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Immediate-Early RNA 2.9 and Early RNA 2.6 of Bovine Herpesvirus 1 Are 3′ Coterminal and Encode a Putative Zinc Finger Transactivator Protein

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Bovine herpesvirus 1 (BHV-1) contains three major immediate-early (IE) genes involved in regulation of the productive cycle of replication. Two spliced IE RNAs, IER4.2 (4.2 kb) and IER2.9 (2.9 kb), are under the control of a single promoter; IER1.7 (1.7 kb) is transcribed from a different promoter in the opposite direction. Examining the kinetics of transcription, we found that the IER4.2/2.9 promoter was turned off at the end of the IE period. An alternative promoter became active, directing synthesis of an unspliced early RNA, ER2.6 (2.6 kb), which was colinear with the second exon of IER2.9 except for its 5′ end in the intron about 10 bases upstream of the splice site. Sequence analysis revealed a single open reading frame common to IER2.9 and ER2.6 with a coding potential of 676 amino acids. The putative protein, named p135, contained a cysteine-rich zinc finger domain near the N terminus with homology to ICP0 of herpes simplex virus type 1, to protein 61 of varicella-zoster virus, to early protein 0 of pseudorabies virus, and to other viral and cellular proteins. The remaining parts of p135 exhibited only limited homology, mainly with pseudorabies virus protein 0, but the entire sequence was highly conserved between two strains of BHV-1 (K22 and Jura). The latency-related antisense transcript covered a large portion of ER2.6 excluding the zinc finger coding region. In transient expression assays, p135 activated a variety of promoters, including that for ER2.6, but repressed the IER1.7 promoter. Thus, p135 combines functional characteristics of ICP0, a strong transactivator, and of protein 61, a repressor. BHV-1 seems to have evolved a subtle mechanism to ensure the continued synthesis of p135 while turning off IER4.2, which encodes p180, the herpes simplex virus type 1 ICP4 homolog.

Bovine herpesvirus 1 (BHV-1) contains three major immediate-early (IE) genes, which are important for the regulation of the productive cycle of replication (44, 55, 57, 58). They are grouped in two divergent transcription units with start sites located in the inverted repeat. Transcription unit 2 runs toward the right from 0.818 to 0.836 map units (m.u.); it specifies a spliced IE RNA of 1.7 kb (IER1.7) which encodes p55, the smallest IE protein. Transcription unit 1 (0.797 to 0.738 m.u.) specifies two alternative spliced BHV-1 transcripts, IER4.2 and IER2.9, with a common noncoding leader sequence (exon 1) under the control of a single promoter. Exon 2 of IER4.2 in the inverted repeat encodes p180, the homolog of ICP4 of herpes simplex virus type 1 (HSV-1) and related IE proteins of other herpesviruses (11, 12, 25, 41, 54). Exon 2 of IER2.9 is located entirely in the unique long (U_L) sequence and encodes p135 (approximately 135 kDa), the homolog of HSV-1 ICP0 (46), as will be shown in this study.

Alternative splicing as a device for coordinating IE gene expression was a novel discovery in alphaherpesviruses, to which BHV-1 and HSV-1 belong; only cytomegaloviruses (betaherpesviruses) were previously known to rely on it (8). Here we report another unusual feature of BHV-1 IE transcription, which we detected by Northern (RNA) blot, S1 nuclease protection, and primer extension analysis. At the end of the IE period, the IER4.2/2.9 promoter was turned off, and a different promoter became active, directing synthesis of an unspliced, early 2.6-kb RNA (ER2.6), which was colinear with exon 2 of IER2.9 except for its 5′ end in the intron about 10 nucleotides (nt) upstream of the splice site. Thus, BHV-1 uses alternative promoters for temporal control of p135 expression. For two BHV-1 strains, we determined the nucleotide sequence of the ER2.6 gene and flanking regions and found that the encoded p135 contains an amino-terminal region with strong homology to a recently discovered variety of zinc finger structures. A large part of the coding sequence except the zinc finger homology was overlapped in the opposite sense by the latency-related (LR) transcriptionally active region (35). Transient expression assays showed that p135 can act as both a transactivator and a repressor.

MATERIALS AND METHODS

Viruses, cells, and isolation of total RNA and viral DNA. The virus strains used in this study were BHV-1.1 Jura (43) and BHV-1.2b K22 (32). Madin-Darby bovine kidney (MDBK) cells were cultured, infected with BHV-1, and treated with metabolic inhibitors if required (55), and total RNA was isolated at the indicated hours postinoculation (p.i.) as described previously (57). Vero cells were cultured in Dulbecco's medium supplemented with 10% fetal bovine serum. Viral DNA was isolated from purified virions (57).

Transcript mapping procedures. Northern blot hybridization, S1 nuclease protection assays, and primer extension analysis were performed as described previously (57).

Plasmids and sequence analysis. Plasmids pJuC, containing

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the BHV-1 Jura *Hin*dIII C fragment (0.733 to 0.852 m.u.), and p601, containing the BHV-1 K22 *Hin*dIII-*Eco*RI c' fragment (0.733 to 0.816 m.u.), and the cloned subfragments pOM75, pMV340, and pMV381 have been described elsewhere (57); additional overlapping subfragments were cloned by the same procedures. The nucleotide sequence was determined for both strands, initially by the chemical method (40) and then by the dideoxy method (50), using double-stranded plasmids as templates for Sequenase (U.S. Biochemical) with 7-deaza-dGTP or dITP instead of dGTP. Additionally, pMV381 was cut with *Nar*I or *Xma*I, and libraries of the resulting fragments were subcloned in M13mp18 for sequence analysis. Some gels contained 15% formamide. Sequences were analyzed with the University of Wisconsin Genetics Computer Group programs (13).

