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Characterization of a Bovine Herpesvirus 4 Immediate-Early RNA Encoding a Homolog of the Epstein-Barr Virus R Transactivator†

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Immediate-early (IE) RNA 2, the less abundant of two bovine herpesvirus 4 (BHV-4) RNAs detected in Madin-Darby bovine kidney cells infected in the presence of cycloheximide, is a 1.8-kb cytoplasmic polyadenylated RNA transcribed from the 8.3-kb *Hin*dIII fragment F. The structure of IE RNA 2 has been determined by S1 nuclease and exonuclease VII mapping, primer extension analysis, and sequencing of a partial cDNA. IE RNA 2 consists of a short, approximately 60-nucleotide 5′ exon spliced to a 1.8-kb 3′ exon. DNA sequence analysis revealed an open reading frame encoding 551 amino acids with sequence homology to the Epstein-Barr virus (EBV) R transactivator and its homolog in herpesvirus saimiri, HVS.R. IE 2 and HVS.R show higher homology to each other than to the EBV R transactivator. The homology is highest in the approximately 320 amino-terminal amino acids. All three proteins have acidic carboxyl termini but have little amino acid sequence homology in this region. In transient expression cotransfection assays, IE 2 activated expression from the BHV-4 early promoter-regulatory region of the major DNA-binding protein homolog over 100-fold in bovine turbinate cells. IE 1 was not necessary for this transactivation and did not augment it. However, IE 2 did not transactivate EBV or herpesvirus saimiri early promoter-regulatory regions that are transactivated by the EBV R transactivator or HVS.R.

Bovine herpesvirus 4 (BHV-4) infection is widespread in cattle in Europe and the United States, but its relationship with disease is unclear (reviewed in reference 39). Herpesviruses have large, complex DNA genomes, and transcription is sequentially regulated during infection in vitro (16). Genes are divided into three kinetic classes, immediate-early (IE), early (E), and late (L), on the basis of their time of expression (15). IE genes are expressed immediately upon infection and do not require prior viral protein synthesis for their expression. Thus, IE RNAs are the only viral RNAs expressed when cells are infected in the presence of inhibitors of protein synthesis, such as cycloheximide. Products of IE genes transactivate E and L genes. In addition to IE proteins, viral DNA synthesis is a prerequisite for expression of L genes (14).

DNA sequence analysis has recently shown that BHV-4 is genetically most closely related to the gammaherpesviruses Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS) (2). During lytic infection, EBV encodes three transactivators: R (also known as BRLF1 or Rta), Z (also known as BZLF1, Zta, ZEBRA, or EB1), and MS (also known as BMLF1, Mta, M-IE, or EB2) (reviewed in reference 18). Specific DNA sequences that mediate transactivation of EBV genes by R and Z have been identified (5, 6, 10, 30). DNA-binding and activation domains of the R transactivator have also been identified (13, 23). MS acts at a posttranscriptional level (3, 17). Identification of IE RNAs during EBV lytic infection is not a straightforward process. However, it is likely that RNAs encoding the R and Z transactivators are IE RNAs, while it is less clear whether the RNA encoding MS is expressed as an IE or E RNA (reviewed in reference 18). HVS encodes two IE RNAs (27). One of these, transcribed from the *Hin*dIII G fragment, encodes a putative protein that does not exhibit amino acid sequence homology with any EBV-encoded proteins (27). The other HVS IE RNA is transcribed from the HVS gene with predicted amino acid sequence homology to EBV MS (26). HVS also encodes a transactivator, HVS.R, exhibiting predicted amino acid sequence homology to the EBV R transactivator. However, HVS.R RNA is expressed as an E, not IE, RNA in HVS-infected cells (25, 27).

In BHV-4-infected cells, cytoplasmic polyadenylated RNA is transcribed from two regions of the BHV-4 genome under IE conditions, i.e., in the presence of cycloheximide. The most abundant IE RNA, IE RNA 1, is a spliced 1.7-kb RNA, which is transcribed from right to left on the restriction map of the BHV-4 genome from DNA contained in the 8.3-kb *Hin*dIII fragment E (Fig. 1). IE RNA 1 contains genetic information encoding a protein of 284 amino acids which could contain zinc finger structures near its amino terminus (42). The predicted amino acid sequence of this protein does not exhibit homology to IE proteins of EBV or HVS. Characterization of the less abundant IE RNA (IE RNA 2), the DNA which encodes it, and the ability of its protein product to transactivate expression of a BHV-4 early gene is the subject of this report. I find that IE RNA 2 is a spliced 1.8-kb RNA, which is transcribed from right to left on the restriction map of the BHV-4 genome from DNA contained in the 8.3-kb *Hin*dIII fragment F. Nucleotide sequence analysis of the DNA encoding IE RNA 2 reveals that it could encode a 61-kDa protein with amino acid sequence homology to the EBV transactivator R and its homolog in HVS, HVS.R. Transactivation studies show that BHV-4 IE 2 protein specifically transactivates expression of a reporter gene linked to the promoter-regulatory region of the BHV-4 E gene encoding the BHV-4 homolog to the herpes simplex virus type 1 (HSV-1) major DNA-binding protein. However, BHV-4 IE 2 protein does not transacti-

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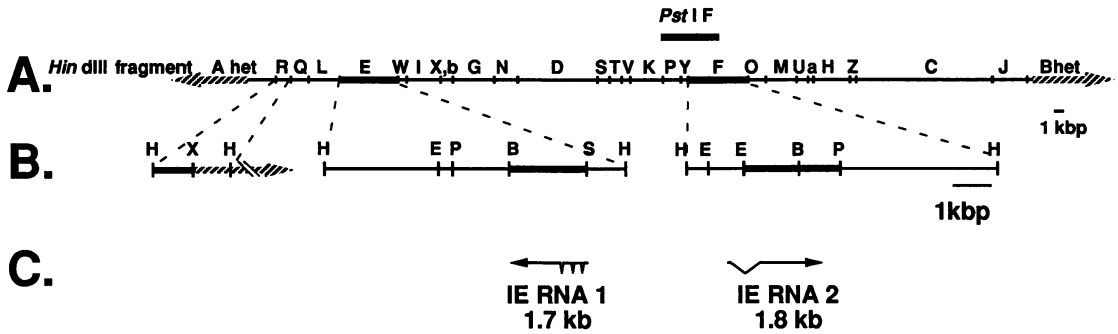

FIG. 1. Map locations of cloned BHV-4 DNA fragments used in this work. (A) *Hin*dIII restriction map of the BHV-4(DN-599) genome (adapted from reference 43), showing fragments that encode IE RNA (bold lines). Not all copies of the 2.5-kb terminal tandem repeats (striped arrows) are shown. The map location of the *Pst*I F fragment used as a probe for the Northern blot shown in Fig. 2 is shown above the map. (B) Expanded maps of *Hin*dIII fragments containing cloned BHV-4 DNA fragments used in this work. Abbreviations: H, *Hin*dIII; X, *Xba*I; E, *Eco*RI; P, *Pst*I, B, *Bgl*II; S, *Sma*I. Only relevant *Xba*I, *Bgl*II, and *Sma*I sites are shown. For *Hin*dIII fragment R, the bold line indicates the 1.1-kb *Xba*I-*Hin*dIII fragment in pBH1HX1.1-CAT, and the striped line indicates the ORF homologous to the major DNA-binding protein of HSV-1. For *Hin*dIII fragment E, the bold line indicates the 2.2-kb *Sma*I-*Bgl*II fragment in pRSVIE1. For *Hin*dIII fragment F, the bold line indicates the 2.6-kb *Eco*RI-*Pst*I fragment used as a uniformly labeled single-stranded probe for S1 nuclease analysis. (C) Structures and directions of transcription of IE RNA 1 and IE RNA 2 relative to the restriction maps shown in panel B.

vate EBV and HVS targets transactivated by the EBV R or HVS.R transactivator.

MATERIALS AND METHODS

Cell culture, virus propagation, preparation of IE RNA, Northern (RNA) blot analysis, S1 nuclease analysis, and primer extension analysis. The DN-599 isolate of BHV-4 was used for all experiments. Madin-Darby bovine kidney cells were used for preparation of RNA. BT (bovine turbinate) cells (ATCC CRL1390) and HeLa cells (JW HeLa cells, obtained from R. Curtis Bird, Department of Pathobiology, Auburn University) were used for transfection experiments. All cell lines were cultured in Dulbecco's modified Eagle medium containing penicillin, streptomycin, and 10% defined, supplemented bovine serum. Virus propagation, preparation of cytoplasmic polyadenylated IE RNA, Northern blot analysis, S1 nuclease analysis, and primer extension analysis were performed as previously described (42).

