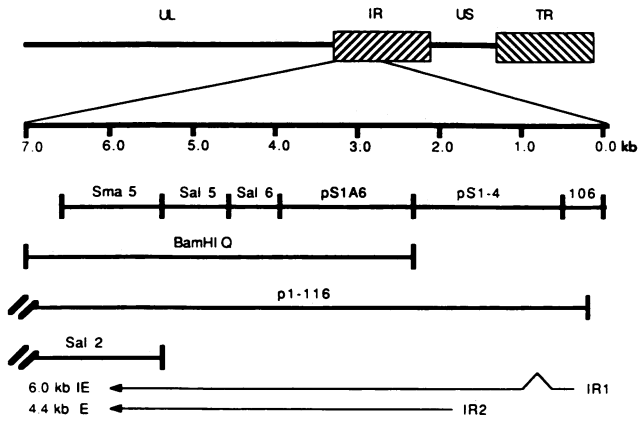

FIG. 1. IE gene region of EHV-1. The EHV-1 genomic structure is shown at the top, with a 7.0-kbp region, containing the IE gene, expanded below. Numerous EHV-1 DNA clones mapping within this region are shown: *Sma* 5, *Sal* 5, *Sal* 6, pS1A6, pS1-4, 106, *Bam*HI Q, p1-116, and *Sal* 2. Clones p1-116 and *Sal* 2 extend into the long region of the genome. The map positions of the EHV-1 6.0-kb IE mRNA (IR1 gene) and EHV-1 4.4-kb E mRNA (IR2 gene) are shown at the bottom. UL, unique long; IR, inverted repeat; US, unique short; TR, terminal repeat.

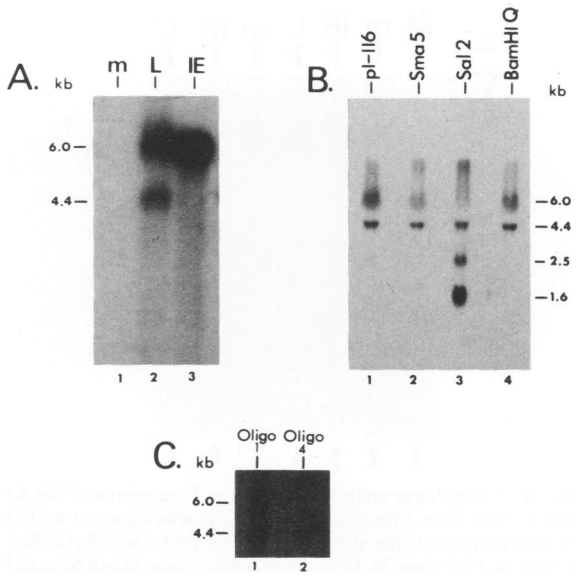

FIG. 2. Northern blot hybridization analyses of the IR2 transcript. (A) Northern blot of poly(A)$^+$ mRNA isolated from mock-infected (m) L-M cells (lane 1) and EHV-1-infected L-M cells (lane 2, L conditions; lane 3, = IE conditions). The probe used was the pS1-4 clone of EHV-1 (see Fig. 1). The 6.0-kb IE mRNA was detected under IE and L conditions, whereas the 4.4-kb E mRNA was not detected under IE conditions. No hybridization was detected with mock mRNA (lane 1). (B) Northern blot of total RNA isolated from EHV-1-infected rabbit kidney cells under L conditions and probed with clones p1-116 (lane 1), *Sma* 5 (lane 2), *Sal* 2 (lane 3), and *Bam*HI Q (lane 4). The 6.0-kb IE and 4.4-kb E mRNAs hybridized to all four clones. The 2.5- and 1.6-kb mRNAs that hybridized to p1-116 and *Sal* 2 map within the long region of the EHV-1 genome (see Fig. 1). (C) Northern blot of poly(A)$^+$ mRNA isolated from EHV-1-infected L-M cells under L conditions and probed with 5′-end-labeled oligodeoxynucleotide 1 (lane 1) or 4 (lane 2). The oligonucleotides were 20-mers derived from opposite strands of the EHV-1 genome, and only oligonucleotide 1 (lane 1) hybridized to both the 6.0-kb IE and 4.4-kb E mRNAs, demonstrating that the two transcripts are synthesized in the same orientation. Oligonucleotide 4 (5′-TGGCGGGAGCGTCGAAGCGC-3′) maps within clone pS1-4 at nt 2018 to 2037 (see Fig. 1; 27). See Materials and Methods for a description of oligonucleotide 1.

