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Characterization of the Latency-Related Transcriptionally Active Region of the Bovine Herpesvirus 1 Genome

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Approximate 5′ and 3′ ends of the bovine herpesvirus 1 (BHV-1) latency-related RNA (LR RNA) were mapped in rabbit trigeminal ganglia (TG) by in situ hybridization. The data provide a size estimate of 0.77 to 1.16 kb for the LR RNA. An LR RNA mapping to a similar location was also detected in TG of cattle latently infected with BHV-1. The BHV-1 LR region is transcriptionally active in bovine cell cultures lytically infected with BHV-1. A 1.15-kb transcript, present at early and late times postinfection, of the same sense and approximate size as that seen in latently infected TG overlaps a 2.9-kb immediate-early and a 2.6-kb early and late transcription unit present on the complementary strand. Sequence analysis of the LR RNA sense strand indicates the presence of a potential polymerase II promoter in close proximity to the 5′ terminus of the LR RNA and two open reading frames within its map positions. The complementary strand contains the 3′ portion of a large open reading frame that almost completely overlaps the map position of the LR RNA present on the opposite strand.

Bovine herpesvirus 1 (BHV-1) remains a significant viral pathogen of cattle, playing a major role in bovine respiratory disease and abortion (14). BHV-1, like other members of the alphaherpesvirus group, establishes reactivatable latent infections in neurons of sensory and autonomic nerve ganglia (8, 9, 22, 28, 29). This pathogenic mechanism is, in all likelihood, solely responsible for the perpetuation and transmission of infection in cattle and must be taken into account when considering control strategies for this viral disease (16, 29). In prior work, aimed at investigating the molecular basis of latent BHV-1 infection, we have used a rabbit latency model to study viral transcription during latent infection (23, 25). Latency-related (LR) transcription was found to be restricted to a 1.9-kb region of the viral genome (map units [m.u.] 0.734 to 0.748) at the left end of *Hin*dIII fragment D (23).

In this report, we further characterize this LR transcriptionally active region (TAR) of the BHV-1 genome, including (i) fine mapping of the LR RNA in rabbit and bovine trigeminal ganglia (TG), (ii) transcriptional analysis of the region in lytically infected bovine cell cultures, and (iii) DNA sequence analysis of the region.

MATERIALS AND METHODS

Cells and virus. Bovine turbinate or bovine lung cells were used for these experiments and maintained as described previously (27). The Cooper strain of BHV-1 as supplied by the National Veterinary Services Laboratory, Ames, Iowa, and used for infection of tissue culture cells at passages 3 to 6 and for animal inoculation at passage 6.

Isolation of RNA from BHV-1-infected tissue culture cells. Total cellular RNA was isolated at various times postinfection (p.i.) from bovine turbinate or bovine lung cells infected with BHV-1 at 10 PFU per cell. For the preparation of immediate-early (IE) RNA, cells were pretreated with cycloheximide (50 μg/ml) 30 min before and throughout infection. RNA extraction was by the guanidinium-cesium chloride procedure (19). Cell pellets were lysed in 6 M guanidinium isothiocyanate–0.5% Sarkosyl–0.1 M β-mercaptoethanol, layered over a 7.5 M CsCl cushion, and centrifuged at 50,000 rpm in an SW50.1 rotor for 18 h at 20°C. The RNA pellet was dissolved in H_2O and ethanol precipitated.

RNA filter hybridization. RNA (10 to 20 μg) was denatured for 60 min at 50°C in a solution containing 10 mM $NaHPO_4$ (pH 7.0) and 2 M glyoxal, separated in 1% agarose gels, and transferred to nitrocellulose (21). Blots were hybridized with single-stranded recombinant M13 bacteriophage DNA radiolabeled by primer extension with [^{32}P]dCTP (3,000 Ci/mmol) to a specific activity of 2×10^8 to 5×10^8 cpm/μg (10). Hybridization was for 36 h at 42°C with approximately 2.5 ng of probe per ml in a hybridization solution containing 10% dextran sulfate, 1× Denhardt solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM $NaHPO_4$ (pH 7.0), 0.1% sodium dodecyl sulfate, and 100 μg of denatured salmon sperm DNA per ml. After hybridization, the nitrocellulose filters were washed in 2× SSC–0.1% sodium dodecyl sulfate followed by 0.1× SSC–0.1% sodium dodecyl sulfate at 65°C. Dried filters were exposed to Kodak XAR-5 film at −70°C for 1 to 3 days.

DNA sequence analysis. Sequencing was performed by the dideoxy-chain termination procedure of Sanger et al. (27a) by a continuous, on-line sequencing method with oligodeoxynucleotide primers with multiple fluorophores (2). In all cases, overlapping sequence information was obtained for all fragments used in the sequencing strategy and the entire sequence was verified by complete analysis of both strands. Sequence data were compiled and analyzed by using the analysis programs of the University of Wisconsin Genetics Computer Group and Staden (31–33).