Exonuclease VII analysis. Exonuclease VII digestion was carried out by using a modification of a previously described procedure (21). After hybridization, carried out under the same conditions as for S1 nuclease analysis, each hybridization mixture (30 μl) was diluted with 0.5 ml of ice-cold buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, and 5 mM dithiothreitol. Exonuclease VII (10 U) was added and allowed to digest DNA for 1 h at room temperature. Samples were purified by phenol-chloroform extraction and ethanol precipitation.

cDNA synthesis and amplification. Polyadenylated IE RNA (10 μg) was hybridized to approximately 0.5 ng of a 325-bp double-stranded DNA fragment primer, the same primer illustrated in Fig. 4B but not labeled with ^{32}P. Hybridization was at 45°C for 16 h in the same buffer used for hybridization for S1 nuclease analysis (35). The hybridization mixture was diluted with water, and nucleic acids were precipitated with ethanol in the presence of glycogen carrier. First- and second-strand cDNA synthesis was carried out by using a cDNA synthesis kit (GIBCO-BRL, Grand Island, N.Y.), omitting the oligo(dT) primer. After phenol-chloroform extraction and ethanol precipitation, the ends of the cDNA were repaired with T4 DNA polymerase (35). The cDNA was again precipitated with ethanol and ligated to UNI-Amp adaptors containing *Eco*RI sites (Clontech Laboratories, Inc., Palo Alto, Calif.). RNA and ligase were destroyed by addition of NaOH to 0.6 M and EDTA to 20 mM and incubation at 68°C for 30 min. Unligated adaptors were removed by using a Sephacryl S-400 spin column (Promega Corporation, Madison, Wis.). cDNA was amplified by the polymerase chain reaction, using reagents from a GeneAmp PCR reagent kit (Perkin-Elmer Corp., Norwalk, Conn.) and UNI-Amp primer (Clontech) under the amplification conditions suggested in instructions supplied with the primer. Amplified cDNA was cleaved with *Eco*RI, purified on a low-melting-point agarose gel, and ligated into *Eco*RI-cleaved pTZ18U (United States Biochemical Corporation, Cleveland, Ohio).

Target and effector plasmids. Effector plasmids containing the IE 2 gene used in transient expression cotransfection assays, pH12PH4.2, pH12PR3.4, and pH12PH4.2ΔBcl, contain the BHV-4 DNA fragments shown in Fig. 9 cloned into pTZ19U vector (United States Biochemical). pH12PH4.2E contains the same BHV-4 DNA *Hin*dIII-*Pst*I fragment as does pH12PH4.2, but the fragment was cloned into a vector containing simian virus 40 (SV40) enhancer sequences. This vector was derived from pCAT-Enhancer (Promega) by deletion of sequences between the *Xba*I and *Hpa*I sites, resulting in deletion of the entire chloramphenicol acetyltransferase (CAT) gene and some SV40 sequences, but not the SV40 enhancer. IE 1 effector plasmid pRSVIE1 contains the 2.2-kb *Sma*I-*Bgl*II fragment containing the BHV-4 IE 1 gene (Fig. 1B) linked to the Rous sarcoma virus long terminal repeat promoter. It was constructed by removing the CAT and SV40 sequences from pRSVCAT (9) by *Hin*dIII and partial *Eco*RI digestion. The 2.2-kb fragment containing the IE 1 gene was ligated to this Rous sarcoma virus long terminal repeat vector after addition of *Hin*dIII linkers to the *Sma*I site of the fragment. The *Eco*RI site at the other end of the fragment was provided by polylinker sequences from the vector into which the 2.2-kb fragment had originally been cloned. Control effector plasmid pSVPE contains the SV40 early promoter and enhancer and was derived from pSV-β-Galactosidase by deletion of the *Bam*HI-*Hin*dIII fragment containing β-galactosidase sequences. Target plasmid pBH1HX1.1-CAT contains the 1.1-kb *Hin*dIII-*Xba*I fragment

approximately 60 bp 5′ to the beginning of the major DNA-binding protein-homologous open reading frame (ORF) (2, 41) (Fig. 1B) cloned into pCAT-Basic vector (Promega). Control target plasmid pCAT-Control and transfection efficiency control plasmid pSV-β-Galactosidase were obtained from Promega. Plasmids pMH48 (12), pMH69, and pMH103 (6) were kindly provided by J. Marie Hardwick, Johns Hopkins University; p110CAT.B (25) was provided by John Nicholas, Johns Hopkins University.

Plasmid DNA to be transfected into cells was purified by the alkaline lysis procedure followed by CsCl-ethidium bromide equilibrium density gradient centrifugation (35). Contaminating RNA was degraded by digestion with RNase A and removed by centrifugation of plasmid DNA through 1 M NaCl (35).

Transient expression cotransfection assays. Effector and target plasmid DNAs were introduced into cells by calcium phosphate-mediated transfection (35). At 18 to 24 h prior to transfection, BT or HeLa cells were plated into six-well plates so that they would be nearly confluent at the time of transfection, and the medium was changed 2 to 5 h before the transfection mixture was added. Each transfection mixture (0.3 ml) contained 2.5 μg of effector plasmid, 2.5 μg of target plasmid, and 2.5 μg of pSV-β-Galactosidase control plasmid and was added directly to the 2 ml of medium in each well of a six-well plate. HeLa cells were subjected to a glycerol shock (15% glycerol, 1 min) 5 h later. The calcium phosphate-DNA coprecipitate was incubated with BT cells for 24 h, after which the medium was changed. Two hours later, the medium was changed again to remove residual transfection mixture and allow the cells to recover from toxic effects. At this time, cells to be infected with BHV-4 were exposed to approximately 20 50% tissue culture infective doses per cell in 0.4 ml of medium for 1 to 2 h, and then 2 ml of medium was added.

Cell extracts to be used for β-galactosidase and CAT assays were prepared 48 to 50 h after application of the calcium phosphate-DNA coprecipitate to cells. Medium was removed, and cells were allowed to detach from the surface of the plate by incubation with 1 ml of 40 mM Tris-HCl (pH 7.4)–150 mM NaCl–1 mM EDTA added to each well. Cells in this buffer were pipetted out of the wells, pelleted by centrifugation, resuspended in 200 μl of ice-cold 0.25 M Tris-HCl (pH 7.8), and lysed by freezing and thawing three times. After removal of cell debris by centrifugation, 30 μl of each sample was removed and set aside for β-galactosidase assays. The remainder of each extract was heated at 60°C for 10 min. A precipitate formed in HeLa cell extracts and was removed by centrifugation. Extracts were stored at −80°C until used in β-galactosidase or CAT assays.

β-Galactosidase assays were performed as previously described (35), using 30 μl of each cell extract in a 60-μl total volume at 37°C for 30 min to 3 h, until a visible yellow color developed. For each length of time, assays were also performed on known amounts of β-galactosidase, to allow comparison of samples within a single experiment assayed for different lengths of time. The relative amount of β-galactosidase activity was used as the relative transfection efficiency.

CAT assays were performed by the phase extraction assay procedure (36), using [^{3}H]chloramphenicol and butyryl coenzyme A. Reaction mixtures contained 5 to 10 μl of cell extract, and reactions were allowed to continue for 1 h. Butyrylated chloramphenicol was extracted with a 2:1 (vol/vol) mixture of tetramethylpentadecane and xylenes and was quantitated by liquid scintillation counting. Background radioactive chloramphenicol found in the organic phase, using extracts from untransfected cells, was about 0.2% of total counts per minute and was subtracted from counts per minute found in the organic phase of each sample before calculation of percent chloramphenicol butyrylated. If CAT activity was extremely low, the assay was repeated for 10 h.

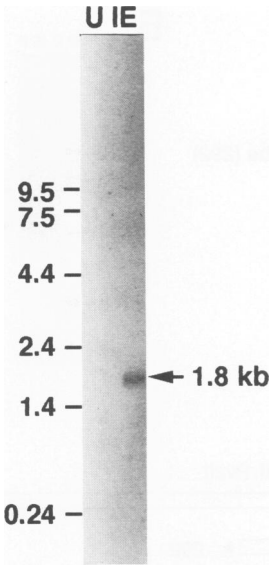

FIG. 2. Northern blot analysis of IE RNA 2. Cloned viral *Pst*I fragment F, identified above the viral DNA restriction map in Fig. 1A, was labeled with ^{32}P by random hexanucleotide-primed synthesis and was hybridized to a Northern blot of polyadenylated uninfected cell RNA (U) and IE RNA (IE). Positions and sizes (in kilobases) of marker RNAs (0.24- to 9.5-kb RNA ladder; GIBCO-BRL) are indicated on the left. The arrowhead on the right indicates the 1.8-kb IE RNA, IE RNA 2, identified by the probe.

Nucleotide sequence accession number. The nucleotide sequence data reported in this article have been submitted to GenBank and assigned accession number L01099.