the presence of 100 μg of phosphonoacetic acid per ml) and L (see Materials and Methods) conditions during a cytolytic infection included a 4.4-kb E transcript (IR2 gene) that was synthesized from the IE gene region, in addition to the 6.0-kb IE mRNA (Fig. 1; Fig. 2A, lane 2) (24, 25, 29, 30, 59). Neither the 6.0-kb IE nor the 4.4-kb E mRNA was detected in mock-infected mRNA samples (Fig. 2A, lane 1). The 4.4-kb E transcript was first observed by Northern blot hybridization at 4 h p.i., whereas the IE mRNA was readily detected at 2 h p.i. (Fig. 3). Both transcripts were observed up to 12 h p.i. (Fig. 3). However, Northern blot experiments revealed that the IR2 mRNA was more readily detected when the viral RNA used was isolated at 6 to 8 h p.i. in the absence of metabolic inhibitors; therefore, this transcript was characterized by using viral mRNA isolated at these times.

The IR2 mRNA was mapped by Northern blot hybridization using numerous EHV-1 clones mapping within the IE gene region (Fig. 1 and 2A; Fig. 2B, lanes 1 to 4; 30). All EHV-1 clones shown in Fig. 1 except clone 106 hybridized to the IR2 mRNA (Fig. 2B, lanes 1 to 4). Clone 106, which lies at the 5′ end of the IE gene, hybridized only to IE mRNA (data not shown). Furthermore, EHV-1 clones mapping entirely within the U_L region failed to detect the IR2 mRNA in Northern blot experiments (data not shown). These observations indicated that the IR2 mRNA mapped entirely within inverted repeat sequences and was embedded within DNA sequences comprising the IR1 gene (Fig. 1; 30). The termini of the IR2 mRNA were mapped within EHV-1 clones pS1-4 and *Sma* 5 by using small subclones of these fragments as probes in Northern blot experiments (Fig. 1; 30). Northern blot hybridization using strand-specific, 5′-end-labeled oligodeoxynucleotides as probes demonstrated that the 4.4-kb IR2 mRNA was transcribed in the same direction as the 6.0-kb IR1 mRNA (Fig. 1 and 2C). Oligodeoxynucleotide 1 hybridized to both IR1 and IR2 mRNAs (Fig. 2C, lane 1), whereas oligodeoxynucleotide 4 did not hybridize to either transcript (Fig. 2C, lane 2). Since both transcripts were synthesized from the same DNA strand (Fig. 2C) and Northern blot experiments mapped their 3′ termini to an identical polyadenylation site on the EHV-1 genome within clone *Sma* 5 (Fig. 1; 29, 30), it was predicted that the IR1 and IR2 mRNAs were 3′ coterminal.

S1 nuclease analysis of the 3′ terminus of the IR2 mRNA. To confirm that the IR1 and IR2 mRNAs were 3′ coterminal, S1 nuclease analysis was performed at their 3′ termini (Fig. 4). The 3′ terminus of the IE mRNA had been mapped previ-

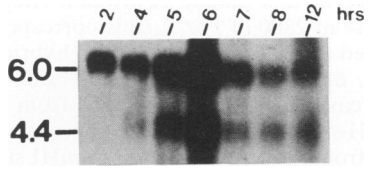

FIG. 3. Northern blot of poly(A)$^+$ mRNA isolated from EHV-1-infected L-M cells at the times postinfection indicated, showing the kinetics of synthesis of the 4.4-kb E mRNA. The probe used was pS1A6 (see Fig. 1).

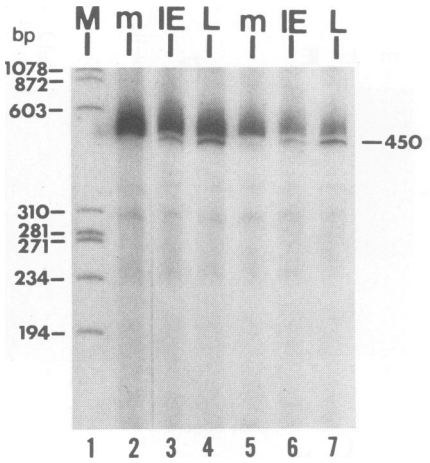

FIG. 4. S1 nuclease analysis to map the 3′ terminus of the 4.4-kb E mRNA. The *Sma* 5 fragment (see Fig. 1) was digested with *Xho*I and 3′ end labeled at this site (29, 30). The probe was hybridized to mock-infected (m; lane 2), IE (lane 3), and L (lane 4) mRNAs at 58°C or to mock-infected (lane 5), IE (lane 6), and late (lane 7) mRNAs at 65°C and digested with S1 nuclease. Molecular weight markers (M) are shown in lane 1.

