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The Three Major Immediate-Early Transcripts of Bovine Herpesvirus 1 Arise from Two Divergent and Spliced Transcription Units

URS V. WIRTH,† BERND VOGT, AND MARTIN SCHWYZER*

Institut für Virologie der Universität Zürich, Winterthurerstrasse 266a, 8057 Zürich, Switzerland

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Among 54 transcripts expressed in a temporal cascade during lytic infection with bovine herpesvirus 1, we have previously identified three major immediate-early (IE) RNAs, IER4.2 (4.2 kb), IER2.9 (2.9 kb), and IER1.7 (1.6 to 1.8 kb depending on the virus strain) transcribed from the *Hin*dIII C genome region (U. V. Wirth, K. Gunkel, M. Engels, and M. Schwyzer, J. Virol. 63:4882–4889, 1989). Northern (RNA) blot, S1 nuclease protection, and primer extension analysis used in the present study demonstrated that all three IE transcripts were spliced and originated from two divergent transcription units with start sites located in the inverted repeat. Transcription unit 1 encoded two alternative spliced transcripts, IER4.2 and IER2.9, with a common exon 1 located at 0.797 to 0.795 map units (m.u.) and an exon 2 for IER4.2 (0.792 to 0.762 m.u.) in the inverted repeat; exon 2 for IER2.9 (0.754 to 0.738 m.u.) was located in the unique long sequence and transcribed in antisense orientation to latency-related RNA. Transcription unit 2 (0.818 to 0.836 m.u.), further characterized by cDNA cloning, encoded the spliced IER1.7 with three exons in the inverted repeat. Additional minor IE transcripts were interpreted as unspliced precursors and splicing variants. With regard to the number and layout of IE genes, bovine herpesvirus 1 occupies an intermediate position between pseudorabies virus and equine herpesvirus 1 on the one hand and varicella-zoster virus and herpes simplex virus type 1 on the other.

Bovine herpesvirus 1 (BHV-1), the causative agent of infectious bovine rhinotracheitis and infectious pustular vulvovaginitis, contains a linear double-stranded DNA genome approximately 140 kb long, composed of a unique long segment, U_L (105 kb), and a unique short segment, U_S (11 kb), the latter being flanked by inverted repeat sequences (IR_S, TR_S) of 11 kb each (76) (see Fig. 1A). A total of 54 viral transcripts have been mapped both temporally and spatially (75). More than 50 viral proteins have been shown to be synthesized during lytic infection, and their expression can be grouped into three kinetic classes, immediate-early (IE), early, and late (52, 75). The genes for thymidine kinase (10), glycoprotein gI (53, 72), glycoprotein gIII (34), several other glycoproteins (cited in reference 75), DNA polymerase (54), and a major DNA-binding protein (7) have been identified and characterized.

The IE proteins are important for the regulation of the productive cycle of herpesvirus replication, and therefore the IE proteins of various herpesviruses, most of human origin, have been studied extensively (for reviews see references 32, 63, and 70). Among animal alphaherpesviruses, pseudorabies virus (PRV) specifies a single unspliced 5.1-kb IE transcript encoding the major 180-kDa IE protein (17, 20, 22, 23, 43, 71) which transactivates various class II and III promoters (4, 33, 35, 39) by interacting with host transcription factors (1, 26, 70a). Equine herpesvirus 1 (EHV-1) specifies a single 6.0-kb transcript containing a short 5′ exon spliced to the coding region, which gives rise to the major 200-kDa IE protein and three smaller, structurally related protein species (18, 37, 38, 40, 41, 60). During lytic infection with BHV-1, we have previously identified three major IE transcripts, IER4.2 (4.2kb), IER2.9 (2.9 kb), and IER1.7 (1.6 to 1.8 kb, depending on the virus strain) specified by the *Hin*dIII C genome region and have shown that IER4.2 cross-hybridizes with the PRV IE gene (75). In the present study, we have used Northern (RNA) blot, S1 nuclease protection, and primer extension analysis to establish a detailed physical map of these transcripts. All three major IE transcripts turned out to be spliced and to originate from two divergent transcription units with start sites located in the inverted repeat. IER4.2 and IER2.9 were shown to belong to a single transcription unit by alternative splicing.

MATERIALS AND METHODS

Viruses, cell, and isolation of total RNA and viral DNA. The virus strains used in this study were BHV-1.1 Jura (51) and BHV-1.2b K22 (46). Madin Darby bovine kidney (MDBK) cells were cultured and infected with BHV-1, and total RNA was isolated at the indicated hours postinoculation (p.i.) as described previously (75). Virus purification and DNA extraction followed procedures described elsewhere (31).

Plasmid cloning procedures. DNA fragments were cloned by established procedures (6, 27, 64). The following vectors were used for subcloning of the BHV-1 *Hin*dIII C subfragments shown in Fig. 1D: pUC9 for pJuC (containing *Hin*dIII DNA fragment C with a size of 16.2 kb), p601 (*Hin*dIII-*Eco*RI, 11.4 kb), p615 (*Eco*RI-*Hin*dIII, 4.8 kb), and pMV340 (*Hin*dIII/*Pst*I-*Eco*RI, 4.1 kb; by deletion of internal *Pst*I fragments from p601); pSPT19 (Pharmacia LKB Biotechnology, Uppsala, Sweden) for pMV381 (*Sal*I-*Pst*I, 4.5 kb); pGEM3Z (Promega) for pOM75 (*Xho*I, 7.5 kb, into *Sal*I). Plasmids were isolated (i) with Triton lysis buffer and CsCl gradients containing ethidium bromide (27) and were additionally precipitated with polyethylene glycol (6) to get rid of interfering RNA for 5′ end labeling, or (ii) by a faster

* Corresponding author.
† Present address: Department of Cancer and Developmental Biology, Syntex Research, Palo Alto, CA 94304.

procedure involving boiling and subsequent polyethylene glycol precipitation essentially as described previously (6).

Northern blot hybridization. Northern blot analysis was carried out as described previously (75) with the modification that some RNA blot strips were reused. In that case the blots were washed for 1 h with 50% formamide–10 mM sodium phosphate (pH 7.0) at 75°C to strip off the probes and then stored in wash solution (0.2% sodium dodecyl sulfate, 5 mM sodium phosphate, 1 mM EDTA [pH 7.0]) at 4°C for at least a month. After this treatment, no signal due to the previous probe could be detected even with long exposures (up to 18 days), and rehybridization efficiency was only slightly reduced.