RESULTS

Characterization of IE RNA 2. Cytoplasmic polyadenylated RNA prepared 6 to 8 h postinoculation from Madin-Darby bovine kidney cells infected with BHV-4(DN-599) in the presence of cycloheximide (100 μg/ml) was used for all experiments to characterize IE RNA. Northern blot analysis using cloned BHV-4 *Pst*I fragment F (Fig. 2) and *Hin*dIII fragment F (data not shown) probes identified a 1.8-kb IE RNA, IE RNA 2. To determine the direction of transcription of IE RNA 2, S1 nuclease analysis was performed by using uniformly labeled single-stranded probes representing each strand of the 2.6-kb *Eco*RI-*Pst*I fragment shown in Fig. 1B. This fragment was cloned into the single-stranded phage vectors M13mp18 and M13mp19 and used as the template for synthesis of uniformly labeled, single-stranded DNA probes. IE RNA protected an approximately 1.7-kb fragment of only one of these probes (data not shown), indicating that IE RNA 2 is transcribed in the direction shown in Fig. 1C. To map the DNA encoding IE RNA 2 within this 2.6-kb fragment, the probe for S1 nuclease analysis was cleaved with *Bgl*II prior to separation of the strands. Results (not shown) indicated that the 5′ and 3′ ends of the RNA protecting this fragment were approximately 0.8 and 0.9 kb, respectively, from the *Bgl*II site.

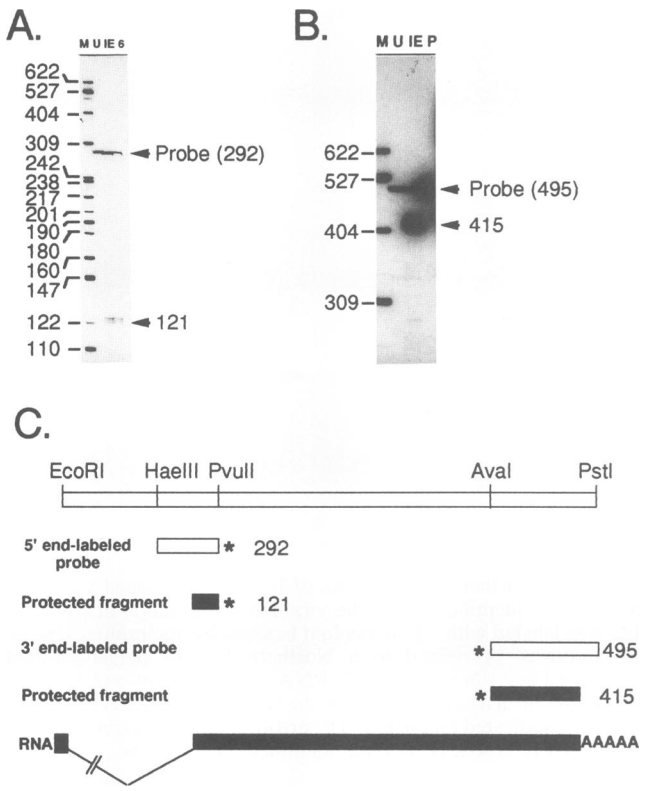

FIG. 3. (A and B) S1 nuclease mapping of the 5′ (A) and 3′ (B) ends of the major exon of IE RNA 2. Hybridization temperature was 44°C. Lanes: M, pBR322 DNA cleaved with *Msp*I; U, uninfected cell RNA control; IE, IE RNA; 6, RNA prepared 6 h postinoculation in the absence of cycloheximide; P, probe. Marker sizes are noted in nucleotides at the left of each panel. Sizes of probes and fragments specifically protected from S1 nuclease digestion by IE RNA are shown next to arrowheads at the right of each panel. (C) Schematic diagrams of the double-stranded, end-labeled probes and protected fragments. The sizes of each are indicated in nucleotides. The ^{32}P label is denoted by an asterisk. The *Eco*RI-*Pst*I fragment shown at the top is the 2.6-kb *Eco*RI-*Pst*I fragment shown as a bold line in Fig. 1B.

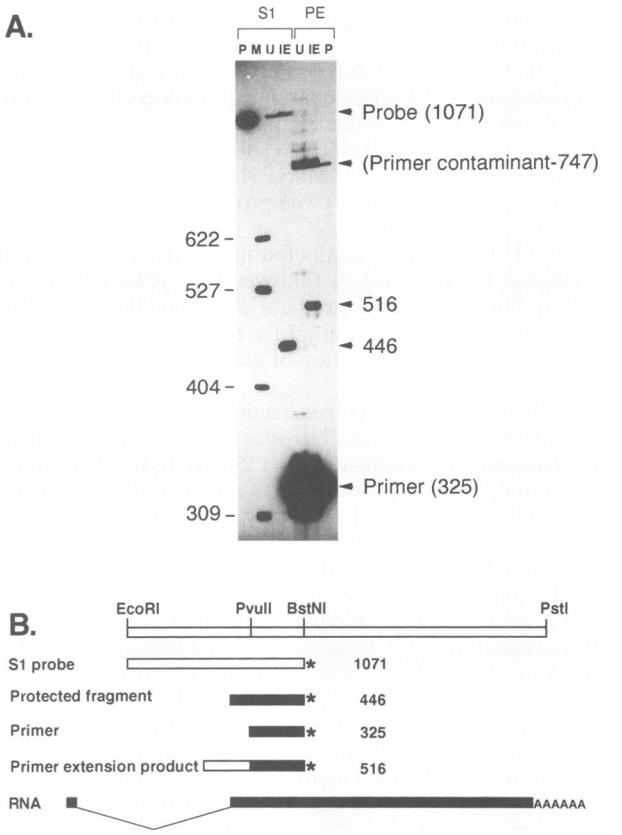

FIG. 4. (A) S1 nuclease and primer extension analysis of the 5′ portion of IE RNA 2. Hybridization temperature was 43°C. Lanes: S1, S1 nuclease analysis; PE, primer extension; P (in S1 lanes), probe; M, pBR322 DNA cleaved with *Msp*I; U, uninfected cell RNA control; IE, IE RNA; P (in PE lanes), primer. Marker sizes in nucleotides are indicated on the left. Sizes of the protected fragment and primer extension product are indicated next to arrowheads on the right. (B) Schematic diagrams of the double-stranded, 5′-end-labeled probe, protected fragment, 5′-end-labeled primer, and primer extension product. The sizes of each are indicated in nucleotides. The 5′ end label is denoted by an asterisk. The *Eco*RI-*Pst*I fragment shown at the top is the 2.6-kb *Eco*RI-*Pst*I fragment shown as a bold line in Fig. 1B.

To map more precisely the ends of IE RNA 2, S1 nuclease analysis using end-labeled DNA probes was undertaken. The 5′ end of the RNA protecting the 1.7-kb fragment of the uniformly labeled *Eco*RI-*Pst*I fragment was mapped by using the 5′ end-labeled probe illustrated in Fig. 3C. IE RNA specifically protected an approximately 121-nucleotide (nt) fragment. Similar amounts of fragment were protected by RNA prepared from cells infected in the absence of cycloheximide (compare lanes IE and 6 in Fig. 3A), indicating that IE RNA 2 is present in similar amounts in the absence and presence of protein synthesis. The 3′ end of the RNA was mapped by S1 nuclease analysis using the double-stranded 3′-end-labeled probe illustrated in Fig. 3C. IE RNA specifically protected a 415-nt fragment (Fig. 3B). This analysis places the polyadenylation site of IE RNA 2 approximately 20 nt 3′ to an AAUAAA consensus polyadenylation signal (see Fig. 6).

To determine whether the 5′ end identified by S1 nuclease analysis represents the actual 5′ end of the RNA or the 5′ end of an exon, primer extension analysis and S1 nuclease analysis were conducted by using a primer and probe 5′ end labeled at the same site (Fig. 4B). IE RNA specifically protected a 446-nt fragment of the probe, while primer extension yielded a 516-nt product (Fig. 4A). This discrepancy indicates that the end of the RNA identified by S1 nuclease analysis is the 5′ end of an exon and that IE RNA 2 is spliced. This finding is consistent with the presence of a consensus 3′ splice site at this location in the nucleotide sequence (see Fig. 6). The difference in size of the fragments identified by S1 nuclease and primer extension analyses is 70 nt, indicating that the approximately 70 5′ nucleotides of IE RNA 2 are made up of an additional exon(s).