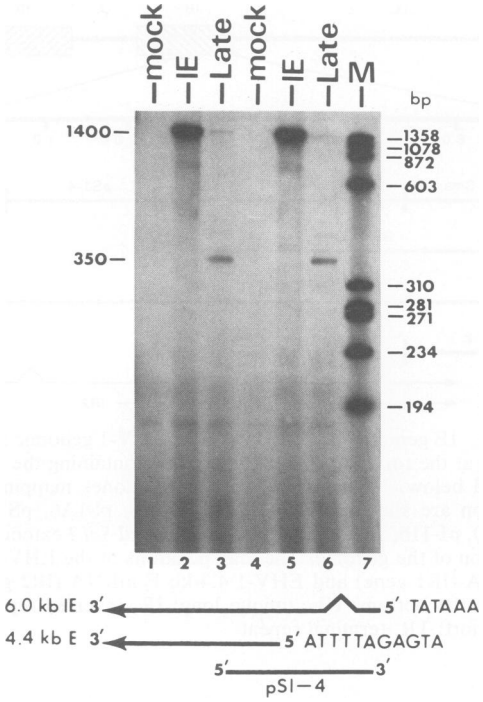

FIG. 5. S1 nucleases analysis to map the 5′ terminus of the 4.4-kb E mRNA. The diagram at the bottom (not drawn to scale) depicts the position of the 5′-end-labeled probe (pS1-4) relative to the 6.0-kb IE and 4.4-kb E mRNAs. The 5′-end-labeled probe was hybridized to mock-infected (lane 1), IE (lane 2), and L (lane 3) mRNAs at 58°C or to mock-infected (lane 4), IE (lane 5), and late (lane 6) mRNAs at 62°C and digested with S1 nuclease. Molecular weight markers (M) are shown in lane 7.

ously by using S1 nuclease analysis and found to lie at nt 635 of the inverted repeat (29, 30). Therefore, if the IR1 and IR2 transcripts were 3′ coterminal, then an S1-resistant fragment of identical size would be observed after S1 nuclease digestion of either DNA-IE mRNA hybrids or DNA-L mRNA hybrids. The *Sma* 5 fragment (Fig. 1) was digested with *Xho*I and 3′ end labeled at this unique site (29, 30), and this probe was hybridized to IE or L mRNA at 58 or 65°C (Fig. 4). As previously determined (29, 30), an S1-resistant fragment of 450 bp was observed following S1 nuclease digestion of the DNA-IE mRNA hybrid (Fig. 4, lanes 3 and 6). An identical 450-bp protected fragment was observed when L mRNA was used instead of IE mRNA (Fig. 4, lanes 4 and 7), and no additional S1-resistant fragments were observed. Indeed, these data confirmed that the IR1 and IR2 transcripts share a polyadenylation signal sequence (27) and were 3′ coterminal. The 450-bp fragment was not detected in mock-infected mRNA samples (Fig. 4, lanes 2 and 5). The occurrence of overlapping, 3′ coterminal transcripts, as demonstrated with the IR1 and IR2 mRNAs of EHV-1, is a feature common to many genes of the family *Herpesviridae* (14, 19, 28, 43, 45, 46, 55, 57, 67, 70).