S1 nuclease analysis. Double-stranded DNA fragments were end labeled and isolated as probes for S1 nuclease analysis as described previously (64) with the modifications indicated below. For 5′ end labeling, polyethylene glycol-precipitated plasmids containing BHV-1 DNA fragments were cut with appropriate restriction endonucleases, dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Mannheim, Federal Republic of Germany), and labeled with [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham) and T4 polynucleotide kinase (New England BioLabs, Beverly, Mass.) by using the forward reaction at 32°C. For 3′ end labeling, plasmids were cut as above, labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham) and Klenow fragment (Boehringer) in H buffer (50 mM Tris hydrochloride, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithioerythritol [Boehringer]). Reactions were stopped by heating (70°C for 10 min). To generate probes with a single labeled end, DNA fragments were extracted with phenol, ethanol precipitated, and cut with a second restriction endonuclease. Fragments were separated by agarose gel electrophoresis, excised, and purified by using a GeneClean DNA elution kit (Bio 101 Inc., La Jolla, Calif.) by following the protocol recommended by the supplier. As size markers, *Hin*dIII- and *Eco*RI-digested λ DNA (Boehringer) or *Hin*fI-digested pGEM4 (Promega) were 5′ end labeled as described above and separated from free nucleotides by using the GeneClean DNA elution kit. For S1 nuclease analysis, essentially as described previously (74), end-labeled probes (10^5 cpm) and total RNA (10 μg) were denatured for 10 min at 90°C. After hybridization at 65°C overnight, samples were immediately expelled into tubes containing the S1 nuclease mixture and incubated for 2 h at 32°C. The S1 nuclease-protected DNA fragments were analyzed together with undigested probes and size markers on alkaline agarose gels or on 6% polyacrylamide–urea gels or both (64).

Primer extension analysis. Oligonucleotide primers, synthesized at the Institute of Zoology, University of Zürich, were 5′ end labeled in minimal reaction volumes as described above and generally used in the subsequent steps without being separated from unincorporated [γ-^{32}P]ATP. Hybridization and subsequent reactions with reverse transcriptase from avian myeloblastosis virus were performed as described previously (16) with some modifications recommended by the supplier (Pharmacia). Briefly, labeled primer (8×10^4 cpm as estimated by electrophoresis of a sample of the labeling reaction mixture) was hybridized with 10 μg of total RNA for 1 h at 45°C in 10 μl of hybridization buffer [400

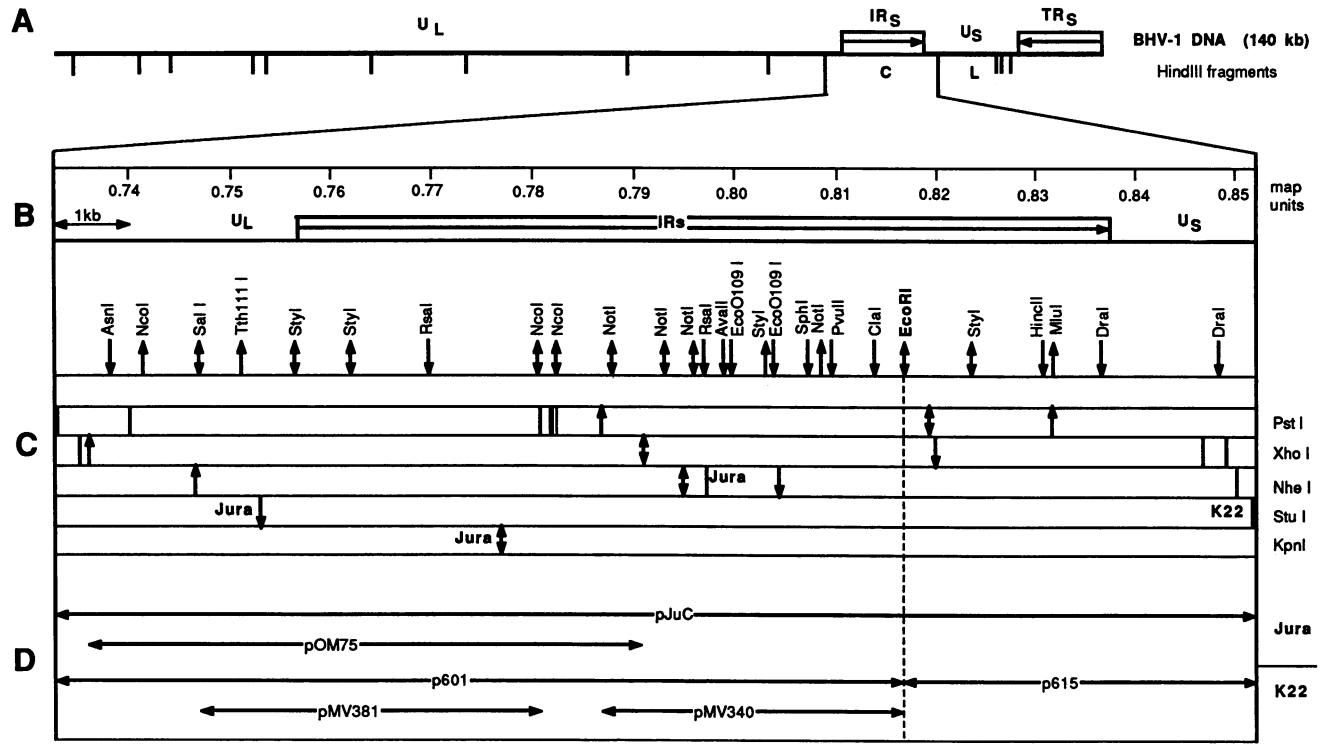

FIG. 1. (A) Structural organization of the BHV-1 genome with unique long (U_L), unique short (U_S), internal short (IR_S), and terminal short (TR_S) repeat regions; location of cloned viral DNA fragments. (B) Expanded *Hin*dIII fragment C genome region with selected restriction endonuclease sites used in this study. (C) Complete map for five restriction endonucleases; sites specific for strain Jura or K22 are indicated. Arrows denote sites used for Northern blot (down), S1 nuclease (up), or both techniques (double). The same arrows are reproduced in Fig. 3 (down) and in Fig. 7 (up) to serve as landmarks for orientation. (D) Cloned subfragments of strains Jura and K22.