Two complementary approaches were used to map the short, approximately 70-nt 5′ exon of IE RNA 2. The 5′ end of this exon was located by exonuclease VII mapping, using the 5′ end-labeled probe illustrated in Fig. 5B. IE RNA specifically protected a 1.1-kb fragment of this probe from exonuclease VII digestion (Fig. 5A). In control experiments (not shown), this 1.1-kb fragment was not produced when the 5′ end of the probe was protected by an unlabeled 1.4-kb *Eco*RI-*Bgl*II DNA fragment (Fig. 5B), indicating that pro-

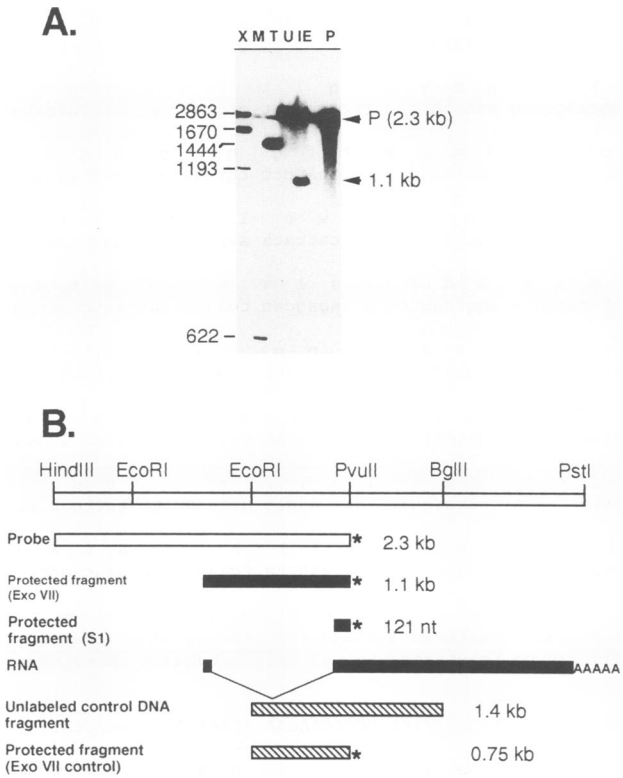

FIG. 5. (A) Exonuclease VII mapping of 5′ end of IE RNA 2. Hybridization temperature was 44°C. Lanes: X, pTZ19U DNA cleaved with *Xba*I and with *Xba*I plus *Pvu*I; M, pBR322 DNA cleaved with *Msp*I; T, pTZ19U DNA cleaved with *Taq*I; U, uninfected cell RNA control; IE, IE RNA; P, probe. Marker sizes are noted in nucleotides at the left. The approximately 2.3-kb fragments visible in lanes M and T are due to leakage of a small amount of probe from lane P into all wells. Sizes of probe and fragment specifically protected from exonuclease VII nuclease digestion by IE RNA are shown next to arrowheads at the right. (B) Schematic diagrams of 5′-end-labeled probe, protected fragments, and unlabeled control DNA fragment. The size of each is noted to the right. Experiments to determine the fragment protected from S1 nuclease digestion by IE RNA and the fragment protected from exonuclease VII by the unlabeled control DNA fragment are not shown in panel A.

tection of a 1.1-kb fragment from exonuclease digestion was not due to secondary structure of the probe. These results suggest that the 5′ end of the short 5′ exon of IE RNA 2 is transcribed from DNA approximately 1.0 kb 5′ to the 5′ end of the exon encoding the remainder of the RNA molecule. To confirm the location of the 5′ exon and to precisely map its 3′ end, a cDNA spanning the exon junction was synthesized and cloned, and the nucleotide sequence of the portion spanning the exon junction was determined. Results confirmed that the 5′ exon is transcribed from DNA approximately 1 kb 5′ to the second exon and showed the 5′ exon ends at a consensus 5′ splice site (Fig. 6). Because the cDNA does not extend to the 5′ end of the RNA, the possibility that the approximately 70 nt at the 5′ end of IE RNA 2 are derived from more than one exon has not been ruled out. However, the absence of a consensus 3′ splice site in this region and the approximate location of the 5′ end of the RNA determined by exonuclease VII mapping are consistent with a single approximately 70-nt 5′ exon.

Nucleotide sequence of the gene encoding IE RNA 2 and the 5′ and 3′ flanking regions. The nucleotide sequence (Fig. 6) of the 4-kb *Hin*dIII-*Pst*I fragment containing the DNA encoding IE RNA 2 plus 1.1 kb of 5′- and approximately 80 nt of 3′-flanking sequences was determined by the dideoxynucleotide chain termination method, using Sequenase 2.0 enzyme (United States Biochemical) and single-stranded templates. Analysis of this sequence revealed that the sequences encoding IE RNA 2 contain an ORF with predicted amino acid sequence homology to EBV ORF BRLF1 and its HVS homology HVS.R, which encode transcriptional transactivators (Fig. 7A). The FASTA (32) "opt" score for alignment of BHV-4 IE 2 with HVS.R, 455, is higher than the opt scores for alignment of BHV-4 IE 2 with EBV R, 270, and alignment of HVS.R with EBV R, 318. The FASTA program identified amino acid sequence homology only in the amino-terminal portions of the three proteins. However, the putative BHV-4 IE 2 protein has an acidic carboxy-terminal portion, as do EBV BRLF1 and HVS.R. The 5′ exon of BHV-4 IE RNA 2 is mostly untranslated but contains the putative translation initiation codon which is spliced onto the remainder of the ORF in exon 2. This translation initiation codon is flanked by nucleotides conforming to the consensus sequence for eukaryotic initiation codons (19, 20). In addition to the ORF contained in IE RNA 2, the 4-kb *Hin*dIII-*Pst*I fragment contains two ORFs in the opposite direction, homologous to EBV BRRF1 and BRRF2 and their homologous ORFs in HVS, EDLF4, and EDLF5 (Fig. 7B and C). Only the amino-terminal portion of the BRRF2 homolog is present in the 4-kb fragment.

Transactivation activity of IE 2. The amino acid sequence homology of the putative BHV-4 IE 2 protein with EBV R and HVS.R transcriptional transactivators made it likely that BHV-4 IE 2 also acts as a transcriptional transactivator. This possibility was tested in transient expression cotransfection assays using putative IE 2 target promoter-regulatory regions linked to the CAT reporter gene. Because herpesvirus IE proteins sometimes act synergistically to activate E and L transcription (6, 7, 8, 28), the effect of IE 2 plus IE 1 on transcription from target promoters was tested, as well as the effect of IE 2 and IE 1 alone. Target promoters tested included a BHV-4 E RNA promoter as well as EBV promoters that had already been shown to be targets of the EBV R transactivator and an HVS promoter that is a target for transactivation by HVS.R. The BHV-4 E promoter region tested by using the target plasmid pBH1HX1.1-CAT was a 1.1-kb *Hin*dIII-*Xba*I fragment containing the transcription start site, approximately 24 bp from the *Xba*I end, for an E RNA containing the BHV-4 ORF homologous to the major DNA-binding protein of HSV-1 (2, 4, 41) (Fig. 1B). Infection by BHV-4 instead of transfection of an effector plasmid served as a positive control for activation of this BHV-4 E target promoter. The EBV R transactivator target plasmids used, pMH69 and pMH103 (6), were both derived from the divergent promoter found in the *Bam*HI H fragment of the EBV genome. pMH103 consists of a 258-bp fragment containing an R-responsive enhancer linked to a heterologous promoter linked to the CAT reporter gene. pMH48 effector plasmid (12), containing the EBV R transactivator gene in an SV40 expression vector, served as a positive control for transactivation of EBV target promoters. The HVS.R transactivator target plasmid used, p110CAT.B (25), contains the promoter-regulatory region for the E RNA encoding the HVS homolog to the HSV-1 major DNA-binding protein. Results of a representative experiment are shown in Fig. 8. Each transfection included plasmid pSV-β-Galactosidase as an internal control for transfection efficiency. However, this

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     g  t  g  l  k  k  l  f  p  l  g  g  e  e  e  p  c  q  p  t  k  c  m  s  e  k  p  e  v  y  y  g  k
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     e  k  l  l  t  l  l  h  k  q  e  r  r  p  p  q  t  t  l  f  n  r  v  t  q  l  t  n  q  l  v  q  f
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     s  d  l  n  y  t  l  e  g  s  g  r  k  t  t  v  i  p  l  l  n  d  y  s  t  f  g  q  s  y  g  n  t  l
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     l  w  n  n  i  s  a  e  l  i  e  f  d  n  q  v  h  k  l  l  m  y  y  f  w  c  l  y  v  c  s  a  v
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     g  r  t  e  l  q  i  d  k  p  l  e  p  f  d  g  m  d  l  v  l  d  s  l  s  l  v  s  v  s  v  r  d
401 ccccttgtct ccagttggat atcttttggg agttctggaa aatcccccat gtccaagaca aggtcagata gtgagagcac tgaaactgag actctatctg

     a  v  v  v  q  n  h  v  t  t  l  f  n  h  a  q  c  v  k  k  h  s  s  l  k  p  p  r  a  d  c  l  s  n
501 ctaccaccac ctggttgtgg acagtagtta aaaagttatg tgcctgacaa acttttttgt gggatgagag cttagggggt ctggcatcac ataatgaatt

     m  l  q  k  t  t  t  i  c  n  l  c  g  c  s  e  s  l  i  l  s  m  r  d  k  c  d  s  f  m  d  e  m
601 cattagttgt tttgttgtgg ttatgcaatt taaacaccca catgattctg ataaaattaa agacattcta tctttacaat cagaaaacat atcctccata

     d  c  s  h  e  q  s  k  i  h  k  y  i  s  q  a  l  f  k  t  q  q  l  i  e  l  l  n  f  r  e  q  s
701 tcacaggaat gttcttgact tttgatgtgc ttgtatatgc tctgagctaa gaactttgtt tgttgtaaaa tctccaggag gttaaacctc tcttgagatt

     k  r  s  t  d  v  f  q  k  l  i  v  l  s  a  l  l  g  t  n  k  q  k  k  f  l  k  h  l  s  s  v  a  e
801 ttctggaggt atccacaaac tgttttaaaa taactaaaga agctaaaagc ccggtgttct tctgtttctt gaataattta tgaagtgaag aaacagcttc

     r  v  n  g  r  k  a  f  t  s  f  i  e  d  f  h  p  r  q  n  e  e  i  g  p  i  y  v  t  i  e  m
901 tctgacattt ccccttttg caaaagtact aaagatttca tcaaagtggg gtctttgatt ttcttcaata cccggaatat atacggtgat ttccatggtg
1001 agtctacgcc ctttaatcca aaaggtatcc ctgaggttaa atatatatgt cagtcactgg tgagtaatgg tgatactcct ccctggaata catcatgcca