Constructs, DNA transfection, and CAT assay. For pCE26X, the 6.3-kb *Hin*dIII-*Pvu*II fragment of pJuC was inserted between the *Hin*dIII and *Hin*cII sites of pBluescript (pBS KS+; Stratagene GmbH, Heidelberg, Germany). From the resulting plasmid (pBHPJ6), most of the coding region of IER4.2 was removed by using *Rsr*II (2.8 kb in three fragments; 0.780 to 0.759 m.u.). The resulting plasmid (pCE26) was truncated once more by removing a 0.5-kb *Xba*I fragment (0.737 to 0.733 m.u.) containing upstream elements of the LR promoter, the LR silencer (30), and the *Hin*dIII site. The resulting construct (pCE26X), encoding the putative zinc finger protein p135, served as an effector plasmid in the transfection experiments. For pCF1CAT, the 1.63-kb *Bgl*II-*Bam*HI fragment from pCAT3M (36) containing the chloramphenicol acetyltransferase (CAT) gene and the simian virus 40 small t intron and early polyadenylation signal was cloned into the *Bam*HI site of pBS KS+ in such an orientation that the 3' end of the SK primer pointed in the direction of the CAT gene. pCF2CAT was identical to pCF1CAT but with the 1.63-kb fragment in the opposite orientation. These two plasmids served as the basis for subsequent promoter-CAT constructs and as negative controls for transfection experiments. For pC29CAT, the 1.7-kb *Not*I fragment from pJuC was cloned into the *Not*I site of pCF1CAT. In this plasmid, the CAT gene is under the control of the BHV-1 IER4.2/2.9 promoter. For pC26CAT, plasmid pCE26X was digested with *Bst*XI, and protruding 3' ends were removed by T4 DNA polymerase. After separation on an agarose gel, the large fragment (4.2 kb) was religated, forming pCE26XX. From pCE26XX, the 0.9-kb *Sac*I-*Kpn*I fragment was excised and, after removal of protruding 3' ends by T4 DNA polymerase, cloned into the *Eco*RV site of pCF2CAT. In the resulting construct, pC26CAT, the CAT gene is regulated by the BHV-1 early 2.6 promoter. For pC17CAT, the 0.6-kb *Pst*I-*Eco*RI fragment of pJuC was cloned into pCF2CAT. This construct includes the IE1.7 promoter but lacks the adjacent ori$_S$ sequences (22). For pCLRCAT, a 0.9-kb *Pst*I fragment of pJuC (0.733 to 0.740 m.u.) was cloned into the *Pst*I site of pCF2CAT. The CAT gene is under the control of the BHV-1 LR promoter (30). pEC3, kindly provided by W. C. Lawrence and L. J. Bello, contains the BHV-1 glycoprotein I promoter upstream of the CAT gene. Plasmids pDs-9 and pJC17, kindly provided by S. Silverstein, contain the CAT gene under the control of the HSV-1 glycoprotein C and thymidine kinase promoters, respectively (9).

Transfection by the calcium phosphate method was performed as described previously (53), using 1.4 to 2.1 pmol of promoter-CAT constructs cotransfected with 0, 0.7, 1.4, 2.1, or 2.8 pmol of pCE2.6X effector plasmid into Vero cells (10^6 cells per dish). Each test series included a dish with 0.7 pmol of pSV2CAT as a standard (24). CAT assays were carried out as described previously (53) except that the thin-layer chromatograms were evaluated by storage phosphor technology (29). After exposure to the thin-layer plates for 3 h, a phosphor screen was scanned with a PhosphorImager (Molecular Dynamics, Inc.), and the data were processed on a personal computer with Image Quant and Excel software. Substrate turnover was calculated as radioactivity of acetylated products divided by the sum of the radioactivities of nonacetylated and acetylated substrate. Relative CAT activity was defined as substrate turnover of the test sample divided by the substrate turnover of the standard (0.7 pmol of pSV2CAT).

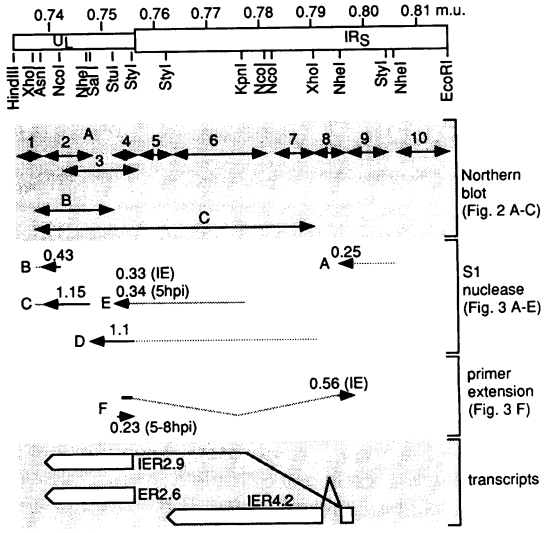

FIG. 1. Map of the BHV-1 *Hin*dIII-*Eco*RI c' genome region previously shown to encode two alternative spliced IE transcripts (IER4.2 and IER2.9). The diagram at the top indicates map units, junction of the U_L and IR_S regions, and restriction endonuclease sites. Below are shown DNA subfragments (1 to 10, B, and C) used as probes for Northern blot analysis (Fig. 2). End-labeled DNA probes were used for S1 nuclease analysis (Fig. 3A to E). Protected fragment parts are shown by arrows pointing in the direction of transcription, and length is indicated in kilobases; S1 nuclease-sensitive parts of the probes are shown by dotted lines. Also shown are a summary of primer extension (Fig. 3F) and locations of transcripts.

Nucleotide sequence accession number. The nucleotide sequence data reported in this article have been submitted to GenBank and assigned accession numbers M84464 (strain K22) and M84465 (strain Jura).

RESULTS

An early 2.6-kb RNA maps to exon 2 of IER2.9 by Northern blot analysis. Total RNA was isolated at 5 h p.i. from BHV-1 Jura-infected cells, loaded into a single slot spanning an entire agarose gel, separated electrophoretically, and blotted onto nylon membranes. The blots were cut into identical strips and probed with various radiolabeled fragments of the *Hin*dIII-*Eco*RI c' genome region (top of Fig. 1; 0.733 to 0.816 m.u.). Autoradiographs of the strips (Fig. 2A) revealed a 2.6-kb transcript which was detected only with fragments overlapping exon 2 of the previously characterized IER2.9 (lanes 2 to 4; see transcript map at the bottom of Fig. 1).

To define the temporal class of this 2.6-kb transcript, infected cells were treated with metabolic inhibitors, and

