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Spatial and Temporal Distribution of Bovine Herpesvirus 1 Transcripts

URS V. WIRTH, KRISTIN GUNKEL, MONIKA ENGELS, AND MARTIN SCHWYZER*

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

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Northern (RNA) blot analysis was used to determine the spatial and temporal distribution of bovine herpesvirus 1 (BHV-1) transcripts. Total RNA was isolated from Madin-Darby bovine kidney cells which had been infected with BHV-1.2b strain K22 or BHV-1.1 strain Jura in the presence or absence of metabolic inhibitors. Cloned restriction fragments representing the entire genome of strain K22 were labeled with ^{32}P and hybridized to immobilized RNA. A total of 54 BHV-1 transcripts were found, ranging in size from 0.4 to larger than 8 kilobases (kb). The inverted repeat regions and an adjacent segment of the unique large part of the BHV-1 genome encoded three major immediate-early (IE) transcripts and one minor IE transcript enriched after cycloheximide treatment of infected cells. Late transcripts were identified by drastically reduced abundance after cytosine arabinoside (araC) treatment. Twelve late transcripts were encoded mainly by the unique long genome region, with a cluster of four transcripts located on *Hin*dIII fragment K (map units 0.677 to 0.733). The 21 transcripts unaffected by araC treatment were defined as early; they showed dispersed locations over the whole genome, with a cluster on the unique short sequence. The 17 remaining transcripts could not be classified unambiguously as early or late by these techniques. The IE transcript with a size of 4.2 kb exhibited homology with the single IE gene of pseudorabies virus, and the IE transcript with a size of 2.9 kb was encoded in part by the genome region known to be transcriptionally active during latency.

Bovine herpesvirus 1 (BHV-1) is an important pathogen of cattle which causes severe respiratory tract infections (infectious bovine rhinotracheitis) as well as harmless infections of the genital tract (infectious pustular vulvovaginitis) (for reviews, see references 20 and 46).

The viral genome is a linear double-stranded DNA molecule with a length of approximately 140 kilobases (kb) composed of a unique long segment (U_L; 105 kb) and a unique short segment (U_S; 11 kb); the latter is flanked by internal repeat (IR) and terminal repeat (TR) sequences of 12 kb each. The repeats enable the U_S component to invert, resulting in two isomeric forms of the BHV-1 genome (23). Figure 1 shows the prototype arrangement of the BHV-1 genome (10). On the basis of its genome structure, BHV-1 has been classified together with equine herpesvirus 1 (EHV-1), pseudorabies virus (PRV), and varicella-zoster virus (VZV) as group D (36) or class 2 (16). Herpes simplex virus type 1 (HSV-1), with two pairs of repeats, has been classified as group E or class 3.

Like other herpesviruses (37), BHV-1 exhibits regulation of viral protein synthesis in a temporal cascade reported first by Misra et al. (26), who identified 40 to 48 electrophoretically distinct proteins and classified four as immediate early (IE) and six as late. Similar experiments carried out by Metzler et al. (24, 25) showed 43 and, more recently (A. E. Metzler, habilitation thesis, University of Zurich, Zurich, Switzerland), at least 53 electrophoretically distinct proteins. A major 180-kilodalton (kDa) IE protein, six minor IE proteins (170, 135, 98, 94, 46, and 35 kDa), and at least six late proteins (260, 180, 87, 68, 22, and 20 kDa) were identified.

The IE proteins are known as important regulatory proteins for the productive cycle of herpesvirus replication (11) and may also play a role in latent infection (38). Recently, IE proteins of HSV-1 were shown to be recognized by cytotoxic T lymphocytes (22) and may be able to stimulate protective immunity, as shown earlier for murine cytomegalovirus (31). EHV-1 DNA encodes a single IE transcript of 6 kb. Surprisingly, four electrophoretically distinct, structurally related IE proteins were identified (8, 13, 34). Similarly, PRV DNA encodes a single 5.1-kb IE transcript (7, 42), and in addition to the major IE protein (17), two other IE proteins have been found (12). In contrast, HSV-1 DNA encodes five different IE transcripts and five corresponding IE proteins (43). A transcription map for BHV-1 is not available, and nothing is known about the number, sizes, and locations of the IE transcripts that encode the four to seven electrophoretically distinct IE proteins. Ample information is available on early and late BHV-1 gene products (Fig. 1C), in particular, the glycoproteins responsible for immune responses during in vivo infections, and therefore they are interesting for vaccine development (2, 41). Transcription maps have been established for HSV-1 (43), EHV-1 (14), and VZV (28, 32).

As a basis for a comparison of existing data about BHV-1 gene products, as well as for a comparison of the transcriptionally active region during latency (35) with transcripts present during productive infection, we determined the spatial and temporal distribution of 54 BHV-1 transcripts by Northern (RNA) blot analysis. The transcript sizes ranged from 0.4 to larger than 8 kb. Of these 54 transcripts, 4 were classified as IE with the protein synthesis inhibitor cycloheximide. With the DNA synthesis inhibitor cytosine arabinoside (araC), 12 transcripts were clearly determined as late and 21 were classified as early, whereas the 17 remaining transcripts could not be classified unambiguously as early or late.

MATERIALS AND METHODS

Virus and cell culture. The virus strains used in this study were BHV-1.1 strain Jura (25) and BHV-1.2b strain K22 (18). Madin-Darby bovine kidney cells were maintained in

* Corresponding author.