                                                                                                        M
1101 ataaccctgc ccacttttgt tcttttaaaa gGGAGCAGAA CAACAGATGG TATGAGTGAC TAGAGCTTCA GCCACAAGAA ACAAACACAC AGACCAGTCA
                                                                                  ---------- ---------- ----------
                     * q  a  k  q  l  i  l  k  c  h  s  l  l  l  r  a  q  f  l  l  i  d  s  s  e  y  t  i
1201 TGgtaagaat gctttattgt gcttttgta gtatcaattt gcaatgtgat aacagcagcc tggcttgaaa taagagaata tctgagctct cgtatgttat
     -- 5'SS
     s  n  v  l  h  e  l  y  k  v  m  a  m  p  k  f  h  i  k  n  i  d  y  w  l  f  f  q  r  v  e  n  k
1301 actgttaact agatgttcta agtattttac cattgccatg ggtttgaaat ggattttgtt gatatcatac catagaaaga actgtctaac ttcatttttc

     l  l  a  i  v  c  q  i  h  d  g  v  m  d  q  r  i  c  e  q  f  m  k  l  v  t  t  w  f  i  a  q  n
1401 aacagggcaa taacacattg tatgtgatct cctaccatat cctgccttat gcactcttga aacatttta agacagttgt ccagaaaatt gcttgattga

     f  f  l  n  f  e  h  d  k  y  e  p  c  h  t  q  s  l  f  l  q  q  m  i  h  c  l  v  q  i  s  p  m  l
1501 aaaacagatt gaattcatga tctttatact ctgggcaatg tgtttgagat aaaaatagtt gttgcatgat gtgacacaaa acttgtatgg aaggcatgag

     t  w  e  r  t  s  l  t  n  r  l  l  n  q  d  t  e  s  i  a  l  m  h  h  l  c  w  e  i  y  d  m  d
1601 ggtccattct cttgttgata gtgtatttct gagtaagttt tgatctgttt cagaaattgc caacatatgg tgcagacacc attcaatata gtccatgtcc

     g  s  n  i  p  s  t  s  f  n  p  y  k  c  l  l  i  e  p  l  l  k  i  l  h  v  g  l  m  q  d  d  s
1701 ccactgttta tgggacttgt ggaaaagttt ggatacttac aaagaagtat ttctggcagt agttttataa ggtggacacc caacatttga tcatcagaaa

     v  l  f  h  l  l  q  p  t  s  c  q  a  d  a  n  n  l  e  n  l  s  t  i  m  y  k  i  i  d  q  i  l  v
1801 ctagaaaatg aagtaattgg ggggtactgc attgtgcatc agcattgttt aactcattta aacttgtgat catatatttt ataatgtctt gaattagtac

     m  i  s  k  e  q  n  k  h  q  i  l  i  q  a  v  d  t  l  f  l  a  r  e  l  a  t  i  p  k
1901 cataatagat ttttcttggt ttttgtgctg aattaatatc tgggctacat ctgttaaaaa aagggccctt tccagagcag tgattggttt ttataatttg
2001 tttccccaga gttaaaactc agcttgtcct tttctttaac tatctgagat aggtcaggga aatgataatt tttgaactct gaaagaatct tattattgac

                                                K  G  I  I  Y  P  A  K  S  S  A  D  R  Y  V  Y  L  S  P  G  V  R
2101 acctgtattc atattggtat ccctttttaaa ttgcagAAGG GGATTATTTA TCCCAGAAAA TCTTCAGCAG ATCGATATGT CTATTTATCA CCCGGAGTCA
                         3'SS          ---- ---------- ---------- ---------- ---------- ---------- ----------
     E  H  L  F  S  L  L  V  K  Y  T  S  S  Q  R  D  S  P  A  G  S  S  T  K  V  A  P  D  T  P  Q  T  N
2201 GAGAGCATCT GTTTTCATTA CTGGTGAAGT ACACCAGCTC CCAACGTGAC TCACCAGCTG GCTCCAGCAC TAAAGTGGCA CCAGATACAC CTCAGACAAA
     ---------- ---------- ---------- ---------T ---------- -------
     F  I  V  E  I  Q  W  V  C  H  K  I  L  L  E  M  E  E  Q  F  G  S  L  G  G  L  V  A  D  I  N  I  C
2301 TTTTATTGTA GAAATACAAT GGGTTTGTCA TAAAATTTTG TTGGAGATGG AAGAACAATT TGGATCTTTG GGGGGTCTTG TGGCAGACAT CAATATTTGT

     L  I  W  T  L  F  R  N  Y  K  H  K  H  R  M  N  N  S  D  T  G  K  S  C  A  E  Y  A  Q  S  V  V  K  H
2401 CTTATATGGA CACTCTTTAG AAACTACAAG CACAAGCACA GAATGAATAA TTCAGACACT GGAAAATCAT GTGCAGAATA TGCTCAATCT GTTGTGAAAC

     L  T  E  R  M  V  Y  C  T  D  K  F  F  I  N  S  A  C  S  G  V  T  V  P  Q  N  L  A  L  V  I  A  S
2501 ATCTCACAGA AAGAATGGTG TATTGCACTG ACAAGTTTTT TATAAACTCT GCGTGTTCTG GCGTCACAGT TCCACAGAAC CTGGCCTTAG TTATAGCCTC

     I  S  Q  V  C  R  N  K  C  Q  G  A  W  R  R  M  G  N  G  R  R  T  L  I  D  L  G  L  Q  L  V  N  T
2601 TATTTCACAA GTGTGCCGGA ATAAATGTCA GGGGGCGTGG AGGCGGATGG GGAATGGAAG AAGGACTCTG ATTGATTTGG GTTTACAGCT GGTTAATACA
```

```
     Y  N  L  L  N  A  C  G  A  I  D  D  K  C  K  A  F  I  K  L  T  F  P  Y  L  N  L  E  T  V  Y  S  P  V
2701 TATAACCTTT TGAATGCATG TGGGGCCATT GATGACAAAT GTAAGGCATT TATAAAGCTC ACCTTTCCTT ACCTTAATCT TGAGACTGTA TACTCCCCAG

       H  A  A  S  T  G  L  H  Q  K  M  A  I  S  L  Y  K  G  Q  E  K  R  K  V  P  N  A  T  I  Y  S  N  L
2801 TCCATGCTGC CTCCACGGGT CTGCACCAAA AAATGGCTAT CTCTCTTTAT AAGGGACAGG AAAAAAGAAA AGTACCCAAT GCAACCATCT ACTCAAACCT

     V  T  Q  E  K  F  A  L  P  E  I  L  L  G  E  I  T  D  E  G  L  L  A  N  K  G  P  D  L  E  K  L  L
2901 TGTAACTCAA GAAAAGTTTG CCCTTCCAGA GATTCTTTTG GGGGAAATAA CAGACGAGGG TCTCTTAGCA AACAAAGGAC CAGATCTGGA GAAACTGTTA

     S  E  P  Q  T  I  L  K  L  V  T  K  L  S  P  V  S  Q  F  Q  V  F  M  A  R  W  G  D  K  L  P  S  H  L
3001 TCTGAACCAC AGACCATTCT CAAACTTGTA ACAAAACTGA GCCCGGTGTC TCAATTTCAA GTGTTTATGG CAAGGTGGGG GGATAAATTA CCCTCCCATC

       K  D  V  C  V  S  D  S  S  Q  L  P  Q  Q  Y  E  N  F  K  V  V  W  P  Q  H  Q  T  W  P  N  D  E  T
3101 TTAAAGATGT ATGTGTAAGT GACTCCTCAC AACTGCCCCA ACAATATGAA AACTTTAAGG TTGTGTGGCC CCAACATCAA ACATGGCCTA ATGATGAAAC

     S  A  L  P  I  A  S  A  A  L  L  S  L  Q  A  E  N  L  P  T  S  V  Q  N  S  V  A  Q  E  T  T  G  L
3201 TTCAGCTCTA CCAATTGCTT CAGCATCTTT GTTGTCACTC CAGGCTGAGA ACCTTCCAAC AAGTGTACAA AATTCAGTAG CCCAGGAAAC AACGGGCCTT

     P  P  Q  V  P  A  G  E  M  P  P  R  G  N  C  P  V  E  D  H  G  L  L  N  S  D  A  P  D  D  L  S  G  K
3301 CCCCCACAAG TACCTGCTGG GGAAATGCCC CCCCGGGGGA ATTGTCCAGT AGAGGACCAT GGTTTATTGA ACAGTGATGC CCCGGATGAT CTCTCTGGGA

