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Mapping Bovine Herpesvirus Type 1 Latency-Related RNA in Trigeminal Ganglia of Latently Infected Rabbits

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Here we have used the bovine herpesvirus type 1 (BHV-1) rabbit model together with in situ nucleic acid hybridization to identify and map viral RNA present in latently infected neurons. Radioactively labeled cloned *Hin*dIII fragments representing most of the BHV-1 genome (Cooper strain) were individually hybridized to sections of trigeminal ganglia taken from rabbits during acute and latent stages of infection. Whereas all viral genomic fragments hybridized to lytically infected tissue culture cells and to acutely infected ganglia, only *Hin*dIII fragment D (map units 0.734 to 0.842) hybridized to latently infected ganglionic neurons. Additional in situ hybridization experiments using subcloned fragments of *Hin*dIII-D further mapped the latency-related viral RNA to a 1.9-kilobase region (map units 0.734 to 0.748) of the viral genome. These results indicate that BHV-1 gene transcription is restricted during the latent phase of infection; further, they suggest that specific viral transcription may be involved in establishment or maintenance of latent BHV-1 infection.

Bovine herpesvirus type 1 (BHV-1) is a significant viral pathogen of cattle, playing a major role in bovine respiratory disease and abortion as well as being responsible for a variety of other disease syndromes: conjunctivitis, vulvovaginitis, meningoencephalitis, and fatal systemic infection (6). Like other members of the alphaherpesvirus group, BHV-1 establishes latent infections in neurons of sensory and autonomic nerve ganglia (4, 5, 10, 17, 19). At this time, little is known about the mechanisms underlying the establishment, maintenance, and reactivation of this latent infection. Recent investigations, using a rabbit latency model, into the molecular basis of this unique virus-cell interaction have shown that the BHV-1 genome is transcriptionally active in latently infected ganglionic tissue (15). However, the nature and significance of this latency-related viral transcription remain unknown. Here we have used the BHV-1 rabbit model together with in situ nucleic acid hybridization to further characterize viral RNA in latently infected ganglionic neurons. Our results indicate that BHV-1 transcription is restricted in latently infected cells to a 1.9-kilobase (kb) region of the viral genome (map units [m.u.] 0.734 to 0.748).

MATERIALS AND METHODS

Cells and viruses. Bovine lung (BLG) cells were used throughout the experiments and were maintained as described previously (16). The Cooper strain of BHV-1 was supplied by the National Veterinary Services Laboratory, Ames, Iowa, and was used for animal inoculation at the sixth passage.

Animals and experimental infection. New Zealand White rabbits (2.0 to 3.0 kg) were lightly anesthetized with methoxyflurane and inoculated in both the right and left conjunctival sac with 10^7 PFU of BHV-1 as described previously (16). Trigeminal ganglia were obtained from acutely infected rabbits at 4 days postinfection and from latently infected animals 30 to 180 days postinfection. Although spontaneous viral reactivation in the BHV-1 rabbit model is uncommon (16), ocular swabs were collected from latently infected animals and cultured for BHV-1 on three consecutive days before sacrifice.

Hybridization probes. The construction and characterization of the *Hin*dIII clones of BHV-1 (Cooper) have been described previously (8). Any necessary subcloning of these fragments was done in PUC 18 or pGEM 1 vectors by using standard recombinant DNA methods (7). Bacterial plasmids were purified by using the alkaline extraction procedure of Birnboim and Doly (1) and then were banded in CsCl-ethidium bromide density gradients. Cloned or gel-purified fragments of the BHV-1 genome were labeled by nick translation with ^{3}H nucleotides to specific activities ranging from 4.0×10^7 to 1.0×10^8 cpm/μg (14). Strand-specific RNA probes were prepared by using pGEM vectors (Promega Biotec) as previously described (3). Restriction mapping of BHV-1 genomic fragments was accomplished by using the procedure of Smith and Birnstiel (18). Pseudorabies virus DNA (strain BE12, obtained from K. B. Platt, Iowa State University) was prepared from purified virions by pronase-sodium dodecyl sulfate treatment followed by phenol-chloroform extraction and two cycles of sodium iodide-ethidium bromide gradient centrifugation (11, 12, 22). Plasmids containing cloned *Bam*HI fragments of the herpes simplex virus type 1 genome (strain F) were obtained from B. Roizman, University of Chicago.