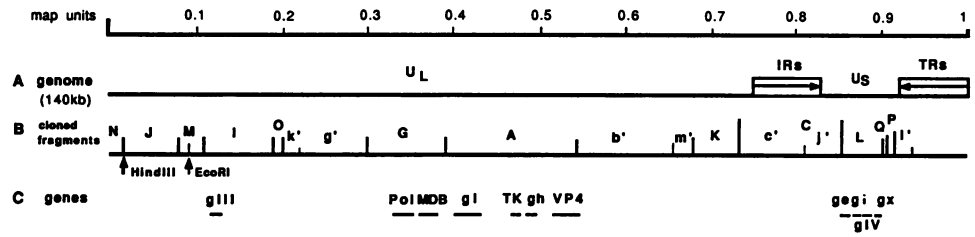

FIG. 1. Map of the BHV-1 genome. (A) U_L and U_S, and IR and TR short repeat regions are indicated. (B) Cloned *Hin*dIII or *Hin*dIII-*Eco*RI restriction endonuclease fragments of BHV-1 strain K22 or strain Jura for fragment C used in this study. (C) Genomic locations of mapped BHV-1 proteins: Pol, homolog of DNA polymerase of HSV-1 (29); gI, homolog of gB of HSV-1 (19, 45); TK, thymidine kinase of BHV-1 (3); MDB, major DNA-binding protein of BHV-1 (S. K. Bandyopadhyay, S. K. Mittal, and H. J. Field, personal communication); gh, homolog to gH of HSV-1 (E. A. Petrovskis and E. L. Post, personal communication); gIII, homolog of gC of HSV-1; VP4, homolog of VP4 of HSV-1; ge, homolog of gE of HSV-1; gi, homolog of gI of HSV-1; gx, homolog of gX of HSV-1 (T. Zamb, personal communication); gIV, homolog of gD of HSV-1 (G. Keil, personal communication).

Eagle minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum.

Infection scheme. Subconfluent cell monolayers were infected with BHV-1 at a multiplicity of infection of 10 to 20 50% tissue culture infective doses per cell or mock infected. After viral adsorption for 1 h at 37°C, Eagle minimum essential medium with 2% fetal bovine serum was added and the cells were incubated at 37°C until harvesting of RNA within 2 to 8 h postinoculation (p.i.). Metabolic inhibitors were used essentially as already described (26). To identify IE transcripts, cycloheximide (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 50 μg/ml for strain K22 was added with the inoculum and again after the adsorption period with the medium; RNA was isolated at 6 h p.i. Strain Jura required 100 μg/ml for complete inhibition of early and late transcription. To identify late transcripts dependent on DNA synthesis, araC (Sigma) was added after adsorption at a concentration of 50 μg/ml, as described for protein studies of BHV-1 (26), or 100 μg/ml, and RNA was isolated at 8 h p.i. At both concentrations, [^{3}H]thymidine (5.0 Ci/mmol; Amersham Corp., Amersham, Buckinghamshire, England) incorporation into acid-insoluble material between 6 and 8 h p.i. was reduced about 85% by araC treatment compared with untreated cells, and similar Northern blot results were obtained; thus, only results of experiments with the higher concentration are shown.

Isolation of DNA and RNA. Cloned *Hin*dIII or *Hin*dIII-*Eco*RI restriction fragments of BHV-1.2b strain K22 (Fig. 1) described previously (10) were used as hybridization probes. Plasmids were isolated with Triton lysis buffer and CsCl gradients containing ethidium bromide as described by Davis et al. (9). Total RNA was isolated by lysis of cells with guanidine isothiocyanate and centrifugation through a CsCl cushion as already described (9, 30). RNA concentrations were determined spectrophotometrically; the integrity and concentration of isolated RNA were verified by agarose gel electrophoresis. RNA preparations were stored as ethanol precipitates at −70°C.