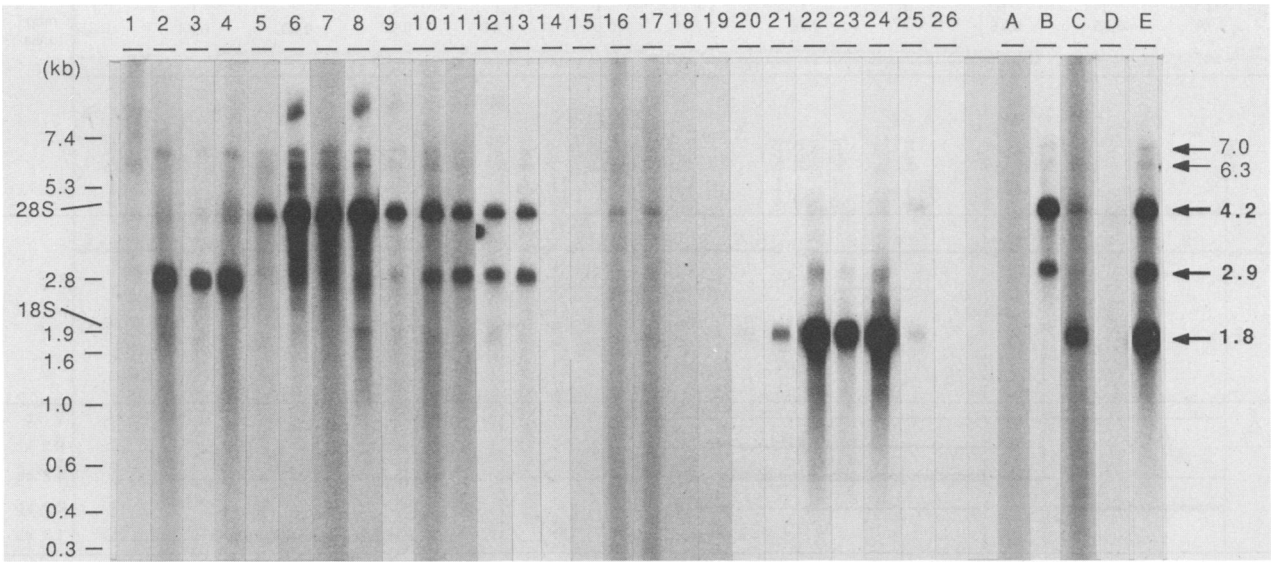

FIG. 2. Northern blot analysis of BHV-1 IE RNA. Labeled DNA subfragments (1 to 26) shown in Fig. 3B were hybridized to identical strips carrying blotted RNA from strain K22-infected and cycloheximide-treated cells. For control lanes (A to E) the same strips except lane D with RNA from mock-infected cells were used and hybridized with the following probes: (A) *Hin*dIII fragment L (see Fig. 1A); (B) plasmid pMV381 (Fig. 1D); (C) genomic DNA of BHV-1 K22; (D and E) *Hin*dIII fragment C. Positions of rRNA (28S and 18S) and RNA size markers are indicated on the left side, and calculated sizes of the most prominent signals are marked on the right side.

mM NaCl, 1 mM EDTA, 40 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); (PIPES; pH 6.4)]. Extension reactions were started by adding a mixture (40 μl) containing 10 U of avian myeloblastosis virus reverse transcriptase in 20 μl of H_2O, 10 μl of 5× avian myeloblastosis virus reverse transcriptase buffer (200 mM KCl, 50 mM $MgCl_2$, 5 mM dithiothreitol, 250 mM Tris [pH 8.6]), and 10 μl of 5× deoxynucleoside triphosphate solution (dATP, dCTP, dGTP, dTTP at 2.5 mM each). After 2 h at 42°C, the cDNA products were phenol extracted, ethanol precipitated, and analyzed on 6% polyacrylamide–8.3 M urea sequencing gels (system S2; BRL Life Technologies, Gaithersburg, Md.). The same, but unlabeled, oligonucleotide primers were used in a Deaza ^{T7}Sequencing reaction (Pharmacia), using [α-^{35}S]dATP and double-stranded plasmids (p601, p615, and pJuC). The reactions were analyzed on the same gel.

RESULTS

Restriction site mapping and plasmid constructions of the BHV-1 *Hin*dIII-C genome region. Figure 1 gives an overview of the BHV-1 genome and the expanded *Hin*dIII-C genome region analyzed in this study and previously shown to encode the major IE transcripts. Selected restriction enzyme sites used for Northern blot analysis (arrows down), S1 nuclease protection assays (arrows up), or both techniques (double arrows) are shown in Fig. 1B; a complete map of cleavage sites appears in Fig. 1C for five enzymes; Fig. 1D indicates the map location of the genome fragments that were cloned.

Fine mapping of three major and two minor IE transcripts on the *Hin*dIII-C genome region of BHV-1 by using Northern blot analysis. Total RNA from BHV-1 K22-infected and cycloheximide-treated cells was loaded into a single slot spanning an entire agarose gel, separated electrophoretically, and blotted on nylon membranes. The blots were cut into identical strips and probed with various radiolabeled fragments of the *Hin*dIII-C genome region. Autoradiographs of the blots are shown in Fig. 2, including the control strips (lanes A to E), which will be described first. The entire *Hin*dIII C fragment was used as a probe for lanes D and E. As a negative control for lane D, a blot strip containing RNA of cycloheximide-treated mock-infected cells was used; therefore, the signals of positive control lane E arise from virus-encoded RNAs. The *Hin*dIII C fragment hybridized to the three major IE RNAs, IER4.2 (4.2 kb), IER2.9 (2.9 kb), and IER1.7 (1.8 kb for strain K22) and to two bands of minor IE RNAs (7 and 6.3 kb). Hybridization with the entire DNA genome of strain K22 (lane C) revealed no additional IE transcripts and indicated that after cycloheximide treatment IER1.7 is most enriched whereas IER2.9 is barely detectable. The *Hin*dIII L fragment (Fig. 1A), known to encode some major early transcripts (75), did not reveal any signal here (Fig. 2, lane A), indicating that inhibition of later classes of transcripts by cycloheximide was effective. To show that identical transcript signals were obtained with probes from different strains, pMV381 derived from strain K22 was used as a probe in lane B; the result should be compared with those for probes 3 to 8 derived from strain Jura (see below).

Lanes 1 to 26 (Fig. 2) illustrate the transcript pattern that was detected by 26 partially overlapping fragments (for map locations, see Fig. 3B) spanning the entire *Hin*dIII-C genome region. These probes were derived from strain K22, except probes 3 to 8, which were from strain Jura because strain K22 lacked some restriction sites in that region (Fig. 1C). Figure 3C summarizes the signal intensities and deduced locations of the three major and two minor IE transcripts (Fig. 2). IER4.2 produced strong signals with fragments 5 to 13 located in the left half of IR_S; the much weaker signals observed with some other fragments (e.g., lanes 16 and 17) may represent cross-hybridization of these G+C-rich probes with IE transcripts or with 28S rRNA, as noted previously for BHV-1 (75) and for other herpesviruses (see reference 66 and references therein). Two discontinuous groups of fragments (lanes 2 to 4 and lanes 10 to 13) gave strong signals