Animal infections. Lightly anesthetized New Zealand White rabbits (2 to 3 kg) were inoculated in the right and left conjunctival sacs with 10^7 PFU of BHV-1 as described previously (27). TG were removed from latently infected rabbits at 30 to 80 days p.i. Five 6- to 8-month-old Hereford calves were inoculated by the conjunctival and intranasal route with 10^7 PFU of BHV-1, and TG were removed from

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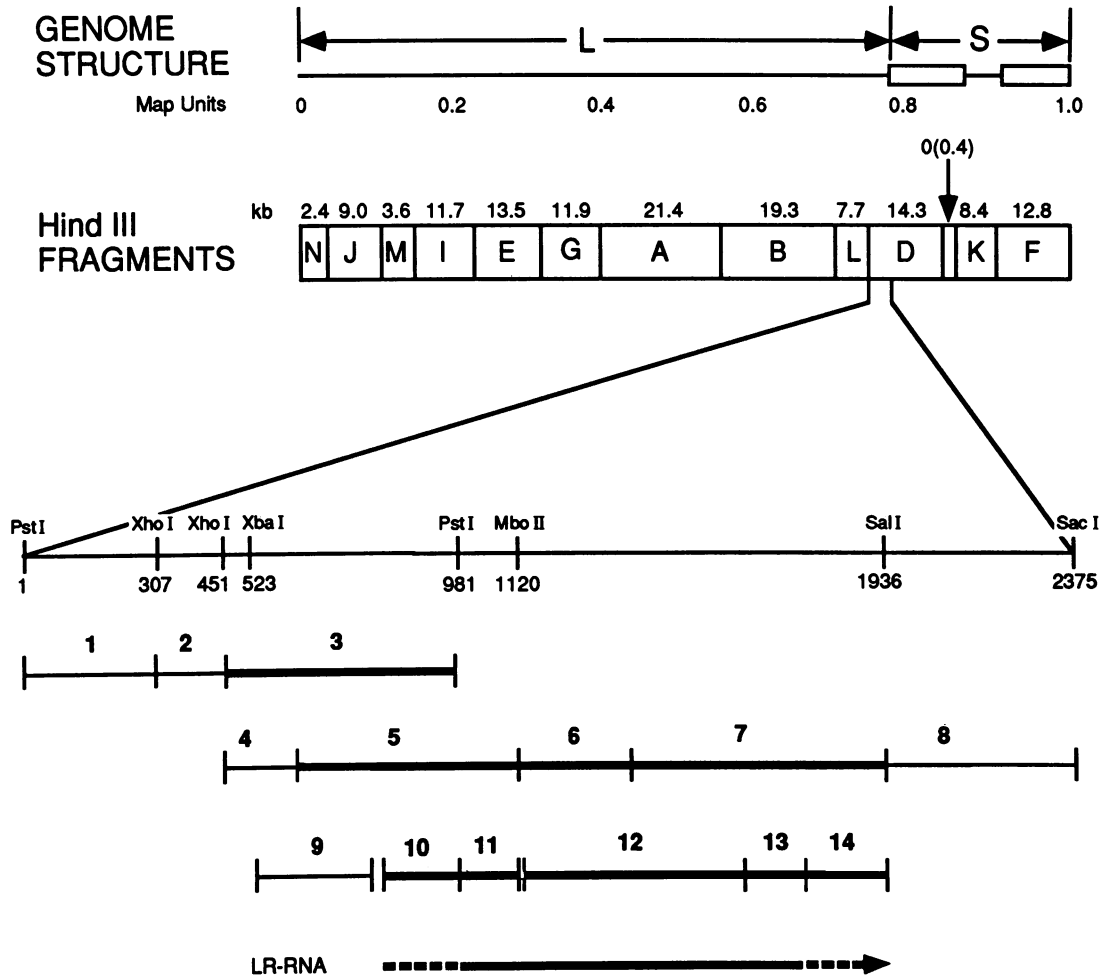


FIG. 1. BHV-1 DNA (Cooper strain). This linear double-stranded molecule of approximately 136 kb consists of two unique sequence regions, the smaller of which is bounded by inverted repeat sequences (20). Probes 1 to 14 used in fine mapping the LR RNA are shown under an expanded map of the BHV-1 LR TAR (23). —, Probes hybridizing to latently infected TG; −, probes failing to hybridize to latently infected ganglionic tissue sections. Approximate map locations of the LR RNA (5′→3′) in rabbit TG are shown at the bottom of the figure. The 5′ end of the RNA is located within nucleotides 773 to 981, with the 3′ end located between nucleotides 1753 and 1936.

latently infected animals at 90 days p.i. Ocular swabs were collected and cultured from latently infected animals for three consecutive days before sacrifice to verify that spontaneous viral reactivation was not occurring (27).