In situ hybridization. Animals were anesthetized and killed by perfusing with 300 to 400 ml of periodate-lysine-paraformaldehyde fixative (9). Trigeminal ganglia were dissected, immersion fixed in periodate-lysine-paraformaldehyde fixative for an additional 24 h at 4°C, and embedded in paraffin for sectioning. In situ hybridization was performed essentially as previously described (2, 15, 21). Tissue sections probed for viral RNA were deparaffinized with xylene, rehydrated in graded ethanol solutions, and pretreated with 0.2 M HCl for 20 min, followed by treatment with proteinase K (1 mg/ml) in 10 mM Tris (pH 7.4)–2 mM $CaCl_2$ for 15 min at 37°C. Tissue sections were hybridized with 1 ng (approximately 10^5 cpm) of a ^{3}H-labeled BHV-1 DNA fragment or RNA probe for 72 h at 45°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 45%

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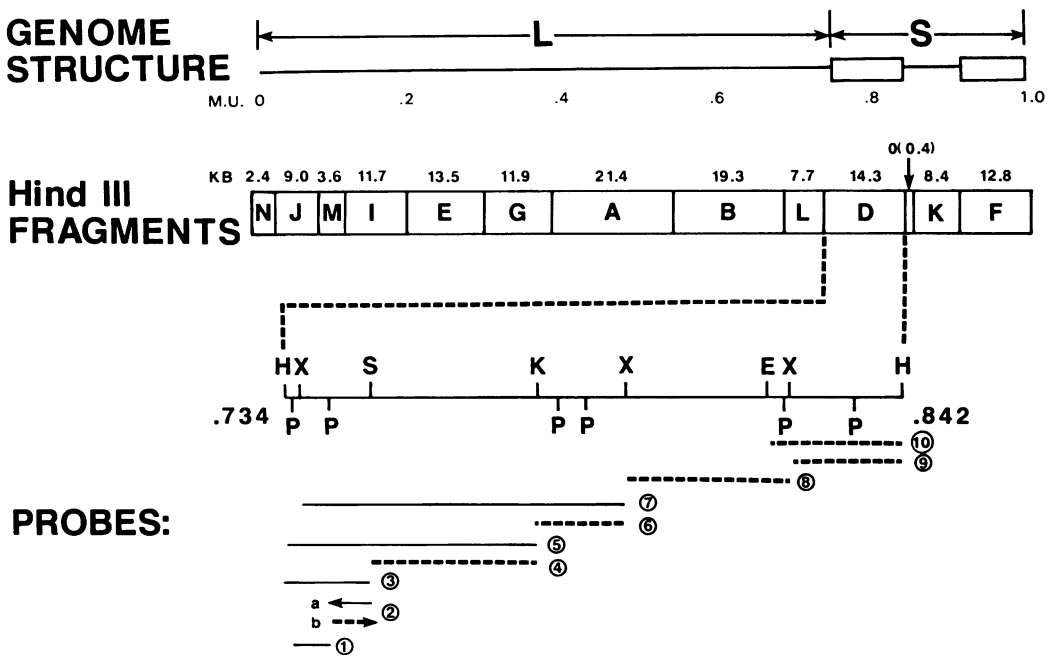

