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Mapping the Termini and Intron of the Spliced Immediate-Early Transcript of Equine Herpesvirus 1

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Equine herpesvirus 1 (EHV-1) has been shown to synthesize a 6.0-kilobase (kb) species of immediate-early (IE) mRNA in productively infected cells. This IE gene region maps within the outer portion (map units 0.79 to 0.83 and 0.96 to 1.00) of the two inverted repeat segments of the short genomic region, and elucidation of its DNA sequence has revealed multiple potential open reading frames (ORFs), including a major ORF of 4,461 nucleotides (F. J. Grundy, R. P. Baumann, and D. J. O'Callaghan, Virology 172:223–236, 1989). Analyses of IE polypeptides synthesized in EHV-1-infected cells (in vivo) and in vitro translation of hybrid-selected IE mRNA indicated that multiple species of IE proteins are encoded by this IE mRNA species. To address the nature of the 6.0-kb IE RNA species, Northern (RNA) blot hybridization, S1 nuclease mapping, and primer extension analyses have been employed. These data revealed that no major introns were detected within the body of the IE transcript. However, the IE mRNA was shown to be spliced at the 5′ terminus, such that a 372-base intron containing two small ORFs (19 and 51 amino acids) was removed from the leader region of the transcript. This splicing event reduced the leader region from 625 to 253 bases. S1 and primer extension analyses of the 5′ terminus of this transcript revealed that the transcription initiation site is located 24 to 26 bases downstream of the consensus TATAAA motif. The 3′ transcription termination site was mapped by S1 nuclease analysis to approximately 10 to 20 bases downstream of the polyadenylation signal, AATAAA. The distance from the stop codon of the major ORF to the polyadenylation site is approximately 300 bases. Results from S1 nuclease experiments indicated that splicing does not occur at the 3′ terminus. These studies indicated that the EHV-1 6.0-kb IE mRNA is spliced at the 5′ terminus and that alternative splicing of this transcript may function in regulating translation of the IE mRNA species.

Equine herpesvirus 1 (EHV-1) (equine abortion virus) serves as a model system for studying herpesvirus gene regulation during both productive and persistent infections (45, 46). Serial, undiluted propagation of standard EHV-1 at a high multiplicity of infection results in the generation of defective interfering particles, which share properties common to classic defective interfering particles (4–6, 27a, 29, 30, 45, 46). EHV-1 DI particles have been shown to possess significant biological activity, since when present with standard EHV-1, they are able to initiate the coestablishment of oncogenic transformation and persistent infection in permissive hamster embryo fibroblasts (18, 59). Numerous EHV-1 persistently infected and oncogenically transformed cell lines and EHV-1 tumor cell lines have been established and have been shown to express viral transcripts and viral proteins, while retaining integrated viral DNA sequences (46, 56–58). The standard EHV-1 genomic structure has been elucidated by restriction endonuclease analysis (7, 31, 68) and electron microscopic analysis (60) (see Fig. 1). The double-stranded DNA molecule is 142 kilobase pairs (kbp) in size and is composed of a unique long (L) region (109 kbp) covalently linked to a short (S) region (32.8 kbp). The S region is composed of unique sequences (U_s; 6.4 kbp) flanked by two identical inverted repeats (13.2 kbp each), which enable the S region to invert relative to the fixed L region, resulting in the formation of two isomeric DNA molecules (7, 8, 31, 45, 46, 48, 60, 68).

EHV-1 transcriptional regulation was first described by Huang et al. (32). DNA-RNA hybridization studies demonstrated that two major classes of viral RNAs were synthesized during a productive infection, and the DNA synthesis inhibitor, 5′-fluoro-2-deoxyuridine, was shown to alter the synthesis and relative molar concentrations of these two classes of RNAs, indicating that regulation of transcription was linked to the initiation of viral DNA synthesis (16, 17). More recently, Caughman et al. (12, 13) have demonstrated by analysis of infected-cell protein synthesis in the presence of metabolic inhibitors that EHV-1 protein synthesis is coordinately regulated into immediate-early (IE), early (E), and late (L) phases. Furthermore, Gray et al. (26) have demonstrated that EHV-1 transcription during a productive infection is also coordinately regulated into IE, E, and L phases.

In the present study, we have analyzed the overall structure of the EHV-1 IE mRNA with respect to its 5′ and 3′ termini, coding region, and potential introns. This genomic region is of interest, since the EHV-1 IE proteins are predicted to be important *trans*-acting regulatory proteins and since the transcriptional pattern from the IE gene region appears to be altered in EHV-1 oncogenically transformed and persistently infected cells (R. N. Harty and D. J. O'Callaghan, unpublished observation).

MATERIALS AND METHODS

Virus and cell culture. The Kentucky A strain of EHV-1 was propagated in L-M cell suspension cultures at a low multiplicity of infection (0.005 PFU per cell) and assayed for infectivity by plaque titration methods (47, 50). Rabbit kidney cells (RK) (RK-13 American Type Culture Collection

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CCL 37) were cultivated in Eagle minimum essential medium (EMEM) supplemented with penicillin (100 U/ml), streptomycin (100 ug/ml), nonessential amino acids, and 5% newborn calf serum (NCS).

Virus infection and use of metabolic inhibitors. Monolayers of RK cells were grown to confluency in 150-cm^2 flasks containing EMEM plus 5% NCS. For the isolation of EHV-1 IE RNA, confluent monolayers of RK cells were preincubated for 1 h at 37°C in EMEM (plus 5% NCS) with 100 μg of cycloheximide per ml (Sigma Chemical Co., St. Louis, Mo.). The cells were subsequently infected with EHV-1 at a multiplicity of infection of 15 PFU per cell in the presence of 100 μg of cycloheximide per ml. After a 90-min adsorption period at 37°C, the inoculum was removed and replaced with EMEM plus 2% NCS, while maintaining the 100-μg/ml concentration of cycloheximide. IE RNA was isolated at 4 h postinfection. The above treatment with cycloheximide has been shown to result in 98% inhibition of total protein synthesis in infected cells (13).

Isolation of RNA. Total cellular RNA was isolated by the guanidinium-cesium chloride centrifugation method of Chirgwin et al. (14), as modified by Maniatis et al. (39). At the appropriate time postinfection, cells were washed twice with phosphate-buffered saline and lysed with 5 ml of the guanidinium isothiocyanate solution (5 M guanidinium isothiocyanate, 5 mM sodium citrate, [pH 7.0], 0.1 M mercaptoethanol, 0.5% *N*-lauryl sarcosine) per 2×10^7 cells. Six milliliters of lysate was layered onto 4 ml of 5.7 M CsCl in 0.1 M EDTA. RNA was pelleted by centrifugation at 32,000 rpm for 20 h in a rotor (model SW41; Beckman Instruments, Inc., Fullerton, Calif.). The RNA pellet was suspended in the guanidinium solution and sterile water and then ethanol precipitated. The RNA was pelleted, suspended in 1 ml of a solution containing 10 mM Tris hydrochloride, 5 mM EDTA, and 1% sodium dodecyl sulfate, extracted with an equal volume of phenol-chloroform, and ethanol precipitated. Poly(A)$^+$ RNA was selected by oligodeoxythymidylate-cellulose chromatography by following the protocol recommended by the supplier (Bethesda Research Laboratories, Inc., Gaithersburg, Md.).