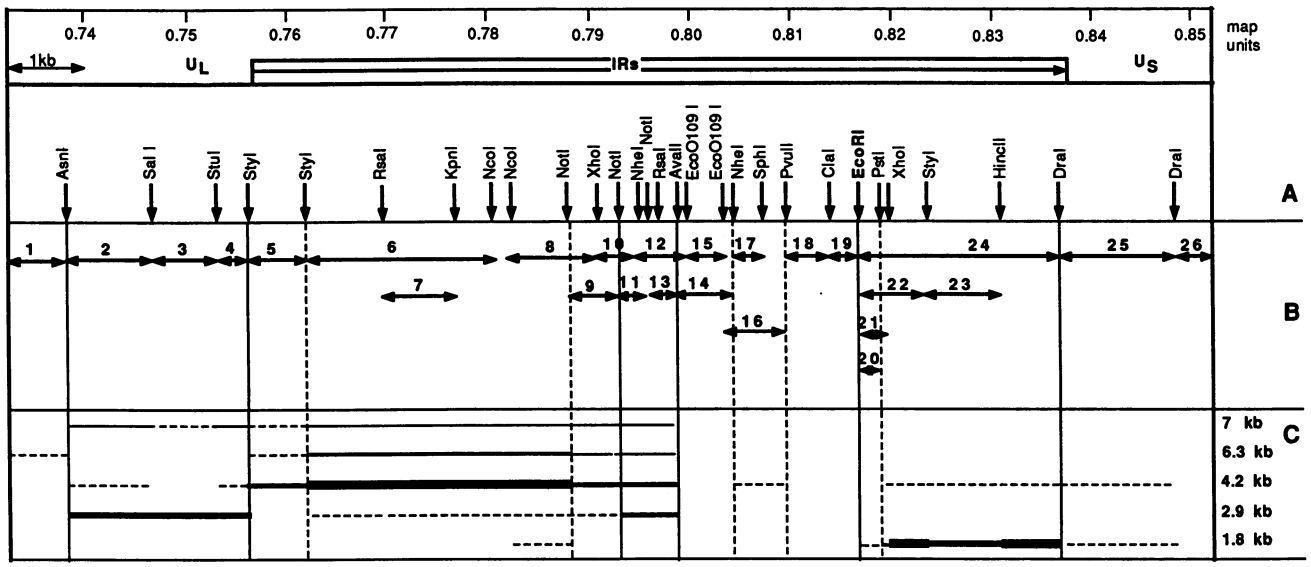

FIG. 3. Location of probes and evaluation of signals from the Northern blot analysis of BHV-1 IE RNA shown in Fig. 2. (A) Genome organization with map units and selected restriction endonuclease sites of *Hin*dIII fragment C. (B) Subfragments (1 to 26) of the *Hin*dIII C region used in Northern blot analysis shown in Fig. 2. (C) Estimation of signal intensities, indicated by thickness of lines, and interpretation of boundaries from the five most prominent transcripts of Fig. 2.

with IER2.9, indicating that this transcript is spliced, as will be confirmed by other means below. The third major transcript, IER1.7, was detected by fragments 21 to 24, located in the right-hand part of IR_S. One of the minor IE transcripts (7 kb) was detected by fragments spanning IER4.2 and IER2.9; the smaller species (6.3 kb) gave a similar pattern but lacked signals from fragments 2 to 4 located in U_L. Comparable results were obtained by using this set of probes with RNA blot strips from strain Jura-infected cells, except that IER1.7 was smaller (1.6 kb), as noted before (75).

S1 nuclease analysis of termini and splice junctions. To map the 5′ and 3′ termini of the IE transcripts more precisely and to determine splice site boundaries, we performed S1 nuclease protection experiments. Figure 4 provides a detailed example of the analyses that were carried out to determine the location of a single splice acceptor site for IER4.2 (0.792 map units [m.u.]). For Fig. 4A, pMV340 (strain K22; Fig. 1D) was digested with *Not*I and labeled with T4 polynucleotide kinase, and a 0.55-kb DNA fragment labeled at both 5′ ends (0.789 to 0.793 m.u.) was isolated. It was hybridized with RNA from mock-infected cells (lane −), from BHV-1 K22-infected cells 2 h after infection (lane 2), or from two infected cultures treated with cycloheximide (lane C). After S1 nuclease digestion, a 5′-end-labeled DNA fragment of 0.46 kb (arrow) remained reproducibly protected by the RNA, as analyzed on a polyacrylamide-urea gel together with undigested probe (lane p; 0.55*). A strong signal was observed with cycloheximide-enriched RNA, a much weaker but clearly detectable signal was observed with RNA from the early stage of infection, and none was observed with RNA from uninfected cells. For Fig. 4B, pMV340 was digested with *Xho*I, 5′ end labeled, and cut again with *Pst*I, and a 6.1-kb fragment carrying only one labeled *Xho*I end at 0.791 m.u. was isolated. This fragment extended until the *Eco*RI site (0.816 m.u.) and then included vector sequences at the unlabeled end. The S1 nuclease digestion products were analyzed in the same way as in Fig. 4A, together with undigested probe (lane p; 6.1*), and were found to give a weak and diffuse signal (arrow) near the bottom of the gel. Aliquots of the same samples were reanalyzed on a sequencing gel (Fig. 4C) together with size markers (M) and a sequencing ladder (not shown) and gave 52 nucleotides (nt) as the precise length of the protected fragment. No protected signal was obtained with a probe containing the same *Xho*I site but labeled at the 5′ end of the opposite strand.

In conclusion, the results shown in Fig. 4A indicated a 5′ transcript boundary situated about 460 nt from one of the labeled *Not*I sites. The same 5′ boundary was defined more precisely in Fig. 4B and C as being situated 52 nt to the right of the *Xho*I site (0.791 m.u.) and belonging to a transcript running in the leftward direction, since only the left-hand 5′ end of the probe was labeled. Northern blots (Fig. 2) showed that IER4.2 was the only major transcript giving a signal near the XhoI site and that the mapped 5′ boundary had to be a splice acceptor site because fragments 10 to 13 upstream of the *Xho*I site also hybridized with IER4.2. Thus, S1 nuclease analysis shows that IER4.2 is spliced.

To search for additional splice sites in IER4.2, the experiment whose results are shown in Fig. 4D and E was carried out in the same way. A 2.6-kb *Sty*I-*Nco*I fragment (0.762 to 0.781 m.u.) from pMV381 was labeled either at both 5′ ends or at both 3′ ends. With the 5′ end-labeled probe, S1 nuclease analysis revealed a large proportion of protected 5′ end-labeled fragment with a size of about 1.9 kb and a small proportion of full-length protected fragment (Fig. 4D). The 3′-end-labeled probe also produced a protected fragment (0.7 kb) and minor amounts of the full-length fragment (Fig. 4E). This result suggested the presence of a very small S1 nuclease-sensitive region around 0.776 m.u., since the two protected fragments together covered the entire 2.6-kb region on IER4.2, previously shown to be transcribed in leftward direction. Although the S1 nuclease-sensitive region might be explained by the presence of a second small intron in IER4.2, we favor as the explanation some unusual secondary structures in DNA-RNA hybrids, because preliminary sequence analysis revealed at that site a cluster of