FIG. 1. BHV-1 DNA (Cooper strain). This linear double-stranded molecule of approximately 136 kb consists of two unique sequence regions, the smaller of which is bounded by inverted repeat sequences (8). The positions of the *Hin*dIII fragments used as probes, including an expanded restriction map of *Hin*dIII-D, are shown under the genome structure. Probes (1 to 10) used in mapping the LR RNA within *Hin*dIII-D are shown under the fragment map. Probes 2a and 2b represent single-stranded RNA probes (5′→3′) made from both strands of this cloned fragment. —, Probes hybridizing to latently infected trigeminal ganglia; ---, probes failing to hybridize to latent ganglionic tissue. Restriction enzyme abbreviations: H, *Hin*dIII; P, *Pst*I; X, *Xho*I; S, *Sal*I; K, *Kpn*I; and E, *Eco*RI.

formamide, 10% dextran sulfate 10 mM Tris (pH 7.4), 1 mM EDTA, 1× Denhardt solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), and 1.0 mg of rabbit brain total nucleic acids per ml. After hybridization, slides were washed in 2× SSC, 45% formamide, 10 mM Tris (pH 7.4), and 1 mM EDTA for 2 to 3 days at room temperature, coated with NTB-2 emulsion, exposed for 18 to 21 days at 4°C, and developed and stained with hematoxylin and eosin as previously described (21). Sections were examined and scored in a masked fashion. Positive cells were considered to be those cells exhibiting grains too numerous to count easily. Sections of trigeminal ganglia from uninfected animals were included in every hybridization experiment as a negative control. BLG cells lytically infected with BHV-1 served as a positive control; cells were infected at a multiplicity of 5 and fixed (20 min at room temperature in 75% ethanol–25% glacial acetic acid) at 18 h postinfection. RNase-treated tissue sections were also included in all hybridization experiments; after standard pretreatment steps, slides were rinsed in 2× SSC and further treated with DNase-free RNase A (100 μg/ml in 2× SSC) for 30 min at 37°C before hybridization.

RESULTS

Identification of an LR transcriptionally active region (TAR) of the BHV-1 genome. In earlier experiments we have shown, using in situ hybridization with a total virion DNA probe, that the BHV-1 genome is transcriptionally active in latently infected ganglionic neurons (15). To further characterize and map this latency-related (LR) viral transcription in these cells, we individually hybridized radioactively labeled *Hin*dIII fragments of the viral genome (8; Fig. 1), representing 97% of all genome sequences, to trigeminal ganglia taken from uninfected, acutely infected, and latently infected rabbits.

The results of these experiments are shown in Table 1. All BHV-1 genomic probes demonstrated strong and specific hybridization to BLG cells lytically infected with BHV-1 (Fig. 2A and B) and to sections of trigeminal ganglion from

TABLE 1. Identification of LR-TAR of BHV-1 genome in rabbit trigeminal ganglia by in situ hybridization

Probe	Hybridization detected in no. of rabbits/total no. examined		
	Uninfected controls	Acutely infected	Latently infected
BHV-1 *Hin*dIII fragment[a] (m.u.)			
N (0–0.018)	ND[b]	ND	ND
J (0.018–0.083)	0/5	2/5	0/10
M (0.083–0.110)	0/3	3/6	0/10
I (0.110–0.195)	0/4	4/5	0/11
E (0.195–0.294)	0/4	3/5	0/9
G (0.294–0.381)	0/5	4/5	1/10
A (0.381–0.537)	0/5	4/5	1/11
B (0.537–0.678)	0/5	4/5	0/11
L (0.678–0.734)	0/4	3/4	0/10
D (0.734–0.842)	0/5	8/9	17/19
O (0.842–0.845)	ND	ND	ND
K (0.845–0.907)	0/5	5/5	0/10
F (0.907–1.0)	ND	ND	ND
Pseudorabies virus (BE12)	0/4	0/6	0/10
HSV F[c]			
pRB 112 (*Bam*HI fragment B)	0/5	0/3	0/9
pRB 113 (*Bam*HI fragment Y)	0/5	0/3	0/5

[a] Mayfield et al. (8).
[b] ND, Not done.
[c] Post et al. (12).