       G  D  Y  D  L  F  T  S  D  D  L  L  P  M  S  S  G  N  V  A  I  P  V  C  E  D  P  L  A  P  P  C  K
3401 AGGGGGACTA TGATTTGTTT ACAAGTGATG ATTTGTTGCC CATGTCATCT GGCAATGTAG CTATACCTGT CTGTGAGGAC CCACTTGCCC CCCCATGTAA

     K  Q  R  M  E  P  P  D  P  L  S  T  P  G  D  N  Q  W  G  D  G  A  A  S  A  W  I  Q  S  Y  M  E  N
3501 GAAGCAAAGG ATGGAACCAC CAGATCCCTT ATCGACTCCC GGGGACAACC AGTGGGGTGA TGGTGCTGCT TCTGCGTGGA TACAATCTTA TATGGAAAAT

     E  D  A  Y  L  E  L  I  L  Q  G  L  Y  H  L  D  E  P  P  K  L  E  D  S  I  Q  V  Q  D  V  S  N  S  T
3601 GAAGATGCAT ACCTAGAGCT TATTCTACAG GGACTATATC ACTTGGATGA GCCCCCCAAA TTAGAAGACA GCATTCAAGT CCAAGATGTC TCCAATTCTA

       N  D  P  L  E  G  P  S  R  S  Q  A  S  C  F  E  D  T  E  N  I  A  N  P  L  D  L  F  V  *
3701 CCAATGACCC ATTGGAGGGC CCATCCAGGT CACAGGCATC ATGTTTTGAG GACACAGAAA ATATTGCGAA CCCCCTCGAT TTGTTTGTGT AAATACTTAT
3801 AAAACTGTAA TTGTGTATAT ATGATGAAAG ATGTTGACAA TAGCCTTGAT AGATTATTGC TCAAACCTAT CTCATTTTAA GCTATTGATA ATTATTGTAC
3901 AGTTTTCAAA TCAATGTTAT ATGTTTTTCA AATAAAGTTT TACTTGGCAG ACTACAAgag gctttggact ttgtatatat tatagtttca ttatgggcat
                                   PA
4001 tttatcatac ctctgcacct ttcaactgac taactgcag
```

FIG. 6. Nucleotide sequence of 4-kb *Hin*dIII-*Pst*I fragment containing the gene encoding BHV-4 IE RNA 2 and flanking sequences. The nucleotide sequence was determined by the dideoxynucleotide chain termination method, using the Sequenase 2.0 sequencing kit (United States Biochemical) and single-stranded templates. The sequence begins near the *Hin*dIII site at the left end of *Hin*dIII-F and extends to the *Pst*I site at the right end of *Pst*I-F. IE RNA 2 exons are in uppercase letters; the intervening sequence and flanking sequences are in lowercase letters. The intervening sequence of IE RNA 2 extends from nt 1203 to 2136. Immediately below the sequence, the sequence of the cDNA determined is shown. Dashes in this sequence indicate identity with the sequence above it. The locations of the 5′ and 3′ ends of IE RNA 2 are approximate. 5′ to the 3′ end of the RNA, sequences conforming to the consensus polyadenylation signal (AAUAAA [34]), are underlined and marked PA. Sequences at the intron-exon junctions conforming to the 5′ splice site consensus sequence (AG/GTAAGT [31]) and 3′ splice site consensus sequence (Py_nNPyAG [31]) are underlined and marked 5′SS and 3′SS. Above the nucleotide sequence, the predicted amino acid sequence encoded by IE RNA 2 is shown in uppercase letters in single-letter code. The predicted amino acid sequences for ORFs homologous to EBV BRRF1 (nt 1990 to 1217) and EBV BRRF2 (nt 996 to 1) are shown in lowercase letters. These two ORFs are encoded on the strand opposite the one shown, and the amino acid sequences are presented right to left.

plasmid might not be a valid internal control for transfection efficiency for at least two reasons: first, the transactivator tested might have an effect on the SV40 control promoter; second, nonactivated and activated target promoters might compete differentially with the SV40 control promoter. Because of these doubts about whether the relative amount of β-galactosidase activity among samples is a valid measure of relative transfection efficiency, results are presented as relative CAT activity per transfected well of cells as well as corrected for relative transfection activity. Regardless of whether the results were corrected for relative transfection efficiency, they clearly show that BHV-4 IE 2 transactivates the BHV-4 major DNA-binding protein-homolog promoter-regulatory region in pBH1HX1.1-CAT up to 100-fold in BT cells (Fig. 8). When activated by IE 2, this promoter was three to four times as active as the SV40 early promoter and enhancer in pCAT-Control (not shown). BHV-4 IE 1 had no stimulatory effect on this promoter and did not increase the stimulatory effect of IE 2 when both effectors were present. Results of S1 nuclease analysis (not shown) of RNA prepared from cells transfected with the IE1 effector plasmid showed that IE RNA 1 was spliced in the IE pattern rather than the L pattern (42). Therefore, the lack of stimulation by IE 1 of the BHV-4 E promoter tested was not due to inappropriate splicing leading to lack of expression of the IE 1 protein. It should be noted that when both IE 1 and IE 2 effector plasmids were transfected into cells, each was present at half the concentration as when IE 2 effector plasmid alone was used, to maintain a constant DNA concentration. This is likely the explanation for the reduction in CAT activity observed when IE 1 and IE 2 were used as effector plasmids. Neither IE 2 nor IE 1 plus IE 2 was able to stimulate expression from EBV R or HVS.R responsive targets to the extent that IE 2 stimulated the BHV-4 E target promoter (Fig. 8).

The transient expression cotransfection experiments just described were performed with bovine (BT) cells. To determine whether BHV-4 IE 2 could stimulate transcription from EBV R and HVS.R targets in primate cells, similar experiments were also performed with HeLa cells. Initial results showed transactivation of the BHV-4 E target promoter by IE 2 to be less effective in HeLa cells than in BT cells. Although there are several possible explanations for this finding, one explanation is that IE 2 was not expressed as

A.

```
initn= 244   init1= 130   opt= 270       22.4% identity in 322 aa overlap

                10        20        30        40        50        60
BHV4IE2  MKGIIYPRKSSADRYVYLSPGVREHLFSLLVKYTSSQRDSPAGSSTKVAPDTPQTNFIVE
               |:|:: : :: |:|:::::| ||: :| :  ::: :::|::::      :: :
EBV R         MRPKKDGLEDFLRLTPEIKKQLGSLVSDYCNVLNKEFTAGSVEIT----LRSYKI-
                      10        20        30        40            50

                70        80        90       100       110       120
BHV4IE2  IQWVCHKILLEMEEQFGSLGGLVADINICLIWTLFRNYKHKHRMNNSDTGKSCAEYAQSV
             |: :: | :::  : |||:|::||| :|:::|| : ::| :|:::: :|:   : |
EBV R    ----CKAFINEAKAHGREWGGLMATLNICNFWAILRNNRVRRRAENAGND-ACSIACPIV
                    60        70        80        90       100

               130       140       150       160       170       180
BHV4IE2  VKHLTERMVYCTDKFFINSACSGVTVPQNLALVIASISQVCRNKCQGAWRRMGNGRRTLI
         :::: ::::  ||:|||:::::| :|:::: :: :: : :| :  :  : :| :| :::
EBV R    MRYVLDHLIVVTDRFFIQAPSNRVMIPATIGTAMYKLLKHSRVRAYTYSKVLGVDRAAIM
          110       120       130       140       150       160

               190       200       210       220       230       240
BHV4IE2  DLGLQLVNTYNLLNACGAIDDKCKAFIKLTFPYLNLETVYSPVHAASTGLHQKMAISLYK
         : | |:|:  | ::  | :::| ||| | :|:|  ||::: :: :::|| | : :::  :
EBV R    ASGKQVVEHLNRMEKEGLLSSKFKAFCKWVFTYPVLEEMFQTMVSSKTG-HLTDDVKDVR
          170       180       190       200       210       220

               250       260             270       280       290
BHV4IE1  GQEKRKVPNATIYSNLVTQEKF---ALPEILL---GEITDEGLLANKGPDL-EKLLSEPQ
         :  | ::|:|: ||: ::| ::   :||: ||   :: ::: :|:: :  : |:|:::
EBV R    ALIK-TLPRAS-YSSHAGQRSYVSGVLPACLLSTKSKAVETPILVSGADRMDEELMGNDG
            230       240       250       260       270       280

            300       310       320       330       340       350
BHV4IE2  TILKLVTKLSPVSQFQVFMARWGDKLPSHLKDVCVSDSSQLPQQYENFKVVWPQHQTWPN . .
         :  :  :: |  :||::| :   ::|||
EBV R    GASHTEARYSESGQFHAFTDEL-ESLPSPTMPLKPGAQSADCGDSSSSSSDSGNSDTEQS . .
            290       300       310       320       330       340
```

B.