Northern RNA blot hybridization. Poly(A)$^+$ or total cellular RNA was fractionated by formaldehyde agarose gel electrophoresis as described by Maniatis et al. (39). First, RNA samples were suspended in a solution consisting of sterile water, gel running buffer (0.02 M morpholinopropanesulfonic acid [pH 7.0], 5 mM sodium acetate, 1 mM EDTA), 50% deionized formamide, and 15% formaldehyde and heated at 55°C for 15 min. The RNA was fractionated by electrophoresis through 1.2% formaldehyde agarose horizontal slab gels containing 1× gel running buffer and 2.2 M formaldehyde. Electrophoresis was performed at 25 mA for 16 h. RNA was transferred to GeneScreen Plus membrane filters (Dupont, NEN Research Products, Boston, Mass.) in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) by the method of Southern (63), using the protocol recommended by the supplier. Cloned EHV-1 DNA fragments, which were ^{32}P labeled by the nick-translation method of Rigby et al. (52) (nick-translation kit from Bethesda Research Laboratories), were used to detect viral RNA on the filters. For M13 clones, the double-stranded DNA (replicative form) was nicktranslated and used to probe viral RNA. Filters were prehybridized for 3 to 12 h at 65°C in a reaction mixture containing 10% dextran sulfate and 1% sodium dodecyl sulfate. Hybridizations were performed for 12 h at 65°C in the same reaction mixture with the addition of 100 μg of salmon sperm DNA per ml and 5×10^5 cpm of the viral DNA probe per ml. After hybridization, filters were rinsed in 2× SSC for 5 min, washed extensively at 65°C in 2× SSC and 1% sodium dodecyl sulfate, and rinsed extensively in 0.1X SSC at room temperature, followed by exposure to X-Omat film (Eastman Kodak Co., Rochester, N.Y.) at −70°C in the presence of Cronex Lightning-Plus (E. I. du Pont de Nemours & Co., Wilmington, Del.) intensifying screens. Molecular size standards 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.

5′ and 3′ end labeling of DNA probes. For 5′ end labeling, 1 to 2 μg of cloned EHV-1 DNA fragment was first digested with the appropriate restriction endonuclease(s), dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), extracted with an equal volume of phenol, and ethanol precipitated. The DNA fragments were suspended at a concentration of 500 μg/ml and 5′ end labeled with [γ-^{32}P] ATP (3,000 Ci/mmol; Dupont NEN) and T4 polynucleotide kinase (Bethesda Research Laboratories) as described by Maxam and Gilbert (40). Labeled DNA fragments were separated from free nucleotides by electrophoresis through 1% agarose slab gels with the proper bands being excised and purified by using the GeneClean DNA elution kit (Bio 101 Inc., La Jolla, Calif.) following the protocol recommended by the supplier. After ethanol precipitation, the DNA was suspended at a concentration of 40 ng/μl.

Oligonucleotide primers (20-mers), purchased from Synthetic Genetics Inc., San Diego, Calif., were 5′ end labeled by using [γ-^{32}P]ATP (6,000 Ci/mmol; Dupont NEN) and T4 polynucleotide kinase (Bethesda Research Laboratories) as described by Maniatis et al. (39), as modified elsewhere (3). In this case, free nucleotides were removed by several precipitations with 10 M ammonium-acetate and ethanol, followed by one precipitation with ethanol and 3 M sodium-acetate. The oligonucleotide primers were then suspended at a concentration of 1 ng/μl.

For 3′ end labeling, specific DNA fragments (5 μg) were first digested to completion with the appropriate restriction endonuclease(s). [α-^{32}P]CTP (800 Ci/mmol; Dupont NEN) was added directly to the reaction mixture along with 10 mM dATP, dGTP, and dTTP (Bethesda Research Laboratories) and 1 U of Klenow fragment (Dupont NEN) as described elsewhere (3). After incubation at 30°C for 15 min, the samples were electrophoresed through a 1% agarose slab gel, with the appropriate bands being excised and purified using GeneClean (Bio 101 Inc.). After ethanol precipitation at −20°C, the DNA was suspended at a concentration of 40 ng/μl.

S1 nuclease mapping. The method of Berk and Sharp (9) as modified by Weaver and Weissman (67) was used. 5′- or 3′-end-labeled DNA probes (100 ng) were mixed with 1 μg of poly(A)$^+$-selected IE mRNA in a solution consisting of 80% phosphate-buffered formamide, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.4), 400 mM NaCl, and 1 mM EDTA in a final volume of 25 μl. After denaturation at 70°C for 10 min, the samples were hybridized for 18 to 20 h at the optimal temperature determined (53°C for 5′-terminal fragments and 57°C for 3′-terminal fragments). After hybridization, 0.3 ml of a solution consisting of ice-cold 250 mM NaCl, 30 mM sodium acetate (pH 4.6), and 1 mM $ZnCl_2$ containing 60 U of S1 nuclease (Bethesda Research Laboratories) was added. Digestion was for 30 min at 37°C, after which the mixture was ethanol precipitated and the S1-resistant products were analyzed on 6% polyacrylamide–urea sequencing gels.

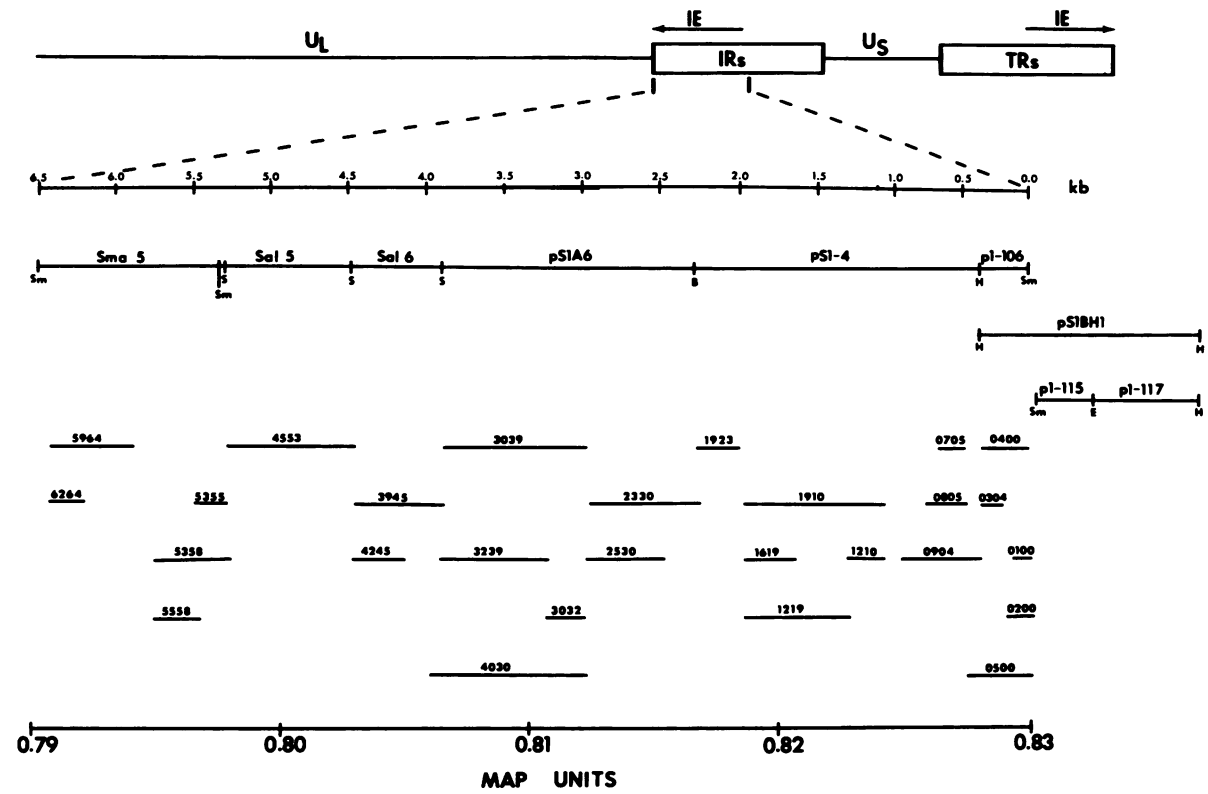

FIG. 1. EHV-1 clones mapping to the IE gene region. The structure of the EHV-1 genome is shown at the top of the figure, illustrating the position and direction of transcription of the EHV-1 6.0-kb IE mRNA. The 6.5-kb IE gene region is expanded, showing the position of several EHV-1 clones that have been inserted into bacterial vectors (Sma5, Sal5, Sal6, pS1A6, pS1-4, p1-106, pS1BH1, p1-115, and p1-117). Restriction endonuclease abbreviations: Sm, *Sma*I; S, *Sal*I; B, *Bam*HI; H, *Hin*dIII; E, *Eco*RI. All of the clones with a four digit number have been inserted into bacteriophage M13 derivatives by using a variety of restriction endonuclease sites. The four-digit number denotes the termini and size of each clone (in kilobase pairs) (i.e., clone 1219 contains EHV-1 IE gene sequences between map position 1.2 and 1.9 kb, and thus the insert is approximately 700 bp in size). The map units of the IE gene region are shown at the bottom of the figure. IRs, Inverted repeats; TRs, terminal repeats.