Northern blot analysis. Northern blot analysis was performed essentially as previously described (21), with minor modifications recommended by the manufacturer of the nylon membranes used (Biodyne A BNNG 3R; Pall Ultrafine Filtration Corp., Glen Cove, N.Y.). Portions (450 μg) of isolated RNA preparations were heated at 55°C for 15 min in electrophoresis buffer (20 mM morpholinepropanesulfonic acid [MOPS], 5 mM sodium acetate, 1 mM EDTA [pH 7.0]) containing 50% (vol/vol) formamide and 6% (vol/vol) formaldehyde. After quick cooling on ice and addition of 5× loading buffer (15% Ficoll type 400, 0.1% bromophenol blue in 0.1 M EDTA [pH 7.5]), the RNA was loaded into a single slot (18 cm) spanning the whole width of a 1.2% agarose gel in electrophoresis buffer containing 6% (vol/vol) formaldehyde. In some experiments, agarose gels with 22 slots were used and 5 to 10 μg of total RNA was loaded per slot. After electrophoresis, the RNA was transferred to a nylon membrane presoaked in water by capillary action with 20× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate [pH 7.0]) as the transfer medium. One edge of the membrane was aligned with the slot to facilitate subsequent identification of the migration origin. After transfer, RNA was UV cross-linked to the membrane by exposition for 2 min on a C-51 mineral light transilluminator (Ultra-Violet Products, Inc., San Gabriel, Calif.). The membrane was baked for 2 h at 80°C under vacuum and, for experiments with single slots, cut into 4-mm strips which were marked with a pencil at the bottom. Mobility of 28S and 18S rRNA was determined for each gel run by staining flanking strips with methylene blue. Sizes of bovine rRNA were determined as 4.6 kb for 28S and 2.0 kb for 18S by comparison with the mobility of rat liver rRNA with a known size (27). Sizes of transcripts and rRNAs were additionally verified by using RNA molecular weight markers with a ladder between 0.3 and 7.4 kb (Boehringer Mannheim Biochemicals, Mannheim, Federal Republic of Germany). Plasmid DNA was labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham) by nick translation to a specific activity of approximately 10^8 cpm/μg as described by Rigby et al. (33). Membrane strips carrying different RNA preparations were prehybridized, hybridized, and washed in 50-ml Falcon tubes in a water bath with constant agitation at 250 rpm. Prehybridization was performed at 42°C for 6 h with prehybridization buffer containing 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA [pH 7.4]), 5× Denhardt solution (1× Denhardt solution is 0.2% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone), 50% formamide, 0.1% sodium dodecyl sulfate, and 0.2 mg of sonicated and heat-denatured calf thymus DNA per ml. For hybridization, heat-denatured ^{32}P-labeled plasmid DNA (5×10^5 cpm/cm^2 of nylon membrane) was added to fresh prehybridization buffer (0.1 ml/cm^2) and incubated for 40 h at 42°C. After hybridization, strips were washed with 0.2% sodium dodecyl sulfate–5 mM sodium phosphate–1 mM EDTA (pH 7.0) once at room temperature and twice at 42°C for 1 h each time (1 ml/cm^2). Wet strips were arranged in a defined order between sheets of Saran Wrap and autoradiographed on Fuji RX films at room temperature.

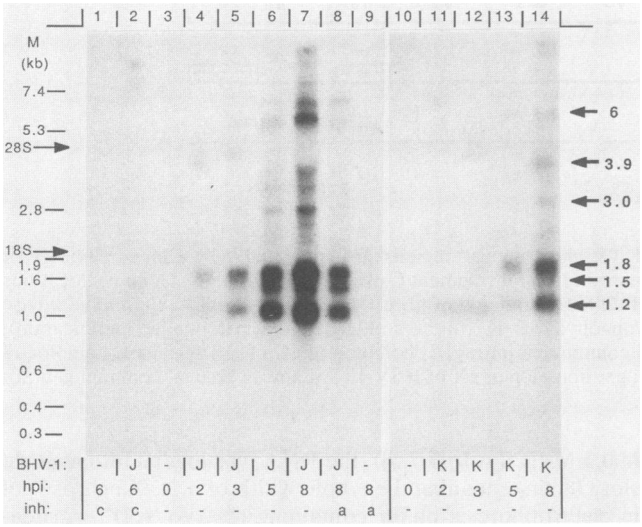

FIG. 2. Northern blot example showing hybridization of labeled fragment J (Fig. 1) to RNA isolated under different conditions. Madin-Darby bovine kidney cells were either mock infected (−) or infected with BHV-1.2b strain K22 (K) or BHV-1.1 strain Jura (J). Total RNA was isolated at the indicated times (hours p.i. [hpi]) and blotted (10 μg per lane) as described in Materials and Methods. As inhibitors (inh), cycloheximide (c) was added at 50 μg/ml for strain K22 or at 100 μg/ml for strain Jura from the time of infection and araC (a) was added after the adsorption period at 100 μg/ml. Autoradiograph exposure time was 1 day for lanes 1 to 9 and 4 days for lanes 10 to 14. RNA size markers (M) are indicated on the left, and calculated transcript sizes are on the right.

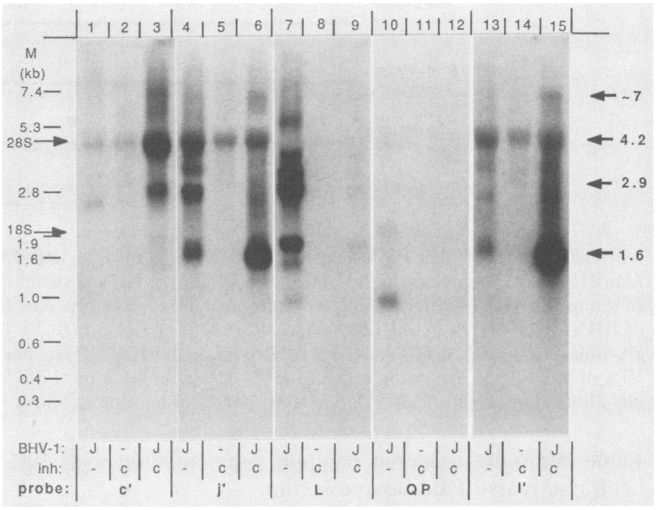

FIG. 3. BHV-1 IE transcripts enriched by cycloheximide. Indicated labeled BHV-1 fragments (c', j', L, QP, and l') were hybridized to 5 μg of total RNA per lane which was isolated at 6 h p.i. from cells infected with strain Jura (J) or mock-infected cells (−) treated with (c) or without (−) cycloheximide. Autoradiograph exposure time was 10 h. RNA size markers (M) are indicated on the left, and calculated sizes of IE transcripts are on the right.