```
initn= 176   init1= 176   opt= 258       24.7% identity in 243 aa overlap

                                                  10        20        30
BHV-4                                        KPITALERALFLTDVAQILIQHKNQEKSIM
                                             :| | ||:: || :: |:|: |:::|: |:
HVSEDLF4  LHKNFHYVDVSVIQSEFKNVILKTVVPKLSQPATHLEKGDFLLKICQLLMIHREEEQQIL
              20        30        40        50        60        70

                 40        50        60          70        80
BHV-4     VLIQDIIKYMITSLNELNNADAQCSTPQLL--HFLVSDDQMLGVHLIKLLPEILLCKYPN
            ::: | |::::| : : :::|  : ::|    | ::|  |:::| : :|:: : |||
HVSEDLF4  NKVKSNIIYFLNELWSAEYGKVQEQVKNILCEVKLDKTDSELSTYLAQEIPKLTVLKYP-
              80        90       100       110       120       130

          90       100       110       120       130       140
BHV-4     FSTSPINSGDMDYIEWCLHHMLAISETDQNLLRNTLSTREWTLMPSIQVLCHIMQQLFLS
           :   : ::::    ||||::|:|::  ::: : :| ::| | : |:|: ::: : || :
HVSEDLF4  -THFKVCEETIPNGRWCLHNLLGIEQYYKDFSNIVLHDPE-TSLGSVQAYSRLSKLLFWC
               140       150       160       170       180       190

         150       160       170       180       190       200
BHV-4     QTHCPE-YKDHEFNLFFNQAIFWTTVLKMFQECIRQDMVGDHIQCVIALLKNEVRQFFLW
          ::   : |  ::||  :||:::|:|:::::     :| ::: |  : ||||:||::|: |
HVSEDLF4  DSFMNKIYPCNAFNSSINQVVLWSTMFHFYSVAHCNDCISESISFTEALLKQEVSAFYEW
                210       220       230       240       250

          210       220       230       240       250
BHV-4     YDINKIHFKPMAMVKYLEHLVNSITYESSDILLFQARLLLSHCKLILQKAQ
          :   : :::   |:|:::  :: ||  |::: |
HVSEDLF4  C--LEEEYEEDRMAKFMKFSADQITILSTHTDLQNLAEYIYSYKKCLINRRFE
                  260       270       280       290       300
```

C.

```
initn= 199   init1= 127   opt= 264       33.3% identity in 129 aa overlap

                          10        20        30        40        50
BHV-4              MEITVYIPGIEENQRPHFDEIFSTFAKRGNVREAVSSLHKLFKKQKNTGLLAS
                   ||::: : |:::::: ::|:|:::|::  ||: :::::::::| : :: |:|::
HVSEDLF5  MIAFSLTMELSLPVFGVTQANKEEWDNIWKNFQQFPNVSRTLGLLRRFFLR-NDLGFLSA
                    10        20        30        40        50

               60        70        80        90        100       110
BHV-4      LVILKQFVDTSRKSQERFNLLEILQQTKFLAQSIYKHIKSQ-EHSCDMEDMFSDCKDRMS
           :|||||:|:: :::::::::||:|  |  ||| :|:|::|||:| : :::: ::| |||:|:
HVSEDLF5   VVILKQYVENLPTTKQKLNLIECTQGLKFLIRSLYEKIKDQCDVKSSIREIFYDCKARLL
          60        70        80        90        100       110