Primer extension analysis. Primer extension analysis was performed by the method of Inoue and Cech (34). Oligonucleotide primers (616 and 986; complementary to nucleotides 254 to 273 and 624 to 643 from the 5′ end of the IE mRNA, respectively) were 5′ end labeled and ethanol precipitated with 1 to 5 μg of poly(A)$^+$-selected IE mRNA. The pellet was suspended in 10 μl of hybridization buffer (60 mM NaCl, 50 mM Tris hydrochloride [pH 8.3], (10 mM dithiothreitol) and placed in a 90°C water bath. The temperature was gradually reduced to 30°C over approximately 45 min. Two microliters of 36 mM $MgCl_2$ in hybridization buffer was then added. The reactions contained 2 μl of the RNA-primer solution and 3 μl of a solution consisting of 375 μM deoxynucleoside triphosphates, 60 mM NaCl, 6 mM $MgCl_2$, 50 mM Tris hydrochloride (pH 8.3), and 10 mM dithiothreitol. Each reaction was placed at 42°C for 2 min, followed by the addition of 1 U of avian myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories). The reactions were allowed to proceed for 30 min at 42°C. The cDNA products were analyzed on 6% denaturing polyacrylamide–urea sequencing gels.

DNA sequencing. DNA sequencing using subclones of the EHV-1 *Eco*RI C fragment in the bacteriophage M13 derivatives (mp8, 9, 18, and 19) was performed by using the dideoxynucleotide chain-termination procedure of Sanger et al. (62), as modified by Messing (43). Protocols were as described by Grundy et al. (28).

RESULTS

Northern hybridization analysis of the EHV-1 IE mRNA. The EHV-1 6.0-kb IE transcript has been mapped to the outer portions of the inverted repeats (map units 0.79 to 0.83 and 0.96 to 1.00), and the direction of transcription was determined by S1 nuclease analysis to be away from the U_s region and toward the termini of the genome (27) (Fig. 1). To define more precisely the termini and overall structure of the EHV-1 IE transcript, numerous EHV-1 IE gene subclones (Fig. 1) were used to probe either total or poly(A)$^+$ IE RNA in Northern hybridization experiments. Clone pS1-4 (Fig. 2A, lane 4), which maps downstream of pS1BH1 (Fig. 1), and clone p1-106 (lane 2), a subclone of pS1BH1, hybridized to the 6.0-kb IE transcript. Clone p1-115 (lane 3), which maps upstream of p1-106 (Fig. 1), did not hybridize to the IE mRNA. Since no viral clones mapping upstream of p1-106 hybridized to the IE transcript (data not shown), the 5′ transcription initiation site of the 6.0-kb IE mRNA mapped within clone p1-106. Clone p1-106 was subsequently sequenced and shown to contain a strong TATAAA consensus sequence and several other potential *cis*-acting regulatory elements, including CCAAT and GGCGGG box homologies (28).

Subclones of p1-106 (0100, 0200, and 0304) (Fig. 1) were used to map the transcription initiation site more precisely (Fig. 2B). Both clone 0304 and p1-106 hybridized to the

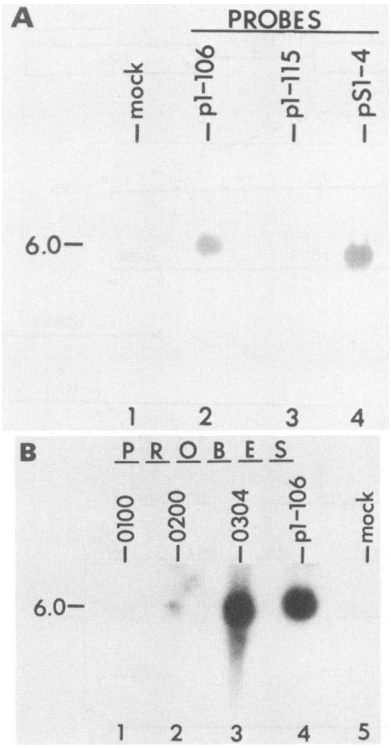

FIG. 2. (A) Northern analysis of EHV-1 IE RNA. Total cellular RNA was isolated at 4 h postinfection from RK cells infected under IE conditions. RNA was transferred to GeneScreen Plus membrane filters, and viral transcripts were detected by hybridization to probes p1-106, p1-115, and pS1-4 (lanes 2 through 4 respectively). Molecular sizes are in kilobases. No hybridization could be detected with RNA from uninfected RK cells (lane 1, mock). (B) Viral transcripts were detected by hybridization to probes 0100, 0200, 0304, and p1-106 (lanes 1 through 4, respectively). Molecular sizes are in kilobases. No hybridization could be detected with RNA from uninfected RK cells (lane 5, mock).

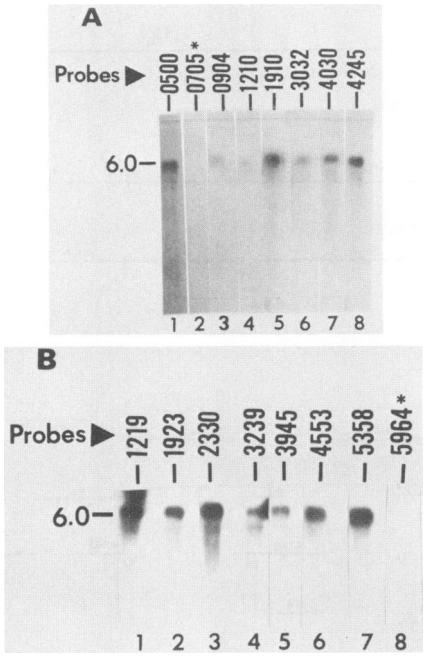

FIG. 3. (A) Northern analysis of EHV-1 IE RNA. Total cellular RNA was isolated at 4 h postinfection from RK cells infected under IE conditions. Poly(A)$^+$ RNA was selected by oligodeoxythymidylate-cellulose chromatography. Poly(A)$^+$ RNA was transferred to GeneScreen Plus membrane filters, and viral transcripts were detected by hybridization to probes 0500, 0705, 0904, 1210, 1910, 3032, 4030, and 4245 (lanes 1 through 8, respectively). No hybridization could be detected with RNA from uninfected RK cells (data not shown). (B) Viral transcripts were detected by hybridization to probes 1219, 1923, 2330, 3239, 3945, 4553, and 5358 (lanes 1 through 7, respectively). Clone 5964 (lane 8) did not hybridize to the IE mRNA. No hybridization could be detected with RNA from uninfected RK cells (data not shown).

6.0-kb IE mRNA, whereas clones 0100 and 0200 did not, indicating that the 5′ transcription initiation site, as determined by Northern blot analysis, mapped within clone 0304. Thus, these data mapped the transcription initiation site to lie within the same subclone that contains the TATAAA motif.