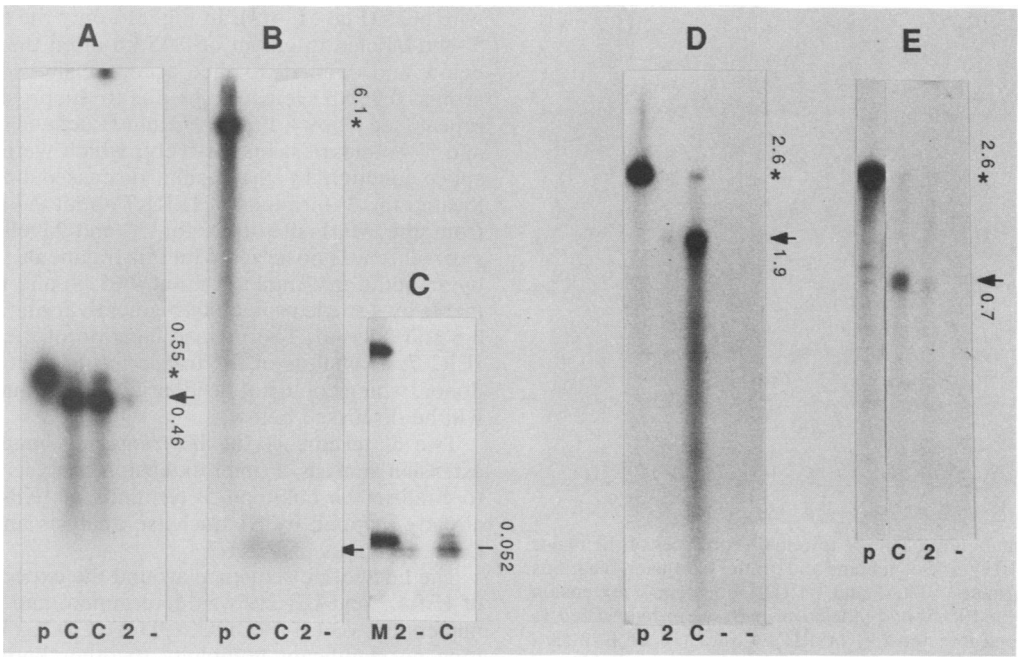

FIG. 4. S1 nuclease analysis to determine DNA sequences protected by IER4.2 of BHV-1. End-labeled DNA probes (for map locations, see Fig. 7B) were hybridized to total RNA from cycloheximide-treated mock-infected (lanes −) or BHV-1-infected (lanes C) cells or from untreated BHV-1-infected cells harvested at 2 h p.i. (2). Fragments protected from S1 nuclease digestion (arrows) and undigested probes (p,*) were analyzed on 1.4% alkaline agarose (D) or on 6% polyacrylamide–urea (A, B, C, and E) gels. End-labeled *Hin*dIII-*Eco*RI fragments of lambda DNA and *Hin*fI fragments of pGEM4 were used as molecular size markers for each gel, and calculated sizes of fragments are indicated in kilobases. Panel C shows similar reactions to those in panel B, but fractionated on a sequencing gel including a marker lane (M) with two *Hin*fI fragments of pGEM4 (53 and 83 nucleotides).

direct and inverted repeat elements lacking convincing splice consensus signals.

The analyses shown in Fig. 5 were performed in the same way as the examples shown above. To facilitate their description, a schematic summary of S1 nuclease protection assays (Fig. 4 and 5 and additional experiments without figures) and primer extension analyses (Fig. 6; see also below) is provided in Fig. 7.

For Fig. 5A, pOM75 was digested with *Nco*I, 3′ end labeled, and cut with *Kpn*I and a 3.5-kb fragment containing vector sequences at the unlabeled end was used as a probe. The protected fragment of 0.43 kb, together with additional

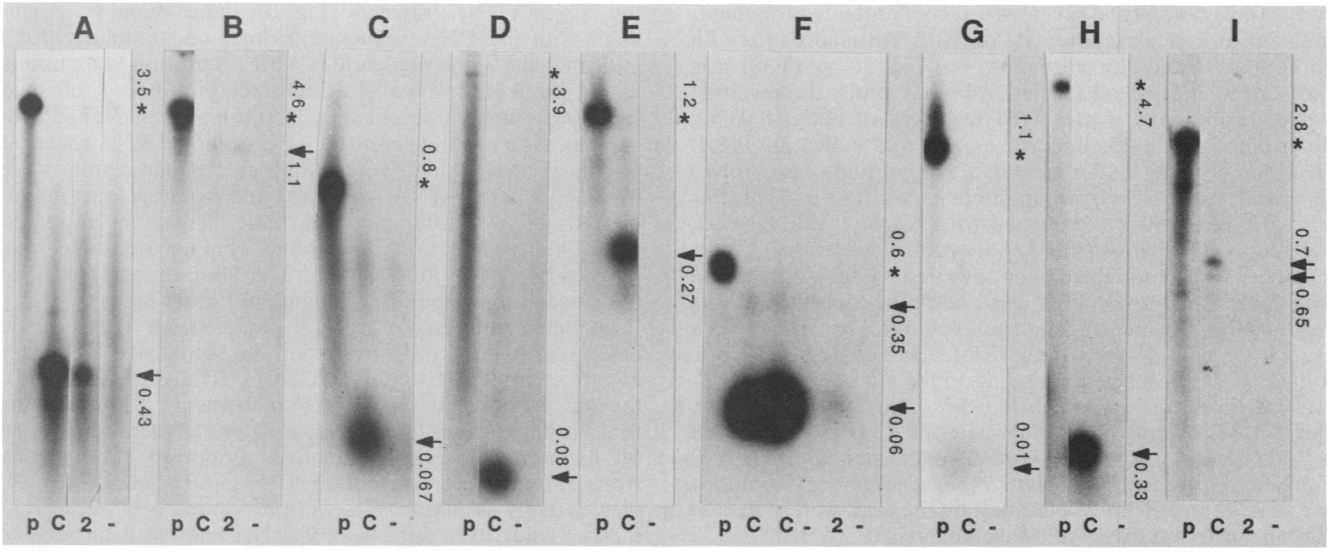

FIG. 5. Additional IE transcript boundaries determined by S1 nuclease analysis. Different end-labeled probes (for map locations, see Fig. 7B) were used for each panel (A to I). The experiments were carried out as described for Fig. 4.

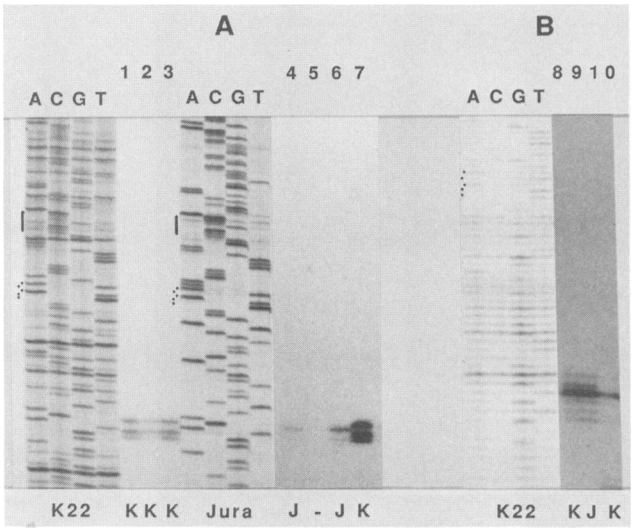

FIG. 6. Determination of the 5'-terminal sequences of the major IE transcripts of BHV-1. Sequencing and primer extension reactions are shown for primers o3 (A) and o4 (B). For primer extension analysis (lanes 1 to 10), 5'-end-labeled primers were hybridized to total RNA of mock-infected (−) or BHV-1 Jura (J)- or K22 (K)-infected cells. Cells had been either treated with cycloheximide and harvested at 6 h p.i. (lanes 1 to 3 and 5 to 10) or harvested untreated at 2 h p.i. (lane 4). End-labeled primers were isolated from sequencing gels (lane 1), purified over a NAP25 column (Pharmacia) (lane 2), or used unpurified (all other lanes). Reverse transcriptase (all lanes from avian myeloblastosis virus except lane 10, from Moloney murine leukemia virus) was used to synthesize cDNA fragments, which were analyzed on 6% polyacrylamide–urea sequencing gels. Molecular size markers were used as described for Fig. 4 (not shown). Unlabeled primers were used to determine the sequences of the cDNA termini and 5'-flanking sequences of the corresponding RNAs. Nucleotides of a putative TATA box are marked with dots, and a possible Sp1 transcription factor consensus sequence is indicated with a line.

