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Characterization of the Bovine Herpesvirus 4 Major Immediate-Early Transcript†

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The major immediate-early (IE) RNA of bovine herpesvirus 4 (BHV-4) has been identified and characterized by analyzing cytoplasmic polyadenylated RNA isolated from Madin-Darby bovine kidney cells infected with BHV-4(DN-599) in the presence of cycloheximide. Hybridization of cDNA to Southern blots of viral DNA, Northern (RNA) blot analysis, and S1 nuclease analyses showed that the major BHV-4 IE RNA 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. The major IE RNA contains three small exons at its 5′ end, spliced to a 1.3-kb 3′ exon. This RNA is present in much-reduced amounts when cells are infected in the absence of cycloheximide. However, late in infection, the major IE RNA gene region encodes abundant RNAs which differ in structure from the major IE RNA. Nucleotide sequence analysis of the gene encoding the major IE RNA revealed an open reading frame encoding 284 amino acids. A homology search of amino acid sequence data bases showed that a 141-amino-acid region near the amino terminus of the predicted amino acid sequence is similar to sequences near the amino terminus of herpes simplex virus type 1 IE110. This region of homology includes CXXC pairs, which could be involved in zinc finger structures. The region encoding this putative zinc finger domain is also found in RNAs transcribed from this IE region late in infection, but it is spliced to different sequences than those used in IE RNA. Thus, the major IE region of the BHV-4 genome could encode a family of proteins sharing a zinc finger domain.

Bovine herpesvirus 4 (BHV-4) has been isolated from cattle affected with a wide variety of disorders, including respiratory diseases, abortion, malignancies, and metritis, as well as from tissues of apparently healthy cattle (reviewed in references 3 and 53). Infection is widespread in Europe and the United States, with an incidence of 86% in a serological survey conducted in Idaho, Oregon, and Washington (20). Experiments designed to test whether BHV-4 is the causal agent of the diseases with which it is associated have yielded mixed results (reviewed in references 3 and 53).

Herpesviruses have large complex DNA genomes, and transcription is sequentially regulated during infection in vitro (27). Genes are divided into three classes, immediate early (IE), early (E), and late (L), on the basis of their time of expression (24). This temporal regulation of herpesvirus gene transcription is accomplished through transactivation of viral transcriptional promoters by products of specific viral genes. IE proteins are among the transactivators. Expression of IE genes requires no prior synthesis of viral proteins; IE genes are the only viral genes transcribed when cells are infected in the presence of protein synthesis inhibitors such as cycloheximide. Under these conditions, only a few genes are expressed. Proteins encoded by the IE genes are required for expression of E and L genes. L genes are expressed after the onset of viral DNA replication and are not expressed when cells are infected in the presence of specific inhibitors of viral DNA polymerase (22).

Regulation of herpesvirus gene expression has been most thoroughly examined for human herpes simplex virus type 1 (HSV-1). HSV-1 produces five IE RNAs, each encoding an IE protein (reviewed in reference 56). Each of the five HSV-1 IE proteins (IE175, IE110, IE68, IE63, and IE12) has a role in regulation of E and L gene expression. IE175 stimulates transcription from many E and L promoters tested, but in most cases, both IE175 and IE110 are necessary for maximal stimulation (6, 16, 17, 34, 38, 39, 43, 45). IE12 may augment the stimulatory effects of IE175 and IE110 on some E promoters (38). IE63 is required in addition to IE175 and IE110 for stimulation of at least some E and L genes (17, 46, 49, 52, 57). However, IE63 can also inhibit activation by IE175 and IE110 of certain other E promoters (49, 52). A role for IE68 in activation of L genes is suggested by the observation that a deletion in the IE68 gene leads to a deficiency in L protein synthesis (48). As expected for transcriptional regulators, HSV-1 IE proteins are nuclear and phosphorylated (42).

The BHV-4 genome contains approximately 144 kb. Both ends of the genome contain multiple copies of a 2- to 3-kb repeating unit flanking a 110-kb unique portion (15). The cloning and generation of a restriction map of the entire BHV-4 genome was recently reported (8). The recent generation of a large set of monoclonal antibodies against BHV-4-infected cells will facilitate characterization of viral proteins (14). No work on regulation of BHV-4 gene expression has been reported.

This article reports the characterization of the major IE RNA in BHV-4(DN-599)-infected Madin-Darby bovine kidney (MDBK) cells. The purpose of this work was to identify BHV-4 IE genes so that their function in regulation of transcription can be studied. We identified two IE RNAs in cells infected in the presence of cycloheximide. The major IE RNA is a spliced 1.8-kb transcript which is transcribed from left to right on the restriction map of the BHV-4 genome. Nucleotide sequence analysis of the DNA encoding this RNA revealed that it could encode a 33-kDa protein. The putative protein contains a cysteine-rich region which could form zinc fingers characteristic of some DNA-binding proteins.

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MATERIALS AND METHODS

Cell culture and virus propagation. MDBK cells and BHV-4(DN-599 isolate) were obtained from David Stringfellow, Department of Pathobiology, Auburn University. Cells were cultured in Dulbecco's modified Eagle medium containing penicillin, streptomycin, and 10% defined, supplemented bovine serum. Virus was propagated by infecting confluent monolayers with 1 50% tissue culture infective dose ($TCID_{50}$) per five cells. After 4.5 days, cultures were frozen and thawed three times to release virus. After removal of cell debris by low-speed centrifugation, the $TCID_{50}$ was determined 10 to 14 days after infection of MDBK cells by limiting dilution. Virus stock thus obtained was stored at −80°C.

Preparation of viral DNA used for Southern blotting. Confluent monolayers of MDBK cells were infected with 0.5 to 1 $TCID_{50}$ of BHV-4 per cell. After most of the cells had detached from the flasks (4 to 5 days), cell-associated virions were liberated by freezing (−80°C) and thawing the flasks three times. Cell debris was removed by low-speed centrifugation, and virions were pelleted by centrifugation in a Beckman SW28 rotor at 25,000 rpm for 1 h at 4°C. The pellet was resuspended in cold 0.1% Nonidet P-40 in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), and virions were pelleted through a 3-ml cushion of 30% sucrose in TE buffer in a Beckman SW41 rotor at 35,000 rpm for 90 min at 4°C. The pellet was resuspended in TE. $MgCl_2$ was added to a final concentration of 10 mM, and DNase I was added to a final concentration of 13 μg/ml; the suspension was incubated at 37°C for 1 to 2 h to destroy contaminating host cell DNA. If DNase treatment was omitted, DNA preparations contained >90% host cell DNA. To inactivate DNase and liberate viral DNA, sodium dodecyl sulfate (SDS) was added to 2%, EDTA to 40 mM, and proteinase K to 300 μg/ml, and the mixture was incubated at 55°C for at least 3 h. After this point, standard precautions were taken to avoid mechanical shearing of viral DNA. After addition of sodium acetate to a final concentration 0.3 M, DNA was purified by extraction with phenol-chloroform (1:1) and with chloroform, then precipitated with ethanol and resuspended in TE buffer. Viral DNA was separated from remaining host cell DNA fragments by pelleting viral DNA through a 4.5-ml cushion of 1 M NaCl in a Quick-Seal bell-top tube (14 by 18 mm) (Beckman Instruments) in a Beckman SW41 rotor using floating spacers at 35,000 rpm for 2.5 h at 20°C. The translucent viral DNA pellet was resuspended in TE buffer, sodium acetate was added to 0.3 M, and the DNA was precipitated with ethanol. Viral DNA was resuspended in TE buffer and stored at 4°C. The yield was 1 to 2 μg of viral DNA per 75-cm^2 flask of infected cells.

Preparation of IE RNA. Confluent monolayers of MDBK cells in 150-mm-diameter plates were infected with 2 to 3 $TCID_{50}$ per cell in medium containing 100 μg of cycloheximide per ml. After 6 to 8 h, cells were washed with ice-cold Dulbecco's balanced salt solution without calcium and magnesium (DBSS) and scraped from the plates into ice-cold DBSS with a rubber policeman. Cells were pelleted at 3,500 rpm in a Beckman JS13.1 rotor at 4°C and lysed by resuspending them in 6 ml of ice-cold DBSS containing 1% Nonidet P-40 and 10 mM vanadyl ribonucleoside complexes (5) for each 10 plates of cells and incubating them on ice for 10 min. Nuclei were removed by centrifugation at 3,500 rpm in a Beckman JS13.1 rotor at 4°C. An equal volume of 7 M urea–2% SDS–0.35 M NaCl–0.01 M Tris-HCl (pH 7.4)–10 mM vanadyl ribonucleoside complexes was added to the cytoplasmic fraction, and nucleic acid was purified by extraction with phenol and chloroform, followed by extraction with chloroform. For each 1 ml of solution, 0.4 g of solid CsCl was added, and RNA was pelleted through 1.5-ml cushions of 5.7 M CsCl in conical ultracentrifuge tubes (14 by 89 mm) in a Beckman SW41 rotor with conical adaptors at 30,000 rpm for 14 to 18 h at 20°C. The RNA pellet was resuspended in 20 mM Tris-HCl (pH 7.4)–0.2% SDS and heated at 68°C for 10 min. NaCl was added to 0.4 M; polyadenylated RNA was bound to oligo(dT) cellulose type 3 (Collaborative Research, Bedford, Mass.) and eluted with 20 mM Tris-HCl (pH 7.4)–0.2% SDS. RNA used in the experiments shown in Fig. 1 was bound to and eluted from oligo(dT) cellulose a second time. *n*-Lauroylsarcosine was added to 0.4% to prevent precipitation of SDS, sodium acetate was added to 0.3 M, and RNA was precipitated with ethanol and resuspended in diethyl pyrocarbonate-treated H_2O at a concentration of 0.5 to 1 mg/ml and stored at −80°C.

RNA harvested at 8, 12, and 24 h postinoculation (p.i.) used in the experiments shown in Fig. 8 and 9 was prepared the same way, except cells were infected with 1 $TCID_{50}$ per cell, and cycloheximide was omitted.

Synthesis and hybridization of cDNA. cDNA was synthesized in 100-μl volume containing 3 μg of polyadenylated RNA; 100 μCi [α-^{32}P]dCTP (3,000 Ci/mmol); 200 μM (each) dATP, dGTP, and dTTP; 20 mM dithiothreitol; 50 mM Tris-HCl (pH 8.3); 3 mM $MgCl_2$; 75 mM KCl; 40 μg of random hexanucleotide primers; 60 U of placental RNase inhibitor; and 600 U of Moloney murine leukemia virus reverse transcriptase for 2 h at 37°C. EDTA was added to 20 mM, and RNA was destroyed by adding NaOH to 0.6 M and incubating for 30 min at 65°C. cDNA was recovered by centrifugation through Sephadex G-50 in TE buffer. cDNA was hybridized to viral DNA restriction fragments bound to nitrocellulose in buffer containing 10× Denhardt's reagent (47), 100 μg of denatured herring sperm DNA per ml, 1 mM EDTA, 0.5% SDS, 6× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate), 20 mM sodium phosphate (pH 7) at 68°C for 20 h. Filters were washed at 68°C in dilutions of SSC down to 0.25× SSC.