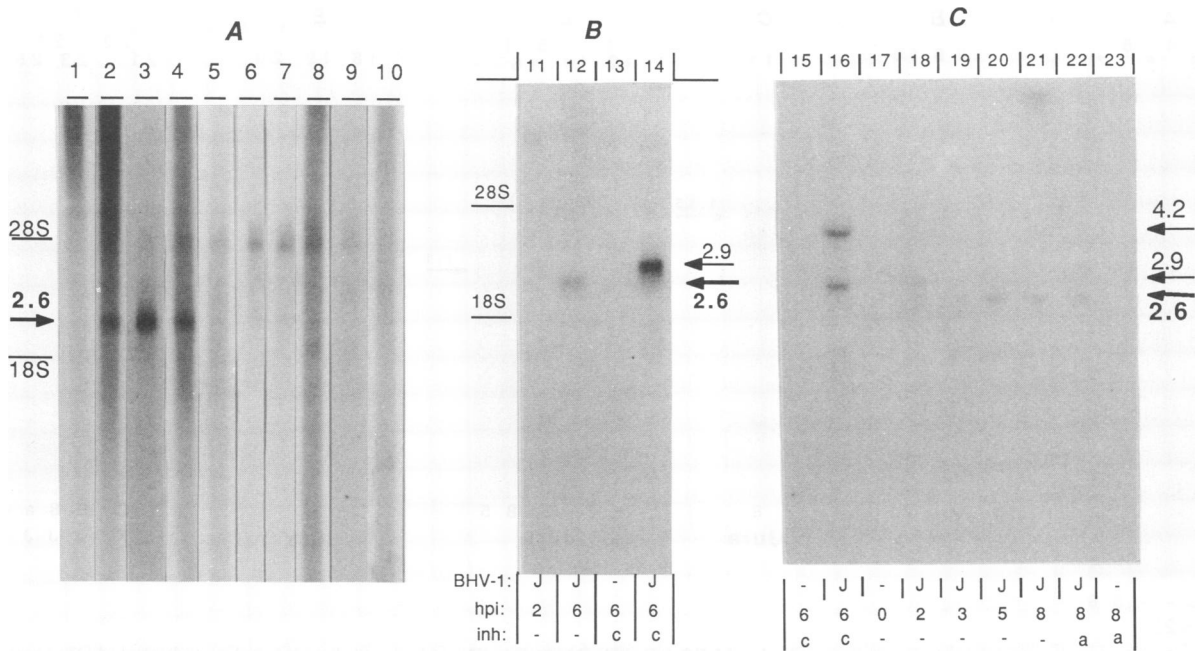

FIG. 2. Northern blot analysis of BHV-1 RNA with probes outlined in Fig. 1. (A) Labeled subfragments (1 to 10) were hybridized to identical strips carrying blotted RNA isolated at 5 h p.i. from BHV-1 Jura-infected MDBK cells. (B and C) Subfragments B and C were hybridized to RNA isolated at different times postinoculation from MDBK cells which had been mock infected (−) or infected with BHV-1 Jura (J) and incubated in the absence (−) or presence of cycloheximide (c; 100 μg/ml) or araC (a; 100 μg/ml). Positions of 18S and 28S rRNAs and transcript sizes (in kilobases) are indicated.

RNA was isolated at different times postinoculation and hybridized with probe B or C (Fig. 1). The 2.6-kb RNA was readily detectable after isolation from untreated cells at 6 h p.i. but not at 2 h p.i. (Fig. 2B), whereas IER2.9 accumulated in cycloheximide-treated cells as reported before (55). Hybridization with probe C spanning both IE transcripts revealed strong signals for IER2.9 and IER4.2 from cycloheximide-treated cells, weak signals from untreated cells at 2 h p.i., and no detectable signals at later times (Fig. 2C). In contrast, the 2.6-kb transcript could be isolated from untreated cells 3 to 8 h p.i., the signal being strongest at 5 h p.i., or from 1-β-D-arabinofuranosylcytosine (araC)-treated cells at 8 h p.i., but not from cycloheximide-treated cells (Fig. 2C). Hence, this transcript belongs to the early class and is designated ER2.6 (early 2.6-kb RNA).

ER2.6 is 3′ coterminal with IER2.9 but carries a different 5′ end. To determine whether ER2.6 is transcribed in the same or opposite sense relative to IER2.9 and to compare the termini of the transcripts, we performed S1 nuclease protection experiments (Fig. 3A to E; see map in Fig. 1). For Fig. 3A, pMV340 (strain K22) was digested with *Nhe*I and labeled with T4 polynucleotide kinase, and a 1.3-kb fragment labeled at both 5′ ends (0.795 to 0.805 m.u.) was isolated. It was hybridized with RNA from two cultures of BHV-1 K22-infected cells treated with cycloheximide (lanes 2 and 3), from mock-infected cells (lanes 4 and 5), or from infected cultures without cycloheximide harvested at 5 or 8 h p.i. (lanes 6 and 7). After S1 nuclease digestion, a 5′-end-labeled DNA fragment of 0.25 kb (arrow) in lanes 2 and 3 remained protected by the RNA, as analyzed on an agarose gel together with undigested probe (*1.3 kb; lane 1). This signal characterizes the common leftward transcription start site of IER4.2 and IER2.9, which has been mapped previously (57). No signal could be detected in lanes 6 and 7, indicating that IER4.2 and IER2.9 are degraded without replenishment at 5 and 8 h p.i. and that ER2.6 is transcribed from a different start site.

To map the 3′ termini, pOM75 (strain Jura) was digested with *Nco*I, labeled with Klenow fragment, and cut with *Xho*I, and a 0.7-kb fragment (0.736 to 0.742 m.u.) 3′ end labeled at the *Nco*I site was isolated. It was hybridized with RNA from BHV-1 Jura-infected cells treated with cycloheximide (Fig. 3B, lane 8), from mock-infected cells (lane 9), or from infected cells without cycloheximide at 8 h p.i. (lane 10). The S1 nuclease digestion products were analyzed on a polyacrylamide-urea gel. The topmost band at 0.43 kb (lane 8) arises from the previously mapped 3′ end of IER2.9 (57). At 8 h p.i. (lane 10), the same 0.43-kb band is observed, suggesting that ER2.6 and IER2.9 are 3′ coterminal. The series of faster-migrating, weaker bands seen in lanes 8 and 10 may indicate some 3′-terminal heterogeneity shared by ER2.6 and IER2.9 or, more likely, the effect of short A+T-rich regions in transcripts of overall high G+C content (see the sequence below). The experiment was repeated with a different probe 3′ end labeled at the *Nhe*I site (0.747 m.u.), resulting in a protected fragment of 1.15 kb (Fig. 3C) and again indicating the same 3′ terminus.

To map the 5′ termini, pOM75 was digested with *Sal*I, labeled with T4 polynucleotide kinase, and cut with *Xho*I, and a 6-kb fragment (0.747 to 0.791 m.u.) 5′ end labeled at the *Sal*I site was isolated. It was hybridized with RNA isolated from BHV-1 Jura-infected cells under the conditions used for Fig. 3A. After S1 nuclease digestion, protected fragments of 1.1 kb were observed (Fig. 3D). For cycloheximide-treated cells (lanes 12 and 13), this size was expected and matched the known map location of the IER2.9 splice acceptor site. The results for cultures without cycloheximide harvested at 8 or 5 h p.i. (lanes 16 and 17) seemed to indicate