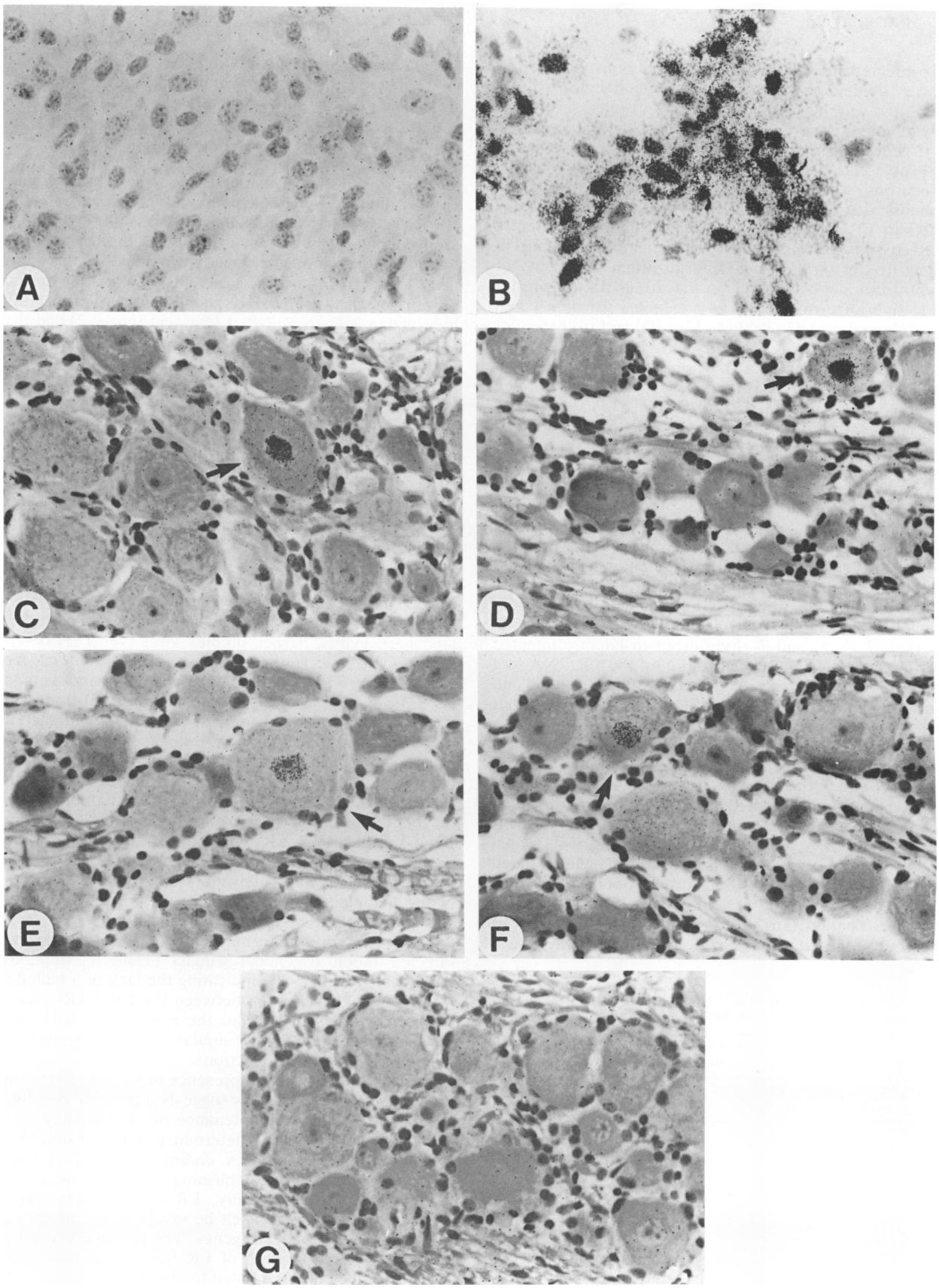

FIG. 2. Detection of BHV-1 LR RNA by in situ hybridization. Uninfected (A) and BHV-1-infected (B) BLG cells hybridized with BHV-1 *Hin*dIII fragment K. (C and D) Sections of latently infected rabbit trigeminal ganglion hybridized with cloned fragment pGH/D-S/P-0.95 (probe 2, Fig. 1). Note in each photo the single latently infected ganglionic neuron containing BHV-1 RNA homologous to this genomic region. (E and F) Sections of latently infected trigeminal ganglion hybridized with cloned fragment pGH/D-P-0.95 (probe 1, Fig. 1). A single neuron with diffuse accumulation of autoradiographic grains is evident in each photo. (G) A section of trigeminal ganglion taken from an uninfected control animal and hybridized with a BHV-1-specific probe (pGH/D-S/P-0.95). Typical background levels of nonspecific grains can be seen.

acutely infected animals. As described previously (15), viral RNA was evident in both the nucleus and cytoplasm of acutely involved neurons. Unlike the results seen with acutely infected tissue, only *Hin*dIII fragment D, m.u. 0.734 to 0.842 (Fig. 1), consistently hybridized to latently infected tissue (Table 1). Hybridizing neurons, approximately 0.25% of all neurons, were morphologically normal in appearance with a marked accumulation of viral RNA in the cell nucleus (Fig. 2C to F). In all cases, RNase treatment of latently infected tissue sections before hybridization resulted in complete loss of viral specific hybridization. Specificity of the hybridization was evident, in that (i) all BHV-1 probes failed to hybridize to sections of trigeminal ganglion taken from uninfected rabbits (Table 1; Fig. 2G) and (ii) heterologous herpesvirus probes, high in %GC content, failed to hybridize to BHV-1-infected ganglionic tissue (Table 1).