Northern (RNA) blot analysis. Polyadenylated RNA (1 to 2 μg) was denatured at 60°C for 5 min in 50% deionized formamide–2.2 M formaldehyde–1× MOPS electrophoresis buffer (20 mM 3-[*N*-morpholino]propanesulfonic acid [pH 7.0], 5 mM sodium acetate, 1 mM EDTA) and electrophoresed in a 0.8% agarose gel in 1× MOPS electrophoresis buffer–2.2 M formaldehyde. Lanes containing marker RNAs were cut off and stained with acridine orange. For the experiment shown in Fig. 1, the remaining lanes were treated with 50 mM NaOH–150 mM NaCl, then with 1 M Tris-HCl (pH 7.0)–3 M NaCl, and transferred to nitrocellulose in 20× SSC by vacuum blotting. For the experiment shown in Fig. 8, the remaining lanes were transferred to nitrocellulose in 20× SSC by capillary blotting without prior treatment. The DNA probe was labeled using a random primers DNA labeling kit (GIBCO-Bethesda Research Laboratories, Grand Island, N.Y.). Probe was hybridized to the transferred RNA in buffer containing 50% deionized formamide, 5× SSC, 20 mM sodium phosphate (pH 7.0), 0.5% SDS, 4× Denhardt's reagent, 100 μg of denatured herring sperm DNA per ml at 42°C for 20 h. Filters were washed at 68°C in dilutions of SSC down to 0.25× SSC.

S1 nuclease analysis. Single-stranded, uniformly labeled DNA probes were synthesized by using DNA cloned into M13 vectors as template. Approximately 2 μg of template DNA was annealed to 5 pmol of universal primer, and DNA

was synthesized in 30 μl containing annealed primer and template, 50 mM NaCl, 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 7 mM dithiothreitol, 50 μCi [α-^{32}P]dCTP (3,000 Ci/mmol), 10 μM unlabeled dCTP, 0.7 mM (each) dATP, dGTP, and dTTP, and 2 to 3 U of Klenow fragment of *Escherichia coli* polymerase I at room temperature for 2 to 3 h. After inactivation of the polymerase at 68°C for 10 min, double-stranded DNA was cleaved with a restriction enzyme cleaving at the 3′ end of the probe fragment and the single-stranded probe fragment was separated from the larger template strand by alkaline low-melting-point agarose gel electrophoresis. S1 nuclease analysis was as previously described (47); hybridization with single-stranded probes was at 30°C and that with double-stranded probes was at 48°C, except in the experiment shown in Fig. 7 (see figure legend). S1 nuclease digestion was at room temperature for 45 min, using 1,000 U of S1 nuclease per ml. Protected fragments were electrophoresed on polyacrylamide gels containing 7 M urea.

Nucleotide sequence accession number. The nucleotide sequence data reported in this article have been submitted to GenBank and assigned the accession number M60043.

RESULTS

Identification of BHV-4 DNA encoding IE RNA. Viral IE genes are experimentally defined as those which are transcribed when cells are infected in the presence of protein synthesis inhibitors, because IE gene expression does not require de novo viral protein synthesis. Under these conditions, RNA transcribed from IE genes usually accumulates to higher levels than in the absence of inhibitors, presumably because of the lack of feedback inhibition. Therefore, BHV-4 IE RNA was characterized by using cytoplasmic polyadenylated RNA prepared 6 to 8 h p.i. from MDBK cells infected with BHV-4 in the presence of 100 μg of cycloheximide per ml. To rid the RNA of viral DNA from virions present in the cytoplasm or on the cytoplasmic membrane, RNA was pelleted through a CsCl cushion prior to analysis. To identify BHV-4 DNA encoding IE RNA, ^{32}P-labeled cDNA to IE RNA was synthesized by using random hexanucleotide primers and hybridized to Southern blots of BHV-4 DNA isolated from virions and cleaved with *Hin*dIII. Results are shown in Fig. 1A. As a negative control, cDNA was synthesized to uninfected cell cytoplasmic polyadenylated RNA and hybridized. cDNA to cytoplasmic polyadenylated RNA harvested at 24 h p.i. in the absence of cycloheximide also was synthesized and hybridized to identify all viral DNA fragments. All viral DNA fragments except Ahet and Bhet were clearly visible upon longer exposure of the autoradiograph shown in Fig. 1A (lane 24 hr.). cDNA to IE RNA specifically identified the band containing two 8.3-kb viral *Hin*dIII fragments, E and F (Fig. 1A, lane IE). There was also a faint signal indicating hybridization of IE cDNA to viral *Hin*dIII fragment G. However, no IE RNA transcribed from *Hin*dIII fragment G could be detected by Northern blot analysis, and the possibility of IE transcription from *Hin*dIII fragment G was not pursued further. Additional experiments (not shown) using cloned viral fragments showed that cDNA to IE RNA hybridizes to both fragments E and F, but it hybridizes 2 to 14 times more to fragment E than to fragment F. The positions of these two fragments on a restriction map of the BHV-4 genome are shown in Fig. 2A. In addition, hybridization of radiolabeled cDNA to cloned viral restriction fragments (not shown) showed that IE RNAs are transcribed from a 2.2-kb *Sma*I-*Bgl*II fragment within *Hin*dIII E and from a 2.6-kb *Eco*RI-*Pst*I fragment within *Hin*dIII F (Fig. 2B). Characterization of the major IE RNA (IE RNA 1 in Fig. 2C) and the DNA which encodes it is the subject of this report.

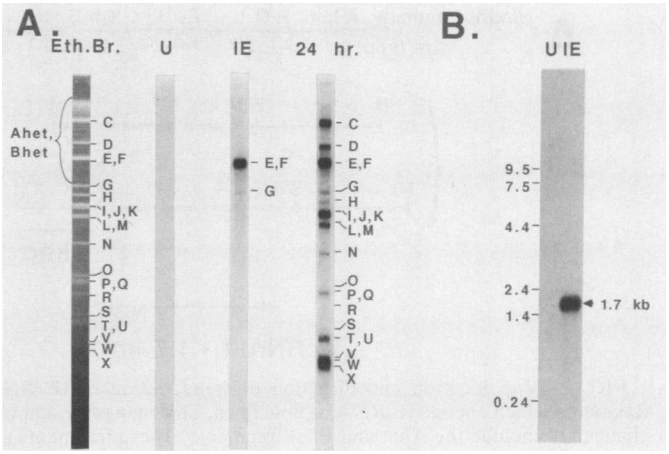


FIG. 1. (A) Identification of BHV-4 DNA encoding IE RNA. ^{32}P-labeled cDNA to the indicated RNA was synthesized by using random hexanucleotide primers and was hybridized to Southern blots of BHV-4 DNA isolated from virions and cleaved with *Hin*dIII. Shown are a photograph of the ethidium bromide-stained gel from which the Southern blot was made (Eth. Br.) and autoradiographs of the blot hybridized to cDNA prepared from uninfected cell RNA (U), IE RNA (IE), and RNA harvested at 24 h p.i. in the absence of cycloheximide (24 hr.). Viral *Hin*dIII fragments are identified by letters beside each lane. (B) Northern blot analysis of *Hin*dIII E-encoded IE RNA. Cloned viral *Hin*dIII fragment E was labeled with ^{32}P by random hexanucleotide-primed synthesis and was hybridized to Northern blots 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-Bethesda Research Laboratories) are indicated on the left. The arrowhead on the right indicates the 1.7-kb IE RNA identified by the probe.

Structure of major IE RNA. Hybridization of ^{32}P-labeled cloned *Hin*dIII E to Northern blots of IE RNA showed that *Hin*dIII E encodes a 1.7-kb IE RNA (Fig. 1B). To determine the structure of this major IE RNA, S1 nuclease analysis was undertaken. First, S1 nuclease analysis was performed by using probes representing each strand of the 2.2-kb *Sma*I-*Bgl*II fragment shown in Fig. 2B. This fragment was cloned into the single-stranded phage vectors M13mp18 and M13mp19 and used as template for synthesis of uniformly labeled, single-stranded DNA probes. Portions of one probe were protected from S1 nuclease digestion by IE RNA (data not shown), indicating that IE RNA 1 is transcribed in the direction shown in Fig. 2C. Further experiments with single-stranded probes used only the strand complementary to IE RNA. In S1 nuclease analysis using the entire 2.2-kb *Sma*I-*Bgl*II fragment as a single-stranded probe, IE RNA specifically protected fragments of approximately 1,300, 186, 151, and 63 nucleotides (nt) (Fig. 3A and B, probe E). To determine roughly where these protected fragments map within the 2.2-kb fragment, additional single-stranded probes were synthesized and used for S1 nuclease analysis. These probes were derived from the 5′ and 3′ portions of the fragment (5′ and 3′ as defined by the RNA), divided by a *Bst*EII restriction site (Fig. 3C). IE RNA protected 225-, 186-, 151-, and 63-nt fragments of the 5′ probe (Fig. 3A, 5′ probe). The 186-, 151-, and 63-nt fragments appear identical to protected fragments of the entire *Sma*I-*Bgl*II fragment