S1 nuclease analysis of the 5′ terminus of the IR2 mRNA. The transcription initiation site of the IR2 transcript was mapped within clone pS1-4 (Fig. 1) by Northern blot hybridization (30). S1 nuclease analysis was performed with pS1-4 as the probe to fine map the transcription start site of the IR2 message (Fig. 5). Clone pS1-4 was digested with *Bam*HI and 5′ end labeled at this unique restriction site, which lies at approximately nt 2300 of Fig. 1 (this corresponds to nt 4132 of the inverted repeat). This probe was hybridized to mock-infected, IE, or L mRNA, and the hybrids were digested with S1 nuclease (Fig. 5). As expected from previous work (29, 30), an S1-resistant fragment of approximately 1,400 bp, which maps from the 5′-end-labeled *Bam*HI site to the splice acceptor site of the IE transcript, was observed with IE mRNA (Fig. 5, lanes 2 and 5). The 1,400-bp protected fragment and an additional fragment of approximately 350 bp were observed with L mRNA (Fig. 5, lanes 3 and 6). This 350-bp fragment was detected only in RNA preparations that contained the IR2 mRNA and thus mapped the transcription initiation site of IR2 at 350 bp upstream of the *Bam*HI site, or approximately 25 nt downstream of the potential TATA-like element shown in Fig. 5. No fragments were detected in mock-infected mRNA samples (Fig. 5, lanes 1 and 4). Thus, the transcription initiation site of IR2, determined by S1 nuclease analysis (Fig. 5), lies at approximately nt 4481 of the inverted repeats, which is 1,548 bp downstream of the transcription initiation site of IR1. This finding is in excellent agreement with the mapping results of Northern blot hybridizations (30). These results suggested that the IR2 mRNA is not simply a degradation product of the IE transcript but rather has a defined transcription initiation site. Furthermore, experiments using the metabolic inhibitors CH and dactinomycin revealed that the 6.0-kb IE mRNA is not degraded to yield the 4.4-kb E mRNA (data not shown), and thus the IR2 message is an independent transcript. Finally, the presence of the 1,400-bp protected fragment in the L mRNA samples (Fig. 5, lanes 3 and 6) demonstrated that the spliced form of the IE mRNA is present late in infection. Preliminary data suggest that an unspliced form of the IE mRNA may exist during L stages of cytolytic infection (32, 36).

Key parameters of the IR2 gene. Several important elements of the IR2 gene are listed in Table 1. The first AUG codon within the IR2 mRNA mapped 43 nt downstream of the transcription initiation site. This ATG-initiated ORF (3,495 bp; 1,165 amino acids) is in frame with the major ORF

TABLE 1. Key elements of the EHV-1 4.4-kb E gene

Element	Nucleotide
TATA sequence	−25[a]
Transcription initiation	+1
1st ATG codon	+43
Stop codon	+3538
Major ORF	3,495 bp
AATAAA sequence	+3824
Transcription termination	+3839

[a] See Grundy et al. (27) for the entire sequence of the 4.4-kb E gene.

of the IE transcript (4,461 bp; 1,487 amino acids) (27), suggesting that the IR2 mRNA may encode a 5′-truncated form of the major IE polypeptide (IE1). On the basis of DNA and protein sequence analyses, the IR2 protein was predicted to have a molecular mass of 123 kDa and to be missing the N-terminal 322 amino acids present in IE1.

Cloning and in vitro transcription of the IR2 gene. To characterize the protein product of the IR2 mRNA, the major ORF of IR2 was cloned into the transcription expression vector pGEM-3Z to yield pGEM44 (Fig. 6A). Plasmid pGEM44 contained the 3,495-bp ORF of IR2, plus a small leader region, cloned behind the Sp6 promoter (Fig. 6A). Plasmid pGEM44 was shown to contain the full-length IR2

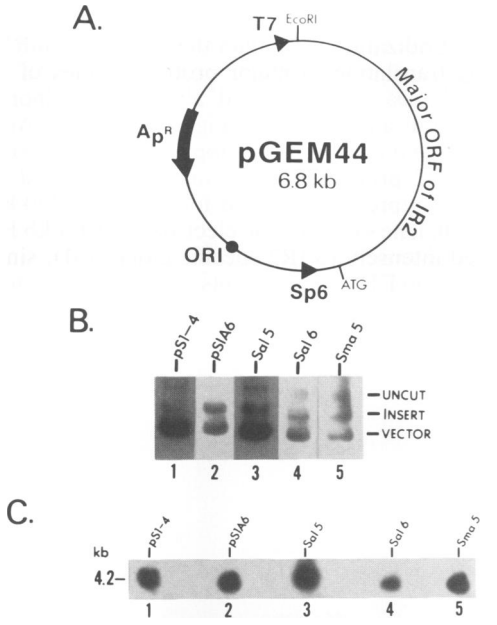

FIG. 6. Cloning of the IR2 gene into the transcription expression vector pGEM-3Z. (A) pGEM44 is an approximately 6.8-kb plasmid containing the major ORF of the IR2 gene cloned behind the Sp6 promoter. The plasmid also contains the T7 promoter, ampicillin resistance gene (Ap^R), and bacterial origin of replication (ORI). The first ATG of the IR2 ORF is shown. (B) Southern blot of pGEM44 digested with *Eco*RI and *Hin*dIII, which flank the IR2 insert (4.0 kb). The DNA was probed with pS1-4 (lane 1), pS1A6 (lane 2), *Sal* 5 (lane 3), *Sal* 6 (lane 4), and *Sma* 5 (lane 5). Cross-hybridization of vector sequences (2.8 kb) was observed, along with a small amount of uncut plasmid (all lanes). (C) Northern blot of RNA transcribed in vitro from pGEM44 and probed with pS1-4 (lane 1), pS1A6 (lane 2), *Sal* 5 (lane 3), *Sal* 6 (lane 4), and *Sma* 5 (lane 5). A transcript of the expected size of approximately 4.2 kb was detected with all five probes that span the IR2 insert.