               120       130       140       150       160       170
BHV-4      LILSESCGCLNCITTTKQLMNSLCDARPPKLSSHKKVCQAHNFLTTVHNQVVVADRVSVS . . .
           |:|:|:|||| :| :|:
HVSEDLF5   LLLEEGCGCGACCATSA . . .
         120       130
```

FIG. 7. Alignment of BHV-4 predicted amino acid sequences with EBV and HVS predicted amino acid sequences. Alignments are the result of homology searches of Genpept and SWISS-PROT protein sequence data bases, using the Pearson and Lipman FASTA program (32) with a "ktup" value of 1. Symbols: |, exact match; :, match with a conservatively replaced amino acid; -, gap introduced to maximize homology. (A) BHV-4 IE 2 aligned with EBV R. Only the amino-terminal portions of the sequences are shown, because the search program did not identify homology in the carboxy-terminal portions. (B) BHV-4 sequences aligned with HVS ORF EDFL4. (C) BHV-4 sequences aligned with HVS EDLF5. Only the portion of the EDLF5 available in the data bases is shown.

effectively in nonpermissive HeLa cells (40, 41) as in BT cells. To possibly increase expression of IE 2 from its own promoter in HeLa cells, thus enhancing its transactivation of homologous and heterologous targets, SV40 enhancer sequences were added to the IE 2 plasmid (pH12PH4.2E). This resulted in increased activation of the BHV-4 E promoter by IE 2 in HeLa cells but did not result in significant transactivation of the EBV or HVS targets (not shown). Addition of IE 1-encoding plasmid did not enable IE 2 to transactivate heterologous targets (not shown).

pH12PH4.2, the IE 2 effector plasmid used in the transient expression cotransfection experiments described thus far, contained, in addition to the ORF encoding IE 2 protein, the BHV-4 ORF homologous to EBV BRRF1 and a portion of the BHV-4 ORF homologous to EBV BRRF2 (Fig. 9). To confirm that the protein encoded by IE 2 ORF was mediating the transactivation of the BHV-4 E gene, two additional IE 2 plasmids were constructed and tested in the transient expression cotransfection assay in BT cells (Fig. 9). pH12PR3.4 contains only the 133 amino-terminal amino acids of the ORF homologous to BRRF2 and would produce only a form of this protein more severely truncated than the protein produced by pH12PH4.2. pH12PH4.2ΔBcl lacks 79 bp of the ORF homologous to BRRF1, resulting in a frameshift after the first 41 codons. This deletion is within the IE 2 intron. Both of these effector plasmids were able to transactivate CAT expression from the BHV-4 E promoter-regulatory region (Fig. 9), supporting the conclusion that IE 2 is responsible for the transactivation. pH12PH3.4 contains 0.5 kbp 5′ to the transcription initiation site of IE RNA 2. The ability of this plasmid to activate CAT expression from the BHV-4 E promoter-regulatory region indicates that sequences contained within this 0.5 kbp and the region encoding IE RNA 2 are sufficient for efficient expression of IE 2 in BT cells.

DISCUSSION

The structure of BHV-4 IE RNA and the nucleotide sequence of the DNA that encodes it have been determined. BHV-4 IE RNA 2 has a short, mostly untranslated, 5′ exon, similar to the structure of the 5′ portion of EBV R RNA (1, 22). The location of consensus splice sites in the nucleotide sequence of the HVS.R gene (25) indicate that HVS.R RNA could also have a similar structure. The introns of the three genes are similar in size (BHV-4 IE 2, 934 nt; EBV R, 940 nt; and HVS.R, 959 nt), and each contains an ORF on the opposite strand, EBV BRRF1 and its homologs. The location of the putative translation initiation codon relative to the intron differs among these three RNAs. The entire ORF, including the translation initiation codon, of EBV R is in the second exon; the initiation codon of BHV-4 IE 2 is contained in the first exon, while the remaining codons are in the second exon; the first exon, if it exists, of HVS.R RNA contains the initiation codon plus codons specifying the next six amino acids of the protein. EBV R mRNA contains an additional ORF 3′ to BRLF1, BZLF1 (1, 22). BHV-4 IE RNA 2 does not contain this additional ORF. In fact, BHV-4 and HVS genomes do not even contain an ORF homologous to EBV BZLF1 (2, 24).

IE RNA 2 is less abundant than IE RNA 1 in the presence of cycloheximide (42). Their relative abundance in the absence of cycloheximide has not been tested. The low abundance of IE RNA 2 in the presence of cycloheximide might suggest that it is not truly an IE RNA, but that small amounts are synthesized under IE conditions as a result of leakiness. However, the fact that the amount of IE RNA 2 is not increased in the absence of cycloheximide suggests that its synthesis is not stimulated by IE protein(s) and that it is truly an IE RNA. Because only one time was tested (6 h), the results do not rule out the possibility that IE RNA 2 expression is increased at other times. However, the expression pattern of IE RNA 2 is clearly different from the expression pattern of its homologous RNA in HVS-infected cells, which is an E RNA (25, 27). The similarity in amount of IE RNA 2 found in infected cells 6 h postinoculation in the absence and presence of cycloheximide also suggests that there is no negative regulation of IE RNA 2 transcription by BHV-4 IE proteins. This is apparently not the case for IE

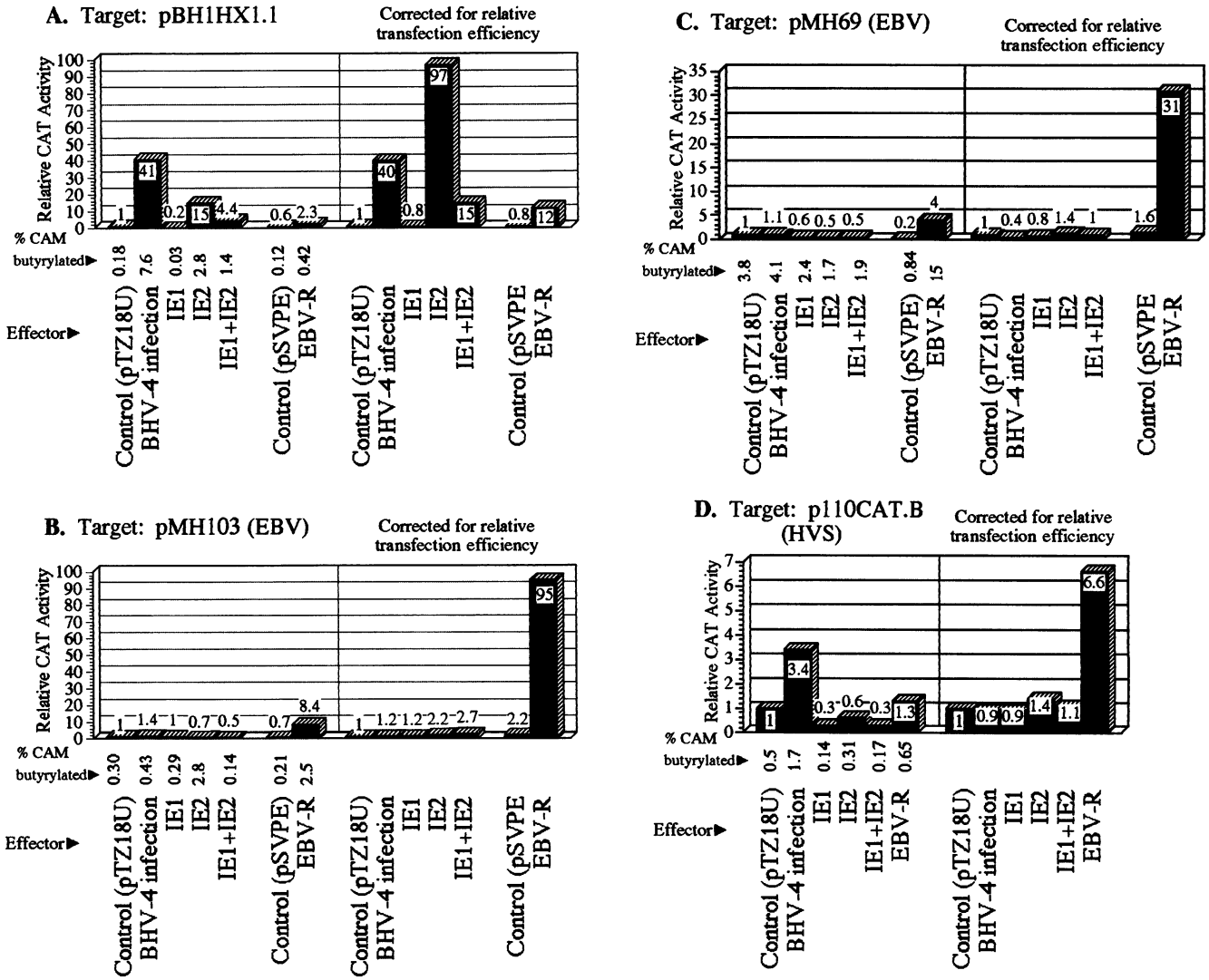

FIG. 8. Results of CAT assays demonstrating activation of a BHV-4 E promoter-regulatory region in BT cells. Results of experiments using four different target plasmids are shown. Experiments shown in panels A to C were conducted in parallel. CAT assays shown in panel D were conducted with cell extracts from a different experiment, and CAT assay reaction mixtures were incubated for 10 h. The left portion of each panel shows the relative CAT activity per well of cells transfected, and the right portion of each panel shows relative CAT activity corrected for relative transfection efficiency. Relative CAT activity is expressed relative to that obtained when the control effector plasmid, pTZ18U vector, was used. An additional control effector plasmid, pSVPE, containing SV40 early promoter and enhancer sequences, was included in experiments to control for competition between the EBV R effector plasmid, which contains the SV40 early promoter and enhancer, and the transfection efficiency control plasmid, pSV-β-Galactosidase.

RNA 1, which is present in greatly reduced amounts in the absence of cycloheximide (42).

BHV-4 IE 2, HVS.R, and EBV R transactivators exhibit amino acid sequence homology. At least BHV-4 IE 2 and HVS.R are also functionally homologous, because each activates expression of the major DNA-binding protein-homologous gene. However, they are not functionally cross-reactive; BHV-4 IE 2 does not transactivate promoters transactivated by HVS.R and EBV R, and EBV R does not transactivate BHV-4 or HVS targets. This finding implies that the transactivator genes and target promoters within each virus have evolved together so that appropriate transactivation has been maintained. In light of these observations, it is not surprising that the BHV-4 major DNA-binding protein-homolog promoter-regulatory region does not contain sequences identified as targets of transactivation by EBV R (5, 6, 10, 11, 41). The specific target sequences for transactivation by BHV-4 IE 2 have not yet been identified.

Although EBV R and BHV-4 IE 2 apparently have different DNA binding specificities, the portion of the EBV R transactivator exhibiting amino acid sequence homology with BHV-4 IE 2 includes the DNA-binding and dimerization domains. The highly basic region noted by Nicholas et al. (25) in HVS.R (50% basic residues in a 28-amino-acid stretch between amino acids 390 and 418) is not present in BHV-4 IE 2. Short, extremely proline-rich segments found in the EBV R transactivator between amino acids 352 and 410 and between amino acids 445 and 449 (23) are also not present in BHV-4 IE 2. However, all three transactivators have acidic carboxy termini. This acidic portion of EBV R

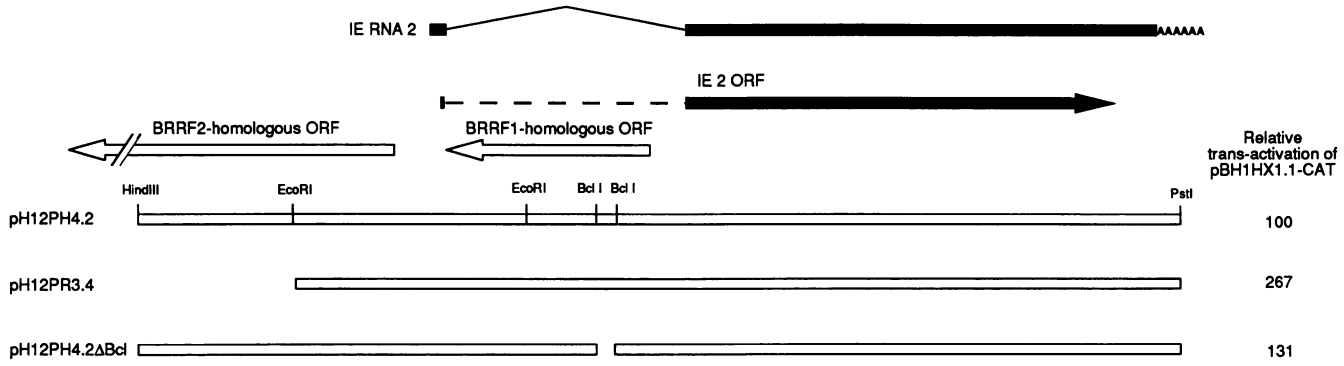

FIG. 9. Relative ability of plasmids encoding BHV-4 IE 2 to transactivate expression of CAT from a BHV-4 E promoter-regulatory region. Diagrams of the BHV-4 DNA included in plasmids transfected are shown at the bottom. Open boxes indicate the DNA sequences present. Above these diagrams, arrows show the positions of ORFs in the 4-kb *Hin*dIII-*Pst*I fragment shown. The map position of IE RNA 2 is shown at the top. To the right of each plasmid map, numbers indicate the relative amount of CAT activity detected when the plasmids were used as effector plasmids with pBH1HX1.1-CAT target plasmid in transient expression cotransfection assays in BT cells. The CAT activity induced by pH12PH4.2 was arbitrarily set at 100. Values shown are the averages obtained from three experiments and are corrected for relative transfection efficiency.

contains transactivation domains (13, 23). Therefore, all three transactivators could act by similar mechanisms.

Transactivation of the promoter-regulatory region of the BHV-4 major DNA-binding protein-homologous gene by BHV-4 IE 2 is extremely specific. This specificity is emphasized by the fact that BHV-4 IE 2 does not transactivate the homologous HVS gene, which is transactivated by the HVS homolog to BHV-4 IE2 (25). In contrast, HSV-1 IE110 and human cytomegalovirus IE2 transactivators transactivate nearly every target tested (29, 33, 37, 38). Between these two extremes of specificity are examples of herpesvirus transactivators exhibiting moderate specificity. For example, both IE genes of HVS are able to transactivate the HSV-1 IE 175K promoter-regulatory region but are unable to transactivate the HVS E promoter-regulatory region tested (25).

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