RESULTS

Hybridization probes derived from BHV-1. Figure 1 shows a map of the cloned *Hin*dIII fragments (capital letters) and *Hin*dIII-*Eco*RI fragments (lowercase letters) of the BHV-1.2b strain K22 used in this study (10). Recombinant plasmids were labeled and used for hybridization without removing the vectors. Control experiments with vectors alone produced no signals after 6 days of autoradiographic exposure with any of the RNA preparations used (data not shown). The same probes from strain K22 were used for hybridization to BHV-1.1 strain Jura transcripts. This was considered legitimate because of the high degree of homology (95%) between the BHV-1.1 and BHV-1.2 strains (39). Identical results were obtained when the cloned *Hin*dIII fragment C of BHV-1.1 strain Jura was substituted for the corresponding fragments c' and j' of strain K22 as a probe. *Eco*RI fragment F on the right-hand end of the genome (10) was not available as cloned, but this part of the sequence is covered by the IR part of *Hin*dIII-*Eco*RI fragment c'.

Strategy used to establish the BHV-1 transcription map. Total RNA was isolated from cells infected with BHV-1 (strain K22 or Jura) or from mock-infected cells at different times between 2 and 8 h p.i.; in parallel experiments, metabolic inhibitors were used to distinguish the different temporal classes of transcripts. The protein biosynthesis inhibitor cycloheximide causes accumulation of IE transcripts, and as a consequence, synthesis of early and late transcripts which are expressed only in the presence of IE proteins is not turned on. The DNA synthesis inhibitor araC should abolish late transcripts which depend on viral DNA synthesis for efficient initiation. After electrophoresis in agarose gels containing formaldehyde, RNA samples were blotted on nylon membranes, hybridized with the DNA probes described above, and detected by autoradiography.

As an example, Fig. 2 shows hybridization of fragment J (map units 0.017 to 0.083) to selected RNA preparations run on a single gel. Lanes with RNA of mock-infected cells with or without inhibitors, included in each hybridization, were blank like lanes 1, 3, 9, and 10 (one exception will be shown below). For determination of transcripts encoded by BHV-1, only bands yielding distinct and reproducible signals in several experiments with both virus strains were considered. Thus, six viral transcripts (1.2, 1.5, 1.8, 3.0, 3.9, and 6 kb) were assigned to fragment J. Additional bands, e.g., those in lane 7, did not meet these criteria and were not included, although some may represent minor BHV-1 transcripts. The 1.2- and 1.8-kb transcripts encoded by fragment J appeared very early in infection, at about 2 h p.i. (lane 4), but they were not IE transcripts, because lane 2 had no corresponding signals that arose from transcripts which were enriched by cycloheximide treatment (see Fig. 3). The other transcripts appeared later in infection, at about 5 h p.i. (lanes 5 to 7); two of these transcripts, with sizes of 3.0 and 6 kb, showed drastically reduced signal intensity after araC treatment (lane 8) compared with untreated cells (lane 7) and were therefore defined as late. Transcripts of strain Jura generally appeared much earlier and with higher abundance at a given time than the corresponding transcripts of strain K22. For example, the two transcripts with sizes of 1.2 and 1.8 kb gave a clear signal at 3 h p.i. for strain Jura (lane 5) but were barely detectable at 3 h p.i. for strain K22 (lane 12) despite a fourfold-increased exposure time. Apart from this general delay in the appearance of transcripts, equivalent results were obtained with both strains. Therefore, only Northern blots derived from strain Jura are shown below.

IE transcripts of BHV-1. Figure 3 shows RNA from cycloheximide-treated BHV-1-infected cells together with control RNA preparations probed with fragments from the right-hand end of the genome. Cycloheximide inhibited the synthesis of early and late transcripts, as demonstrated, for example, by fragment L, a region encoding abundant tran-

TABLE 1. Spatial and temporal distribution of BHV-1 transcripts

Probe	Transcript sizes (kb)[a]
N	1.1,[b] 1.7,[c] (3.0),[bg] (~7)[dh]
J	**1.2**, (1.5),[c] **1.8**, (3.0),[bg] (3.9), (6)[b]
M	**2.7**,[i] **4.5**[bj]
I	**1.4**, **2.7**,[i] **3.7**,[ck] (4.5)[bj]
O	1.7,[cl] **3.7**[ck]
k′	1.7,[cl] (3.9), 8[bm]
g′	(4.0),[cn] 8[bm]
G	(0.4),[b] 1.7[c] 4.0,[cn] (4.3),[c] (7.5)[b]
A	(1.4),[c] 3.4,[c] 3.9,[c] (5)[o]
b′	(1.3),[c] (1.8), (3.8),[c] (5)[o]
m′	(1.5),[c] (3.5), (4.0), (6–7)[bp]
K	0.5,[b] (1.1),[b] (1.7), **2.5**,[c] **3.2**,[c] 4.1,[b] 6–7[bp]
c′	2.6, 2.9,[d] 4.2,[d] (~7),[dh] (>8)[bq]
j′	**1.6**,[dr] (2.9),[s] 3.0,[ct] 3.6,[u] 4.0,[v] (4.7),[cw] (~7),[dh] (>8)[bq]
L	(1.0),[cx] (1.5), **1.8**,[c] (2.1),[cy] **3.0**,[ct] **3.5**,[c] 4.0,[v] (5.6)
QP[f]	1.0,[cx] 2.1,[cy] (4.7)[cw]
l′	**1.6**,[dr] (2.9),[s] (3.6),[u] (4.7),[cw] (~7),[dh] (>8)[bq]

[a] Bold letters indicate transcripts of highest abundance, and parentheses indicate those of lowest abundance. For transcripts with superscripts *g* to *y*, those with the same superscript may be presumed to cross fragment boundaries or to arise from repeated sequences.
[b] Late transcript drastically reduced by araC.
[c] Early transcript not significantly reduced by araC treatment.
[d] Immediate-early transcript enriched by cycloheximide treatment.
[e] 1.8 kb for strain K22.
[f] Identical results for both BHV-1 fragments.