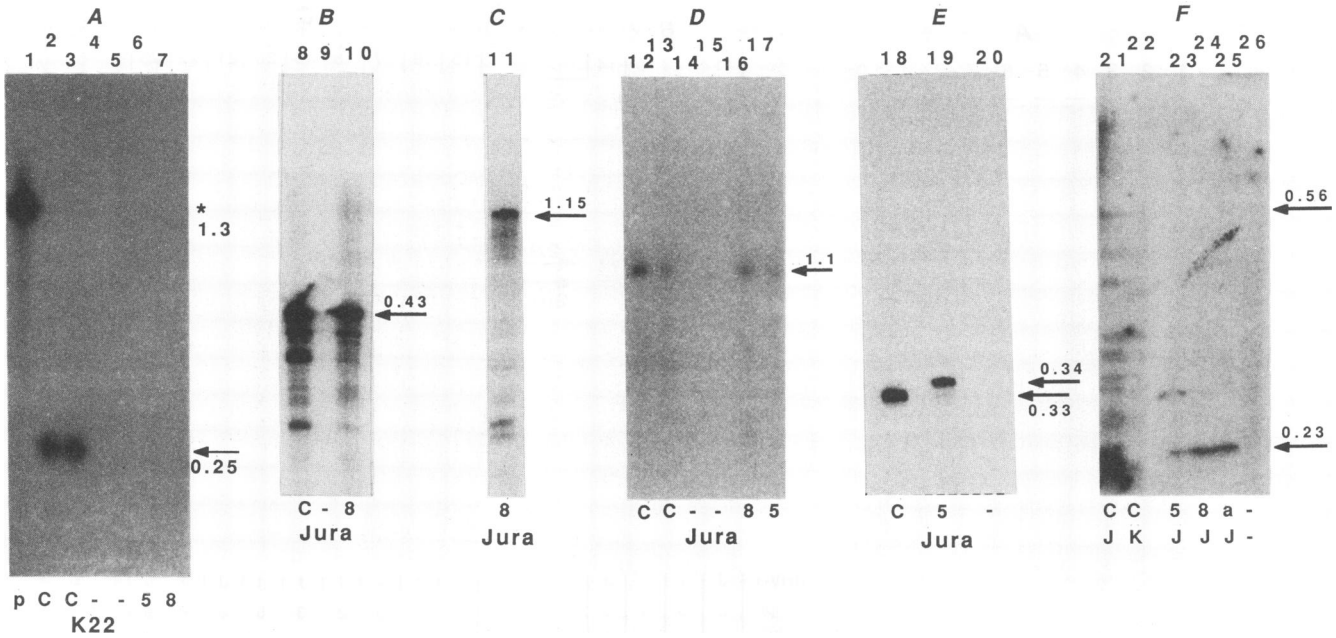

FIG. 3. S1 nuclease and primer extension analysis of ER2.6 and IE RNA. For S1 nuclease analysis (A to E), end-labeled DNA probes (Fig. 1) were hybridized to total RNA from mock-infected (−) or BHV-1 K22 (K)- or Jura (J)-infected cells, either treated with the metabolic inhibitor cycloheximide (C) or araC (a) for 6 h or untreated, and harvested at the indicated hours postinfection. Fragments protected from S1 nuclease digestion (arrows) and undigested probes (p, *) were analyzed on 6% polyacrylamide–urea (B, C, and E) or on 1.4% alkaline agarose (A and D) gels. End-labeled *Hin*dIII-*Eco*RI fragments of lambda DNA and *Hin*fI fragments of pGEM4 were used as molecular weight markers (M) for each gel, and calculated sizes of fragments are indicated in kilobases. (F) For primer extension reactions, primer o9 was 5′ end labeled and hybridized to RNA samples as described above. Reverse transcriptase from avian myeloblastosis virus was used to synthesize cDNA fragments, which were analyzed on a 6% polyacrylamide-urea sequencing gel.

that the same splice acceptor site was employed. However, Fig. 3E shows that these 5′ ends are different. The experiment was repeated with pOM75 cut and labeled at the *Stu*I site (0.753 m.u.). RNA from cycloheximide-treated cells protected a 0.33-kb fragment (lane 18), whereas RNA from untreated cells harvested at 5 h p.i. protected a fragment which was about 10 nt longer and migrated with distinctly lower mobility on the polyacrylamide-urea gel (lane 19).

Taking results of the Northern and S1 nuclease analyses together, we concluded that ER2.6 is colinear with exon 2 of IER2.9 and shares the same 3′ terminus, but that ER2.6 is not spliced and is transcribed from a start site located just upstream of exon 2 of IER2.9. This conclusion was confirmed by primer extension analysis (Fig. 3F). A 22-mer oligonucleotide, o9 (5′-AGATCTCCTCGAAGCACTCATC-3′), based on the BHV-1 nucleotide sequence (except for the 5′-terminal A; see below) was labeled at the 5′ end. After hybridization to total RNA from BHV-1 K22 or Jura-infected, cycloheximide-treated cells, the primer could be extended by reverse transcription to a maximal length of 0.56 kb (lanes 21 and 22), corresponding to a distance of 0.22 kb from the primer to the splice acceptor site plus the 0.34-kb leader sequence of IER2.9. In contrast, when hybridized to RNA from untreated cells (5 or 8 h p.i.; lanes 23 and 24) or treated with araC (lane 25), the same primer was extended to a single 0.23-kb species. Thus, the 5′ end of ER2.6 is located approximately 10 nt upstream of the IER2.9 splice acceptor site.

IER2.9 and ER2.6 encode the same putative zinc finger protein. We determined the nucleotide sequences of BHV-1 K22 and Jura in the region specifying ER2.6 (Fig. 4). The K22 sequence (3,330 nt) is displayed as the prototype in the sense of ER2.6 transcription and includes 300 nt of 5′-flanking sequence containing the short inverted repeat (IR_S)-U_L junction, as well as 750 nt of 3′-flanking sequence extending to the *Hin*dIII boundary (0.733 m.u.) of the *Hin*dIII-*Eco*RI c′ fragment. The line below the K22 sequence shows only those nucleotides found to be different from K22 in the Jura sequence (5′-flanking and coding regions) or in the published strain Cooper sequence (3′-flanking region) (35). The same line contains roman numerals which identify underlined sequence features to be described here.

The sequence begins at a *Nar*I site (i), located in IR_S at 0.757 m.u., 119 nt from the IR_S-U_L junction. Promoter elements for ER2.6 appear shortly after the junction and include potential NFκB (ii) and Sp1 (iii) binding sites and a TATA box (iv). The ER2.6 start site (>>>) is located approximately 10 nt upstream of the IER2.9 splice acceptor site, shown with the terminal AG of the intron underlined (v). The oligonucleotide o9 used to locate these sites is complementary to sequence (vii). Just 4 nt downstream from the IER2.9 splice acceptor site is an ATG codon (vi) in proper sequence context for translation initiation (33). It must serve as the initiation codon in both RNAs, IER2.9 and ER2.6, because the common leader of IER4.2 and IER2.9 (exon 1) does not contain a single ATG (54, 57). An open reading frame predicted to encode p135, a polypeptide of 676 amino acids (see below), extends to a TAA codon (xiii). The common 3′ terminus of IER2.9 and ER2.6 (−>) is located about 220 nt after the stop codon and is preceded by a polyadenylation signal (xv). Several underlined features belong to the opposite DNA strand. Part of an unidentified open reading frame runs across the *Hin*dIII site (xx) and