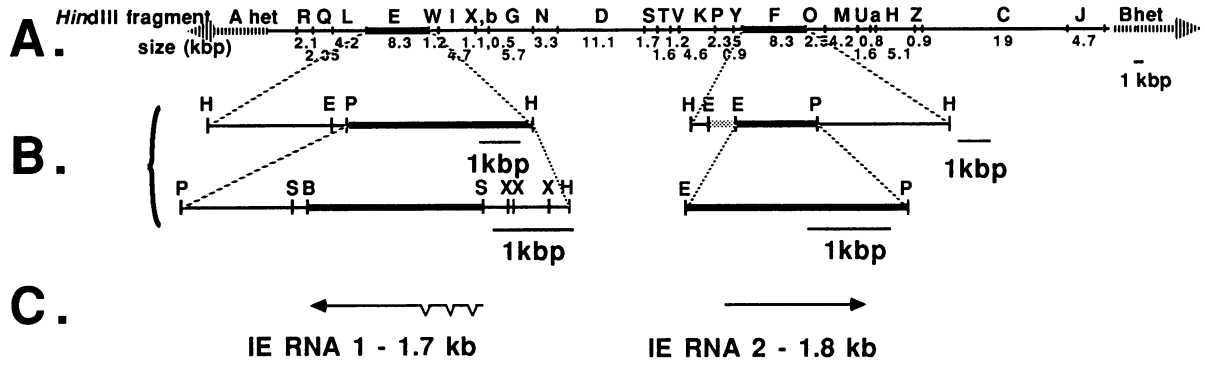


FIG. 2. Map location and direction of transcription of IE RNA. (A) *Hin*dIII restriction map of BHV-4(DN-599) genome, showing fragments which encode IE RNA as bold lines. This map is an adaptation of the one published by Bublot et al. (8). Fragment names have been changed to include the Ahet and Bhet fragments. These fragments are heterogeneous in size because of variable numbers of 2.5-kb terminal repeats (shown as striped arrows). (B) Restriction maps of fragments encoding IE RNA. Portions to which cDNA synthesized to IE RNA hybridized (data not shown) are indicated as bold lines. The stippled line indicates a small amount of hybridization. Abbreviations: H, *Hin*dIII; E, *Eco*RI; P, *Pst*I; S, *Sma*I; B, *Bgl*II; X, *Xba*I. (C) Structure and direction of transcription of IE RNA 1 and IE RNA 2. The map positions of the RNAs are shown relative to the restriction maps shown in the bottom line of panel B. The structure, direction of transcription, and map position of IE RNA 1 (the major IE RNA) were determined by the experiments shown in Fig. 3 through 7. Preliminary S1 nuclease analyses of IE RNA 2 (not shown) have shown its direction of transcription and approximate map location.

probe; the 225-nt protected fragment is novel to the 5′ probe. IE RNA protected a single 1.1-kb fragment of the 3′ probe (Fig. 3B, 3′ probe). This is smaller than the 1.3-kb protected fragment produced when the entire *Sma*I-*Bgl*II fragment probe was used. If all protected fragments are protected by a single RNA species, these results suggest that the RNA consists of three small exons (186, 151, and 63 nt) in the 5′ portion of the molecule and a 1.3-kb exon at the 3′ end. IE RNA protects 225- and 1,100-nt fragments of probes consisting of sequences on the 5′ and 3′ side of the *Bst*EII site, respectively, suggesting that the 1.3-kb 3′ exon begins approximately 225 nt 5′ to the *Bst*EII site. Less of each protected fragment was produced when RNA from cells infected in the absence of cycloheximide was used (Fig. 3A and B), indicating that the major IE RNA may be subject to negative regulation by viral proteins during the normal course of infection.

To determine whether the four fragments protected from S1 nuclease digestion by IE RNA were protected by a single RNA molecule, two-dimensional S1 nuclease analysis was performed with the 5′ probe illustrated in Fig. 3C. In the first dimension, electrophoresis of S1 nuclease digestion products was in a nondenaturing agarose gel without prior disruption of RNA-probe hybrids. After destruction of the RNA by soaking the gel in alkaline electrophoresis buffer, the protected probe fragments were electrophoresed in the second dimension under alkaline conditions. In the autoradiograph shown in Fig. 4, dots falling on the vertical line below the diagonal indicate probe fragments protected by a single IE RNA species. The source of the dot above the diagonal is unknown, but since it represents a molecule which migrates more slowly in the alkaline dimension than the neutral dimension, it cannot represent DNA protected from nuclease digestion by RNA. The autoradiograph was overexposed to make the smallest protected fragment visible. However, even on shorter exposures, the 225-, 186-, and 151-base protected fragments identified in Fig. 3A were not visible as discrete dots. Nevertheless, all the protected fragments appear to fall on a single vertical line, showing that all probe fragments protected from S1 nuclease digestion by IE RNA, including the small, approximately 63-nt fragment, are protected by a single RNA species.

To confirm that the 5′ portion of BHV-4 major IE RNA is spliced and to determine whether all the 5′ exons had been identified by the S1 nuclease analyses illustrated in Fig. 3, the primer extension analysis shown in Fig. 5 was performed. At the same time, an S1 nuclease analysis was performed with a double-stranded 5′-end-labeled probe labeled at the same site as the 5′-end-labeled primer. Primer and probe are illustrated in Fig. 5B. Primer extension yielded a 622-nt product, while the fragment protected from S1 nuclease digestion was 225 nt (Fig. 3). This discrepancy in size indicates that the 5′ portion of the major IE RNA is spliced. The size of the primer extension product (622 nt) is in good agreement with the sum of the protected fragments identified by using the 5′ probe in Fig. 3 (225 + 186 + 151 + 63 = 625 nt), indicating that all the 5′ exons of this RNA have been identified.

The map positions of the 5′ exons of the major IE RNA were determined by the S1 nuclease analyses shown in Fig. 6. The probe was the double-stranded *Sma*I-*Dra*I fragment shown in Fig. 6B, uniformly labeled on one strand. The restriction sites indicated in Fig. 6B were determined by the nucleotide sequence (see Fig. 10). Aliquots of the probe were cleaved with one of five restriction enzymes prior to hybridization or were used uncleaved. The results are shown in Fig. 6A and interpreted in Fig. 6B. The 129-nt protected fragment was present regardless of whether the probe was cleaved. This fragment is protected by a portion of the fourth exon, the 5′ end of which had already been located 225 nt 5′ to the *Bst*EII site by the S1 nuclease analysis shown in Fig. 5. The 186-nt protected fragment was not produced when the probe was cleaved with *Ava*I. Instead, 132- and 54-nt protected fragments appeared. Therefore, one end of the 186-nt exon is 69 nt from an *Ava*I restriction site and the other end is 83 nt from the restriction site. Indeed, 69 nt 5′ to the second *Ava*I restriction site is a 5′ splice site consensus sequence, and 83 nt 3′ to this *Ava*I restriction site is a 3′ splice site consensus sequence (see Fig. 10). This indicates placement of the 186-nt exon as shown in Fig. 6B. Cleavage of the probe with *Tha*I resulted in absence of the 151-nt protected fragment and appearance of 83- and 69-nt protected fragments. This observation and location of splice site consensus sequences indicate the location of the 151-nt exon

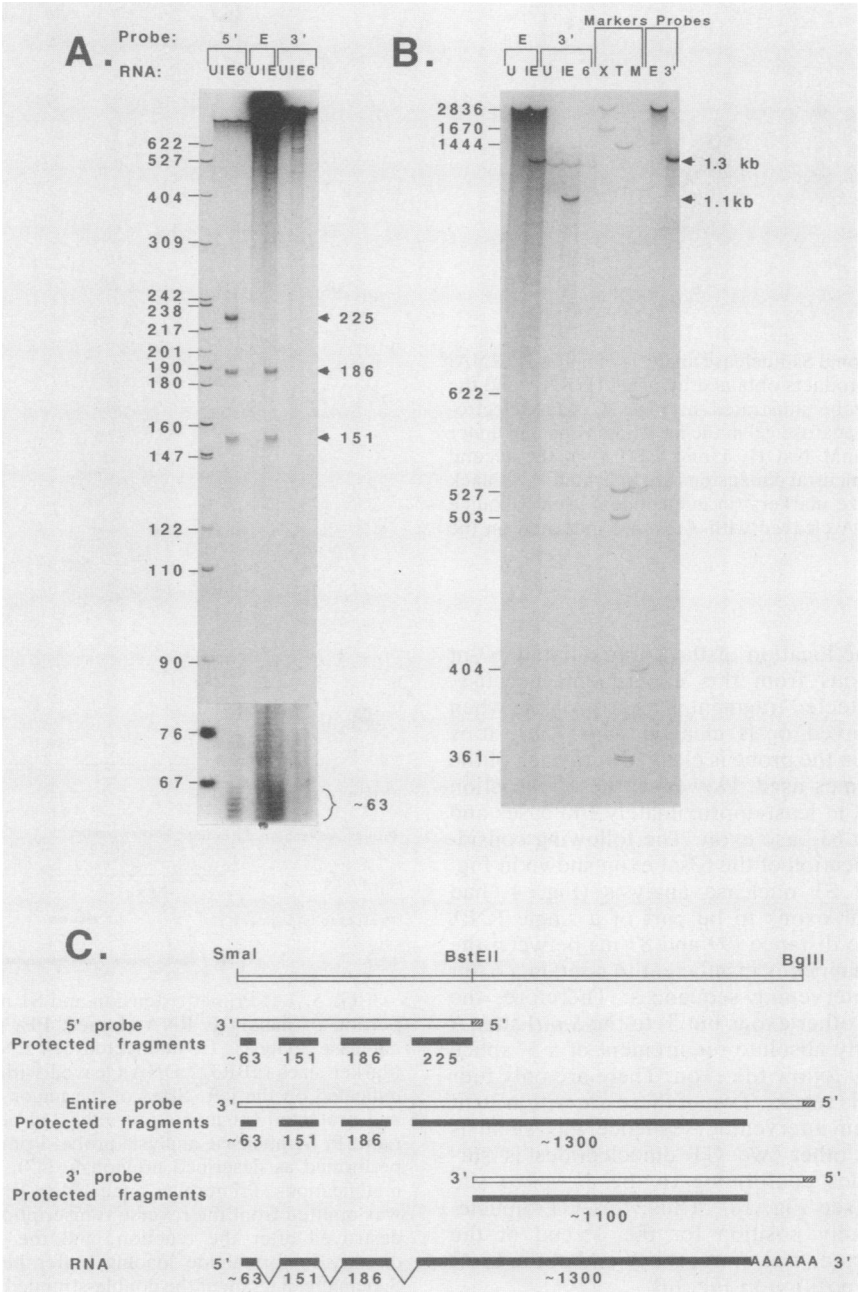


FIG. 3. S1 nuclease mapping of major IE RNA. Uniformly labeled single-stranded probes shown in panel C were synthesized by using the 2.2-kb *Sma*I-*Bgl*II fragment of *Hin*dIII E shown in Fig. 2B and subfragments cloned into the single-stranded phage vectors M13mp18 and M13mp19 as templates. (A and B) Autoradiographs of denaturing polyacrylamide gels of fragments protected from S1 nuclease digestion. (A) 6% gel. (B) 4% gel to show large fragments. Three different probes were used, representing the 5′ portion of, 3′ portion of, and the entire (E) gene. Lanes: U, uninfected cell RNA control; IE, IE RNA; 6, RNA harvested at 6 h p.i. in the absence of cycloheximide; X, pTZ19U DNA cleaved with *Xba*I and with *Xba*I + *Pvu*I; T, pTZ19U DNA cleaved with *Taq*I; M, pBR322 DNA cleaved with *Msp*I. Marker sizes are noted in nucleotides at the left of each panel. Sizes (in nucleotides for panel A, in kilobases for panel B) of fragments specifically protected from S1 nuclease digestion by IE RNA are shown next to arrowheads and the bracket at the right of each panel. The lower portion of panel A is overexposed to make the ~63-nt protected fragments clearly visible. These small fragments were observed reproducibly, even in 2-dimensional S1 nuclease analysis (Fig. 4) and are not artifacts of incomplete S1 digestion. (C) Schematic diagram of probes used, protected fragments, and structure of major IE RNA. The probes are illustrated in the opposite orientation to that shown in the map of the entire BHV-4 genome in Fig. 2 so that the RNA could be drawn left to right in the standard 5′→3′ orientation. The hatched portion at the 5′ end of each probe represents M13 vector sequences. The sizes of each protected fragment are shown in nucleotides. The positions of the 3 small exons in the 5′ portion of the RNA were determined in the experiment shown in Fig. 6.