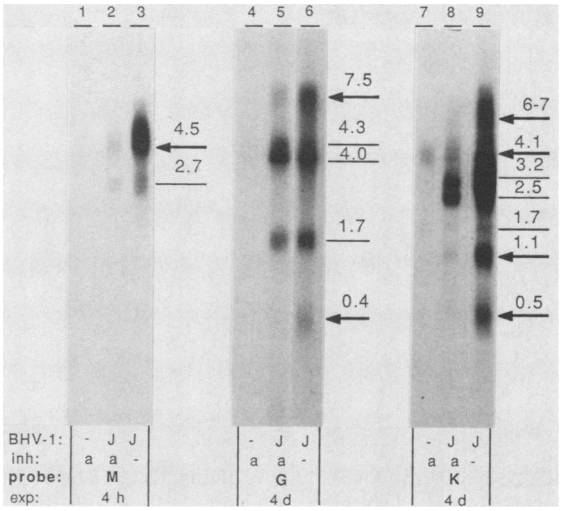

FIG. 4. Examples of BHV-1 late transcripts. Indicated labeled BHV-1 fragments (M, G, and K) were hybridized to 10 μg of total RNA per lane from araC-treated (a) or untreated (−) cells infected with strain Jura (J) or mock-infected cells isolated at 8 h p.i. Identical strips were used for each condition. Sizes of transcripts classified as late are indicated by arrows, and others are indicated by thin lines. Autoradiograph exposure time (exp) is indicated for each probe in hours (h) or days (d).

scripts in the absence of cycloheximide (lane 7) but lacking virtually any detectable signals in its presence (lane 9). All transcripts enriched by cycloheximide can therefore be interpreted as IE transcripts whose expression is independent of protein biosynthesis.

Fragment c′ encoded two major IE transcripts of 4.2 and 2.9 kb (Fig. 3, lane 3). Fragment j′ encoded one major IE transcript which exhibited a size of 1.6 kb for strain Jura (lane 6) and 1.8 kb for strain K22 (data not shown). This was the only significant transcript size difference observed between the two strains. Fragments j′ and l′ gave rise to identical signals (lanes 6 and 15), indicating the main location of the 1.6-kb transcript on the repeats. RNA from mock-infected cells showed a weak broad signal coinciding with bovine 28S rRNA, particularly when fragments j′, l′, and, to some extent, c′ were used as probes. This background signal may be due to cross-hybridization of these regions of the genome with rRNA as has been observed for human herpesviruses (40). Therefore, the band that appeared at around 4.6 kb in lanes 6 and 15 was not interpreted as an IE transcript. In addition to the three major IE transcripts, a minor, sometimes diffuse band was regularly detected at about 7 kb with all three probes (c′, j′, and l′; see also Fig. 6). All IE transcripts except the 7-kb species were detected at 2 h p.i. in cells not treated with cycloheximide (data not shown). Their signals were faint, and cycloheximide caused an estimated 10- to 30-fold enrichment. At 6 h p.i. in the absence of cycloheximide, only a minor 2.6-kb transcript was detected with fragment c′ (lane 1), whereas fragments j′ and l′ encoded several major transcripts not belonging to the IE class (lanes 4 and 13).

Other BHV-1 DNA fragments did not give a signal with cycloheximide treatment, except for fragment N, which showed a 7-kb transcript as just described. Complete data concerning IE transcripts are summarized in Table 1.

Late transcripts of BHV-1. For classification as late transcripts, drastic reduction or abolishment of the corresponding transcript by araC treatment was demanded. Figure 4 shows late transcripts encoded by fragments M, G, and K as examples. Fragment M encoded two transcripts with apparent sizes of 2.7 and 4.5 kb. Only the latter was reduced sufficiently by araC treatment to be interpreted as a late transcript. Of the five transcripts encoded by fragment G, the 7.5-kb transcript was drastically reduced and the 0.4-kb transcript was absent after araC treatment, justifying classification as late transcripts. Of the seven transcripts encoded by fragment K, two were drastically reduced (4.1 and 1.1 kb) and two were completely abolished (0.5 and 6 to 7 kb) by araC treatment and therefore classified as late transcripts. Twelve transcripts were considered to fall into this category (Table 1).

Early transcripts of BHV-1. All transcripts which did not meet the criteria discussed above for IE or late transcripts were considered to be potential early transcripts. Transcripts which were only slightly reduced by araC treatment (e.g., the 1.7-kb transcript encoded by fragment G [Fig. 4]) or not reduced at all (e.g., the 4.0- and 4.3-kb transcripts encoded by fragment G [Fig. 4]) were classified as early. Twenty-one transcripts (Table 1) were placed in this category. The remaining 17 transcripts (Table 1) exhibited intermediate sensitivity to araC and could not be classified unambiguously as early or late. The 1.5-kb transcript in Fig. 2, for example, was classified as early and the 6-kb transcript was defined as late, whereas the 1.2- and 1.8-kb species did not fit either category.