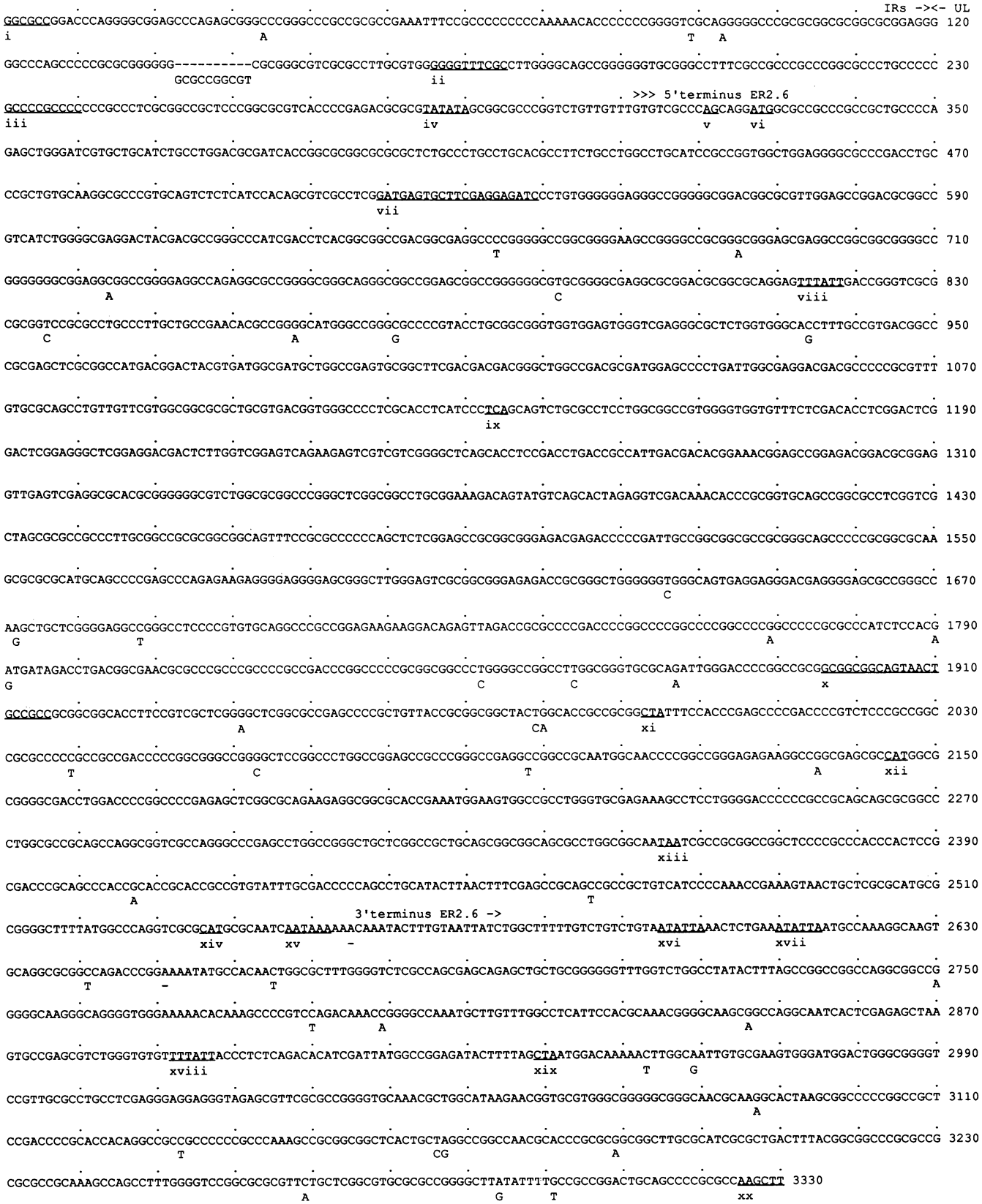

FIG. 4. Nucleotide sequence of BHV-1 K22 in the sense of ER2.6 transcription. The IR_S-U_L junction (−><−) and the 5′ terminus (>>>) and 3′ terminus (−>) of ER2.6 are marked in the line above the sequence. The line below displays only those bases which are different from K22 or absent (−) in strain Jura (1 to 2630) or Cooper (2631 to 3330). The same line contains roman numerals which identify underlined sequence features described in the text.

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  1 MAPPAAAPELGSCCICLDAITGAARALPCLHAFCLACIRRWLEGRPTCPL 50
 51 CKAPVQSLIHSVASDECFEEIPVGGGPGADGALEPDAAVIWGEDYDAGPI 100
101 DLTAADGEAPGAGGEAGAAGGSEAGGGAGGAEAAGEARGAGAGRAAGAAG 150
151 GRAGRGADAAQEFIDRVARGPRLPLLPNTPGHGPGAPYLRRVVEWVEGAL 200
201 VGTFAVTARELAAMTDYVMAMLAECGFDDDGLADAMEPLIGEDDAPAFVR 250
251 SLLFVAARCVTVGPSHLIPQQSAPPGGRGVVFLDTSDSDSEGSEDDSWSE 300
301 SEESSSGLSTSDLTAIDDTETEPETDAEVESRRTRGASGAARARRPAERQ 350
351 YVSTRGRQTPAVQPAPRSLARRPCGRAAAVSAPPSSRSRGGRRDPRLPAA 400
401 PRAAPAAQARACSPEPREEGRGAGLGVAAGETAGWGVGSEEGRGERRAKL 450
451 LGEAGPPRVQARRRRRTELDRAPTPAPAPAPAPISTMIDLTANAPARP 500
501 ADPAPAAALGPALAGAQIGTPAAAAAVTAAAAAPSVARGSAPSPAVTAAA 550
551 TGTAAAISTRAPTPSPAGRAPAADPRRAGAPALAGAARAEAGRNGNPGRE 600
601 RRPASAMARGDLDPGPESSAQKRRRTEMEVAAWVRESLLGTPRRSSAALA 650
651 PQPGGRQGPSLAGLLGRCSGGSAWRQ 676
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FIG. 5. Deduced amino acid sequence of p135 encoded by IER2.9 and ER2.6. The main sequence (in the single-letter code) is for strain K22; letters below show only those amino acids which are different in strain Jura. The zinc finger, acidic region, and Cooper duplication are underlined and discussed in the text.

continues for 129 codons to a stop codon (xix), which is followed by a polyadenylation signal (xviii). The LR transcriptionally active region (35) exhibits two possible TATA boxes (xvii and xvi), two open reading frames (xiv to xi and xii to ix), and a polyadenylation signal (viii). In about 1,700 nt of overlapping sequence, strains Jura (this report) and Cooper (35) exhibit no base differences, with a single exception: the underlined 21-nt motif (x) is present as a direct repeat in strain Cooper only. Presumably the two strains have a virtually identical sequence in this region because they both belong to subtype 1; even K22 (subtype 2b) exhibits fewer than 2% base differences compared with Jura and Cooper.