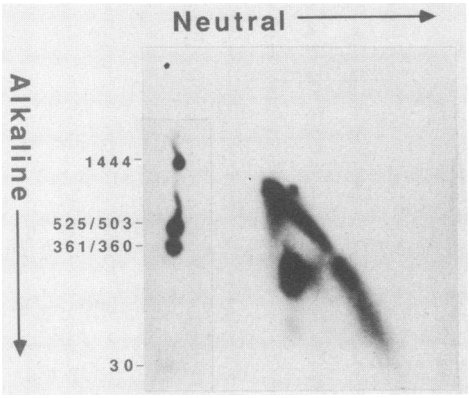


FIG. 4. Two-dimensional S1 nuclease analysis of major IE RNA. S1 nuclease protection products obtained by using IE RNA and the uniformly labeled 5′ probe illustrated in Fig. 3C were electrophoresed in a neutral 2% agarose gel in the first dimension and under alkaline conditions (50 mM NaOH, 1 mM EDTA) in the second dimension. Alkaline and neutral dimensions are indicated. The black dot marks the origin. Size markers (in nucleotides) in the alkaline dimension (pTZ19U DNA cleaved with *Taq*I) are indicated on the left.

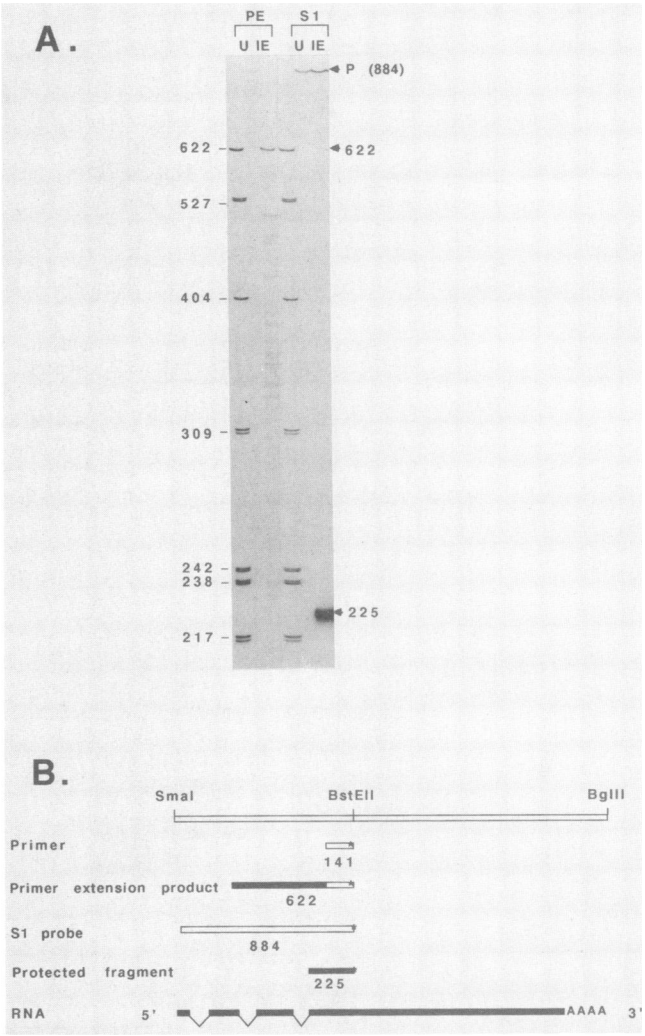


FIG. 5. (A) Primer extension and S1 nuclease analysis of the 5′ portion of major IE RNA. Lanes: PE, primer extension, S1, S1 nuclease analysis; U, uninfected cell RNA control; IE, IE RNA. Marker sizes (pBR322 DNA cleaved with *Msp*I) in nucleotides are indicated on the left. Sizes of the major primer extension product and protected fragments are indicated next to arrowheads on the right. P, S1 nuclease analysis probe. Primer extension analysis was performed as described previously (47), with the following minor modifications. Primer was annealed to RNA at 48°C, dactinomycin was omitted from the reverse transcription reaction, RNA was not destroyed after the reaction, and the products were dissolved directly in formamide loading buffer before electrophoresis. (B) Schematic diagram of the double-stranded 5′-end-labeled primer and S1 nuclease probe, primer extension products, and S1 nuclease protection products. 5′-end label is indicated by an asterisk. The map positions of the 5′ exons in the RNA shown at the bottom were determined by the experiment shown in Fig. 6.

shown in Fig. 6B. The location of the approximately 63-nt exon is not as obvious from this experiment, because, although the 63-nt protected fragment is clearly visible when the probe is not cleaved or is cleaved with *Tha*I, it is apparently absent when the probe is cleaved with each of the other restriction enzymes used. However, these restriction sites cover a region of at least approximately 140 bases and cannot occur within a 63-base exon. The following considerations suggest the location of the 63-nt exon shown in Fig. 6B. Two-dimensional S1 nuclease analysis (Fig. 4) had already shown all four exons to be part of a single RNA species. However, the distance (99 and 87 nt) between the other exons already mapped is insufficient to contain a 63-nt exon plus flanking intervening sequences. Therefore, the 63-nt exon is 5′ to the other exons but 3′ to the *Sma*I site. A GT dinucleotide (nearly absolute requirement of a 5′ splice site) must immediately follow the exon. There are only four GT dinucleotides in this region. Two of these are within 30 nt of the 151-nt exon; an intervening sequence this small is unlikely. One of the other two GT dinucleotides is surrounded by nucleotides conforming to the 5′ splice site consensus sequence (see Fig. 10). Thus, this GT dinucleotide is the most likely position for the 3′ end of the approximately 63-nt exon. Therefore, the 3′ end of the 63-nt exon is shown at this position in Fig. 6B.

The 3′ end of the 1.3-kb exon was mapped by S1 nuclease analysis using the double-stranded 3′-end-labeled probe illustrated in Fig. 7B. As shown in Fig. 7A, IE RNA specifically protected a 248-nt fragment. This analysis locates a single polyadenylation site of the major IE RNA approximately 31 and 18 nt 3′ to two closely spaced AAUAAA consensus polyadenylation signals (see Fig. 10).

Transcription from BHV-4 IE-1 gene late in infection. Results shown in Fig. 3 indicated that very little of the major IE RNA is present 6 h p.i. in the absence of cycloheximide. To determine whether the BHV-4 IE-1 gene region is transcribed later in infection, we performed Northern blot and S1 nuclease analyses of cytoplasmic polyadenylated RNA prepared 8, 12, and 24 h p.i. in the absence of cycloheximide. This RNA represents late transcription because viral DNA synthesis begins about 6 to 8 h p.i. (55). The 2.2-kb *Sma*I-*Bgl*II fragment (see Fig. 3C) probe hybridized to multiple species in RNA prepared at these late times. The largest amount of these RNAs was detected at 24 h p.i. Northern blot analysis using RNA prepared 24 h p.i. is shown in Fig. 8. These RNAs belong to the late kinetic class because they were either not detected or greatly reduced in amount in RNA prepared from cells infected in the presence of phos-