In situ hybridization. In situ hybridization analysis was performed as described previously (1, 23, 25, 36). Briefly, TG were removed from latently infected rabbits and cattle and immersion fixed in periodate-lysine-paraformaldehyde fixative for 24 h at 4°C before being embedded in paraffin for sectioning. All bovine ganglia were removed and placed in fixative within 10 to 20 min of the death of the animals. Tissue sections were deparaffinized with xylene, rehydrated in graded ethanol solutions, and pretreated with 0.2 N HCl for 20 min followed by treatment with proteinase K (1 mg/ml) in 10 mM Tris (pH 7.4)–2 mM CaCl for 15 min at 37°C. The sections were then probed with various ^{35}S-labeled BHV-1 DNA fragments (~10^5 cpm) in a hybridization solution containing 2× SSC, 45% formamide, 10% dextran sulfate, 10 mM EDTA, 1× Denhardt solution, and 0.25 mg of control rabbit brain total nucleic acids per ml. After hybridization, sections were washed in 2× SSC–45% formamide–10 mM Tris (pH 7.4)–1 mM EDTA for 1 day at room temperature, coated with NTB-2 emulsion, exposed for 4 to 7 days at 4°C, and developed and stained with hematoxylin and eosin as previously described (36). Sections were examined and scored in a masked fashion. Positive cells were considered to be those cells exhibiting grains too numerous to count easily.

RESULTS

Transcription from the LR TAR of the BHV-1 genome in latently infected rabbit and bovine TG. The TAR of the BHV-1 genome in latently infected rabbit ganglionic neurons was previously mapped by in situ hybridization analysis to a 1.9-kb region at the left end of *Hin*dIII fragment D (m.u. 0.734 to 0.748). Within this region, hybridization was most intense with the 955-bp *Pst*I-*Sal*I fragment probe (Fig. 1, nucleotides 981 to 1936) and weaker, although consistently present, with the adjacent 981-bp *Pst*I-*Pst*I fragment (nucleotides 1 to 981) (23). To further fine map the LR RNA present within rabbit ganglionic neurons and to examine the transcription of this region in latently infected bovine ganglionic neurons, a series of overlapping restriction fragments from this region were individually radiolabled and hybrid-

TABLE 1. Fine mapping of BHV-1 LR RNA in bovine and rabbit TG by in situ hybridization

Probe			Hybridization detected (no. of animals/total no. examined)			
			Rabbit		Bovine	
No.[a]	Fragment	Nucleotide position	Uninfected controls	Latently infected	Uninfected controls	Latently infected
1	*Pst*I-*Xho*I	1–306	0/2	0/10	0/2	0/5
2	*Xho*I-*Xho*I	307–451	0/2	0/10	0/2	0/5
3	*Xho*I-*Pst*I	452–981	0/2	10/10	0/2	3/5
4	*Alu*I-*Alu*I	448–617	0/2	0/10	0/2	0/5
5	*Alu*I-*Alu*I	618–1132	0/2	10/10	0/2	5/5
6	*Sac*I-*Sal*I	1132–1368	0/2	10/10	0/2	5/5
7	*Sac*I-*Sal*I	1369–1936	0/2	10/10	0/2	5/5
8	*Sal*I-*Sac*I	1937–2375	0/3	0/15	0/2	1/5
9	*Xba*I-*Sph*I	523–773	0/3	0/15		
10	*Sph*I-*Pst*I	805–981	0/3	12/15		
11	*Pst*I-*Mbo*II	982–1120	0/1	5/5		
12	*Sac*I-*Mbo*II	1132–1613	0/3	15/15		
13	*Mbo*II-*Mbo*II	1614–1752	0/1	5/5		
14	*Mbo*II-*Sal*I	1753–1936	0/3	12/15		
15	*Sac*I-*Kpn*I	2375–~6050	0/1	0/5		
16	*Sal*I-*Kpn*I	1936–~6050	0/1	0/5	0/3	0/5

[a] For probe map locations, see Fig. 1.

ized to sections of TG from uninfected and latently infected rabbits and cattle.

Positive hybridization was observed in latently infected rabbit TG with probes 3, 5, 6, 7, 10, 11, 12, 13, and 14 (Fig. 1 and Table 1). Hybridization was not detected in uninfected rabbit TG with any of the probes tested (Table 1). The hybridizing probes spanned a continuous 1.16-kb region within the previously identified LR TAR (Fig. 1). The failure of probes 1, 2, 4, 8, 9, 15, and 16 to hybridize further localizes the ends of the LR RNA. Since LR RNA transcription occurs in the rightward direction (23), these data locate the 5′ end of the LR RNA in a 208-bp *Sph*I-*Pst*I fragment (nucleotides 773 to 981) and the 3′ end in a 183-bp *Mbo*II-*Sal*I fragment (nucleotides 1753 to 1936). The strong positive hybridization seen with all the probes throughout the 1.1-kb region, together with the degree of overlap provided by the probe set used, suggests that if splicing of this transcript occurs, the spliced portion(s) consists of only short sequences. Assuming that no splicing occurs, these data provide a minimum size estimate of 772 bases and a maximum one of 1163 bases for the LR RNA.