Figure 5 shows the deduced amino acid sequence of p135 encoded by IER2.9 and ER2.6. Its most conspicuous feature is a cysteine-rich region near the N terminus constituting a potential zinc finger motif (see below). Amino acids 280 to 330 are notably rich in acidic (D and E) and hydroxyl (S and T) residues. They contribute to a large excess of acidic residues (53 to 56 acidic versus 18 basic residues) in the N-terminal half of the molecule (amino acids 1 to 330), which is compensated for by a similar excess of basic residues (55 basic versus 23 acidic residues) in the C-terminal half. Residues 70 to 160 are rich in glycine and alanine and overlap with a cluster of amino acid differences between K22 and Jura, noted below the prototype K22 sequence. A second cluster of sequence divergence, including the underlined seven amino acids which are duplicated only in Cooper, is located in the C-terminal half in a region notably rich in alanine.

Analysis of the predicted polypeptide using the protein data bases MIPSX and Swissprot revealed a cluster of homologous amino acid residues near the N terminus. The alignment (Fig. 6) shows residues 10 to 60 of BHV-1 p135 in the top line. The lines below represent the early protein 0 of pseudorabies virus (PRV; residues 43 to 93), the ORF61 gene product of varicella-zoster virus (VZV; residues 16 to 66), and ICP0 of HSV-1 (residues 113 to 165), followed by 16 additional polypeptide sequences of widely different origin, mostly from regulatory proteins. The consensus sequence (bottom line) shows perfect alignment of one histidine and seven cysteine residues, a motif which has been recognized recently (23) as a novel variety of the well-known zinc finger structures (2). The remaining sequence of p135 did not contain any convincing homologies to viral or nonviral proteins in the data base, with the exception of residues 61 to 110 and 181 to 350, which exhibited homology (not shown) to residues 94 to 146 and 147 to 315 of PRV protein 0, including the acidic domain.

To determine whether other herpesviruses might encode proteins related to p135, various bovid herpesvirus genomes were cut with restriction enzymes, blotted, and hybridized under low stringency with labeled subfragments of pJuC. Most fragments did not hybridize (not shown), but the *Sty*I-*Stu*I fragment (Fig. 1, fragment 4; nt 175 to 654) encoding the first 110 amino acid residues of p135 including