Similar experiments utilizing Northern blot analysis were performed to map the body of the 6.0-kb IE mRNA. In all, a total of 27 subclones (Fig. 1) from the EHV-1 IE gene region, which were used to sequence the IE gene (28), were used to probe the 6.0-kb IE transcript in Northern hybridization experiments (Fig. 3). All of the clones tested hybridized to the 6.0-kb IE transcript, except for clone 0705 (Fig. 3A, lane 2), which is an approximately 200-bp fragment mapping at the 5′ region of the IE gene (Fig. 1). Similarly, clone 0805, which maps to the same region of the genome as clone 0705 (Fig. 1), did not hybridize to the 6.0-kb IE transcript (data not shown). Analysis of the DNA sequence from this region revealed the presence of a potential splice donor site and splice acceptor site. These findings suggested that the 6.0-kb IE mRNA may be spliced at the 5′ terminus, as has been reported for two IE mRNAs (IE mRNAs 4 and 5) of herpes simplex virus type 1 (HSV-1) (53). Finally, the 3′ terminus of the IE mRNA was mapped to clone 5358 (Fig. 3B, lane 7), which was also shown to contain the consensus polyadenylation signal sequence AATAAA (28).

S1 nuclease analysis of the 5′ terminus of the IE mRNA. To position the 5′ transcription initiation site and to detect any splicing within this transcript, S1 nuclease analysis was employed. Two (p1-106 and pS1BH1) of the three probes used in these S1 analyses are shown in Fig. 1, while the other probe, pFG65, is described in the legend to Fig. 4. Clones p1-106 and pS1BH1 were 5′ end labeled at their shared *Hin*dIII site (Fig. 1) and thus should yield similarly sized protected fragments upon S1 nuclease digestion after hybridization to the EHV-1 IE transcript (Fig. 4, lanes 2 and 3). A fragment of less than 100 bp was predicted on the basis of the results of Northern hybridization experiments. A series of protected fragments migrating between 72 and 79 nucleotides in size (lanes 2 and 3) was observed. This pattern was observed repeatedly and may indicate multiple transcription initiation sites for the IE transcript. Identical samples were analyzed on a 15% polyacrylamide–urea gel, which resulted in a sharp band migrating at approximately 75 to 77 nucleotides in size for both probes (Fig. 4, lanes 11 and 12). These results mapped the 5′ transcription initiation site of the 6.0-kb IE mRNA approximately 24 to 26 bases downstream of the TATAAA motif (Fig. 5), which is a distance similar to that of other eucaryotic genes (42), including the ICP4 gene of HSV-1 (24, 37, 38).

Plasmid pFG65 was 5′ end labeled at its unique *Nhe*I site (Fig. 5) and after hybridization to the IE transcript, was found to yield two major S1 nuclease-resistant fragments of

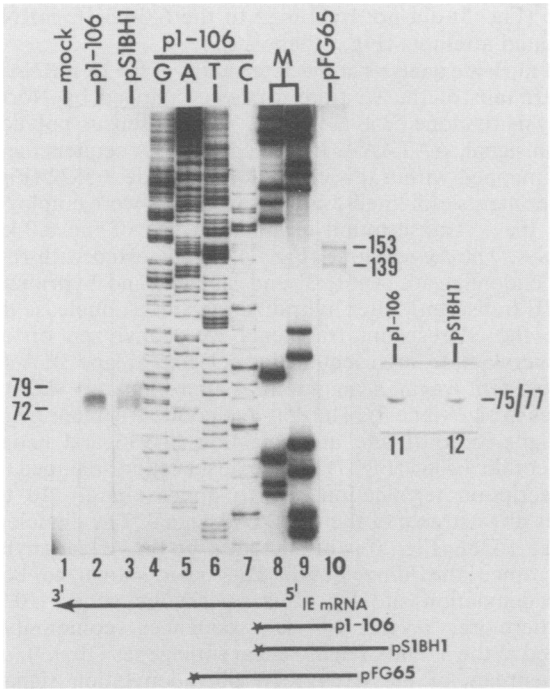

FIG. 4. S1 nuclease analysis of the 5′ end of the EHV-1 IE mRNA. DNA clones labeled at the 5′ ends, denoted by the asterisks (*), were hybridized to poly(A)$^+$ IE RNA and digested with S1 nuclease, and the protected fragments were fractionated on a 6% polyacrylamide–urea gel (lanes 2, 3, and 10). Clones p1-106 and pS1BH1 are illustrated in Fig. 1. DNA sequencing and Southern blot analysis revealed that clone pFG65 contained 5′-terminal IE gene sequences from −813 to +637. The DNA sequences of EHV-1 clone p1-106 terminating at G, A, T, and C (lanes 4 through 7) and *Hae*II and *Alu*I fragments of pBR322 (lanes 8 and 9, respectively) were included as molecular weight markers. The DNA sequencing bands are offset by the length of the primer. Identical samples in lanes 2 and 3 were analyzed on a 15% gel (lanes 11 and 12). No hybridization was detected with RNA from uninfected RK cells (lane 1, mock). Molecular sizes are in nucleotides. The approximate position of the 5′ termini of each probe relative to the IE mRNA is depicted under the gel.

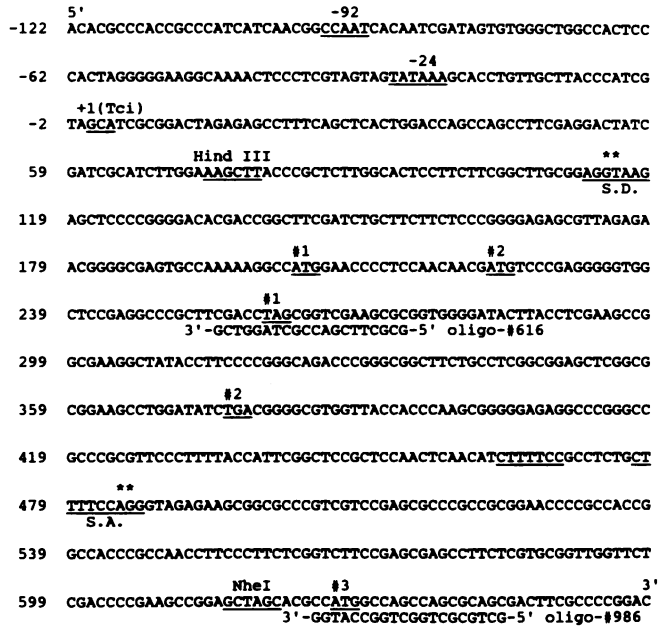

FIG. 5. Nucleotide sequence at the 5′ terminus of the EHV-1 IE gene. The sequence between map units 0.824 and 0.828 of the Kentucky A strain of EHV-1 is shown. The transcription initiation site (Tci) of the 6.0-kb IE mRNA is at nucleotide +1. The CCAAT box and TATAAA box homologies are located at nucleotides −92 and −24, respectively. The splice donor (S.D.) (nucleotide +113) and splice acceptor (S.A.) (nucleotide +485) sites are indicated by asterisks (*). The sequences of primers 616 (nucleotides +254 to +273) and 986 (nucleotides +624 to +643), used in primer extension experiments, are shown. The 7-mer sequence, CTTTTCC, which is part of the splice acceptor site (nucleotide +477), is repeated upstream at nucleotide +463. The start and stop codons for ORFs 1 (nucleotides +202 to +259; 19 amino acids) and 2 (nucleotides +222 to +375; 51 amino acids) are underlined. ATG 3, at nucleotide +626, is the translational start codon for the major ORF of the IE gene. The *Nhe*I site, which is present in pFG65 and was 5′ end labeled for S1 nuclease analysis, is at nucleotide +615. The *Hin*dIII site, which was 5′ end labeled in probes p1-106 and pS1BH1 for S1 nuclease analysis, is at nucleotide +73.

approximately 139 and 153 bases (lane 10). The S1-resistant 139-nucleotide fragment had its 3′ end lie within several bases of the consensus splice acceptor (S.A.) site, determined by DNA sequencing (28) to map at nucleotide +485 (Fig. 5). The S1-resistant 153-nucleotide fragment had its 3′ end lie at nucleotide +466. A 7-mer sequence centered at nucleotide +466 with the sequence CTTTTCC is repeated immediately adjacent to the splice acceptor site at nucleotide +485. This repeat 7-mer sequence may allow an intron of variable size to be spliced from the 5′ terminus of the 6.0-kb IE mRNA. An S1-resistant fragment of approximately 620 nucleotides was predicted using probe pFG65; if the IE mRNA were not spliced, however, a fragment of this size was never observed. These results strongly suggest that the 6.0-kb IE mRNA isolated at 4 h postinfection is spliced at the 5′ terminus, upstream of the major ORF.