On examination of latently infected bovine TG, strong positive hybridization was detected in neurons with probes 3, 5, 6, and 7 but not with probes 1, 2, 4, 8, and 16 (Table 1). These results would place the 5′ end of the RNA between nucleotides 617 and 981 and the 3′ end between nucleotides 1369 and 1936. The similarities in map location of the LR RNA(s) in latently infected rabbit and bovine TG suggest that they are both structurally and functionally quite similar in nature. Similar to past observations made in latently infected rabbit TG (23, 27), involved bovine neurons were morphologically normal in appearance, with LR RNA localized to the cell nucleus (Fig. 2). Qualitatively, the number of LR RNA-containing neurons in bovine ganglia appeared to be increased over that seen in rabbit ganglia; however, the number of involved neurons was still less than 1% of total neurons.

Transcription from the LR TAR in infected bovine cell cultures. To further characterize the LR TAR of the BHV-1 genome, we examined lytically infected bovine tissue culture cells for RNA transcription from this region at various times p.i. by Northern (RNA) hybridization analysis of total cellular RNA with strand-specific probes (*Pst*I-*Sal*I fragment, nucleotides 981 to 1936) from this region. With a probe that would detect RNA of the same sense as the LR RNA seen in latently infected neurons, a 1.15-kb transcript was detected in BHV-1-infected bovine cells (Fig. 3A); the RNA was first present at 6 h p.i. and maximally expressed at 16 h p.i. It was not detected in IE or control cell RNA preparations. Northern hybridization analysis of infected cell RNA with the complementary strand-specific probe from this region is shown in Fig. 3B. An abundant 2.9-kb transcript was seen in IE RNA preparations and at 3 h p.i. in time course experiments. This transcript is replaced by 6 h p.i. with an abundant, slightly smaller transcript of 2.6 kb.

These data indicate that the LR TAR is transcriptionally active in bovine cell cultures lytically infected with BHV-1. A 1.15-kb transcript of the same sense and approximate size as that seen in latently infected TG overlaps to some degree a 2.9-kb IE and a 2.6-kb early and late transcription unit present on the complementary strand.

Sequence analysis of BHV-1 LR TAR. The 2,611-bp region containing the BHV-1 LR TAR from the *Pst*I site (m.u. 0.734) to 675 bases to the right of the *Sal*I site (m.u. 0.748) as illustrated in Fig. 1 was sequenced, and the results are shown in Fig. 4 and 5. The 5′ end of the LR RNA detected in latently infected TG is located between nucleotides 773 and 981, while the 3′ end maps between nucleotides 1753 and 1936. The sequence of the LR strand 5′ to the start of the LR RNA contains a consensus Sp1 binding site (TGGGCGGGG) located between nucleotides 173 and 184, as well as multiple Sp1-like binding sites at nucleotides 243 to 249, 250 to 256, and 343 to 349 (6, 12, 13). The region between nucleotides 250 and 450—that containing the Sp1-like binding sites—and the region between nucleotides 650 and 800 containing the putative LR RNA start site are both A+T-rich (60%) relative to the average base composition (70% G+C) of the entire 2.6-kb sequence. No regions with homology to glucocorticoid or cyclic AMP response elements were found within the region upstream of the mapped location of the LR RNA.

Potential RNA polymerase II promoter sequences within the 208-bp region containing the 5′ start site of the LR RNA include a CAAT box (CCAAT at position 648) and TATA boxes (TTAATA at position 699 and TAATATA at position