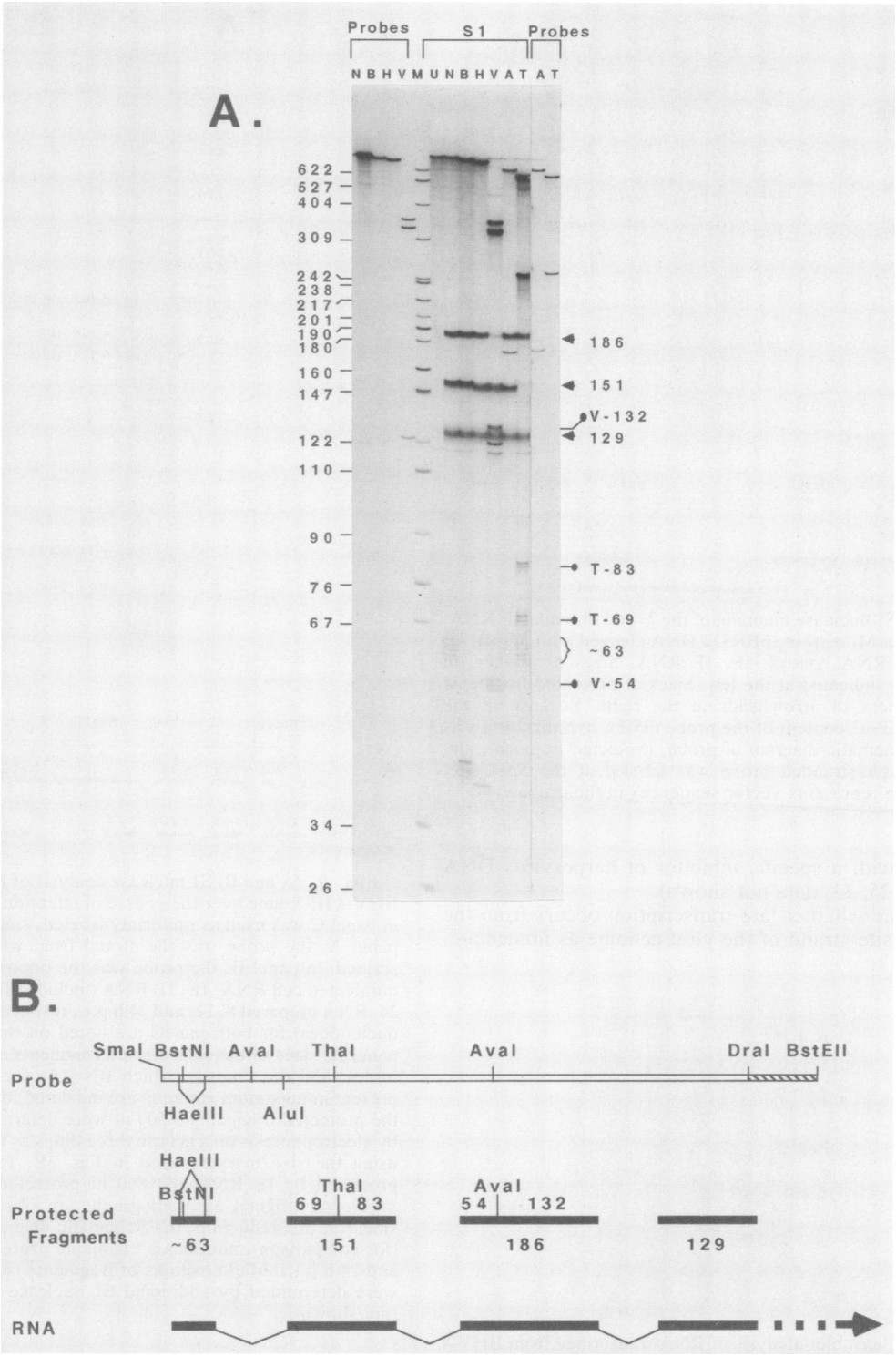


FIG. 6. S1 nuclease mapping of the 5′ exons of major IE RNA. (A) The double-stranded *Sma*I-*Dra*I fragment shown in panel B, uniformly labeled on one strand, was used as probe. Prior to hybridization, the probe was cleaved with a restriction enzyme as indicated over the lanes. Lanes: N, none; B, *Bst*NI; H, *Hae*III; V, *Ava*I; A, *Alu*I; T, *Tha*I. In the lanes labeled Probes, an aliquot of each cleaved probe was electrophoresed to confirm that cleavage had occurred and to show the sizes of the resulting fragments. In the lanes labeled S1 are the fragments of each probe protected during S1 nuclease analysis. Lane U, uninfected cell RNA control, uncleaved probe. Positions and sizes (in nucleotides) of marker (pBR322 DNA cleaved with *Msp*I) are indicated on the left. On the right, sizes (in nucleotides) of uncleaved probe fragments protected by IE RNA are shown next to arrowheads and the bracket. Sizes of cleaved probe fragments protected are shown next to black circles, and the letter indicates the restriction enzyme (V, *Ava*I; T, *Tha*I). (B) Diagram of probe, showing restriction sites, and interpretation of results (see text). The hatched *Dra*I-*Bst*EII portion is shown to enable comparison to the probes shown in Fig. 3 and 5 but was not included in this probe.

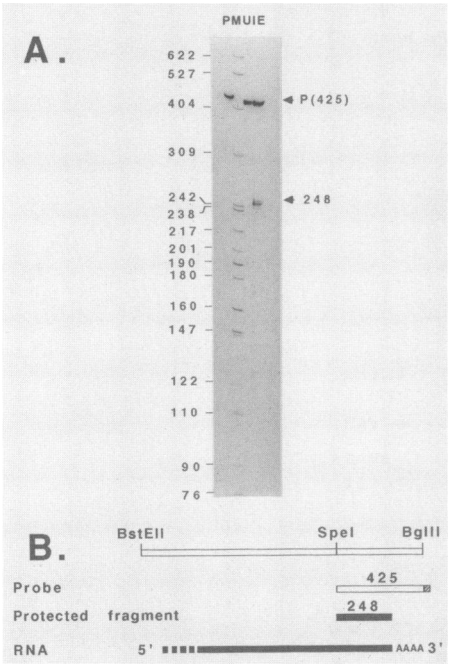


FIG. 7. (A) S1 nuclease mapping of the 3′ end of major IE RNA. Lanes: P, probe; M, marker (pBR322 DNA cleaved with *Msp*I); U, uninfected cell RNA control; IE, IE RNA. Sizes of marker (in nucleotides) are indicated at the left. Sizes of protected fragments are indicated next to arrowheads at the right. Because of the extremely low G+C content of the probe (35%), hybridization was at 40°C. (B) Schematic diagram of probe, protected fragment, and RNA. The double-stranded probe was labeled at the *Spe*I site. Hatched portion represents vector sequences in the probe.

phonoacetic acid, a specific inhibitor of herpesvirus DNA polymerases (25, 33; data not shown).

To determine whether late transcription occurs from the same or opposite strand of the viral genome as immediate-

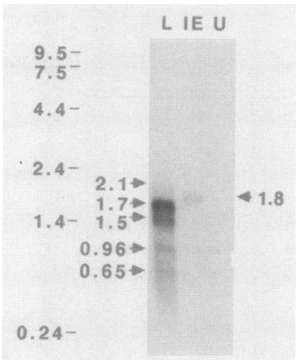


FIG. 8. Northern blot analysis of RNA transcribed from BHV-4 IE-1 gene late in infection. The 2.2-kb *Sma*I-*Bgl*II fragment (Fig. 3C), labeled by random hexanucleotide-primed synthesis, was hybridized to a Northern blot of cytoplasmic polyadenylated RNA. Positions and sizes (in kilobases) of marker RNAs (0.24- to 9.5-kb RNA ladder; GIBCO-Bethesda Research Laboratories) are indicated at the far left. Sizes (in kilobases) of L RNAs identified by the probe are indicated next to arrowheads on the left. Lane L, RNA prepared 24 h p.i. IE and uninfected (U) cell RNA were included for comparison. The position of the IE RNA identified by the probe is indicated by the arrowhead on the right. In this gel, the position of the IE RNA indicates a size of 1.8 kb rather than 1.7 kb.

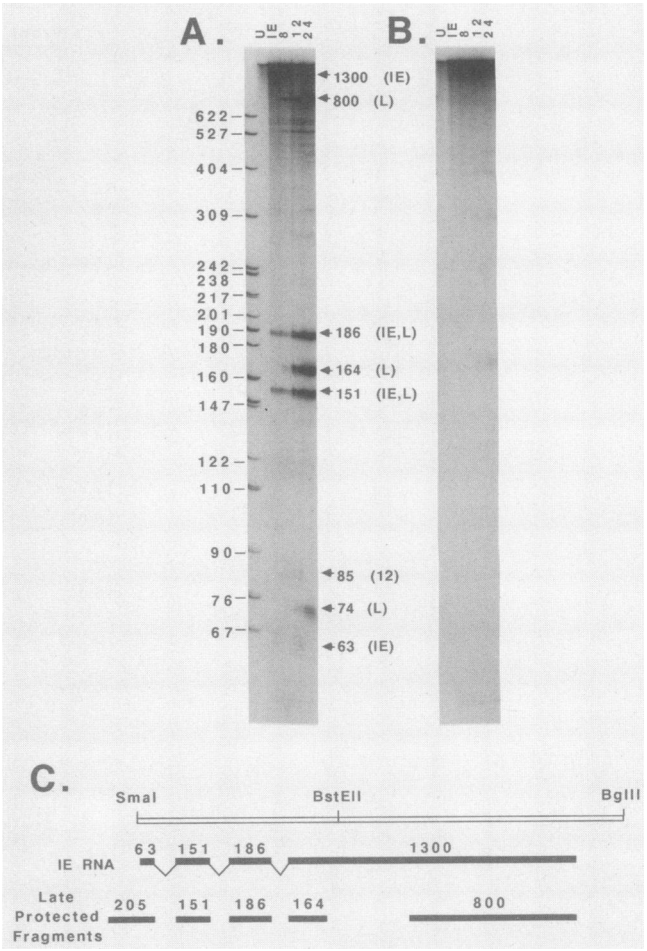


FIG. 9. (A and B) S1 nuclease analysis of RNA transcribed from BHV-4 IE-1 gene over the course of infection. The fragment shown in panel C was used as uniformly labeled, single-stranded probe. In panel A, the probe was the strand from which IE RNA is transcribed; in panel B, the probe was the opposite strand. Lanes: U, uninfected cell RNA; IE, IE RNA (included for comparison); 8, 12, 24, RNA prepared 8, 12, and 24 h p.i., respectively. Marker sizes (in nucleotides) for both panels are noted on the left. At the right of panel A, sizes of major protected fragments are indicated in nucleotides, and the times at which RNA protecting each fragment is present in maximum amounts are indicated in parentheses. Sizes of the protected fragments >500 nt were determined more accurately by electrophoresis on a gel run three times as long as the one shown, using the size markers used in Fig. 3B. Fragments of 1,300 nt protected by IE RNA and 800 nt protected by L RNA, clearly visible on original autoradiograph, are obscured by background smear in this reduction. (C) Schematic diagram of probe, IE RNA (for comparison), and major fragments protected by late RNA (12 and 24 h p.i.). Map positions of fragments protected by late RNAs were determined by additional S1 nuclease mapping experiments (not shown).

early transcription, we performed S1 nuclease analysis by using single-stranded uniformly labeled DNA probes representing each strand of the 2.2-kb *Sma*I-*Bgl*II fragment. Results (Fig. 9A) showed that most of the late transcription occurs from the same strand as IE transcription. Very little DNA of the opposite strand was protected (Fig. 9B). S1 nuclease analysis of late RNA using the 2.2-kb *Sma*I-*Bgl*II fragment from which the major IE RNA is transcribed yielded an additional interesting result. While two of the

probe fragments (63 and 1,300 nt) protected by IE RNA showed a decrease or modest increase in amount protected by RNA prepared 12 and 24 h p.i. compared with IE RNA, the other two fragments (151 and 186 nt) were protected in larger amounts by RNA prepared 12 and 24 h p.i. compared with IE RNA (Fig. 9A). Additional S1 nuclease analyses (not shown) using fragments of the 2.2-kb *Sma*I-*Bgl*II fragment as probes showed that the 151- and 186-nt fragments protected by late RNA map within the same restriction fragment as the 151- and 186-nt fragments protected by IE RNA and are thus likely to be the same fragments. This suggests that the 151- and 186-nt IE exons are also used in late transcripts, but, because the 63- and 1,300-nt IE exons do not exhibit the same increase in amount during the late stage of infection, they are used in a different context. In addition to the fragments shared with IE RNA, late RNA protected major fragments of approximately 800, 165, and 74 nt; several minor fragments were also protected. In addition, an approximately 85-nt fragment was protected by RNA isolated at 12 h p.i. but was not as evident 24 h p.i. Additional S1 nuclease analyses (not shown) using fragments of the 2.2-kb *Sma*I-*Bgl*II fragment as well as the entire 3.2-kb *Hin*dIII-*Bgl*II fragment (see Fig. 2B) yielded results consistent with the placement of the major late exons shown in Fig. 9C. Instead of the IE exon 1, late RNA contains an exon beginning approximately 140 nt 5′ to the beginning of IE RNA. This was shown by primer extension analysis (not shown) to represent the 5′ end of a late transcript rather than a splice site. Another primer extension analysis (not shown) showed that the 5′ end of the fifth late exon (800 nt) shown in Fig. 9C is not the 5′ end of a late transcript; this exon is spliced to upstream sequences. As is evident from Fig. 8, multiple RNAs are transcribed from this region late in infection and it is not possible to determine from these experiments which exons are part of which RNA.