S1 nuclease results (Fig. 7B) and the Northern blot data of Fig. 2, locates the 3'terminus of IER2.9 (Fig. 7D). For Fig. 5B, p601 was digested with *Sal*I, 5' end labeled, and cut with *Nco*I; the 4.6-kb probe revealed a 1.1-kb protected fragment, indicating a splice acceptor site of IER2.9 in accordance with additional S1 and Northern data. For Fig. 5C, pMV381 was digested with *Sty*I and labeled at both 3' ends; the resulting 0.8-kb probe gave a protected fragment of about 0.07 kb determined on an alkaline agarose gel and indicating the 3' terminus of IER4.2. On a sequencing gel (data not shown) this band was resolved into multiple bands of 63 to 67 nt. For Fig. 5D, pMV340 was digested with *Nhe*I, 3' end labeled, and cut with *Eco*RI; the 3.9-kb probe containing vector sequences at the unlabeled end revealed a 0.08-kb protected fragment. Since no other 3' ends could be identified in this region (Fig. 7B), the fragment protected in Fig. 5D defines a common splice donor site of IER4.2 and IER2.9, which is joined to the described splice acceptor sites (Fig. 4A to C and 5B) in accordance with Northern blot results (Fig. 2). For Fig. 5E, the protected fragment of 0.27 kb obtained with a 1.2-kb probe derived from pMV340 digested with *Nhe*I, 5' end labeled, and cut with *Sty*I reveals a common 5' terminus for IER2.9 and IER4.2. No additional exons were detected further upstream by S1 nuclease analysis (Fig. 7B).

For IER1.7, a total of four transcript boundaries could be mapped in a similar way by S1 nuclease protection assays with p615 (Fig. 5F to I). In Fig. 5F, only the minor protected 5'-end-labeled fragment of 0.35 kb fitted the data described below and defined a splice acceptor site; the major signal around 0.06 kb seemed to be due to the presence of a direct repeat (see below). Figure 5G and H defined unambiguous 3' and 5' boundaries, respectively, which were identified as a splice junction by the results discussed below. Figure 5I located the 3' terminus of IER1.7 about 0.7 kb downstream from the *Mlu*I site used for 3' end labeling. Some size variability was observed with this fragment, shown in Fig. 5I by an additional minor band at 0.65 kb and in other experiments by a single protected fragment varying in size between 0.6 and 0.85 kb. The precise location of the 3' terminus of IER1.7, as well as of the transcription start and first splice donor, which could not be identified by S1 nuclease analysis, will be discussed below.

Two 5' termini of the IE transcripts mapped by primer extension analysis. Primer extension analysis was performed to confirm the common 5' terminus of IER4.2 and IER2.9 already mapped by S1 nuclease analysis and to determine the 5' terminus of IER1.7.

The nucleotide sequence around the expected 5' terminus of IER4.2 and IER2.9 was determined, and a 19-mer oligonucleotide, o3 (5'-TTGGCCGTCTCCTCCTTCC-3'), exhibiting no significant homology to other known herpesvirus sequences was synthesized. It was labeled at the 5'end, hybridized to total RNA, and used as primer for reverse transcription to analyze the 5'-terminal sequences of both BHV-1 strains. With strain K22 RNA, three cDNA species were obtained, of 97, 95, and 94 nt including the primer (Fig. 6A, lanes 1 to 3 and 7). In parallel lanes on the left-hand side, unlabeled o3 was used as the primer for sequence analysis, with pMV340 as the template. The alignment suggested that the longest primer extension product ended with the sequence-GGAGGTA-3' and that some products were terminated 2 to 3 nt earlier. This may represent multiple initiation sites, which are not unusual for eucaryotic RNA polymerase II (74), the enzyme transcribing herpesvirus genes (25). No significant additional bands except the unextended primer were detected above or below the three cDNA bands, indicating a common 5' terminus for both IER4.2 and IER2.9 (Fig. 7C). With strain Jura RNA, a similar pattern of three cDNA species was obtained, but with a higher abundance of the longest cDNA fragment (Fig. 6A, lanes 4 and 6). Alignment with the DNA sequence (primer o3; template pJuC) indicated the same nucleotides as transcription start points as for strain K22; the slight difference in mobility reflected two single-nucleotide changes in the leader sequence (not shown). As a negative control, mock-infected RNA revealed no signal at all (lane 5). Qualitatively similar results were obtained whether RNA was harvested from cycloheximide-treated cells 6 h after infection (lane 6) or from untreated cells 2 h after infection (lane 4) and whether the primer was purified by electrophoresis (lane 1) or chromatography (lane 2) or used unpurified after labeling (all other lanes).

Sequence analysis of cDNA clones from IER1.7 (see below) provided the information to synthesize a 19-mer oligonucleotide, o4 (5'-GGCTGGGGATCGGTGAAGG-3'), located just downstream of the *Pst*I site near 0.82 m.u. Using the procedure described above, a major cDNA product of 56 nt, including the primer and five minor products ranging from 53 to 58 nt, was obtained (Fig. 6B, lanes 8 to 10). Qualitatively similar results were produced with strain K22 (lanes 8 and 10) or Jura (lane 9) RNA and by using reverse transcriptase from avian myeloblastosis virus (lanes 8 and 9) or murine leukemia virus (lane 10). Thus, the transcription