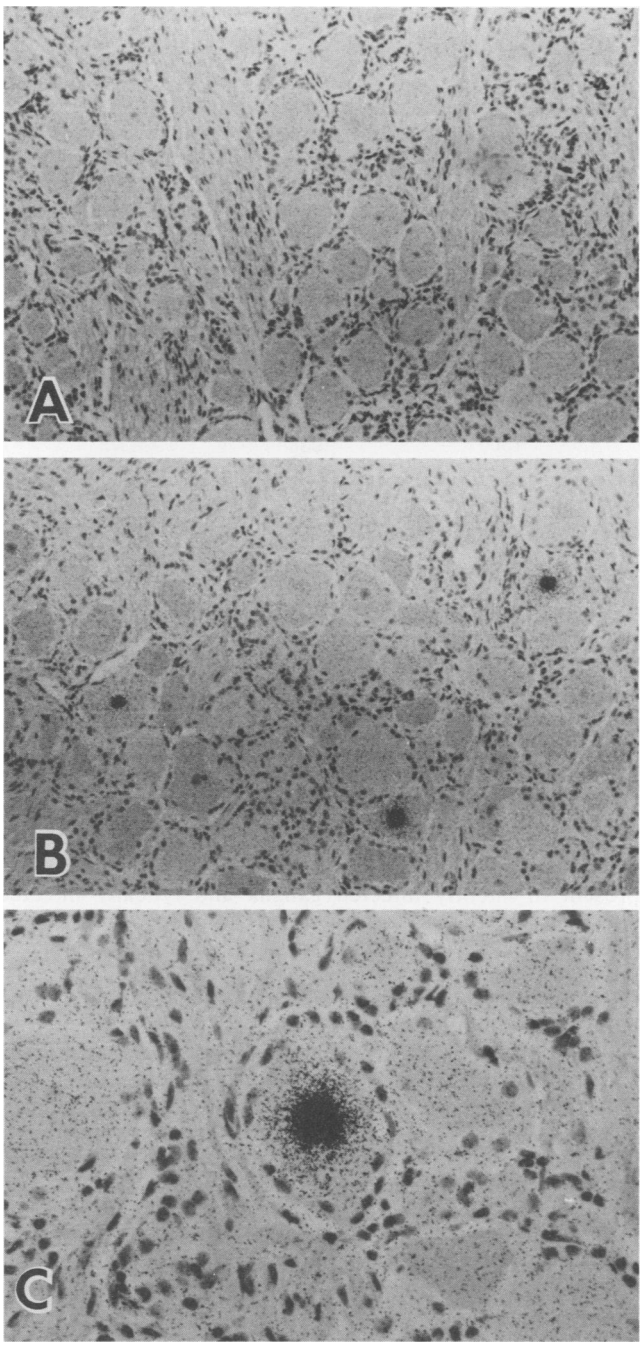


FIG. 2. Detection of BHV-1 LR RNA in latently infected bovine TG by in situ hybridization. (A) Section of bovine TG taken from an uninfected control animal and hybridized with BHV-1 probe 7 (a 567-bp *Sac*I-*Sal*I fragment, nucleotides 1369 to 1936, Fig. 1). (B) Section of latently infected bovine TG hybridized with BHV-1 probe 7. Three LR RNA-containing neurons are evident in this tissue section. (C) Higher magnification of an LR RNA-containing neuron shown in panel B. Note the dense accumulation of autoradiographic grains in the cell nucleus.

718). Analysis in all frames of the LR strand indicated the presence of significant open reading frames (ORFs) within the map locations of the LR RNA and in close proximity to the putative polymerase II (Pol II) promoter site. An ORF, LR-ORF-2, starts 58 bases downstream from the second TATA box at nucleotide 776 and remains open for 545 bases. The high frequency of alternating purine-pyrimidine bases at the start of LR-ORF-2 (nucleotides 771 to 820) suggests a high probability of Z-DNA formation in this region. A second ORF, LR-ORF-1, begins at nucleotide 1168 with a consensus translational initiation site (GCGCCATGG) (15) and remains open for 854 bases. LR-ORF-1 is out of frame relative to LR-ORF-2 and overlaps it by 153 nucleotides.

Based on the analysis method of Fickett (5), the region between nucleotides 1200 and 2200 has a high probability of being protein coding. Consistent with this prediction, the positional base preference analysis of Staden (31, 33) suggests that regions including portions of both LR-ORF-1 and LR-ORF-2 are likely to be protein coding. Putative proteins from these ORFs would contain large amounts of proline, arginine, lysine, and alanine and would be highly basic in nature.

Additional sequence features of the LR sense strand include (i) an RNA polymerase III A element sequence homology (17) beginning at nucleotide 921 and an associated B-like element homology located 50 bp downstream from the A element homology and (ii) a consensus polyadenylation signal (AATAAA) that occurs at nucleotide 2521, 585 to 768 bases downstream from the 3′ end of the LR RNA.

The complementary strand of the LR TAR, from which the IE 2.9-kb and the early and late 2.6-kb transcripts are synthesized in lytically infected bovine cells, contains a single large ORF that begins open and ends at nucleotide 956. A consensus polyadenylation signal is present immediately downstream at nucleotide 762. This ORF almost completely overlaps the map position of the LR RNA on the opposite strand.

DISCUSSION

Our in situ hybridization analysis in latently infected rabbit TG further localized both the 5′ and 3′ ends of the BHV-1 LR RNA within *Hin*dIII fragment D. The 5′ end of the transcript occurs within a 208-bp *Sph*I-*Pst*I fragment (nucleotides 773 to 981), while the 3′ end is located within a 183-bp *Mbo*II-*Sal*I fragment (nucleotides 1753 to 1936). These results are consistent with and expand upon our previous analysis which placed the 5′ end within the 0.98-kb *Pst*I-*Pst*I fragment (nucleotides 1 to 981) and the 3′ end within the 0.96-kb *Pst*I-*Sal*I fragment (nucleotides 981 to 1936) (23). These data provide a size estimate of between 771 and 1,163 bases for the LR RNA.

LR RNA mapping to this same region was also detected in TG from cattle latently infected with BHV-1. We believe this to be the first report of latent BHV-1 gene transcription in the natural host species. The similarities in map location of the LR RNA(s) in the two species suggest that they are structurally and likely functionally similar. The rabbit model for latent BHV-1 infection has previously been shown to be consistent with observations made for latently infected cattle (25, 27). The findings reported here further underscore the relevance and the potential usefulness of the rabbit model to the study of the molecular mechanisms that underlie latent BHV-1 infection.