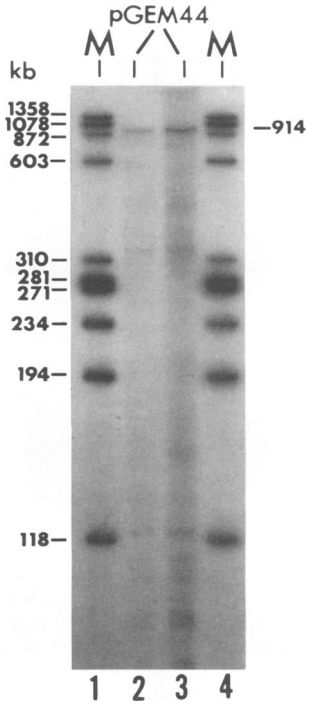

FIG. 7. Primer extension analysis of the 4.2-kb RNA transcribed in vitro from pGEM44. 5′-End-labeled oligodeoxynucleotide 1 (see Materials and Methods) was hybridized to 3 (lane 2) or 6 (lane 3) μg of the 4.2-kb mRNA and extended with avian myeloblastosis virus reverse transcriptase. The resultant full-length cDNA was approximately 914 bases in size (lanes 2 and 3), which confirmed the integrity of the IR2 insert at the 5′ end. Molecular weight markers (M) are shown in lanes 1 and 4.

insert (approximately 4.0 kb) by Southern blot hybridization using EHV-1 probes (Fig. 1) spanning the IR2 gene (Fig. 6B). Furthermore, an mRNA of approximately 4.2 kb was synthesized from pGEM44 and shown to hybridize to EHV-1 clones spanning the IR2 gene (Fig. 6C). Finally, primer extension analysis (Fig. 7) was used to confirm that the 5′ terminus of the 4.2-kb transcript synthesized from pGEM44 was intact and contained the ATG of the major IR2 ORF. Oligodeoxynucleotide 1 (see Materials and Methods) was hybridized to the pGEM44-derived RNA and extended with avian myeloblastosis virus reverse transcriptase to yield a full-length cDNA of the expected size of approximately 914 nt (Fig. 7, lanes 2 and 3). Indeed, the intensity of the cDNA fragment of 914 nt increased as the amount of pGEM44-derived RNA was increased (Fig. 7, lane 3).

In vitro translation of pGEM44-derived RNA. Various amounts of pGEM44-derived RNA were translated in a rabbit reticulocyte lysate for 90 min, and the protein products were analyzed by SDS-PAGE (Fig. 8). Two large protein species of 150 and 130 kDa were observed when 1 (lane 4), 3 (lane 5), or 6 (lane 6) μg of pGEM44-derived RNA was translated in vitro. The 4.4-kb mRNA has the potential to encode proteins in the size range of 130 to 150 kDa. The 130-kDa protein was similar in size to that predicted to be encoded by IR2 (123 kDa), whereas the 150-kDa protein may represent a modified form of the 130-kDa protein. Numerous smaller proteins were also observed (lanes 4 to 6) that may be (i) degradation products of the 150- or 130-kDa protein, (ii) generated from shorter RNA molecules within the sample

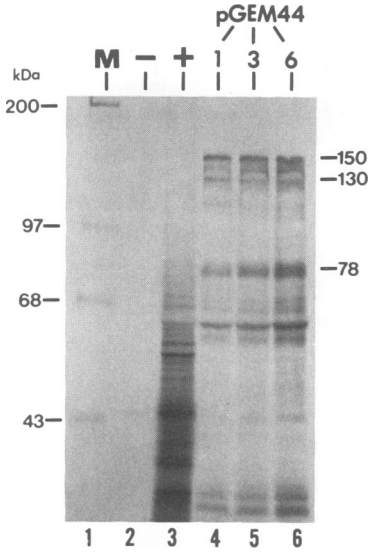

FIG. 8. SDS-PAGE of in vitro translation products synthesized from pGEM44-derived RNA. One (lane 4), three (lane 5), and six (lane 6) micrograms of RNA was translated in a rabbit reticulocyte lysate to yield two major protein species of 150 and 130 kDa. Molecular weight markers (M) are shown in lane 1; negative (no mRNA added; lane 2) and positive (adenovirus mRNAs; lane 3) controls are also shown.