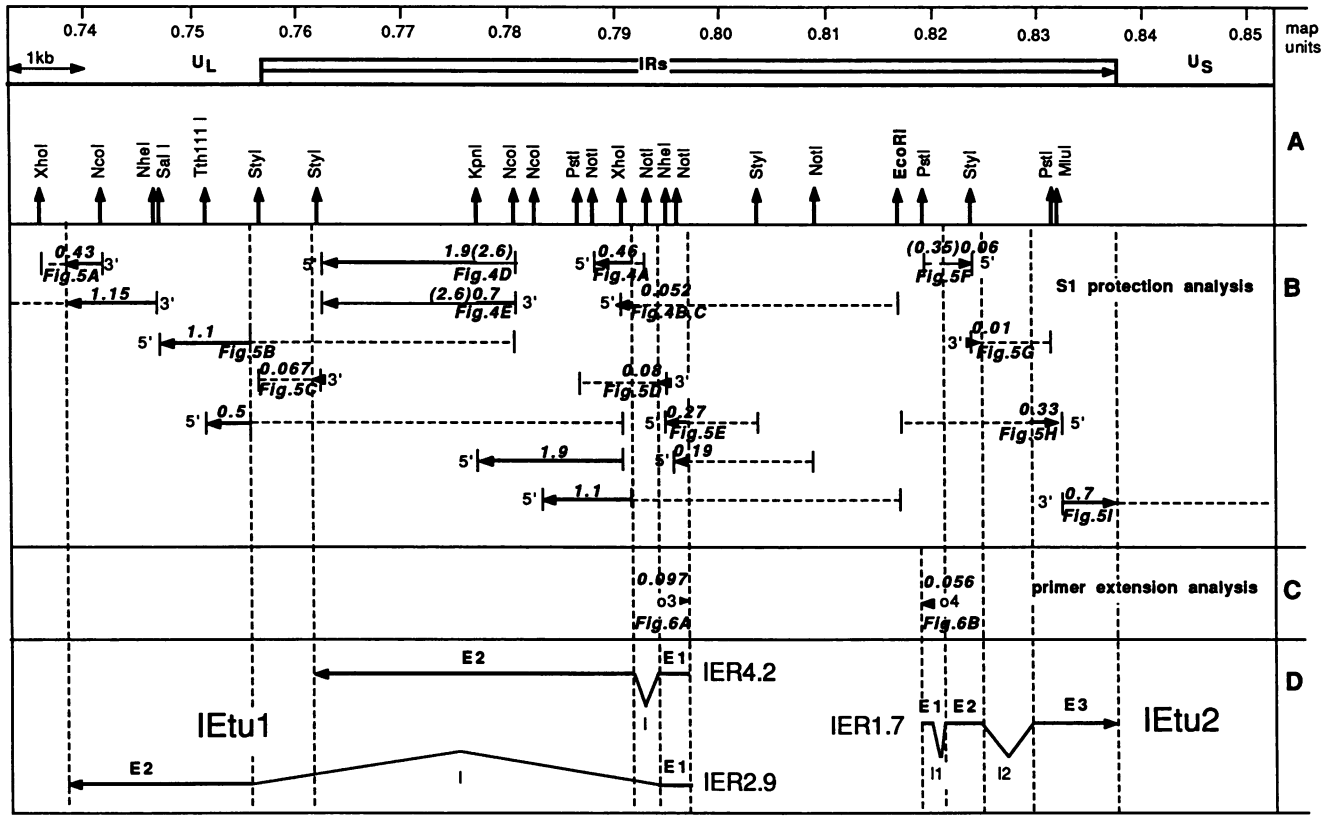

FIG. 7. Summary of S1 protection and primer extension analysis; deduced physical map of the major IE transcripts from BHV-1. (A) Genome organization with selected restriction endonuclease sites of *Hin*dIII fragment C. (B) Interpretation of the S1 nuclease experiments displayed in Fig. 4A to E and Fig. 5A to I and of additional control S1 nuclease analyses carried out in the same way. DNA fragments labeled at the indicated ends (5′ and 3′) were hybridized to IE RNA and digested with S1 nuclease, and the results are described as follows. Protected fragment parts are shown by continuous lines with the arrow pointing in the direction of transcription and the length indicated in kilobases; S1 nuclease-sensitive parts of the probes are shown by dashed lines. (C) Summary of primer extension results described in Fig. 6A and B. The position of primers and direction of synthesized cDNAs are given by arrows; sizes of produced cDNAs are indicated in kilobases. (D) Interpretation of these results by comparison with the data obtained by the Northern blot technique (Fig. 3). Directions of transcripts are indicated; exon (E) and intron (I) sequences are numbered from the 5′ termini for each IE RNA. IER4.2 and IER2.9 share a common exon (E1) at the 5′ end and arise by alternative splicing from the same transcription unit (IEtu1) with different 3′-terminal exons located, for IER4.2 and IER2.9, in the IR_S and the U_L genome regions of BHV-1, respectively. A second divergent transcription unit (IEtu2) encodes the spliced IER1.7 with three exons in the IR_S genome region.

start site of IER1.7 is heterogeneous, like that of IER4.2 and IER2.9, and is located 29 to 34 nt to the left of the *Pst*I site (Fig. 7C).

Map of the three major IE transcripts of BHV-1. The nucleotide sequence ladders of Fig. 6 were displayed to indicate the precise position of the transcription start sites. Additionally, they revealed some preliminary information about promoter structure. The IER1.7 and the IER4.2/2.9 promoters both contained a TATA box element (12, 24), and the latter also contained an Sp1 box (30). Further sequence data (64b) were in full agreement with the transcription patterns proposed here; i.e., the appropriate splicing (55) and polyadenylation (58) signals could be identified.

From IER1.7, some nearly full-length cDNA clones were obtained (64c), which were examined to clarify missing points in the S1 nuclease analysis shown above. These clones permitted us to identify a splice donor site, not seen by S1 nuclease analysis, 70 nt downstream from the major 5′ initiation site. The corresponding splice acceptor site forming the junction was located 29 nt to the right of the *Xho*I site at 0.82 m.u. and about 350 nt to the left of the *Sty*I site, in accordance with the minor S1 nuclease-protected 0.35-kb fragment (Fig. 5F). In exon 2, a direct repeat (21 nt reiterated 10 times) was discovered, which presumably accounted for the strong 0.06-kb signal observed in Fig. 5F by creating an S1 nuclease-sensitive site as already discussed for Fig. 4D and E. The *Dra*I site near 0.836 m.u. was present in the cDNA clones and overlapped with a polyadenylation signal AATAAA, from which the sequence extended for another 26 nt to the actual polyadenylated 3′ end. Comparison of the IR_S-U_S and TR_S-U_S junctions in cloned genome fragments by restriction enzyme mapping showed that the *Dra*I site was located close to the ends of IR_S and TR_S and that the first U_S-specific site was only 130 nt away. Thus, IER1.7 down to the polyadenylation signal is located entirely in the inverted repeats. Furthermore, probes from IR_S and TR_S gave protected 3′-terminal fragments that were apparently identical, but we cannot exclude the possibility that the last few nucleotides are encoded by U_S.

Figure 7D summarizes the deduced map location of the three major IE transcripts (IER4.2, IER2.9, and IER1.7) of BHV-1. They are all spliced and arise from two divergent

transcription units in *Hin*dIII fragment C. Transcription unit IEtu1 encodes two alternative spliced transcripts with a common exon 1 and an exon 2 for IER4.2 and IER2.9 in IR_S and U_L, respectively. For IER2.9, additional small introns could be excluded by unambiguous S1 nuclease protection (Fig. 7B) over both exon sequences, whereas for IER4.2 the presence of another small intron in exon 2 near 0.78 m.u., at least in a fraction of the transcripts, could not be excluded definitely (Fig. 4D and E). The divergent transcription unit IEtu2 encodes a spliced transcript, IER1.7, with three exons in IR_S. From the arrangement of these genes, it can be deduced that TR_S encodes copies of IER4.2 and IER1.7 but not of IER2.9; this has been verified for IER1.7 (75).

The majority of results were reproduced for both analyzed BHV-1 strains K22 and Jura and did not reveal any significant differences; both subtypes therefore exhibit the same transcription pattern for IEtu1 as shown in Fig. 7D. However, a complete analysis of IEtu2 has been performed only for strain K22. For strain Jura, primer extension analysis revealed the same 5′ end, but no cDNA clones were available, and S1 nuclease analysis missed one of the splice donor sites as mentioned above. Therefore, the different transcript size of 1.8 kb for strain K22 and 1.6 kb for strain Jura previously observed in Northern blots (75) has not yet been traced to a specific map location. No significant qualitative differences in the transcription patterns of the three major IE transcripts were detected by using either RNA from infected cycloheximide-treated cells or untreated cells harvested 2 h after infection. This indicates that all major transcripts enriched after cycloheximide treatment are transcribed at very early times of BHV-1 infection and that transcription patterns of major IE transcripts are not significantly changed by cycloheximide treatment.

DISCUSSION

Homology of IER4.2 with major IE transcripts of other alphaherpesviruses. The map location of IER4.2 established in this study (Fig. 7D) is equivalent to that of the IE transcripts of four alphaherpesviruses, namely EHV-1 (40), PRV (22, 23, 71), herpes simplex virus type 1 (HSV-1) (49), and varicella-zoster virus (VZV) (29). All of these IE transcripts are diploid and start approximately in the center of the IR_S and TR_S sequences; their 3′ ends map within 0.1 to 1.2 kb of the termini of the short genome segment. The observed similarity also extends to the structure and function of the encoded proteins. Nucleotide sequence analysis of IER4.2 points to strongly conserved regions at the amino acid level, and a major 180-kDa BHV-1 IE protein has been identified as a phosphoprotein (51a) resembling IE1 (EHV-1), IE180 (PRV), ICP4 (HSV-1), and p140 (VZV). The last three are known to be potent transactivator proteins for a variety of viral and cellular promoters (15, 26, 33, 35, 39, 65). The BHV-1 IE protein also seems to have this property, since plasmids encoding IER4.2 are capable of transactivating the simian virus 40 early promoter (64b).