The following features of the DNA sequence encoding the LR RNA suggest that it is transcribed by RNA Pol II. (i) Consensus TATA and CAAT box sequences are located within a highly A+T-rich region (60%) which contains the LR RNA transcriptional start site, and (ii) Sp1 and multiple Sp1-like enhancer binding sequences are located within a second A+T-rich region located 200 bases upstream of the

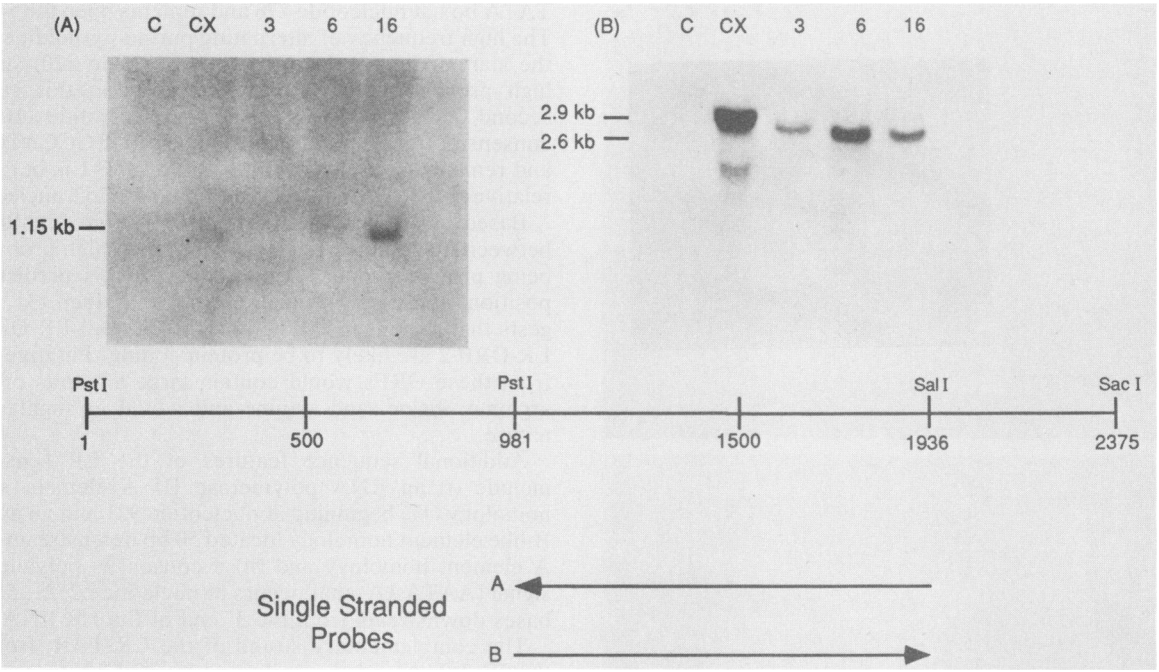


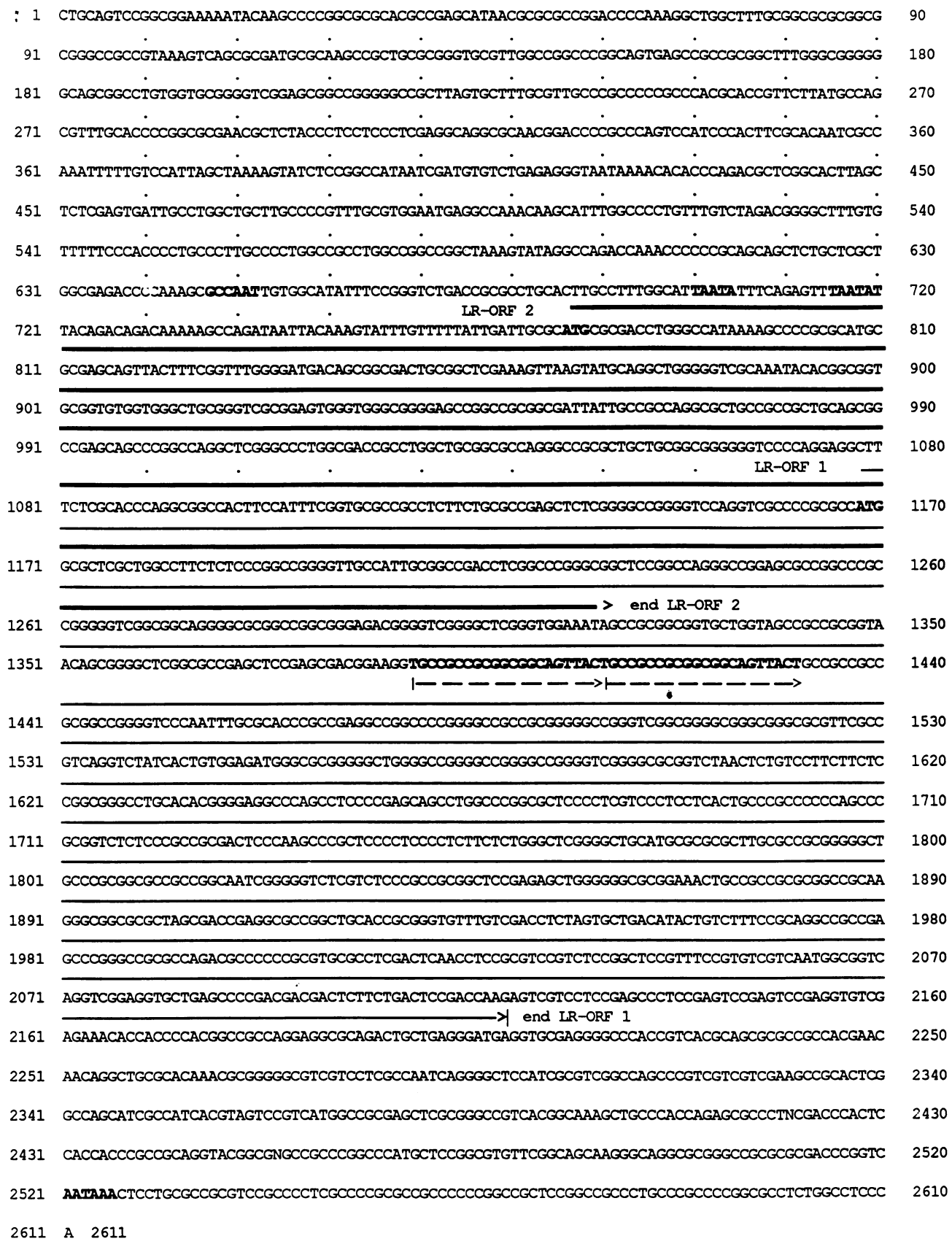
FIG. 3. Northern hybridization analysis of transcription from the LR region in BHV-1-infected bovine turbinate cell cultures. (A) Northern blot of total cellular RNA (20 μg) isolated from uninfected cells (lane C) or virus-infected cells at 3, 6, and 16 h p.i. or at 6 h p.i. in the presence of cycloheximide (lane CX), hybridized with a strand-specific probe (probe A, shown below) that would detect RNAs of the same sense as LR RNA detected in infected ganglia. (B) Northern blot identical to that described in panel A hybridized with the complementary strand-specific probe from this region (probe B, shown below).

CAAT box. Moreover, this same region has been shown recently to contain a complex transcriptional promoter that is efficiently expressed in primary cultures of rabbit sensory neurons (11). Consistent with the possibility that LR RNA is protein coding is the presence of sizable ORFs within the mapped transcript. LR-ORF-1, located between 187 and 395 bases downstream from the 5′ end of the RNA, begins with a consensus (GGGCCATGG) translational start site (15). In addition, the LR RNA contains a second significant ORF, LR-ORF-2. The ATG initiation codon for this ORF is 58 bases downstream from the second TATA box and is located within the 208-base region containing the 5′ end of the LR RNA. Positional base preference analysis (31, 33) indicated that portions of the DNA region encoding LR-ORF-1 and LR-ORF-2 are likely protein coding. The possible use of both ORFs in the synthesis of a single polypeptide would suggest that a small, as yet undetected, splice(s) of the transcript results in a joining of the two ORFs.