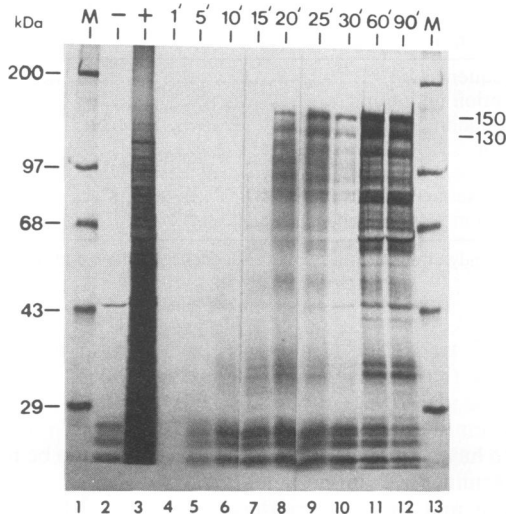

FIG. 9. SDS-PAGE of a time course experiment using the in vitro translation products of the pGEM44-derived RNA. Three micrograms of RNA was translated in a rabbit reticulocyte lysate and terminated at the indicated time points by adding 3 volumes of 2× Laemmli sample buffer. Time points span 1 to 90 min (lanes 4 to 12). Molecular weight markers (M; lanes 1 and 13) as well as negative (lane 2) and positive (lane 3) controls (see legend to Fig. 8) are shown.

as a result of RNA degradation, or (iii) translated from internal AUG codons within the pGEM44-derived RNA. Indeed, the DNA sequence of the IR2 gene predicts that there would be numerous internal AUG codons and ORFs within the EHV-1 IR2 transcript (27).

To determine whether the 130-kDa protein was a breakdown product of the 150-kDa protein (Fig. 8, lanes 4 to 6), the time course of the synthesis of both polypeptides was monitored at intervals of up to 90 min (Fig. 9). The 150- and 130-kDa proteins appeared simultaneously in samples in which translation was terminated at 20 min (Fig. 9, lane 8), and both polypeptides were detected through the 90-min time point (lanes 8 to 12), indicating that a slow degradation of the 150-kDa protein to yield the 130-kDa protein was unlikely. Although the possibility exists that a rapid degradation or modification of the 150-kDa species occurs, the observation that the relative amounts of the 150- and 130-kDa proteins remain constant during the 90-min period suggests that the 130-kDa species is not derived solely from the 150-kDa species. Interestingly, the smaller proteins appear before the larger species, suggesting perhaps that they are not generated from the degradation of the larger protein species (Fig. 9, lanes 4 to 7).

In vitro translation of electroeluted IR2 mRNA. Since the IR1 mRNA completely overlaps the IR2 mRNA and the synthesis of the IR2 E mRNA is dependent on a functional IE gene, the IR2 transcript could not be isolated by hybrid selection techniques. Therefore, a method employing the electroelution of in vivo-synthesized IR2 mRNA from a nondenaturing agarose gel was used as a means to isolate the in vivo IR2 transcript for in vitro translation. EHV-1-infected cell mRNA (isolated under L conditions), migrating at approximately 4.4 kb on a 1.2% nondenaturing agarose gel, was electroeluted and purified from a gel slice (31) and translated in a rabbit reticulocyte lysate system (Fig. 10, lane 4). mRNA isolated from this gel slice was shown by Northern blot hybridization to contain the 4.4-kb IR2 mRNA (31). Following translation, a major protein species of approximately 130 kDa was observed along with minor protein species of 140 and 150 kDa (Fig. 10, lane 4). Again, the 130-kDa protein observed was approximately the size of the predicted IR2 protein product, and the 140- and 150-kDa species may represent modified forms of this 130-kDa protein (Fig. 10, lane 4). Since the electroeluted 4.4-kb E mRNA hybridized intensely to IR2-specific clones (31), since there are virtually no EHV-1 transcripts identical in size to the IR2

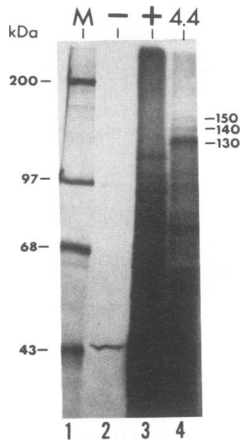

FIG. 10. SDS-PAGE of in vitro translation products synthesized from the 4.4-kb E mRNA, isolated from infected cells and electroeluted from an agarose gel. A major protein species of 130 kDa was synthesized from the electroeluted mRNA, along with minor species of 150 and 140 kDa (lane 4). Molecular weight markers (M) are shown in lane 1; negative (lane 2) and positive (lane 3) controls (see legend to Fig. 8) are also shown.