Primer extension analysis of the 5′ terminus of the IE mRNA. Primer extension analysis was performed at the 5′ terminus of the 6.0-kb IE transcript to corroborate results obtained by S1 nuclease analysis. Both the 5′ transcription initiation site and the splice acceptor site were mapped using primer extension. Two 20-mer deoxy-oligonucleotides (616, complementary to nucleotides +254 to +273; 986, complementary to nucleotides +624 to +643) (Fig. 5) were 5′ end labeled, hybridized to poly(A)$^+$ IE RNA, and extended with avian myeloblastosis virus reverse transcriptase. When primer 986 (Fig. 5) was hybridized to various concentrations of the 6.0-kb IE mRNA and extended with reverse transcriptase, a single cDNA fragment was visible on the gel migrating at approximately 270 nucleotides in size (Fig. 6, lanes 5 through 7), which was in excellent agreement with results obtained by S1 nuclease analysis. The intensity of the cDNA fragment increased with increasing concentration of the IE transcript. In the absence of splicing, a cDNA with a size of 642 bases would be expected to be synthesized by using oligonucleotide 986 as the primer. A fragment of this size was not visible, even upon overexposure of the gel. These findings confirmed that a 372-base intron was spliced from the 5′ leader region of the 6.0-kb IE mRNA and that the 5′ transcription initiation site mapped 24 to 26 bases downstream of the TATAAA motif. These results also indicated that the splice acceptor site at nucleotide +485 appeared to be the major splice acceptor site or possibly the only splice acceptor site, since only a single band was visible on the gel. Finally, as further evidence of splicing, primer 616, which is complementary to intron sequences at the 5′ end of the IE

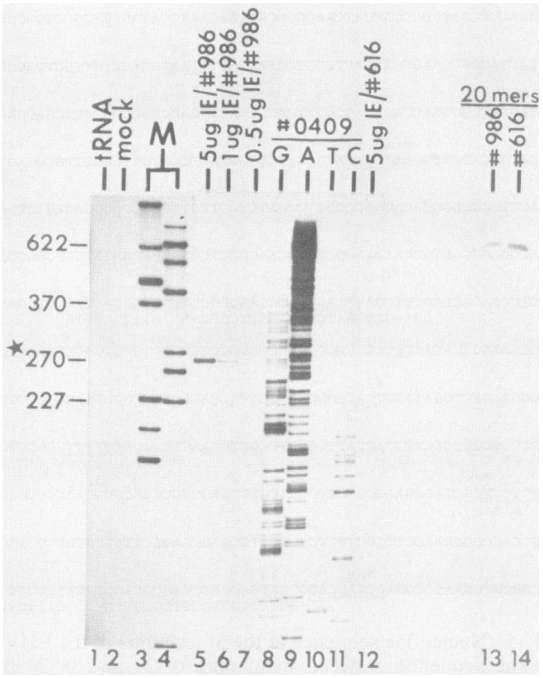

FIG. 6. Primer extension analysis of the 5′ end of the EHV-1 IE mRNA. Poly(A)$^+$ IE RNA was hybridized to 5′-end-labeled primers 616 (lane 12) or 986 (lanes 5 through 7) (see Fig. 5 for the primer sequences). Reverse transcriptase was used to synthesize a cDNA fragment which was fractionated on a 6% polyacrylamide–urea gel. The DNA sequence of EHV-1 subclone 0409 terminating at G, A, T, and C (lanes 8 through 11), and *Hae*II and *Alu*I fragments of pBR322 (lanes 3 and 4, respectively) were used as molecular weight standards. Yeast tRNA and RNA from uninfected RK cells (mock) did not hybridize to either primer (lanes 1 and 2, respectively). Both primers were shown to be labeled and intact (lanes 13 and 14). Lanes M, Molecular size markers. Asterisk indicates the size of the cDNA product. Molecular sizes are in nucleotides.

gene (Fig. 5) did not hybridize to the 6.0-kb IE mRNA in repeated attempts (Fig. 6, lane 12).

S1 nuclease analysis of the 3′ terminus of the IE mRNA. The 3′ terminus of the IE transcript was mapped by Northern analysis to clone 5358 (Fig. 3B). The consensus polyadenylation signal, AATAAA, identified by DNA sequencing (28), also mapped within this clone at nucleotide +5372 (Fig. 7). Experiments utilizing S1 nuclease analysis were employed to map the 3′ transcription termination site of the 6.0-kb IE mRNA. The *Sma*5 clone (Fig. 1) was digested with restriction endonuclease *Xho*I, 3′ end labeled, and hybridized to the IE transcript. After hybridization and S1 nuclease digestion, the S1-resistant fragments were analyzed on a 6% polyacrylamide–urea sequencing gel (Fig. 8, lane 3). A single S1-resistant fragment migrated at approximately 450 nucleotides in size (lane 3). The 450-nucleotide fragment began at the labeled *Xho*I site at nucleotide +4936 and ended at nucleotide +5385 (Fig. 7). These observations mapped the 3′ transcription termination site to approximately 10 to 20 bases downstream of the AATAAA signal. The dinucleotide TA at +5386 (Fig. 7) is predicted to be the polyadenylation site, since this dinucleotide has been shown to be the polyadenylation site for other eucaryotic genes (10, 69). Furthermore, several GT-rich sequences, commonly observed at the 3′ end of many eucaryotic genes (10, 69), occur downstream of the EHV-1 IE polyadenylation signal sequence (Fig. 7). Finally, although potential splice donor and splice acceptor sites were identified at the 3′ terminus of the IE gene by DNA sequencing (28), splicing in this region was not detected by using S1 nuclease analysis; thus, the untranslated 3′ end of the 6.0-kb IE transcript is approximately 300 bases in length.

DISCUSSION

Previous studies from this laboratory have indicated that a single 6.0-kb transcript is synthesized under IE conditions, yet four IE polypeptide species (IE1, IE2, IE3, and IE4) are

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     5'              XhoI
4918 GAGCCCGACGACGAGCTCGAGATCGACGGGGCCGGCGATGTGGAGCTGGTGGTTGAGCAC

4978 CCCGGCCCGAGCCCCGGCGTGGCCCAGGCCCTCCGCCGCGCTCCCATCAAGATCGAGGTG

5038 GTGTCGGACGACGAGGACGGAGGAGACTGGTGCAATCCGTACCTCTCCTGAACACGATGG

5098 AGCGCCTCCCTGCGGCCGAAAACAAGAAAAATCAGTACATCCACAACTATGTGTCCGCCC

5158 AGCACAACGCAGACTCCGCCTAGACTCCCGCCTCCATCCGCTGACGCTGAACCCCGCCCC

5218 GCCCTCTGCTGACGCGAAGACAAGGCCCTCCCCGGACGACATGTGAGGAACGAAGGGGGC

5278 GTTGTATCTAGCAGCCCACGTTCCTTATTGCTCACATGTCTGCCCAATCGGTGGGCACTT

                                          @@@@@@        ++
5338 CCAGGCTTTCCCCTATCGCTGAGTGGTTGTTTTTAATAAAGTTTTTTTTAAATTTTGATT

                                                                3'
5398 GACCGCGTGGTCTTTGTTTACTGGGCGGGTTGATGGGCGGGTTGATGGGCGGGTTGATGG
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FIG. 7. Nucleotide sequence of the 3′ terminus of the EHV-1 IE gene. The sequence of the Kentucky A strain of EHV-1 between map units 0.795 and 0.797 is shown. The position of the unique *Xho*I site, that was 3′ end labeled for S1 nuclease analysis, begins at nucleotide +4933. The stop codon for the major ORF of the IE gene is shown at nucleotide +5086. The polyadenylation signal sequence, AATAAA, (@) is shown at nucleotide +5372. The dinucleotide TA (+) (nucleotide +5386) and several GT-clusters (underlined) are shown down stream of the AATAAA motif.