Nucleotide sequence analysis of the gene encoding major IE RNA and 5′ and 3′ flanking regions. The nucleotide sequence (Fig. 10) of the entire 3,241-nt *Hin*dIII-*Bgl*II fragment encoding the major IE RNA (Fig. 2B) shown in Fig. 8 was determined by the dideoxynucleotide chain termination method, using the Sequenase 2.0 sequencing kit (United States Biochemical Corp., Cleveland, Ohio) and single-stranded templates. It includes approximately 1.1 kb of 5′ flanking sequences and 0.15 kb of 3′ flanking sequences. This 3.2-kb portion of the BHV-4 genome contains 60% A+T and 40% G+C. At 20 to 27 nt 5′ to the approximate start site of the major IE transcript is an A+T-rich sequence (TATATAA) which might serve as a TATA box (35). No CAAT box-like sequence (35) is apparent. The portion of the sequence contained in the major IE RNA contains an open reading frame encoding 284 amino acids and a long (approximately 817 nt) 3′ untranslated region. The sequences including the first ATG codon in this open reading frame conform to the sequence (A/GNNATGG) shown to be necessary for efficient translation initiation (31, 32). Each intervening sequence disrupts a codon in this open reading frame, but splicing preserves the reading frame.

Comparison of the predicted amino acid sequence of BHV-4 IE-1 to other proteins. We refer to the putative protein encoded by the 284-codon open reading frame of the major IE RNA as BHV-4 IE-1. The predicted amino acid sequence of BHV-4 IE-1 was compared with the amino acid sequences in the GenPept and SWISS-PROT protein sequence data bases using the Pearson and Lipman FASTA program (41). The protein most similar to BHV-4 IE-1, as indicated by the highest "opt" score, 109, was an HSV-1 IE protein, IE110. The region of similarity is a 141-amino-acid region near the amino terminus of the predicted amino acid sequence of BHV-4 IE-1 which shows similarity to sequences near the amino terminus of HSV-1 IE110 (Fig. 11). The region of similarity includes a cysteine-rich region of HSV-1 IE110 (residues 99 to 156). Eight of the nine cysteine residues in this region are present in the same position in BHV-4 IE-1 (shown in boldface letters in Fig. 11). These include three CXXC motifs, which could be involved in zinc or other metal binding, as found in the zinc finger DNA binding domains of several proteins (4, 28). Although the mRNAs encoding BHV-4 IE-1 and HSV-1 IE110 are both spliced, the exon junctions occur at different points in the region of similar amino acid sequence, as indicated in Fig. 11. In addition, whereas HSV-1 IE110 RNA is spliced between codons in the region of similarity, BHV-4 IE-1 RNA is spliced within codons.

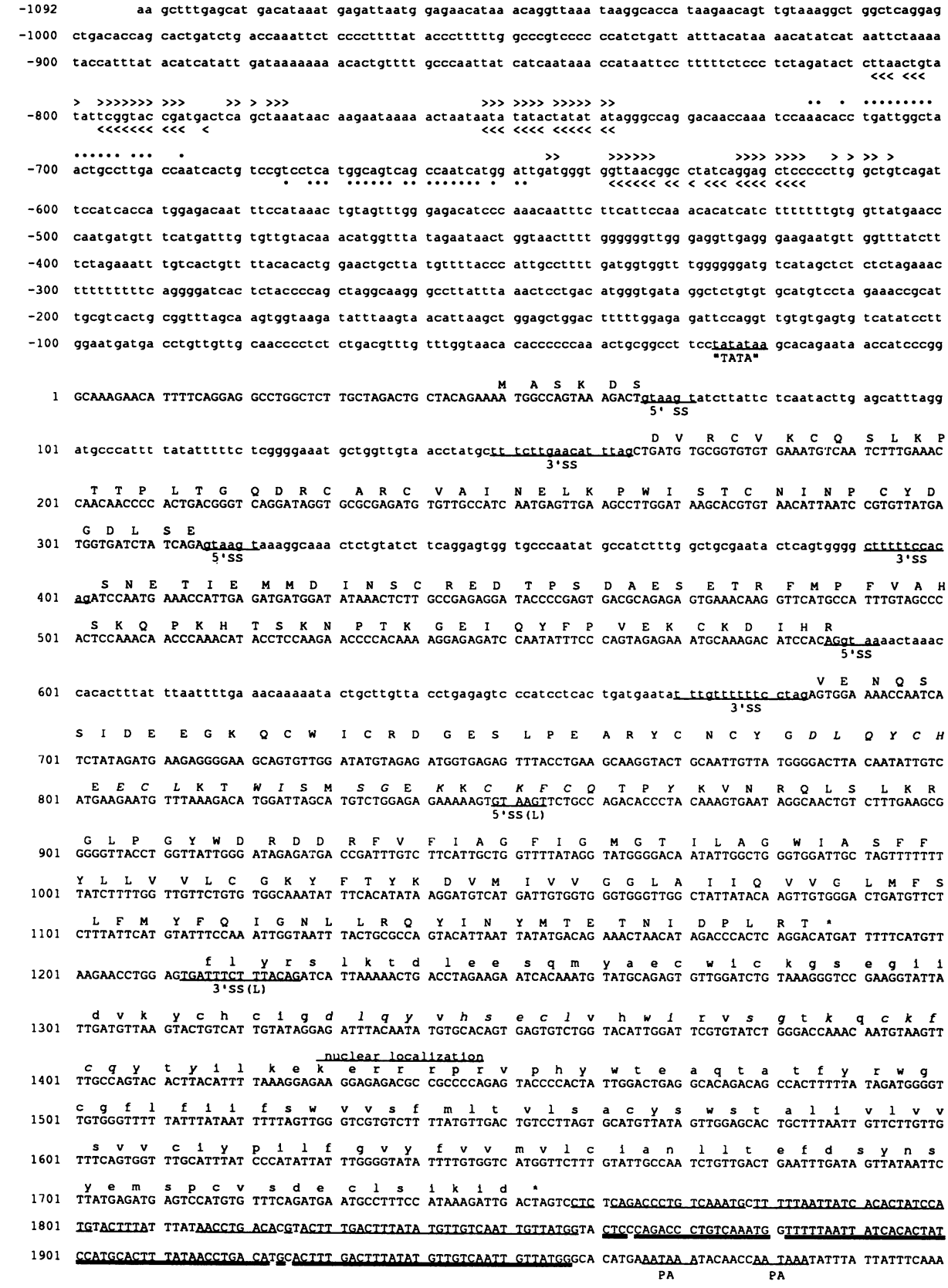
DISCUSSION

Features of BHV-4 major IE RNA. We have characterized the BHV-4 major IE RNA. This is the most abundant viral RNA species found in MDBK cells under IE conditions, i.e., when cells are infected in the presence of cycloheximide as an inhibitor of protein synthesis. The amount of the major IE RNA is greatly reduced at 6 h p.i. in the absence of inhibitors, relative to its abundance in the absence of protein synthesis. This suggests that transcription of this RNA is subject to negative regulation by newly synthesized viral proteins and that during infection in the absence of inhibitors, it might not be the most abundant of the IE transcripts, even at earlier times. Our experiments do not directly address the issue of whether this is the most abundant RNA very soon in infection when cells are infected in the absence of inhibitors. However, since it is the major RNA found under IE conditions, we refer to it as the major IE RNA.

The BHV-4 major IE RNA is a spliced, 1.7-kb RNA 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 of the DN-599 isolate. It contains three small exons at its 5′ end, spliced to a 1.3-kb 3′ exon. No evidence has been obtained for minor IE RNAs transcribed from the same region and generated by alternative splicing, as was found for the spliced major IE RNA of human cytomegalovirus (50). However, additional RNA species are generated from this region at late times after infection (see below).

Features of the nucleotide sequence of DNA encoding major IE RNA and flanking sequences. The nucleotide sequence of the DNA encoding the BHV-4 major IE early RNA, approximately 1.1-kb 5′ flanking sequences, and 0.15-kb 3′ flanking sequences contains 60% A+T and 40% G+C nucleotides. The region exhibits depression in frequency of CpG dinucleotides and a corresponding excess of TpG and CpA dinucleotides, as is expected for an IE gene of a betaherpesvirus or for any region of the genome of a gammaherpesvirus (23). The 5′ flanking sequences of this BHV-4 IE gene do not contain multiple repeat motifs characteristic of the major IE promoter region of human (7, 54), murine (12), and simian cytomegaloviruses (26). However, a 29-nt inverted repeat with seven mismatches is found between 716 and 648 nt 5′ to the beginning of transcription, and several interrupted palindromes extending 22 to 34 nt are present between 809 and 607 nt 5′ to the start of transcription (Fig. 10). The significance of these sequences for transcription of this IE gene is not yet known.

The nucleotide sequence of the portion encoding the major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2101 cccgagtgtg gcaacattca tggaatcctc ccaggttata gttagatct

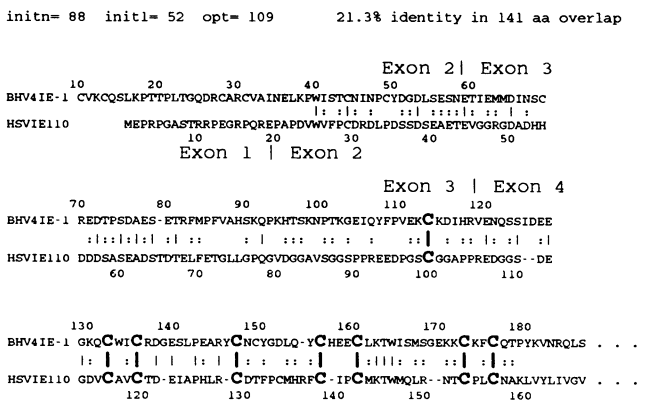

FIG. 11. Homology of BHV-4 IE protein predicted amino acid sequence with that of HSV-1 IE110: GenPept (release 64.0) and SWISS-PROT (release 14.0) protein sequence data bases were searched for proteins with similarity to the predicted amino acid sequence of proteins encoded by the BHV-4 major IE RNA using the Pearson and Lipman FASTA program (41) with a "ktup" value of 1. The resulting alignment yielding the highest opt score is shown. Only the regions of each protein exhibiting similarity are shown. |, exact match; :, match with a conservatively replaced amino acid. Conserved cysteine residues are emphasized by large, bold type. Dashes indicate gaps introduced to maximize homology. Exon junctions of the RNA are noted above the BHV-4 IE-1 sequence and below the HSV-1 IE110 sequence.