Mapping of LR RNA within *Hin*dIII-D. Based on the restriction enzyme map of *Hin*dIII-D (Fig. 1), a series of 10 overlapping restriction fragments were either subcloned or purified from restriction digests of *Hin*dIII-D for use as probes in in situ hybridization experiments to map the LR RNA. Fragments 1 to 10 (Fig. 1) were nick translated and individually hybridized to sections of trigeminal ganglion from uninfected, acutely infected, and latently infected rabbits. Summary results from these experiments are shown in Table 2. All probes hybridized efficiently with lytically infected BLG cells and ganglion sections from acutely infected rabbits, while failing to hybridize to tissue from uninfected control animals. Only a subset of the probes—numbers 1, 2, 3, 5, and 7—hybridized to latently infected tissue. These results allow localization of the LR RNA to a 1.9-kb region, made up of probes 1 and 2 at the left-hand end of *Hin*dIII-D, m.u. 0.734 to 0.748 (Fig. 1). Within this region, hybridization with probe 2, a *Sal*I-*Pst*I fragment of 950 base pairs, was particularly intense and detectable in tissue from 95% (20 to 21) of latently infected animals examined (Fig. 2C and D). In contrast, the hybridization observed with probe 1, a *Pst*I fragment of approximately 950 base pairs, was much weaker and detectable in only 63% of the animals (Fig. 2E and F).

To determine the sense of this LR RNA, single-stranded RNA probes were made from both strands of probe 2 (cloned in pGEM 1) by in vitro transcription using either SP6 or T7 polymerase. The leftward probe (2a), but not the rightward synthesized probe (2b), hybridized to viral RNA present in ganglia from 13 of 17 latently infected animals (Fig. 1; Table 2). Thus, the LR RNA, being of complementary sense to the hybridizing probe, is transcribed in a rightward direction.