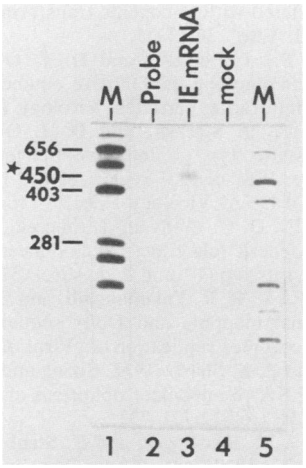

FIG. 8. S1 nuclease analysis at the 3′ terminus of the EHV-1 IE transcript. The *Sma*5 clone (Fig. 1) was 3′ end labeled at its unique *Xho*I site. (Fig. 7) and hybridized to poly(A)$^+$ IE mRNA. The hybrids were digested with S1 nuclease and fractionated on a 6% polyacrylamide–urea gel (lane 3). *Alu*I and *Hae*II digests of pBR322 (lanes 1 and 5, respectively) served as approximate molecular weight markers. The probe alone was shown to be digested with S1 nuclease (lane 2), and RNA isolated from uninfected RK cells did not hybridize to the probe (lane 4, mock). Lanes M, Molecular size markers. Asterisk indicates the size of the S1-resistant fragment. Molecular sizes are in nucleotides.

detected in the infected cell and are synthesized in vitro from hybrid-selected IE mRNA (12, 13, 26, 27, 55). A similar phenomenon of multiple proteins from a single transcript has been reported for pseudorabies virus (23). EHV-1 IE1 has an apparent molecular mass of 200 kDa and has been shown to be phosphorylated and localized in the nucleus during a productive infection (G. B. Caughman, personal communication). The four EHV-1 IE polypeptides are antigenically related (12), and are predicted to be important *trans*-acting regulatory proteins, as are those of other herpesviruses (21, 22, 24, 25, 33, 36, 38, 44, 49, 51, 54).

In this report, the physical structure of the 6.0-kb IE transcript was analyzed by Northern hybridization, S1 nuclease, and primer extension analyses to examine IE gene regulation at the transcriptional level. Northern hybridization analysis demonstrated that the 6.0-kb IE transcript of EHV-1 hybridized only to sequences of the inverted repeats of the S region. Thus, the genomic loci of the EHV-1 IE gene are similar to those of the ICP4 gene of HSV-1 (2, 15, 37, 66) and the IE gene of varicella zoster virus (19, 20, 41). Northern blot analysis revealed that the EHV-1 6.0-kb mRNA was contiguous on the genome and possessed no major introns, although splicing was detected at the 5′ terminus. In agreement with these findings, analysis of the DNA sequence at the 5′ terminus revealed strong consensus splice donor and splice acceptor sites.

S1 nuclease analysis revealed that the 5′ transcription initiation site (Tci) of the 6.0-kb IE transcript was located 24 to 26 bases downstream of the TATAAA sequence and 92 to 94 bases downstream of the CCAAT sequence (Fig. 5). These distances are in excellent agreement with those commonly observed for other eucaryotic genes (42). S1 nuclease analysis using probe pFG65 resulted in two S1 nuclease-resistant fragments, approximately 139 and 153 nucleotides in size (Fig. 4). The 139-bp protected fragment terminated within several bases of the splice acceptor site at nucleotide +485, while the 153-bp fragment terminated in the center of a 7-mer sequence, CTTTTCC, located just upstream of the splice acceptor site. This 7-mer sequence is homologous to the sequence immediately adjacent to the splice acceptor site at nucleotide +485 (Fig. 5). Splicing of the 5′ untranslated region of two HSV-1 IE mRNAs (IE mRNAs 4 and 5) has been reported by Rixon and Clements (53), whereby different copy numbers of a 22-bp tandem reiteration at the 5′ termini of two HSV-1 IE mRNAs result in the variation in length of a single spliced intron.

A corresponding splice donor site was identified by DNA sequencing (28) at the 5′ terminus of the IE transcript, such that a 372-base intron was spliced from this transcript (Fig. 5). Within this intron are two small ORFs (ORF 1, 19 amino acids; ORF 2, 51 amino acids) that would be removed upon splicing. The ATGs for ORFs 1 and 2 are in a consensus context sequence to be efficiently translated (35). These two ORFs, if translated, may function to down regulate translation from the major ORF of the IE gene. Therefore, splicing of this intron would not affect the major ORF of the IE gene but would remove the two upstream ORFs and reduce the leader region from 625 bases to 253 bases, which would likely increase translational efficiency from the ATG of the major ORF. Finally, preliminary data suggest that this 372-base intron may not be spliced from the IE transcript as efficiently during late stages of a productive infection as it is during IE stages, suggesting that this splicing event may regulate the synthesis of the IE polypeptides.

Primer extension analysis was utilized to corroborate the splicing event at the 5′ end of this transcript and to position the transcription initiation site. Extension of a synthetic oligonucleotide (primer 986), following its hybridization to the IE transcript, yielded a cDNA of 270, not 642, nucleotides, which would be the size cDNA fragment expected were splicing not to occur. That this cDNA fragment was 270 nucleotides confirmed the position of the transcription initiation site, as determined by S1 nuclease mapping. This result differs with that of S1 nuclease mapping using probe pFG65, which resulted in two major bands on the gel, approximately 153 and 139 nucleotides in size (Fig. 4). The primer extension analysis, which yielded a single band, indicates that there may be only one splice acceptor site and that the 7-mer sequence beginning at nucleotide +463 may not represent a true splice acceptor site. This 7-mer sequence does not contain the dinucleotide AG common to all splice acceptor sites and may represent an S1 nuclease-sensitive site. Alternatively, this region may represent the transcription initiation site of a second viral transcript. As further evidence of splicing, primer 616, which is complementary to intron sequences, did not hybridize to the IE transcript as expected. It is important to note that there are numerous potential ORFs (total number of ORFs, 38) throughout the EHV-1 IE gene (28), and thus the possibility that small-scale splicing within the body of the IE transcript to generate a family of similarly sized transcripts, cannot be ruled out. The 3′ terminus of the 6.0-kb IE mRNA was mapped by Northern analysis to subclone 5358, which also contained the polyadenylation signal sequence, AATAAA. S1 nuclease analysis revealed an S1-resistant fragment approximately 450 nucleotides in size (Fig. 8). These results mapped the polyadenylation site of the 6.0-kb IE mRNA 10 to 20 bases downstream of the polyadenylation signal sequence (Fig. 7). This distance is in good agreement with values reported for other eucaryotic genes (10, 69). The dinucleotide TA and several GT-rich clusters that are conserved at the 3′ termini of many eucaryotic genes are present at the 3′ terminus of the 6.0-kb IE mRNA (Fig. 7).