A previously unreported 1.15-kb RNA transcript of the same sense as the LR RNA seen in latently infected neurons was detected from this region at early and late times p.i. in BHV-1-infected bovine tissue culture cells (Fig. 3A). The function of this transcript and/or its protein product in the lytic infection cycle has not been determined. While the exact degree of structural similarity between the RNAs present in latently infected ganglia and lytically infected tissue culture cells is unknown, their apparent similarity in size and relative map location is particularly interesting. If these RNAs represent similar transcripts, it would suggest a role for LR RNA and/or its protein product in both lytic and latent viral infection.

As with other alphaherpesviruses (3, 4, 26, 30, 35), BHV-1 LR transcription overlaps an IE gene coded for on the complementary strand. Although not detected in latently infected ganglia (23), an abundant 2.9-kb IE transcript is detected in lytically infected bovine cells by Northern analysis with the complementary strand of the *Pst*I-*Sal*I fragment (nucleotides 981 to 1936) as a probe (Fig. 3B). While the 3′ end of the transcript was not mapped here, Wirth et al. (37) have described a similar-sized transcript from this region that terminates in the vicinity upstream of the polyadenylation signal at nucleotide 762. Given the map locations of LR RNA in latently infected ganglia, this IE gene transcription would almost completely overlap the LR transcription unit present on the other strand. The BHV-1 IE protein encoded by this RNA has not been described, and its function in the viral replicative cycle is unknown. Of some interest is the replacement of the 2.9-kb IE transcript from