IE RNA includes a short (50-nt) 5′ untranslated sequence, an open reading frame with the potential to encode a 285-amino-acid polypeptide, and a long (816-nt) 3′ untranslated region. The 3′ untranslated region contains a second open reading frame; this is unlikely to be translated from the IE RNA because its putative initiation codon is 1,258 nt from the 5′ end of the mRNA. However, S1 nuclease analysis shows that the region containing this open reading frame is represented in late transcripts. Therefore, this open reading frame may encode all or part of a late protein. The amino acid sequence predicted by the second open reading frame exhibits no significant similarity to any amino acid sequence in the GenPept or SWISS-PROT data bases. Immediately following the termination codon of the second open reading frame is a 97-bp tandem repeat with only five mismatches. The first copy contains a 5-bp insertion relative to the second copy (Fig. 10). The significance of this long repeated sequence near the 3′ end of the RNA is unknown.

Protein encoded by the major IE RNA. The nucleotide sequence of the DNA encoding the major IE RNA predicts that it encodes a protein of 33 kDa. IE proteins have not yet been identified in BHV-4-infected cells (13). To aid in identification of this IE protein in infected cells, we plan to produce antibodies to this putative IE protein expressed from cDNA in bacteria. Antibodies to this protein will also be useful in determining the location of the protein in infected cells. The predicted amino acid sequence of IE-1 does not contain a clear nuclear localization signal of several basic amino acids flanked by helix-breaking amino acids (29) as several other herpesvirus IE proteins do (36).

The amino-terminal region of BHV-4 IE-1 shows amino acid sequence similarity to the IE protein IE110 of HSV-1. Although the similarity is not especially striking, the region of similarity includes a cysteine-rich region which includes CXXC pairs which could be involved in metal binding to form finger structures which are found in some DNA-binding proteins (4, 28). These cysteine residues are also conserved in the HSV-1 IE110 homolog in varicella-zoster virus (42a), further suggesting functional importance. HSV-1 IE110 is a DNA-binding protein (21), but it is not known whether its DNA binding is mediated by this region of the protein. Although similarity searches showed that the amino acid sequence of BHV-4 IE-1 is more similar to that of HSV-1 IE110 than to any other protein for which the amino acid sequence is known, these two proteins are not likely to be evolutionary homologs because the intron-exon junctions fall at different positions within the similar amino acid sequence. Also, splicing of HSV-1 IE110 mRNA occurs between codons, while splicing of BHV-4 IE-1 mRNA occurs within codons. In addition, HSV-1 IE110 contains nearly three times the number of amino acids as the putative BHV-4 IE-1 polypeptide. Nevertheless, the two proteins could be functionally homologous. IE110 is a nonspecific transactivator, activating transcription from nearly every promoter tested, including other viral and cellular promoters as well as HSV-1 promoters (39, 49). In addition, IE110 acts synergistically with another HSV-1 IE protein, IE175, to activate transcription from HSV-1 E and L promoters (16, 17, 45, 49). Mutational analyses have shown that the region

FIG. 10. Nucleotide sequence of the gene encoding BHV-4 major IE RNA and flanking sequences. Numbers of nucleotides is relative to the approximate start of transcription. Exons, as determined by experiments shown in Fig. 3 through 7, are in uppercase letters; intervening sequences and 3′ and 5′ flanking sequences are in lowercase letters. The precise locations of the 5′ and 3′ ends of the RNA are not known. Sequences at the intron-exon junctions conforming to the 5′ splice site consensus sequence (AG/GTAAGT; 40) and 3′ splice site consensus sequence (Py_nNPyAG; 40) are underlined and marked 5′SS and 3′SS. Splice site consensus sequences which might be used in late transcripts, based on preliminary S1 nuclease analyses, but not used in IE RNA, are marked (L). 5′ to the 3′ ends of the RNA, sequences conforming to the consensus polyadenylation signal (AAUAAA; 44) are underlined and marked PA. In the 3′ untranslated region, a 97-nt tandem repeat is underlined, the first copy with single underlining, and the second copy with double underlining. The first copy contains a 5-nt insertion relative to the second copy at nt 1810 to 1814. 5′ to the transcription start site, a sequence which might function as a TATA box (35) is underlined and marked "TATA." In the 5′ flanking sequences, an inverted repeat is marked with dots. Dots above the sequence mark the first copy; dots below the sequence mark the inverted copy on the complementary strand. Several palindromic sequences are marked with arrowheads. Arrowheads above the sequence mark nucleotides on the strand shown; arrowheads below the sequence mark nucleotides on the complementary strand. Palindromes located at −647 to −626 and −634 to −607 are overlapping. The repeats and palindromes were identified by Pustell matrix analysis with MacVector sequence analysis software (International Biotechnologies, Inc., New Haven, Conn.) using the following settings: hash value = 6, window size = 30, minimum % score = 65. Above the nucleotide sequence, the predicted amino acid sequence of exons is shown in single-letter code. Uppercase letters indicate the predicted amino acid sequence of the protein presumably encoded by the major IE RNA. The translation of a second open reading frame (see Discussion) is shown in lowercase letters. A possible nuclear localization signal in the second open reading frame is marked. Homology between amino acid sequences in the putative IE protein encoded by nt 785 to 871 and the amino acid sequence encoded by nt 1330 to 1416 in the second open reading frame is denoted by italics. Only identical amino acids are italicized. Similar amino acids are not marked.

of IE110 containing the putative metal-binding fingers is absolutely required for stimulation of transcription by IE110 alone and is necessary for full activation in the presence of IE175 as well (18, 19). The region immediately amino terminal to the putative metal-binding fingers, which is also similar to the sequence of BHV-4 IE-1, appears nonessential to IE110 function (18, 19). IE110 appears to be a functionally complex protein, with different domains involved in activation of different promoters (9). No homology of other regions of BHV-4 IE-1 with other regions of HSV-1 IE110 is apparent. The function of BHV-4 IE-1 remains to be determined. On the basis of the functions of other herpesvirus IE proteins, it is likely to function in transcriptional activation of E and L genes.

Proteins encoded by late transcripts from the IE-1 region. Multiple RNAs are transcribed from the BHV-4 IE-1 region late in infection. These RNAs have not been characterized in detail. However, limited S1 nuclease analyses suggest that a longer exon 1, exons 2 and 3 of IE RNA, the 5′ portion of exon 4 of IE RNA, and an additional fifth exon as shown in Fig. 9C are abundant in late mRNAs. We do not know whether these exons form a single late mRNA. Many late RNAs are transcribed from this region. For this reason, interpretation of two-dimensional S1 nuclease analysis to determine which exons are linked together would be difficult; mapping and sequencing of cDNAs would be more informative and is currently under way. However, if the abundant late exons comprise a single mRNA, the RNA would be approximately 1.5 kb; an RNA of this size is present late in infection (Fig. 8). In such an RNA, the additional sequences at the 5′ end of exon 1 compared with those of IE RNA would not encode additional amino acids at the amino terminus; the RNA would just have a longer 5′ untranslated region. The alternative transcriptional start site might be responsible for the alternative splicing of late compared with that of IE transcripts. On the basis of the location of splice site consensus sequences predicted to be used by S1 nuclease analysis, such an RNA would encode a truncated version of the IE protein, containing only the first 172 amino acids and two additional amino acids. However, the truncated protein would contain the putative metal-binding finger domain. If this is a DNA-binding domain, the same DNA-binding domain would be used in more than one protein.

In a late RNA with the structure just proposed (the five major late exons linked together), the open reading frame found in the 3′ untranslated portion of the IE RNA would not be translated. Our analyses do not show whether this open reading frame is in a position in a late RNA with a different structure in which it could be translated. However, a 29-amino-acid portion of this second open reading frame exhibits amino acid homology to a portion of the putative IE protein encoded by part of exon 4 (Fig. 10). This homology is evident at both the nucleotide and amino acid sequence levels, but similarity is stronger at the amino acid sequence level, suggesting functional significance. Alternative splicing could produce an RNA encoding a late protein with the same amino acid terminus as the IE protein and a similar, but different, carboxyl terminus. Such a protein might have a similar, but different, function as the IE protein. The predicted amino acid sequence of this second open reading frame, in contrast to the predicted IE amino acid sequence, does contain a possible nuclear localization signal (29), KGRRRPR, encoded by nt 1429 to 1449 (Fig. 10).

Classification of BHV-4. BHV-4 was at one time classified as a bovine cytomegalovirus on the basis of biological properties (51). However, several recent findings at the DNA sequence level call this classification into question. First, several segments of the genome not containing IE genes show a depression in CpG dinucleotide frequency and a corresponding relative excess of TpG and CpA dinucleotides (8) characteristic of the gammaherpesviruses (23). Second, this work shows that the nucleotide sequence 5′ to the DNA encoding the major IE RNA does not contain the multiple repeat motifs characteristic of human, simian, and murine cytomegaloviruses (7, 12, 26, 54). In addition, the protein encoded by the BHV-4 major IE RNA is much smaller (predicted molecular weight 33,000) than the predicted molecular weight of proteins encoded by the major IE RNAs of human and murine cytomegaloviruses (55,000 and 67,000; 1, 30). Third, the nucleotide sequence (55) of the other BHV-4 IE gene we have identified, BHV-4 IE-2, predicts that it encodes a protein with amino acid sequence homology to transactivating proteins of two gammaherpesviruses, the Epstein-Barr virus BRLF1 transactivator (2, 10, 11) and an IE protein encoded by the herpesvirus saimiri *Eco*RI D fragment (37). Together, these findings suggest that BHV-4 might be most closely related to the gammaherpesviruses rather than the cytomegaloviruses (betaherpesviruses).