mRNA (24, 25), and since electroeluted mock-infected mRNA migrating at 4.4 kb did not yield a protein of 130 kDa following in vitro translation, the 130-kDa protein may be the major product of the IR2 mRNA; however, further experimentation with IE-specific antisera will be necessary to confirm the identity of the primary translation product of the IR2 mRNA. Experiments have been initiated to prepare antisera to synthetic peptides located at the shared 3′ end of the IR1 and IR2 proteins. It will be of interest to determine whether these antibodies react with in vitro-translated products of the IR1 and IR2 mRNAs.

DISCUSSION

The EHV-1 IE gene has been shown to encode a single, spliced 6.0-kb IE mRNA that, when translated (in vivo or in vitro), yields multiple antigenically related IE polypeptide species (12, 13, 24, 25, 27, 29–31, 59). In this report, we describe a 4.4-kb E mRNA that is synthesized from the IE gene region during E and L stages of EHV-1 productive infection. The 4.4-kb E mRNA (IR2) was shown by Northern blot analysis to (i) map within the EHV-1 inverted repeat sequences that encode the IE mRNA, (ii) be transcribed in the same direction as the IE mRNA, (iii) terminate at the polyadenylation site used by the IE mRNA and thus be 3′ coterminal with the IE transcript, and (iv) first appear at approximately 4 h p.i. Splicing of the IR2 mRNA was not detected, and the first 15 AUG-initiated ORFs within the IR2 mRNA would be translated in frame with the major IE ORF (27).

To confirm that both the IR1 and IR2 mRNAs were 3′ coterminal, S1 nuclease mapping of their 3′ termini was performed by using the *Sma* 5 probe 3′ end labeled at a unique *Xho*I site (29, 30). A single S1-resistant fragment of 450 bp was observed on the gel following hybridization and S1 nuclease digestion of the 3′-end-labeled probe hybridized to IE (6.0 kb) or L (6.0 and 4.4 kb) mRNA, confirming that IR1 and IR2 mRNAs were 3′ coterminal. Numerous examples of 3′-coterminal transcripts have been described for various genes of members of the herpesvirus family (14, 19, 28, 43, 45, 46, 55, 57, 67, 70). Rixon and McGeoch (57) were the first to describe a 3′-coterminal family of mRNAs, encoded by herpes simplex virus type 1 (HSV-1), involving the spliced IE mRNA-5 and two E mRNAs. The major difference between the EHV-1 3′-coterminal mRNAs and those described by Rixon and McGeoch (57) is that the major ORF of the EHV-1 IR2 mRNA (Table 1; 1,165 amino acids) is in frame with that of the EHV-1 IR1 mRNA, whereas the three HSV-1 3′-coterminal mRNAs were shown to utilize different ORFs. The EHV-1 IR2 mRNA is predicted to encode a 5′ truncated form (approximately 123 kDa on the basis of sequence analysis) of the major EHV-1 IE polypeptide (IE1 = 200 kDa), which would have the first 322 N-terminal amino acids of IE1 deleted. Indeed, a protein of approximately 130 kDa was observed upon in vitro translation of both in vitro-transcribed IR2 mRNA (Fig. 6, 8, and 9) and IR2 mRNA isolated from EHV-1-infected cells (Fig. 10). Interestingly, other examples of overlapping, 3′-coterminal mRNAs utilizing all or part of the same ORF, yet having unique 5′ transcription initiation sites, have been described for the herpesviruses (14, 19, 43). The 5′ transcription initiation site for the IR2 mRNA was mapped by S1 nuclease analysis (Fig. 5) and shown to be approximately 1.5 kb downstream of the 5′ terminus of the IR1 mRNA. Thus, the data suggest that the promoter region of IR2, which is functional at E and L stages of EHV-1 cytolytic infection, is embedded within the IE gene coding sequences. A similar phenomenon has been reported for the UL26 ORF of HSV-1, which contains two distinct transcriptional units that utilize the same ORF to encode proteins with shared amino acid sequences (43). Furthermore, an E gene of human cytomegalovirus was shown to synthesize three 3′-coterminal mRNAs that encode the same protein but have three separate promoters that are induced at various times postinfection (14).