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LGSCCICLD------AITGAAR---------ALPCLHAFCLACI-RRWLEGR------PTCPLCKAPVQSLIH    p135    BHV1
VMDCPICLD------VAATEAQ---------TLPCMHKFCLDCI-QRWTLTS------TACPLCKARVTSILH    EP0     PRV
DNTCTICMS------TVSDLGK---------TMPCLHDFCFVCI-RAWTSTS------VQCPLCRCPVQSILH    ORF61   VZV
GDVCAVCTD------EIAPHLR-------CDTFPCMHRFCIPCM-KTWMQLR------NTCPLCNAKLVYLIV    ICP0    HSV1
PLSCKSCWQ------KFDSLVR----------CHDHYLCRHCLNLLLSVSDR-------CPLCKYPLPTRLK    Z       LCMV
KFECSVCLE------TYSQQSNDTC--PFLIPTTCDHGFCFKCVINLQSNAMNIPHSTVCCPLCNTQVKMWRS    PE38    Baculovirus
KLQCNICFSVAEIKNYFLQPIDRLTIIPVLELDTCKHQLCSMCI-RKIRKRK-----KVPCPLCRVE---SLH    CG30    Baculovirus
RLQCHICCSVGEIKNYFLQPVDAITILPIVELHTCRHQLCVMCV-RKIAQRGR--DKRVECPMCRRK---NAH    CG30r   Baculovirus
LLRCHICKD------FLKVPV----------LTPCGHTFCSLCI-RTHLNNQ------PNCPLCLFEFRESLL    RAD18   Yeast
YGMCAVCRE------PWAEGA--------VELLPCRHVFCTACVVQRW----------RCPSCQRRI          L-rep   Trypanosome
LITCRLCRG------YMIDPTT---------VDYCYHTYCRSCILKHLLRA-------VYCPECKASGGKEIN    Su(z)2  Drosophila
HIICHLCQG------YLINATT---------IVECLHSFCHSCLINHLRKE-------RFCPRCEMVINNAKP    Psc     Drosophila
HLMCVLCGG------YFIDATT---------IIECLHSFCKTCIVRYLETS-------KYCPICDVQVHKTRP    bmi-1   Mouse
HLMCALCGG------YFIDATT---------IVECLHSFCKTCIVRYLETN-------KYCPMCDVQVHKTRP    mel-18  Mouse
ELMCPICLD------MLKNTMT---------TKECLHRFCSDCIVTALRSGN------KECPTCRKKLVS       RING1  Human
SISCQICEH------ILADPVE---------TNCKHVFCRVCILRCLKVMG------SYCPSCRYPCFP        RAG-1   Human
ETTCPVCLQ------YFAEPM----------MLDCGHNICCACLARCWGTA----ETNVSCPQCRETFP-QRH    RFP     Human
EVTCPICLE------LLKEPVS---------ADCNHSFCRACITLNYESNRNT-DGKGNCPVCRVPYPFGNL    RPT-1   Human
FLRCQQCQA------EAKCPKL---------LPCLHTLCSGCLEASG---------MQCPICQAPWPLGAD    PML     Human
EVTCPICLD------PFVEPVS---------IECGHSFCQECISQVGKGGG------SVCAVCRQRF         SS-ARo Human
---C-•C---------•----------------C-H-•C--C•---•----------CP-C*--•-----    Consensus
```

FIG. 6. Alignment of p135 (top line; residues 10 to 60) with putative zinc finger motifs. The lines below represent the homologous regions of protein 0, ORF61, and ICP0 (see text). The remaining 16 polypeptide sequences are the Z protein of lymphocytic choriomeningitis virus (49), PE38, CG30, and CG30-related IE proteins of baculoviruses (3, 34, 51), and nonviral polypeptides listed in reference 23 (RAD18, RING1, RAG-1, RFP, and RPT-1), reference 6 (L-rep, Su(z)2, Psc, bmi-1, and SS-ARo), and reference 31 (mel-18 and PML). Hyphens indicate gaps added to maintain alignment of one histidine and seven cysteine residues (shaded), dots mark large hydrophobic residues, and the asterisk marks basic, acidic, or amide residues.

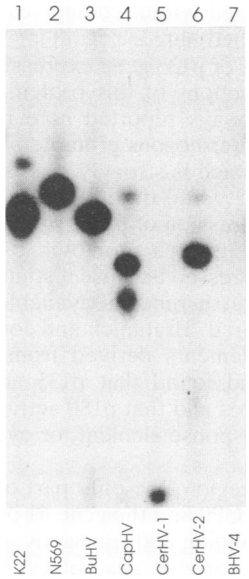

FIG. 7. Southern blot of various herpesvirus genomes cut with *Pst*I and probed with the *Sty*I-*Stu*I fragment (nt 175 to 654) encoding the first 110 amino acid residues of p135 including the zinc finger domain. Lanes: 1, BHV-1 K22; 2, BHV-1 N569; 3, BuHV-b6; 4, CapHV-1 E/CH; 5, CerHV-1; 6, CerHV-2; 7, BHV-4 Movar.

the zinc finger gave prominent cross-hybridization signals (Fig. 7). The *Sty*I-*Stu*I fragment hybridized with a specific fragment of BHV-1 K22 as expected, since the sequences of subtypes 1 and 2b are identical in that region (lane 1). With the more distantly related, neurovirulent BHV-1 strain N569 (subtype 3a) (43), and with a herpesvirus isolate obtained from buffalo (BuHV-b6) (4), hybridization was equally strong (lanes 2 and 3). Weaker hybridization signals were obtained with other alphaherpesviruses: a goat herpesvirus (CapHV-1 E/CH; lane 4) (17) and with two herpesvirus isolates from red deer (CerHV-1; lane 5) (47) and reindeer (CerHV-2; lane 6) (16). As a negative control, BHV-4 Movar (1), which belongs to the gammaherpesviruses, did not show any cross-hybridization (lane 7). Thus, in addition to VZV and HSV-1, a variety of animal alphaherpesviruses may be expected to encode the p135 zinc finger motif.

Transactivation by the zinc finger protein. To elucidate possible functions of p135, we constructed a subfragment of pJuC which contained the ER2.6 promoter, coding sequence, and polyadenylation signal but had all other genes removed or disrupted. A plasmid (pCE26X) containing this subfragment was cotransfected with various promoter-CAT constructs into Vero cells and tested for stimulation of CAT activity in transient expression assays (Fig. 8). An example is shown in Fig. 8A, in which different amounts of an indicator plasmid (pC29CAT) containing the IER4.2/2.9 promoter linked to CAT exhibited a basal activity of 0.15 to 0.45 relative to pSV2CAT used as a standard in each test series. Saturation was reached toward 2.1 pmol of indicator plasmid. Cotransfection of the effector plasmid pCE26X resulted in a dose-dependent increase of the basal promoter activity (up to 10-fold), with a peak at 2.1 pmol, larger amounts being inhibitory. Thus, p135 transactivates the promoter governing synthesis of its own RNA (IER2.9) and, coordinately, of the RNA for another IE gene product (IER4.2), as summarized in Fig. 9 together with the results described below.

Similar data sets were collected for other promoter-CAT

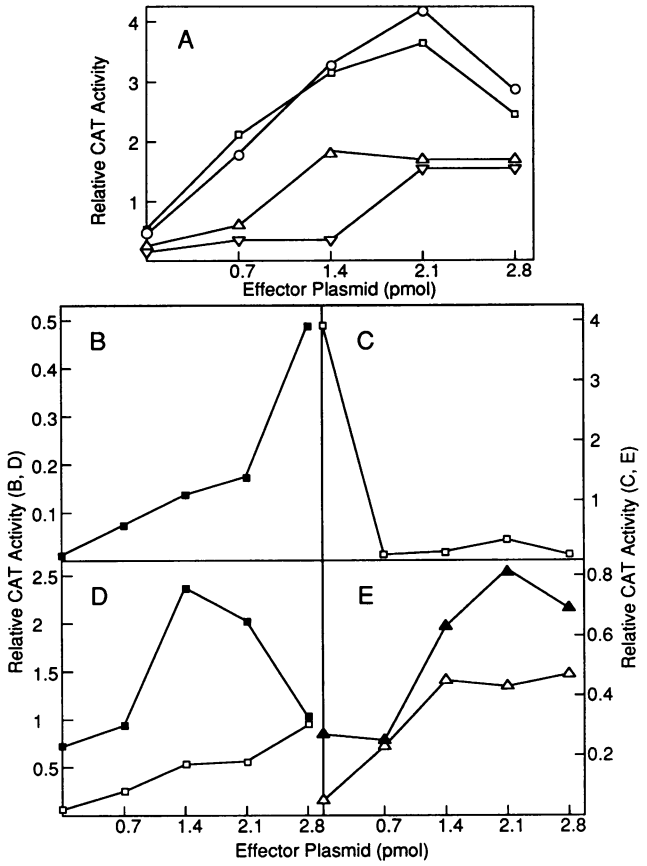

FIG. 8. Effect of p135 on various promoter-CAT constructs measured by transient expression assays. (A) 0.7 (▽), 1.4 (△), 2.1 (□), and 2.8 (○) pmol of pC29CAT indicator plasmid were cotransfected with various amounts of pCE26X effector plasmid as marked on the *x* axis. The *y* axis shows relative CAT activity (see Materials and Methods). (B to E) Cotransfection of various amounts of pCE26X effector plasmid as in panel A, using 2.1 (B to D) or 1.4 (E) pmol of pC26CAT (■) (B), pC17CAT (□) (C), pEC3 (■) or pCLR CAT (□) (D), and pDs-9 (▲) or pJC17 (△) (E) as indicator plasmids.

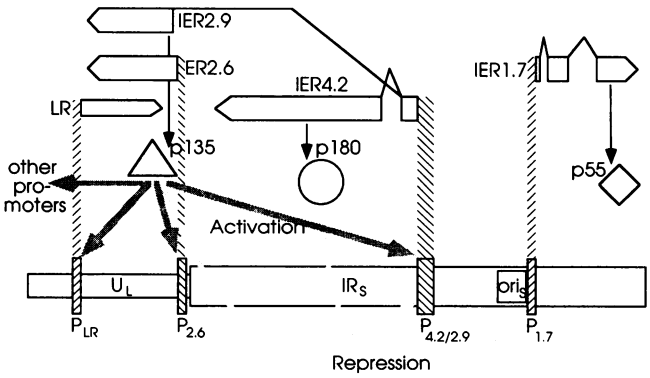

FIG. 9. Summary of transactivating (arrows from above) and repressing (arrows from below) functions of IE gene products. P, promoters (subscripts designate the RNAs transcribed) (see text).

constructs, from which only the curves obtained with optimal indicator plasmid amounts (1.4 or 2.1 pmol) are shown. The ER2.6 promoter (pC26CAT) exhibited rather low basal activity but, on the other hand, the strongest transactivation by p135 (50-fold) of any promoter tested (Fig. 8B). Since the effector plasmid pCE26X used in all of these tests contains the ER2.6 promoter, its own gene product p135 may be amplified by autostimulation. In contrast, p135 was found to repress IER1.7 promoter activity. The IER1.7/CAT construct (pC17CAT) exhibited the strongest basal activity of all indicator plasmids tested; this promoter activity was specifically inhibited by p135 (Fig. 8C) but not by control effector plasmids. The BHV-1 early promoters for glycoprotein I (pEC3) and for the LR transcripts (pCLRCAT) were stimulated 3- and 10-fold, respectively, by p135 (Fig. 8D). Furthermore, p135 was capable of stimulating heterologous promoters such as early (thymidine kinase; pJC17) and late (glycoprotein C; pDs-9) promoters of HSV-1 (Fig. 8E).

DISCUSSION