TABLE 2. Fine mapping of LR RNA within BHV-1 *Hin*dIII fragment D (m.u. 0.734 to 0.842) using in situ hybridization

Probe[a]	Hybridization detected in no. of rabbits/total no. examined		
	Uninfected controls	Acutely infected	Latently infected
1	0/3	7/9	12/19
2	0/3	7/8	20/21
a	0/6		13/17
b	0/7		0/18
3	0/4	4/5	16/16
4	0/5	4/6	1/18
5	0/3	3/4	8/9
6	0/3	4/4	1/9
7	0/3	2/4	10/10
8	0/3	2/4	0/10
9	0/3	2/4	0/10
10	0/3	6/8	1/14

[a] Probe map locations shown on Fig. 1.

DISCUSSION

Here we have shown that BHV-1 gene transcription in latently infected ganglionic neurons is restricted and that the LR RNA seen in these cells maps to a 1.9-kb region of the viral genome (m.u. 0.734 to 0.748). Although this in situ hybridization mapping technique provides only an approximate location for the LR RNA, it is clear that the major portion of this LR TAR is contained within the strong hybridizing 0.95-kb *Sal*I-*Pst*I fragment (probe 2) and that to some extent it overlaps into the adjacent, more weakly hybridizing *Pst*I fragment (probe 1). This region of the BHV-1 genome is poorly characterized at the molecular level, making it difficult to speculate which specific viral gene(s) are being transcribed in latently infected neurons. Preliminary results have shown, however, that this region is abundantly transcribed at immediate-early times in infected BLG cells (T. L. Lewis and D. L. Rock, unpublished data), indicating the presence of a major immediate-early gene (IE) within this LR region. Whether the LR RNA represents transcripts from this IE gene or transcripts from another, as yet unidentified gene contained within this region is presently unknown.

The observations reported here for BHV-1 latency are strikingly similar to results from latency studies with another alphaherpesvirus, herpes simplex virus type 1 (HSV-1) (13, 15a, 20). In these studies, using both the rabbit and mouse model, HSV-1 LR-RNA was mapped to an area containing the IE gene ICPO. Thus, with both viruses, transcription from the latent viral genome is restricted, with LR RNA mapping to a region of the viral genome encoding an IE gene. These similarities suggest that a common latency-regulatory mechanism, perhaps involving the uncoupling of normal regulation of IE gene expression, may exist among members of the alphaherpesvirus group. Interestingly, the major BHV-1 and HSV-1 LR TAR do not cross hybridize with each other's LR RNA. By using identical in situ hybridization conditions, which allowed strong hybridization in both of the homologous systems, probes containing the HSV-1 LR TAR (*Bam*HI-B) and the BHV-1 LR TAR (probes 1 and 2) failed to hybridize to heterologous LR RNA in latently infected rabbit trigeminal ganglia (Table 1; unpublished data). This result, while indicating the lack of a high degree of sequence conservation between the LR TARs, does not discount the possibility that these RNAs or their protein products fulfill functionally similar roles in establishment or maintenance of latent infections.

Although the consistent presence of specific BHV-1 RNA in latently infected neurons suggests a possible role for it in the establishment or maintenance of viral latency, its real significance remains to be determined. It is plausible that LR RNA functions as an mRNA, coding for a critical regulatory protein responsible for establishing or maintaining the latent viral infection. Alternatively, LR RNA, functioning as a regulatory RNA, could itself be involved in controlling the expression of critical viral genes. The possibility also exists, however, that expression of LR RNA in latently infected neurons is not causally related to viral latency but is rather a resulting effect of the latent virus-cell interaction.

Similarly, the significance of HSV-1 LR RNA is also poorly understood. HSV-1 LR RNA maps in the vicinity of the IE gene ICP0, but is transcribed off the noncoding strand

(15a, 20). It remains unclear whether this RNA is a transcript from a gene colinear with ICP0 located on the other strand, or whether it represents a regulatory RNA capable of regulating ICP0 expression.

A more thorough characterization of both the BHV-1 LR TAR and the RNA transcripts from it will be necessary before it can be determined what functional role transcription of this region has in establishment or maintenance of the latent infection.

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