Aside from these similarities, a surprising difference has been discovered in this study. The cognate IE transcripts of PRV (20, 71) and HSV-1 (49) are known to be unspliced. In contrast, IER4.2 of BHV-1 has been shown to consist of a leader RNA (0.35 kb) spliced to the main body (3.8 kb) after removal of a 0.45-kb intron. Between this established splice site and the 3′ terminus located 0.4 kb to the right of the U_L-IR_S junction, IER4.2 probably does not contain any further splice sites. Recently, the major IE transcript of EHV-1 has been shown to consist of a small (253-nt) untranslated exon, separated by a 372-nt intron from the main body of the 6-kb RNA (41), thus resembling the IER4.2 of BHV-1 in this regard.

Alternative splicing produces IER2.9, which overlaps latency-related transcripts in the antisense orientation. The map location and orientation of IER2.9 recall those of the transcripts encoding ICP0 in HSV-1 and ORF61 in VZV, but sequence analysis is not sufficiently complete to decide whether there are any potential homologies and whether the BHV-1 gene encodes the previously detected 135-kDa IE protein (75). The observation that IER2.9 shares its promoter and exon 1 with IER4.2 stands in clear contrast to the situation in HSV-1, in which the ICP0 gene is spliced but possesses its own promoter (57), and in VZV, in which the ORF61 gene has a promoter near the IR-U_L junction and is presumably unspliced (29).

At present, we can only speculate about the functions served by alternative splicing. The common IER4.2 and IER2.9 promoter suggests coordinate regulation of transcription initiation. The next level of regulation would be provided by transcription termination and the relative frequencies of splice site selection. Northern blots indicate that the two sites are used with approximately equal frequency in the cell types tested (MDBK cells, Fig. 2; bovine embryonic lung cells, not shown). Regulated alternative splicing might be particularly suited to terminally differentiated, long-lived cells that have lost replicating capacity and must respond to environmental stimuli (13). It would therefore be interesting to test whether the splicing pattern is different in nerve cells, which are the site of latency for BHV-1 (2, 3). Translation initiation provides a further level of regulation in gene expression. For IER4.2 and IER2.9, both open reading frames seem to start in exon 2, encoding entirely different proteins (50a). The long 5′ noncoding sequence may affect export to the cytoplasm or translation initiation, for which there are precedents such as the late mRNA for VP16 from HSV-1 (11) or an early transcript of human cytomegalovirus (36). Similar long leader sequences are present on the single IE transcripts of EHV-1 (41) and PRV (17, 71) and on all five HSV-1 IE transcripts (50).

Exon 2 of IER2.9 overlaps in antisense orientation with the latency-related transcript (61), the 3′ terminus of IER2.9 being located within the latency-related transcript promoter (44). Analogous situations have been observed for HSV-1 (reviewed in reference 67) and PRV (21, 70b). This overlap in the coding of potentially important regulatory functions calls for high nucleotide sequence conservation, especially for BHV-1, because the same region seems to encode a third, early transcript (74b). Indeed, for BHV-1 K22 (subtype 2b) (64a) and Cooper (subtype 1) (60a) the nucleotide sequence of this region, as far as it has been determined, is virtually identical. In contrast, this genomic region exhibits low conservation in other animal alphaherpesviruses; it contains multiple repeats (40) and a heterogeneous DNA sequence (8) for EHV-1 and is deleted or substituted in PRV, resulting in a growth advantage in chicken embryo fibroblasts (48).

Northern blot analysis (Fig. 2) revealed two distinct minor RNA species which overlapped with transcription unit IEtu1. The larger RNA (IER7) represents an unspliced precursor of IER2.9, and the smaller RNA (IER6.3) seems to arise by transcription over fused genome ends (74b).

A second divergent transcription unit encodes IER1.7. The map location and orientation of IER1.7 suggest possible homology with transcripts encoding ICP22 in HSV-1 and ORF63 in VZV. The last two proteins have been shown to contain a limited region of homology (28). The HSV-1

transcript for ICP22 is spliced, but the protein-coding part is located in U_S; the VZV transcript for ORF63 is encoded by IR_S and TR_S but is presumably unspliced. Thus, the layout of both transcripts differs from that of IER1.7, which consists of three exons and confines its protein coding part to IR_S and TR_S. The protein specified by IER1.7 is likely to be the recently described 52 to 57-kDa major IE phosphoprotein (42). Genomic locations corresponding to that of IER1.7 are transcribed in other animal alphaherpesviruses, but they do not seem to encode IE transcripts. EHV-1 encodes a 1.2-kb very early transcript (9, 40), whereas PRV encodes a 2.2-kb transcript later in infection (63a).

The two divergent BHV-1 IE transcription units are separated by a 3-kb sequence which does not seem to encode any significant transcripts during lytic infection (74a). This arrangement resembles a shorter intergenic genome region at a similar location on IR_S of HSV-1, which contains many regulatory sequences for transcription initiation of the divergently transcribed IE genes for ICP4 and ICP22/47 (5, 59, 73), as well as an origin for viral DNA replication (*oriS* [69]). *oriS* sequences have been identified at similar locations for EHV-1 (9), VZV (28, 68), and BHV-1 (64a). The proximity of these elements suggests that IE transcription and viral DNA replication may share some regulatory mechanisms.

The BHV-1 IE transcription pattern exhibits features of animal and human herpesviruses. The IE transcription pattern presented in this study will provide an opportunity to study regulatory functions of the three major IE gene products on selected viral and cellular genes during productive and latent infection with BHV-1. This virus seems to occupy an intermediate position in the evolution of alphaherpesviruses with regard to the number and layout of IE genes. Whereas PRV and EHV1 produce a single IE transcript encoding different related species of IE proteins, the human herpesviruses VZV and HSV-1 encode four or five IE proteins from different IE transcripts arranged similarly to the arrangement in BHV-1. If IE gene products of BHV-1 and HSV-1 shared some functional roles, BHV-1 would be an excellent model system with which to study latency and neurovirulence in its natural host in comparison with HSV-1 in humans. Contrary to HSV-1, reactivation from latency can be studied in vivo for BHV-1 by using corticosteroids in cattle or rabbits (56, 62).

The alternative splicing described here is a novel feature for IE genes of alphaherpesviruses but has been observed in betaherpesviruses. The IE genes of human (70) and murine (45) cytomegaloviruses arise in part by alternative splicing and have a layout which is reminiscent of that presented here. The beta- and alphaherpesviruses are biologically distinct, and their IE genes are probably not related by evolution (49). Nevertheless, herpesviruses classified in different subfamilies based on biological criteria may exhibit surprising similarities in gene organization, as exemplified by recent data on Marek's disease virus (14), human herpesvirus 6 (47), and human cytomegalovirus (19).

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