It has been reported that an acidic carboxyl-terminal domain is a feature common to many eucaryotic and procaryotic *trans*-activating proteins (1, 11, 61, 64, 65). Indeed, several regions at the carboxyl-terminal end of the IE protein are composed of acidic amino acid residues. This observation supports the prediction of a regulatory function for the EHV-1 IE polypeptide(s). Interestingly, since the 3′ terminus of the IE transcript does not appear to be altered by splicing, the untranslated region at the 3′ end is unusually long (300 bases) compared with that of the ICP4 gene of HSV-1 (45 bases) (1).

The data presented in this paper suggest that splicing of the 6.0-kb IE mRNA, at the 5′ terminus, may function to regulate expression of the IE polypeptide species. No major introns were detected by Northern hybridization within the body of the 6.0-kb IE transcript; however, small-scale splicing events to generate a family of similarly sized transcripts cannot be ruled out. A second transcript, that arises from the IE gene region, is a 4.4-kb mRNA that can be detected during early and late stages of a productive infection (27; R. N. Harty, C. F. Colle, and D. J. O'Callaghan, *in* E. K. Wagner, ed., *Herpesvirus transcription and its regulation*, in press). Finally, the transcriptional pattern from the IE gene region during a productive infection, appears to be altered in EHV-1 oncogenically transformed and persistently infected cells (Harty and O'Callaghan, unpublished observation). Experiments are now in progress to understand how the EHV-1 IE gene sequences are differentially regulated during IE, E, and L stages of a productive infection and how this regulation is altered in EHV-1 oncogenically transformed and persistently infected cells.

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ADDENDUM IN PROOF

Primer extension analyses of large quantities (15 μg) of Poly(A)-selected IE mRNA with oligonucleotide 616 revealed a faint cDNA fragment of approximately 270 nucleotides, suggesting that a small population of the IE transcripts is not spliced.

LITERATURE CITED

1. **Ace, C. I., T. A. McKee, J. M. Ryan, J. M. Cameron, and C. M. Preston.** 1989. Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. J. Virol. **63**:2260–2269.
2. **Anderson, K. P., R. H. Costa, L. E. Holland, and E. K. Wagner.** 1980. Characterization of herpes simplex virus type 1 RNA present in the absence of de novo protein synthesis. J. Virol. **34**:9–27.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. Greene Publishing Associates and John Wiley & Sons, Inc., New York.
4. **Baumann, R. P., S. A. Dauenhauer, G. B. Caughman, J. Staczek, and D. J. O'Callaghan.** 1984. Structure and genetic complexity of the genomes of herpesvirus defective-interfering particles associated with oncogenic transformation and persistent infection. J. Virol. **50**:13–21.
5. **Baumann, R. P., J. Staczek, and D. J. O'Callaghan.** 1986. Cloning and fine mapping the DNA of equine herpesvirus type one defective interfering particles. Virology **153**:188–200.
6. **Baumann, R. P., J. Staczek, and D. J. O'Callaghan.** 1987. Equine herpesvirus type 1 defective-interfering (DI) particle DNA structure: The central region of the inverted repeat is deleted from DI DNA. Virology **159**:137–146.
7. **Baumann, R. P., D. C. Sullivan, J. Staczek, and D. J. O'Callaghan.** 1986. Genetic relatedness and colinearity of genomes of equine herpesvirus types 1 and 3. J. Virol. **57**:816–825.
8. **Baumann, R. P., V. R. R. Yalamanchili, and D. J. O'Callaghan.** 1989. Functional mapping and DNA sequence of an equine herpesvirus 1 origin of replication. J. Virol. **63**:1275–1283.
9. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
10. **Birnstiel, M. L., M. Busslinger, and K. Strub.** 1985. Transcription termination and 3′ processing: The end is in site. Cell **41**:349–359.
11. **Brent, R., and M. Ptashne.** 1985. A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. Cell **43**:729–736.
12. **Caughman, G. B., A. T. Robertson, W. L. Gray, D. C. Sullivan, and D. J. O'Callaghan.** 1988. Characterization of equine herpesvirus type 1 immediate early proteins. Virology **163**:563–571.
13. **Caughman, G. B., J. Staczek, and D. J. O'Callaghan.** 1985. Equine herpesvirus type 1 infected cell polypeptides: evidence for immediate-early/early/late regulation of viral gene expression. Virology **145**:49–61.
14. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
15. **Clements, J. B., R. J. Watson, and N. M. Wilkie.** 1977. Temporal regulation of herpes simplex virus type 1 transcription: location of transcripts on the viral genome. Cell **12**:275–285.
16. **Cohen, J. C., M. L. Perdue, C. C. Randall, and D. J. O'Callaghan.** 1977. Herpesvirus transcription: altered regulation induced by FUdR. Virology **76**:621–633.
17. **Cohen, J. C., C. C. Randall, and D. J. O'Callaghan.** 1975. Transcription of equine herpesvirus type 1: evidence for classes of transcripts differing in abundance. Virology **68**:561–565.
18. **Dauenhauer, S. A., R. A. Robinson, and D. J. O'Callaghan.** 1982. Chronic production of defective-interfering particles by hamster embryo cultures of herpesvirus persistently infected and oncogenically transformed cells. J. Gen. Virol. **60**:1–14.
19. **Davison, A. J., and D. J. McGeoch.** 1986. Evolutionary comparisons of the S segments in the genomes of herpes simplex virus type 1 and varicella-zoster virus. J. Gen. Virol. **67**:597–611.
20. **Davison, A. J., and J. E. Scott.** 1985. DNA sequence of the major inverted repeat in the varicella-zoster virus genome. J. Gen. Virol. **66**:207–220.
21. **DeLuca, N. A., and P. A. Schaffer.** 1985. Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. Mol. Cell. Biol. **5**:1997–2008.
22. **DeLuca, N. A., and P. A. Schaffer.** 1988. Physical and functional domains of the herpes simplex virus transcriptional regulatory protein ICP4. J. Virol. **62**:732–743.
23. **Fenwick, M. M., and M. McMenamin.** 1984. Synthesis of alpha (immediate-early) proteins in vero cells infected with pseudorabies virus. J. Gen. Virol. **65**:1449–1456.
24. **Gelman, I. H., and S. Silverstein.** 1987. Dissection of immediate-early gene promoters from herpes simplex virus: sequences that respond to the virus transcriptional activators. J. Virol. **61**: 3167–3172.
25. **Godowski, P. J., and D. M. Knipe.** 1986. Transcriptional control of herpes virus gene expression: gene functions required for positive and negative regulation. Proc. Natl. Acad. Sci. USA **83**:256–260.
26. **Gray, W. L., R. P. Baumann, A. T. Robertson, G. B. Caughman,**