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REFERENCES

1. **Akrigg, A., G. W. G. Wilkinson, and J. D. Oram.** 1985. The structure of the major immediate early gene of human cytomegalovirus strain AD169. Virus Res. **2**:107–121.
2. **Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnill, and B. G. Barrell.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310**:207–211.
3. **Barta, A., A. M. Fadol, H. Liebermann, H. Ludwig, S. B. Mohanty, F. A. Osorio, D. E. Reed, J. Stortz, O. C. Straub, M. J. Van der Maaten, and G. Wellermans.** 1987. Problems concerning the taxonomy of the "Movar-type" bovine herpesvirus. Intervirology **28**:1–7.
4. **Berg, J. M.** 1986. Potential metal-binding domains in nucleic acid binding proteins. Science **232**:485–487.
5. **Berger, S., and C. Birkenmeier.** 1979. Inhibition of intractable nucleases with ribonucleoside-vanadyl complexes: isolation of RNA from resting lymphocytes. Biochemistry **18**:5143–5149.
6. **Blair, E. D., and E. K. Wagner.** 1986. A single regulatory region modulates both *cis* activation and *trans* activation of the herpes simplex virus VP5 promoter in transient-expression assays in vivo. J. Virol. **60**:460–469.
7. **Boshart, M., F. Weber, G. Jahn, Dorsch-Hasler, B. Fleckenstein, and W. Schaffner.** 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell **41**:521–530.
8. **Bublot, M., M.-F. van Bressem, E. Thiry, J. Dubuisson, and P.-P. Pastoret.** 1990. Bovine herpesvirus 4 genome: cloning, mapping and strain variation analysis. J. Gen. Virol. **71**:133–142.
9. **Cai, W., and P. A. Schaffer.** 1989. Herpes simplex virus type 1 ICP0 plays a critical role in the de novo synthesis of infectious virus following transfection of viral DNA. J. Virol. **63**:4579–4589.
10. **Chevallier-Greco, A., H. Gruffa, E. Manet, A. Calender, and A. Sergeant.** 1989. The Epstein-Barr virus (EBV) DR enhancer contains two functionally different domains: domain A is constitutive and cell specific, domain B is transactivated by the

EBV early protein R. J. Virol. 63:615–623.
11. **Cox, M. A., J. Leahy, and J. M. Hardwick.** 1990. An enhancer within the divergent promoter of Epstein-Barr virus responds synergistically to the R and Z transactivators. J. Virol. **64**:313–321.
12. **Dorsch-Haesler, K., G. M. Keil, F. Weber, M. Jasin, W. Schaffer, and U. H. Koszinowski.** 1985. A long and complex enhancer activates transcription of the gene coding for the highly abundant immediate early mRNA in murine cytomegalovirus. Proc. Natl. Acad. Sci. USA **82**:8325–8329.
13. **Dubuisson, J.** Submitted for publication.
14. **Dubuisson, J., E. Thiry, M. Bublot, M. Sneyers, D. Boulanger, J. Guillaume, and P.-P. Pastoret.** 1989. Production and characterization of monoclonal antibodies to bovid herpesvirus-4. Vet. Microbiol. **19**:305–315.
15. **Ehlers, B., H.-J. Buhk, and H. Ludwig.** 1985. Analysis of bovine cytomegalovirus genome structure: cloning and mapping of the monomeric polyrepetitive DNA unit, and comparison of European and American strains. J. Gen. Virol. **66**:55–68.
16. **Everett, R. D.** 1984. *trans* activation of transcription by herpesvirus products: requirements for two HSV-1 immediate-early polypeptides for maximum activity. EMBO J. **3**:3135–3141.
17. **Everett, R. D.** 1986. The products of herpes simplex virus type 1 HSV-1 immediate early genes 1, 2 and 3 can activate HSV-1 gene expression in trans. J. Gen. Virol. **67**:2507–2513.
18. **Everett, R. D.** 1987. A detailed mutational analysis of Vmw110, a *trans*-acting transcriptional activator encoded by herpes simplex virus type 1. EMBO J. **6**:2069–2076.
19. **Everett, R. D.** 1988. Analysis of the functional domains of herpes simplex virus type 1 immediate-early polypeptide Vmw110. J. Mol. Biol. **202**:87–96.
20. **Guo, W. Z., D. T. Shen, J. F. Evermann, and J. R. Gorham.** 1988. Comparison of an enzyme-linked immunoabsorbance assay and a complement fixation test for the detection of IgG to bovine herpesvirus type 4 bovine cytomegalovirus. Am. J. Vet. Res. **49**:667–670.
21. **Hay, R. T., and J. Hay.** 1980. Properties of herpesvirus-induced "immediate early" polypeptides. Virology **104**:230–234.
22. **Holland, L. E., K. P. Anderson, C. Shipman, and E. K. Wagner.** 1980. Viral DNA synthesis is required for the efficient expression of specific herpes simplex virus type 1 mRNA species. Virology **101**:10–24.
23. **Honess, R. W., U. A. Gompels, B. G. Barrell, M. Craxton, K. R. Cameron, R. Staden, Y.-N. Chang, and G. S. Hayward.** 1989. Deviations from expected frequencies of CpG dinucleotides in herpesvirus DNAs may be diagnostic of differences in the states of their latent genomes. J. Gen. Virol. **70**:837–855.
24. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14**:8–19.
25. **Huang, E.-S.** 1975. Human cytomegalovirus. IV. Specific inhibition of virus-induced DNA polymerase activity and viral DNA replication by phosphonoacetic acid. J. Virol. **16**:1560–1565.
26. **Jeang, K.-T., D. R. Rawlins, P. J. Rosenfeld, J. H. Shero, T. J. Kelly, and G. S. Hayward.** 1987. Multiple tandemly repeated binding sites for cellular nuclear factor 1 that surround the major immediate-early promoters of simian and human cytomegalovirus. J. Virol. **61**:1559–1570.
27. **Jones, P. C., and B. Roizman.** 1979. Regulation of herpesvirus macromolecular synthesis. VII. The transcription program consists of three phases during which both extent of transcription and accumulation of RNA in the cytoplasm are regulated. J. Virol. **31**:299–314.
28. **Kadonaga, J. T., K. R. Carner, F. R. Masiarz, and R. Tjian.** 1987. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell **51**:1079–1090.
29. **Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith.** 1984. A short amino acid sequence able to specify nuclear location. Cell **39**:499–509.
30. **Keil, G. M., A. Ebeling-Keil, and U. H. Kozinowski.** 1987. Sequence and structural organization of the immediate-early gene of murine cytomegalovirus. J. Virol. **61**:1901–1908.
31. **Kozak, M.** 1984. Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nucleic Acids Res. **12**:857–872.
32. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44**:283–292.
33. **Mao, J. C.-H., E. E. Robishaw, and L. R. Overby.** 1975. Inhibition of DNA polymerase from herpes simplex virus-infected Wi-38 cells by phosphonoacetic acid. J. Virol. **15**:1281–1283.
34. **Mavromara-Nazos, P., S. Silver, J. Hubenthal-Voss, J. L. C. McKnight, and B. Roizman.** 1986. Regulation of herpes simplex virus 1 genes: α gene sequence requirements for transient induction of indicator genes regulated by β or late γ2 promoters. Virology **149**:152–164.
35. **McKnight, S. L., and R. Kingsbury.** 1982. Transcriptional control signals of a eukaryotic protein-coding gene. Science **217**:316–324.
36. **Mullen, M. A., and G. S. Hayward. (John Hopkins University).** 1990. Personal communication.
37. **Nicholas, J., L. S. Coles, C. Newman, and R. W. Honess.** 1991. Regulation of the herpesvirus saimiri (HVS) delayed-early 110-kilodalton promoter by HVS immediate-early gene products and a homolog of the Epstein-Barr virus R *trans* activator. J. Virol. **65**:2457–2466.
38. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53**:751–760.
39. **O'Hare, P., J. D. Mosca, and G. S. Hayward.** 1986. Multiple *trans*-activating proteins of herpes simplex virus that have different target promoter specificities and exhibit both positive and negative regulatory functions. Cancer Cells **4**:175–188.
40. **Padgett, R. A., P. J. Grabowski, M. M. Konarska, S. Seiler, and P. A. Sharp.** 1986. Splicing of messenger RNA precursors. Annu. Rev. Biochem. **55**:1119–1150.
41. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA. **85**:2444–2448.
42. **Pereira, L., M. H. Wolff, M. Fenwick, and B. Roizman.** 1977. Regulation of herpesvirus macromolecular synthesis. V. Properties of α polypeptides made in HSV-1 and HSV-2 infected cells. Virology **77**:733–749.
42a. **Perry, L. J., F. J. Rixon, R. D. Everett, M. C. Frame, and D. J. McGeoch.** 1986. Characterization of the IE110 gene of herpes simplex virus type 1. J. Gen. Virol. **67**:2365–2380.
43. **Persson, R. H., S. Bacchetti, and J. R. Smiley.** 1985. Cells that constitutively express the herpes simplex virus immediate-early protein ICP4 allow efficient activation of viral delayed-early genes in *trans*. J. Virol. **54**:414–421.
44. **Proudfoot, N. J.** 1989. How RNA polymerase II terminates transcription in higher eukaryotes. Trends Biochem. Sci. **14**:105–110.
45. **Quinlan, M. P., and D. M. Knipe.** 1985. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. Mol. Cell. Biol. **5**:957–963.
46. **Rice, S. A., and D. M. Knipe.** 1988. Gene-specific transactivation by herpes simplex virus type 1 α protein ICP27. J. Virol. **62**:3814–3823.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
48. **Sears, A. E., I. W. Halliburton, B. Meignier, S. Silver, and B. Roizman.** 1985. Herpes simplex virus 1 mutant deleted in the α22 gene: growth and gene expression in permissive and restrictive cells and establishment of latency in mice. J. Virol. **55**:338–346.
49. **Sekulovich, R. E., K. Leary, and R. M. Sandri-Goldin.** 1988. The herpes simplex virus type 1 α protein ICP27 can act as a *trans*-repressor or a *trans*-activator in combination with ICP4 and ICP0. J. Virol. **62**:4510–4522.
50. **Stenberg, R. M., P. R. Witte, and M. F. Stinski.** 1985. Multiple spliced and unspliced transcripts of human cytomegalovirus

immediate early region 2 and evidence for a common initiation site within immediate-early region 1. J. Virol. **56**:665–675.

51. **Stortz, J., B. Ehlers, W. J. Toss, and H. Ludwig.** 1984. Bovine cytomegaloviruses: identification and differential properties. J. Gen. Virol. **65**:697–706.
52. **Su, L., and D. M. Knipe.** 1989. Herpes simplex virus α protein ICP27 can inhibit or augment viral gene transactivation. Virology **170**:496–504.
53. **Thiry, E., M. Bublot, J. Dubuisson, and P.-P. Pastoret.** 1989. Bovine herpesvirus-4 (BHV-4) infections of cattle, p. 96–115. *In* G. Wittmann and Y. Becker (ed.), Herpesvirus diseases of cattle, horses, and pigs. Kluwer Academic Publishers, Boston.
54. **Thomsen, D. R., R. M. Stenberg, W. F. Goins, and M. F. Stinski.** 1984. Promoter-regulatory region of the major immediate-early gene of human cytomegalovirus. Proc. Natl. Acad. Sci. USA **81**:659–663.
55. **van Santen, V. L.** Unpublished data.
56. **Wagner, E. K.** 1985. Individual HSV transcripts: characterization of specific genes, p. 45–104. *In* B. Roizman (ed.), The herpesviruses. Plenum Press, New York.
57. **Wu, C. A., N. J. Nelson, D. L. McGeoch, and M. D. Challberg.** 1988. Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. J. Virol. **62**:435–443.