Figure 5 shows a particular RNA preparation obtained under conditions of early transcription (araC treatment), electrophoresed in a single gel, blotted, cut into identical strips, and hybridized with the entire set of BHV-1 DNA fragments. This approach is optimal for identifying transcripts which cross fragment boundaries. For reasons discussed above, IE transcripts with continued expression until late times of infection (e.g., the 1.6-kb transcript encoded by fragments j′ and l′) and late transcripts not completely abolished by araC treatment (e.g., the 4.5-kb transcript

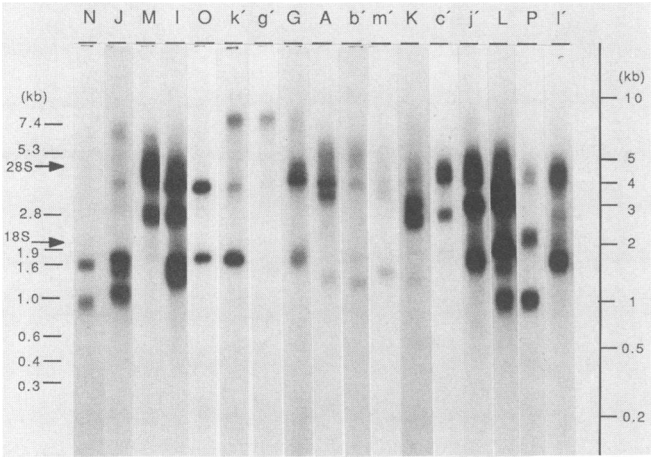

FIG. 5. Northern blot analysis of the indicated labeled BHV-1 fragments (N to l') hybridized to identical strips with RNA from cells infected with strain Jura and treated with araC (100 μg/ml). RNA size markers are indicated on the left, and a deduced scale is on the right. Autoradiograph exposure time was 4 days for all strips.

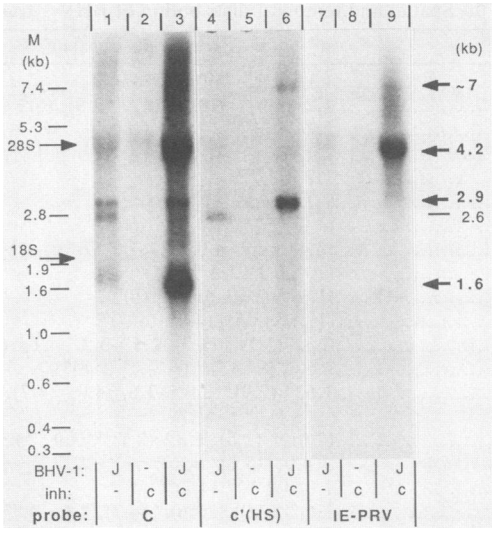

FIG. 6. Hybridization of indicated labeled DNA fragments C (c' and j'), c' (HS) (*Hin*dIII-*Sal*I subfragment of fragment c'; map units 0.734 to 0.748), and IE-PRV (*Bam*HI fragment 8 of PRV; 17) to 5 μg of total RNA (per lane) of cells infected with strain Jura (J) or mock infected and treated with (c) or without (−) cycloheximide and isolated at 6 h p.i. Autoradiograph exposure time was 4 days. RNA size markers (M) are indicated on the left, and calculated sizes of transcripts are on the right.

encoded by fragment M) also appear in Fig. 5. Figure 5 conveys an overview of the great diversity of BHV-1 transcripts with regard to size, intensity, and spatial distribution. The intensities of transcript signals encoded by fragments J, M, and I (map units 0.017 to 0.188) and fragments j' and L (map units 0.811 to 0.904) indicate the highest abundances of transcripts encoded by these regions.

Examination of the RNA strips from BHV-1-infected cells between 2 and 8 h p.i. probed with all of the cloned BHV-1 fragments revealed that almost every transcript followed its individual kinetics. These results are not presented but are illustrated by Fig. 2.

Spatial and temporal distribution of BHV-1 transcripts. Table 1 shows a list of all transcripts determined in this study and expressed during lytic infection of Madin-Darby bovine kidney cells. Data about the number, sizes and locations of transcripts were collected by comparing and summarizing all of the results relating to temporal appearance (exemplified in Fig. 2) and spatial distribution (exemplified in Fig. 5). Transcripts with similar sizes encoded by the same fragment at different times (Fig. 2) were presumed to be identical, and size values were averaged. Transcripts with similar sizes and appearances (e.g., diffuse or discrete bands) encoded by neighboring fragments were presumed to cross fragment boundaries.

Temporal classification of transcripts as IE, early, or late was done as long as it was unambiguous with respect to the criteria discussed above. Three highly abundant transcripts that accumulated during cycloheximide treatment were classified as major IE transcripts. The minor 7-kb signal that arose from fragments c', j', and N may originate from a single IE transcript detected by partially homologous sequences within these fragments (see Discussion). Generally, the IE transcripts were clustered around the IRs and TRs represented by fragments c', j', and l' (Fig. 1). Twelve transcripts were classified as late by araC treatment. Late transcripts were encoded mainly by the U_L sequence of the genome; four of them were clustered on fragment K (map units 0.677 to 0.733). Many of the smallest and largest transcripts belonged to the late class. Among the 38 transcripts which could not be classified as IE or late, 21 were considered early transcripts, whereas 17 transcripts remained unassigned. The early transcripts showed dispersed locations over the entire genome, with a cluster on the U_S genome region. Thus, a total of 54 transcripts, ranging in size from 0.4 to larger than 8 kb, were mapped.