The BHV-1 IE gene described here has clear counterparts in the ICP0 gene of HSV-1 (46) and in the IE gene 61 of VZV (12). The genes have equivalent locations at one end (or both ends for HSV-1) of the large genome segment. The proteins which they encode are all involved in gene regulation and exhibit strong amino acid sequence homology in a cysteine-rich region of about 50 residues near the N terminus. Even the biphasic kinetics of gene expression that we have observed for BHV-1 appear to resemble, at first sight, the pattern described for the corresponding HSV-1 ICP0 gene, which exhibits a transitory decline in RNA levels at the end of the IE period followed by an increase toward 5 h p.i. (60). The mechanisms underlying these kinetics, however, are different.

Previous work has demonstrated synthesis of two distinct BHV-1 transcripts, IER4.2 and IER2.9, under the control of a single promoter due to alternative splicing, which is without precedent among alphaherpesviruses (57). As a consequence, synthesis of IER4.2 and IER2.9 must be tightly coupled, and autorepression of the IER4.2/2.9 promoter at the end of the IE period should affect both transcripts in the same way (Fig. 2C and 3). In contrast, the HSV-1 genes for ICP4 and ICP0 are governed by separate promoters. Although both promoters are subject to autorepression in vitro through specific binding sites for ICP4, which share partial sequence homology but are oriented differently (14), the ICP0 gene is turned off less efficiently in vivo than is the ICP4 gene (26), and protein levels of ICP0 increase steadily throughout infection (21). The present report shows that BHV-1 carries an alternative promoter of the early kinetic class, specifying ER2.6, which contains the entire exon 2 of IER2.9 and is predicted to express an identical IE protein. Thus, synthesis of p180, encoded by IER4.2, and of p135 would remain coupled only under IE conditions. Later during infection, p180 would be turned off while the alternative ER2.6 promoter would restore p135 expression. In PRV, a simplified version of this mechanism may exist, because the recently discovered gene 0 appears to be restricted to the early class even though the encoded zinc finger protein (10) is closely related to p135 of BHV-1. Alternative promoters governing transcripts which are 3′ coterminal and shortened at their 5′ ends have recently been identified in the IE region of equine herpesvirus 1 (27) and the U_L26 open reading frame of HSV-1 (37). However, these promoters are located within coding sequences, not within an intron as described here.

The maintenance of p135 gene expression suggests important regulatory functions of this protein. Indeed, the transient expression assays reported here reveal a variety of homologous and heterologous promoters that are stimulated by p135 and one which is strongly repressed (Fig. 9). At IE times, the IER4.2/2.9 and the IER1.7 promoters are active (57), leading to expression of p135, p55, and p180; the latter is able to turn off its own promoter (54). However, p135 continues to be expressed because it stimulates its own early promoter; therefore, it remains available to activate other promoters as required. Bratanich and Jones (5) tested additional promoter elements derived from a retroviral large terminal repeat and found that p135 activated promoters containing AP-1 sites and that p180 activated those containing AP-1, serum response element, or cyclic AMP response sites.

The zinc finger region, the only part of p135 with significant homology to ICP0 and the gene 61 product, is probably crucial for DNA binding and transactivation (18, 19). Functional requirement for zinc has not yet been demonstrated for any of these viral proteins but has been shown for transcription factor Sp1 (59). A large number of putative zinc finger proteins have been recognized, which can be grouped into several families depending on the arrangement of the cysteine and histidine residues and on functional properties such as binding to double-stranded DNA, to single-stranded nucleic acids, or between proteins (2). The viral members of this set, e.g., adenovirus E1A (39), simian virus 40 large T antigen (38), and retroviral nucleocapsid proteins, are dispersed among all of these different families. The herpesvirus IE proteins discussed here do not belong to any of the established families but rather belong to a newly recognized family of proteins sharing a novel zinc finger motif and a potential involvement with DNA (23). The other viral proteins belonging to the new family (3, 34, 49, 51) are from baculoviruses and lymphocytic choriomeningitis virus, which bear no obvious relation to herpesviruses. The recently reported zinc finger domain of the major IE protein of BHV-4 does not seem to fit into the family reported here, because it lacks the central cysteine-histidine pair (52). The absence of cross-hybridization (Fig. 7) is thus explained.

ICP0 is often termed a promiscuous transactivator (7), the gene 61 product seems to act mainly as a repressor (28), and p135 combines both properties. These differences probably reside in the nonhomologous parts of the molecules. For p135, the strongly acidic region (residues 280 to 330) might serve as a transcriptional activation domain (45), possibly regulated through phosphorylation by casein kinase II, for which there are several sites with consensus SXXE (42). An acidic region is also present in ICP0, but it is much shorter (residues 229 to 243), and its deletion does not affect function (20). Alternatively, the acidic region may constitute a PEST (proline, glutamate, serine, threonine) sequence implicated in short intracellular half-life (48). The C-terminal region of ICP0 has been shown to be functionally important (18). The corresponding region of p135 exhibits slight homology with ICP0 and may be important because it is perfectly conserved between two subtypes of BHV-1, as are the zinc finger and acidic domains. At present, such notions are based entirely on the deduced amino acid sequence of p135 and will require mutational analysis and physical characterization of the protein to be confirmed. As a first step, using Western immunoblots or immunoprecipitation with hyperimmune sera, we identified in infected cells a BHV-1-specific protein

(approximately 135 kDa; additional minor bands nearby), which appeared after a cycloheximide-actinomycin D block and could be labeled with [^{35}S]methionine or $^{32}P_i$ (56). Production of antipeptide antibodies and expression of the p135 gene in a baculovirus system are in progress.

LR transcripts of BHV-1 have been detected in rabbit trigeminal ganglia by in situ hybridization (35); their 5′ and 3′ ends map within nt 2327 to 2535 and 1393 to 1578, respectively (numbered as in Fig. 4). The TATA boxes (xvii and xvi) and the polyadenylation signal (viii) suggest a longer, primary LR transcript which would extend over a large part of the p135 coding sequence. This has, in fact, been observed for HSV-1 (15), with a significant difference: the zinc finger homology would be covered in HSV-1, but not in BHV-1, by the antisense LR transcript.

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