Even though this complex transcription unit of EHV-1 is not a unique gene arrangement within the herpesvirus family, the question of why EHV-1 would encode a 5′-truncated form of the major IE polypeptide at E and L stages postinfection remains. Preliminary data generated to date suggest that transcription or translation of the EHV-1 IE mRNA and IE polypeptides may be inhibited or reduced during L stages of EHV-1 cytolytic infection by two possible mechanisms. First, preliminary data indicate that unspliced IE mRNA is present at L stages of infection (32, 36). The 372-bp intron spliced from the leader region of the IE mRNA contains two small ORFs of 19 and 51 amino acids (27, 29, 30). The presence of these intron sequences in the IE mRNA would increase the size of the leader region to approximately 625 bases, and the presence of the two small ORFs may decrease the efficiency of translation from the AUG that begins the IE ORF (22, 41). Second, a delayed-early transcript of 0.9 kb originating from a gene (IR3) upstream of the IR1-IR2 complex has been mapped by Northern blot hybridization, S1 nuclease, and primer extension analyses and found to be synthesized in the opposite direction of IR1 and IR2, such that its 5′ terminus overlaps the 5′ terminus of the IR1 mRNA (36). The potential overlapping promoter regions of IR1 and IR3 may diminish expression of the IE polypeptides during E and L stages of a productive infection. Thus, for both possibilities described, EHV-1 could overcome the possible reduced efficiency of the IE promoter during late stages of infection by utilizing an alternative promoter (IR2) that is available and functional at E and L stages of infection.

The protein(s) synthesized from the IR2 mRNA would not include the majority of domain 1 of the EHV-1 IE polypeptide but would retain domains 2 through 5 (27). Several reports have attempted to determine the functions of the five domains of the ICP4 IE protein of HSV-1 (a homolog of IE1 of EHV-1) (21, 27, 38). DeLuca and Schaffer (21), using nonsense and deletion mutants of ICP4, demonstrated that the majority of domain 1 of ICP4 was nonessential for ICP4 function in tissue culture. Interestingly, a serine-rich region is conserved in domain 1 of IE1 of EHV-1, ICP4 of HSV-1, and the 180-kDa IE protein of varicella-zoster virus (27) and is thought to be a site of phosphorylation. However, this conserved region of serine residues was also shown to be nonessential for ICP4 function in tissue culture (21, 53). Thus, the function of domain 1 of ICP4 has yet to be determined precisely; however, possible functions for domains 2 through 5, including DNA binding and transactivation, have been proposed (21, 38). If domain 1 of the EHV-1 IE protein is also presumed to be nonessential in tissue culture, then the IR2 protein(s) may possess similar functions associated with the EHV-1 IE protein, including transactivation of viral genes (65). However, further experimentation, including Western immunoblot analysis using EHV-1 IE-specific monoclonal antibodies, will be necessary to demonstrate whether IE1 is antigenically related to the IR2 gene product. Also, functional analyses of the IR2 gene product (now in progress) will be necessary to ascertain whether the IR1 and IR2 gene products have similar func-

tions. The possibility exists that the protein product(s) of IR2 possesses a repressor function to down-regulate expression of some EHV-1 promoters at the E or L stage of infection. Since EHV-1 encodes a single IE gene, the IR2 product(s) may have functions analogous to those of some IE proteins of HSV-1 which have repressor activity. Lastly, the IR2 gene has been cloned into a simian virus 40 expression vector (32) to address the potential transactivating or transrepressing function(s) of the IR2 gene product (65).

Repeated analyses of EHV-1 RNA isolated under IE conditions have consistently revealed the presence of only the single 6.0-kb IE mRNA species (24–26, 29–31, 58, 59). Therefore, it is unlikely that the IR2 mRNA encodes one of the IE polypeptides since the IR2 message could not be detected under IE conditions. The possibility does exist, however, that AUG-initiated ORFs located within the 3′ portion of the 6.0-kb IE mRNA and corresponding to ORFs encoded by the IR2 mRNA are utilized to synthesize minor species of IE polypeptides (12, 13, 59).

Last, it has been reported that new 5′ transcription initiation sites for mRNAs encoding similar or identical proteins can become available during different stages of virus infection, such as infections with human cytomegalovirus (14), simian virus 40 (11, 23), adenovirus (52), and JC virus (40). It has been suggested that DNA replication may change the viral DNA structure; these changes in conjunction with newly synthesized regulatory proteins that could bind to alternative promoters may result in differential activation of specific viral genes (11, 15, 64). Possibly this type of mechanism is responsible for the induction of IR2 transcription at E and L stages of EHV-1 infection.

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