Further characterization and localization of two BHV-1 IE transcripts. A plasmid containing *Bam*HI fragment 8 of the PRV IE protein-coding sequence (17) was labeled and hybridized to blotted RNA from cycloheximide-treated cells. This probe hybridized specifically with the 4.2-kb IE transcript of BHV-1 encoded by fragment c' (Fig. 6, lanes 9 and 3). Partial sequence homology between IE genes of BHV-1 and PRV was also inferred from Southern and dot blots exhibiting cross-hybridization at the genome level (data not shown). A *Hin*dIII-*Sal*I subfragment (map units 0.734 to 0.748) of BHV-1 fragment c' had been shown to be transcriptionally active during latency in the BHV-1 rabbit model by in situ nucleic acid hybridization (35). This *Hin*dIII-*Sal*I fragment was subcloned, labeled, and hybridized to blotted RNA from cycloheximide-treated cells. The probe detected the 2.9-kb IE transcript encoded by fragment c' (lanes 6 and 3). In untreated cells, however, the 2.9-kb IE transcript was not detected by this probe; instead a 2.6-kb early transcript was revealed (lane 4). The same blot was subsequently reprobed with the entire *Hin*dIII fragment C of strain Jura (Fig. 1) for comparison (lanes 1 to 3). The 2.9-kb transcript detected by probe C in untreated cells (lane 1) is encoded by fragment j' (Fig. 3, lane 4) and does not belong to the IE class (Table 1). This transcript is not related to the 2.9-kb IE transcript revealed in lanes 3 and 6.

DISCUSSION

We mapped 54 transcripts expressed during productive infection of BHV-1 by Northern blot hybridization with ^{32}P-labeled cloned fragments of the viral genome. On the basis of analyses using cycloheximide as the inhibitor of translation, we identified three major transcripts and one

minor IE transcript encoded by the repeats and some adjacent unique sequences. The IE proteins observed by A. E. Metzler (see above) were not assigned to individual IE transcripts, and the precise locations and orientations of the transcripts remain to be determined. A similar approach has been used to map the transcripts of EHV-1 (8, 13, 14). The researchers found a single 6-kb transcript and four structurally related proteins under IE conditions. Similar findings have also been reported for PRV (5, 12), which possesses the same genomic structure as BHV-1 and EHV-1. The IE proteins of BHV-1, however, are encoded by more than one IE transcript, like HSV-1 (43), despite the different genomic structures of these viruses. Generally clustering of IE transcripts around repeats can be observed for all alphaherpesviruses examined so far (7, 13, 14, 17, 28, 32, 43). Preliminary results (data not shown) indicate that the 4.2-kb IE transcript of BHV-1 is encoded by a segment of IRs that borders upon U_L (TRs were not tested). An equivalent genomic location has been reported for the 4.2-kb transcript of HSV-1 encoding IE infected-cell protein 4 (43), as well as for the unique IE transcripts of EHV-1 (13, 14) and PRV (7, 42). In addition, the coding region of the latter has been found to cross-hybridize with the 4.2-kb IE transcript of BHV-1 (Fig. 6). A similar cross-hybridization between HSV-1 DNA sequences that encode IE infected-cell protein 4 and PRV DNA sequences that encode the 180-kDa IE protein has been described (6). Transfection experiments showed that gene products encoded by the corresponding segments of BHV-1 and PRV were both capable of *trans*-activating a simian virus 40 promoter; preliminary sequence data revealed a well-conserved domain at the amino acid level, indicating the same orientation of the corresponding transcripts of BHV-1, PRV, HSV-1, and VZV (O. Menekse and M. Schwyzer, unpublished data). The size of the major BHV-1 IE protein (180 kDa) corresponds well to the 180-kDa IE protein of PRV (5, 17) and the 175-kDa IE infected-cell protein 4 of HSV-1 (43). These data thus suggest that the 4.2-kb IE transcript of BHV-1 codes for a structural and functional analog of the PRV IE protein, which itself has strong amino acid sequence homology with infected-cell protein 4 of HSV-1 and p140 of VZV (42).

The U_L part of BHV-1 fragment c′, which is the region transcribed during latency (35), hybridized only to the 2.9-kb IE transcript (Fig. 6). T. Mainprize, G. Kutish, and D. L. Rock (personal communication) determined a similar 2.9-kb polyadenylated transcript in BHV-1.1 strain Cooper-infected and cycloheximide-treated bovine lung cells for the same region. Fragments j′ and l′, which span the borders between the repeats and U_S, hybridized both to an IE transcript of 1.6 kb for strain Jura and a 1.8-kb IE transcript for strain K22. This strain difference may be related to the observed size heterogeneities of IRs and TRs near the junction with U_S (46). The minor 7-kb IE transcript detected by fragments that also encode the major IE transcripts (Fig. 3) might represent a precursor to some of the shorter IE transcripts. Surprisingly, an apparently identical 7-kb transcript from cycloheximide-treated cells was detected with fragment N (2.4 kb) from the left end of the genome (Table 1) but not with the adjacent fragment J (Fig. 2). Homologous sequences between fragments N and c′ were found by sequence analysis (15) and might cause cross-hybridization.