FIG. 4. Nucleotide sequence of the DNA encoding the BHV-1 LR RNA. The sequence of the Cooper strain of BHV-1 from a *Pst*I site (m.u. 0.734) to 675 bases to the right of the *Sal*I site (m.u. 0.748) is shown. The 5′ end of the LR RNA maps between nucleotides 773 and 981, with the 3′ end located between nucleotides 1753 and 1936. A putative Pol II promoter including a CCAAT box (648) and TATA boxes (TAATA [699] and TAATAT [718]) is present within the 208-bp region containing the 5′ start site of the LR RNA. Two sizable ORFs are located within the map positions of the LR RNA. LR-ORF-1 (1168 to 2022, shown as a thin line above the sequence) begins with a consensus translational start site (GCCATGG). LR-ORF-2, in closer proximity to the region containing the 5′ start site, is open for 545 bases (776 to 1321, shown as a thick line above the sequence). A 21-bp tandem direct repeat (1389 and 1410) is indicated by arrows beneath the sequence. A consensus polyadenylation signal (AATAAA) occurs at nucleotide 2521, downstream from the 3′ end of the LR RNA.


LR-ORF 2
LR-ORF 1
> end LR-ORF 2
>| end LR-ORF 1

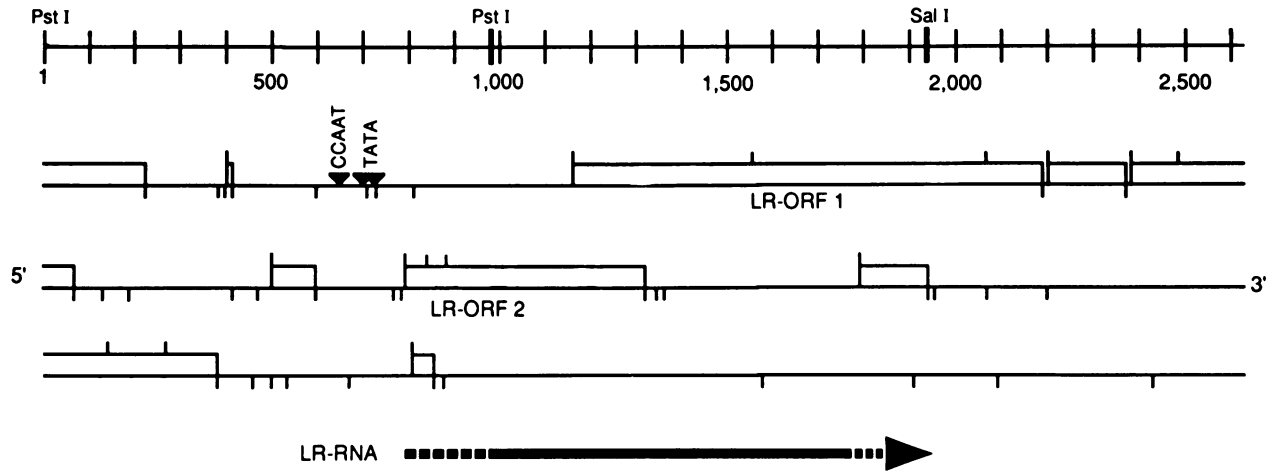


FIG. 5. Reading frame analysis of the DNA encoding the BHV-1 LR RNA. All three translational frames on the LR RNA sense strand are shown. Possible translational initiation codons are shown as dashes above the lines, while stop codons are represented as dashes below the line. The putative Pol II promoter and the ORFs discussed in the text are illustrated. The map position of the LR RNA is shown at the bottom of the figure. The approximate ends of the RNA as determined by in situ hybridization analysis are illustrated by a dashed line, the 5′ end falling between bases 773 and 981 and the 3′ end between bases 1752 and 1936.

this region with a smaller 2.6-kb transcript at early and late times p.i. Although further mapping will be required, this region may contain a nested set of viral transcripts with a common 3′ end.

The functional significance of BHV-1 LR transcription in latently infected ganglionic neurons, like that seen in herpes simplex virus and pseudorabies virus (3, 4, 24, 26, 30, 35), remains unknown. Experiments examining the ganglionic events that occur during dexamethasone-induced BHV-1 reactivation do, however, suggest a role for LR RNA in some aspect of viral latency or reactivation. The number of LR RNA-containing neurons in latently infected ganglia decreased significantly (60%) 24 to 48 h after dexamethasone treatment with a return to control values by 72 h posttreatment (D. L. Rock, J. Lokensgard, T. Lewis, and G. Kutish, unpublished data). The fact that dexamethasone had a profound negative effect on BHV-1 LR promoter activity in vitro (11) would explain this transient decrease as down-regulation of LR RNA expression in these cells. The positive correlation of this decrease with viral reactivation suggests that altered regulation of LR RNA transcription in neurons is a significant event in the process of latent viral reactivation. Similarly, with herpes simplex virus, evidence is available supporting a role for latency-associated transcripts in viral reactivation (7, 18, 34).

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