D. J. O'Callaghan, and J. Staczek. 1987. Regulation of equine herpesvirus type 1 gene expression: characterization of immediate-early, early, and late transcription. Virology 158:79–87.
27. Gray, W. L., R. P. Baumann, A. T. Robertson, D. J. O'Callaghan, and J. Staczek. 1987. Characterization and mapping of equine herpesvirus type 1 immediate early, early, and late transcripts. Virus Res. 8:233–244.
27a. Gray, W. L., R. Yalamanchili, B. Raengsakulrach, R. P. Baumann, J. Staczek, and D. J. O'Callaghan. 1989. Defective interfering particles. Virology 172:1–10.
28. Grundy, F. J., R. P. Baumann, and D. J. O'Callaghan. 1989. DNA sequence and comparative analyses of the equine herpesvirus type 1 immediate-early gene. Virology 172:223–236.
29. Henry, B. E., W. W. Newcomb, and D. J. O'Callaghan. 1979. Biological and biochemical properties of defective interfering particles of equine herpesvirus type 1. Virology 92:495–506.
30. Henry, B. E., W. W. Newcomb, and D. J. O'Callaghan. 1980. Alterations in virus protein synthesis and capsid production in infection with DI particles of herpesvirus. J. Gen. Virol. 47: 343–353.
31. Henry, B. E., R. A. Robinson, S. A. Dauenhauer, S. S. Atherton, G. S. Hayward, and D. J. O'Callaghan. 1981. Structure of the genome of equine herpesvirus type 1. Virology 115:97–114.
32. Huang, H. L., J. M. Szabocsik, C. C. Randall, and G. A. Gentry. 1971. Equine abortion (herpes) virus-specific RNA. Virology 45:381–389.
33. Ihara, S., L. Feldman, S. Watanabe, and T. Ben-Porat. 1983. Characterization of the immediate-early functions of pseudorabies virus. Virology 131:437–454.
34. Inoue, T., and T. R. Cech. 1985. Secondary structure of the circular form of the *Tetrahymena* rRNA intervening sequence: a technique for RNA structure analysis using chemical probes and reverse transcriptase. Proc. Natl. Acad. Sci. USA 82:648–652.
35. Kozak, M. 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44:283–292.
36. Kristie, T. M., and B. Roizman. 1986. Alpha-4, the major regulatory protein of herpes simplex virus type 1, is stably and specifically associated with promoter-regulatory domains of alpha genes and of selected other viral genes. Proc. Natl. Acad. Sci. USA 83:3218–3222.
37. Mackem, S., and B. Roizman. 1980. Regulation of herpesvirus macromolecular synthesis: transcription-initiation sites and domains of alpha genes. Proc. Natl. Acad. Sci. USA 77:7122–7126.
38. Mackem, S., and B. Roizman. 1982. Differentiation between alpha promoter and regulator regions of herpes simplex virus 1: the functional domains and sequence of a movable alpha regulator. Proc. Natl. Acad. Sci. USA 79:4917–4921.
39. Maniatis, T., E. R. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. 65:499–560.
41. McGeoch, D. J., A. Dolan, S. Donald, and D. H. K. Brauer. 1986. Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1. Nucleic Acids Res. 14:1727–1745.
42. McKnight, S. L., and R. Kingsbury. 1982. Transcriptional control signals of a eukaryotic protein-coding gene. Science 217:316–324.
43. Messing, J. 1983. New M13 vectors for cloning. Methods Enzymol. 101:20–78.
44. Muller, M. T. 1987. Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site. J. Virol. 61:858–865.
45. O'Callaghan, D. J., G. A. Gentry, and C. C. Randall. 1983. The equine herpesviruses, p. 215–318. *In* B. Roizman (ed.), The herpesviruses, vol. 2. Comprehensive virology, series 2. Plenum Publishing Corp., New York.
46. O'Callaghan, D. J., B. E. Henry, J. H. Wharton, S. A. Dauenhauer, R. B. Vance, J. Staczek, and R. A. Robinson. 1981. Equine herpesviruses: biochemical studies on genomic structure, DI particles, oncogenic transformation and persistent infection, p. 387–418. *In* Y. Becker (ed.), Developments in molecular virology, vol. 1. Herpesvirus DNA. Martinus Nijhoff, The Hague, The Netherlands.
47. O'Callaghan, D. J., J. M. Hyde, G. A. Gentry, and C. C. Randall. 1968. Kinetics of viral deoxyribonucleic acid, protein, and infectious particle production and alterations in host macromolecular syntheses in equine abortion (herpes) virus-infected cells. J. Virol. 2:793–804.
48. O'Callaghan, D. J., D. C. Sullivan, R. P. Baumann, G. B. Caughman, C. Clay Flowers, A. T. Robertson, and J. Staczek. 1984. Genomes of the equine herpesviruses: molecular structure, regions of homology and DNA sequences associated with transformation. UCLA Symp. Mol. Cell. Biol. 21:507–525.
49. O'Hare, P., and G. S. Hayward. 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. 53:751–760.
50. Perdue, M. L., M. C. Kemp, C. C. Randall, and D. J. O'Callaghan. 1974. Studies of the molecular anatomy of the L-M cell strain of equine herpes virus type 1: proteins of the nucleocapsid and intact virion. Virology 59:201–216.
51. Preston, C. M., M. G. Cordingley, and N. D. Stow. 1984. Analysis of DNA sequences which regulate the transcription of a herpes simplex virus immediate-early gene. J. Virol. 50: 708–716.
52. Rigby, P. W., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. 113:237–251.
53. Rixon, F. J., and J. B. Clements. 1982. Detailed structural analysis of two spliced HSV-1 immediate-early mRNAs. Nucleic Acids Res. 10:2241–2256.
54. Roberts, M. S, A. Boundy, P. O'Hare, M. C. Pizzorno, D. M. Ciufo, and G. S. Hayward. 1988. Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (alpha 4) promoter and a specific binding site for the IE175 (ICP4) protein. J. Virol. 62:4307–4320.
55. Robertson, A. T., G. B. Caughman, W. L. Gray, R. P. Baumann, J. Staczek, and D. J. O'Callaghan. 1988. Analysis of the *in vitro* translation products of the equine herpesvirus type 1 immediate early mRNA. Virology 166:451–462.
56. Robinson, R. A., and D. J. O'Callaghan. 1981. The organization of integrated herpesvirus DNA sequences in equine herpesvirus type 1 transformed and tumor cells, p. 419–436. *In* Y. Becker (ed.), Developments in molecular virology, vol. 1. Herpesvirus DNA. Martinus Nijhoff, The Hague, The Netherlands.
57. Robinson, R. A., and D. J. O'Callaghan. 1983. A specific viral DNA sequence is stably integrated in herpesvirus oncogenically transformed cells. Cell 32:569–578.
58. Robinson, R. A., P. W. Tucker, S. A. Dauenhauer, and D. J. O'Callaghan. 1981. Molecular cloning of equine herpesvirus type 1 DNA: analysis of standard and defective viral genomes and viral sequences in oncogenically transformed cells. Proc. Natl. Acad. Sci. USA 78:6684–6688.
59. Robinson, R. A., R. B. Vance, and D. J. O'Callaghan. 1980. Oncogenic transformation by equine herpesviruses. II. Coestablishment of persistent infection and oncogenic transformation of hamster embryo cells by equine herpesvirus type 1 preparations enriched for defective interfering particles. J. Virol. 36:204–219.
60. Ruyechan, W. T., S. A. Dauenhauer, and D. J. O'Callaghan. 1982. Electron microscopic study of equine herpesvirus type 1 DNA. J. Virol. 42:297–300.
61. Sadowski, I., J. Ma, S. Triezenberg, and M. Ptashne. 1988. GAL4-VP16 is an unusually potent transcriptional activator. Nature (London) 335:563–564.
62. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.
63. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.

64. **Struhl, K.** 1987. The DNA-binding domains of the *jun* oncoprotein and the yeast GCN4 transcriptional activator protein are functionally homologous. Cell **50**:841–846.
65. **Triezenberg, S. J., R. C. Kingsbury, and S. L. McKnight.** 1988. Functional dissection of VP16, the transactivator of herpes simplex virus immediate early gene expression. Genes Dev. **2**:718–729.
66. **Watson, R. J., C. M. Preston, and J. B. Clements.** 1979. Separation and characterization of herpes simplex virus type 1 immediate-early mRNAs. J. Virol. **31**:42–52.
67. **Weaver, R. F., and C. Weissman.** 1979. Mapping of RNA by a modification of the Berk-Sharp procedure: 5′ termini of 15-S beta-globin messenger-RNA precursor and mature 10-S beta-globin messenger RNA have identical map coordinates. Nucleic Acids Res. **7**:1175–1193.
68. **Whalley, J. M., G. R. Robertson, and A. J. Davidson.** 1981. Analyses of the genome of equine herpesvirus type 1: arrangement of cleavage for restriction endonucleases *Eco*RI, *Bgl*II, and *Bam*HI. J. Gen. Virol. **57**:307–323.
69. **Zarkower, D., and M. Wickens.** 1987. Formation of mRNA 3′ termini: stability and dissociation of a complex involving the AAUAAA sequence. EMBO J. **6**:177–186.