Of the 12 defined late transcripts of BHV-1, 4 were clustered on fragment K (map units 0.677 to 0.733), corresponding to a map position known for many late transcripts of HSV-1, and as in HSV-1, no late transcripts were found to be encoded by the U_S genome region (43). Six BHV-1 late transcripts appeared in high abundance, and six appeared in low abundance. Late transcripts showed great diversity in size; four were very short (0.4, 0.5, 1.1, and 1.1 kb), and six were very long (>8, 8, 7.5, 6 to 7, 6, and 4.5 kb). The six late BHV-1 proteins identified by A. E. Metzler (see above) showed similar characteristics, with molecular masses of 260 kDa for the largest one and 22 and 20 kDa for the smallest ones. The 0.4- and 0.5-kb late transcripts may have approximately the coding capacity of the late proteins of 20 and 22 kDa if they are assumed to be glycosylated. The 4.5-kb late transcript encoded by fragments M and I is one of the most abundant transcripts of BHV-1. Its characteristics may be appropriate for the BHV-1 gIII protein, a major late protein that has been mapped on fragment I (Fig. 1). The lack of complete reduction of signals observed for some late transcripts after araC treatment might be due to incomplete inhibition of DNA synthesis (see Material and Methods), but this would be surprising in view of published data indicating abolishment of progeny virus at even lower araC concentrations (1).

Among the 38 transcripts not classified as IE or late by inhibitors, 21 were identified as early. The remaining 17 transcripts exhibited intermediate sensitivities to araC and could not be assigned to the early or late class by the techniques used. It must be kept in mind that Northern blot analysis provides only a measurement of the relative abundance of transcripts, which is determined in turn by transcription rate and mRNA stability. For HSV-1, late transcripts which were detectable before viral DNA synthesis were defined as a subclass termed "early-late" (43). However, Northern blot analysis by Weinheimer and McKnight (44) revealed significant inhibition by phosphonoacetic acid of two transcripts which had been defined as early (43) and on the other hand, incomplete inhibition of two transcripts which had been defined as early-late (43). Zhang and Wagner (47) demonstrated that transcription rates of early-late and late genes did not decline significantly at late times of infection, in contrast to those of early genes. Until such detailed studies are performed for BHV-1, final classification of the remaining 17 transcripts or subdivision into more than three temporal classes does not seem to be justified. In any event, the numbers of 21 early, 12 late, and 17 unassigned transcripts found for BHV-1 fall within the range observed with other alphaherpesviruses, namely, 20 early and 44 late (including early-late) transcripts for HSV-1 (43) or 41 to 45 early and 18 to 20 late transcripts for EHV-1 (14).

The total of 54 BHV-1 transcripts described in this study compares favorably with the number of BHV-1 proteins determined by Misra et al. (26) and A. E. Metzler (see above), but several factors could have led to overestimates or underestimates of the number of transcripts. Specifically, minor RNA species may have escaped detection, comigrating RNAs with similar sizes cannot be differentiated, interpretation of transcripts that cross fragment boundaries may be ambiguous, different transcripts may share coding sequences, different transcript sizes may arise by splicing or from different 5′ and 3′ ends, and a few weak signals not regularly reproduced or not present for both strains were neglected (see, e.g., Fig. 2). Accordingly, for VZV, 33 minor transcripts were not included in the final transcription map (32), and for HSV-1, eight novel RNA species were found recently by closer examination of the *Bam*HI B DNA fragment (4).

Figure 1C shows the genomic locations of previously mapped BHV-1 gene products. Tentative correlations between available data on mapped gene products and tran-

scripts determined in this work are possible. This is illustrated by the fact that the *Hin*dIII A fragment encoded four transcripts with sizes of 1.4, 3.4, 3.9, and 5 kb. Two subclones of the *Hin*dIII A fragment (map units 0.388 to 0.455) covering the location of the mapped BHV-1 gI protein hybridized only to the early 3.9-kb transcript (data not shown). For BHV-1 thymidine kinase, an early protein encoded by the *Hin*dIII A region, S. Kit (personal communication) determined a transcript length of 1.3 kb, which would fit well with the 1.4-kb early transcript determined in this work. E. A. Petrovskis and L. E. Post (personal communication) determined the location and sequence of a BHV-1 protein homologous to protein gH of HSV-1. Deduced from initiation and termination signals in the sequence, this gene is probably transcribed from a 2.7-kb long sequence which may correspond to a 3.4-kb [putative poly(A) tail included] transcript determined in this work. On the junction of the *Hin*dIII A and *Hin*dIII-*Eco*RI b′ fragments, T. Zamb (personal communication) has localized a region homologous to the VP4 sequence of HSV-1; this protein might be encoded by the 5-kb transcript detected by these fragments.

The transcription map presented provides a basis for further examination of genes and gene products that regulate lytic and latent infections or elicit immune responses in hosts. Selected genes may be mutated in vitro and recombined into the virus to study their functions. Some recombinant viruses may prove useful as vaccine strains with the advantage that they may be unequivocally distinguished from challenge virus in protection studies.

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