Detection of Latency-Related Viral RNAs in Trigeminal Ganglia of Rabbits Latently Infected with Herpes Simplex Virus Type 1

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Using a combination of in situ hybridization and Northern (RNA) blot analysis, we investigated herpes simplex virus type 1 (HSV-1) transcriptional activity in an ocular rabbit model of HSV-1 latency. Radioactively labeled cloned fragments, representing virtually the entire HSV-1 genome, were individually hybridized to RNA in sections of trigeminal ganglia taken from rabbits during the latent phase of infection with HSV-1 (McKrae). Our results suggest that two discrete latency-related RNAs (LR-RNAs) may be present. The LR-RNAs were localized mainly in the nuclei of neurons. The more abundant LR-RNA was detected in approximately 3% of all neurons examined and was designated major LR-RNA. The other LR-RNA, designated minor LR-RNA, was detected in approximately 0.3% of neurons from latently infected rabbits. The genes for the LR-RNAs mapped in the vicinity of the immediate-early gene ICP0 (also designated IE110). The gene for the major LR-RNA partially overlapped the left (3') end of the ICP0 gene. In situ hybridization with single-stranded RNA probes showed that this LR-RNA was of complementary sense to that of ICP0 mRNA. Northern blot analysis gave an approximate size for this LR-RNA of 1.8 to 2.2 kilobases. The minor LR-RNA mapped to or near the right (5') end of the ICP0 gene. The detection of LR-RNAs suggests the possibility that these RNAs or their products may play significant roles in the initiation and/or maintenance of HSV-1 latency.

Like all herpesviruses, herpes simplex virus type 1 (HSV-1) establishes latent infections with subsequent periods of reactivation. Virologists and clinicians alike have been intrigued by the enigma of HSV recurrences. A wealth of data has been published on the subject (2, 10, 12, 14, 19, 20, 28). After primary infection at a peripheral site, the virus ascends through the nerves and becomes latent in ganglionic neurons, where it persists throughout life. As a result of poorly understood mechanisms, HSV is reactivated in ganglionic cells, and the virus travels via axons to peripheral sites. This results in virus shedding with or without clinical disease and has been called the latency-reactivation recurrence cycle (14).

HSV probably persists as a latent infection in which the host cells harbor viral genetic material without replicating fully infectious virus. The majority of evidence confirms the nervous system, and in particular the sensory ganglia, as the major reservoir of latent HSV (14).

Little is known about the molecular basis of HSV-1 latency and reactivation. HSV DNA has been demonstrated in latently infected animals and humans (1, 3, 5, 24-26). HSV RNA has been detected in neurons from latently infected animals (6, 7, 31, 32). This latency-related RNA (LR-RNA) is present in a small percentage of neurons and is restricted to the nucleus (31). Recent work with mouse neurons has suggested that transcription during latency is restricted to the region of the HSV-1 genome that contains the immediate-early gene ICP0 (4, 23, 30). All three reports concluded that there was one RNA transcript. Two of the reports concluded that the RNA was either ICP0 mRNA or a gene colinear with ICP0 (4, 23). The other report (30) demonstrated that the RNA had a sense complementary to that of

the ICP0 mRNA, and the possibility of antisense control was suggested.

In this report, we confirm that only the region in the vicinity of ICP0 is transcriptionally active during HSV-1 latency. We describe the presence of a major HSV-1 LR-RNA and the possibility of a second, minor LR-RNA in trigeminal ganglia of latently infected rabbits. The first LR-RNA is approximately 1.8 to 2.2 kilobases (kb) in length and is complementary to ICP0 mRNA. The gene for this LR-RNA appears to partially overlap the left (3') end of the ICP0 gene. The second LR-RNA is less abundant and probably maps to or near the right (5') end of the ICP0 gene. The presence of LR-RNAs in HSV-1 latency suggests the possibility that these RNAs or their products may play significant roles in the initiation and/or maintenance of HSV-1 latency.

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MATERIALS AND METHODS

Virus and cells. Plaque-purified HSV-1 (McKrae) and Vero cells were grown as previously described (15, 33).

Rabbits. New Zealand White male rabbits (approximately 2 kg each) were used for all experiments. These animals develop a primary and recurrent herpetic disease (15-18, 29), which mimics HSV keratitis in man.

Acute ocular and latent ganglionic HSV infections. Rabbits were bilaterally infected without corneal scarification by placing approximately 1×10^5 to 2×10^5 PFU of virus into the conjunctival cul-de-sac, closing the eye, and rubbing gently for 30 s. Acute ocular infection was verified by swab cultures and monitored by examination with slit lamp biomicroscopy, with 1% methylene blue to delineate epithe-

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lial ulceration. Conjunctivitis, iritis, and stromal keratitis were also determined to confirm the acute infection. Acute ocular infection resolved by 10 to 14 days postinoculation. Rabbits surviving after 4 weeks were considered latently infected (15–18, 29) unless they had spontaneously reactivated.

Spontaneous reactivation. To confirm that latently infected rabbits had not spontaneously reactivated, tear film cultures were done just before sacrifice to look for the presence of HSV. Rabbits in which virus was detected were not considered latently infected, and their trigeminal ganglia were not used.

Rabbit trigeminal ganglia. Rabbit trigeminal ganglia were taken from sacrificed rabbits and immediately placed in liquid nitrogen (for RNA extractions for Northern blots [RNA blots]) or into preservative (periodate-lysine-paraformaldehyde [31]; for sectioning before in situ hybridizations).

In situ hybridizations. The fixing, embedding, and cutting of sections of trigeminal ganglia were done as described elsewhere (27). Tissue sections were deparaffinized in xylene, rehydrated in graded ethanol solutions, and sequentially pretreated with 0.2 N HCl, heat, and proteinase K as described elsewhere (27, 31). Slides probed for viral RNA were rinsed in $2\times$ SSC ($1\times$ is 0.15 M NaCl plus 0.015 M sodium citrate) and further treated with DNase A (RNase free; 100 μ g/ml of $2\times$ SSC) for 30 min at 37°C. The slides were refixed in 5% paraformaldehyde, dehydrated in graded ethanol solutions, and stored and desiccated at room temperature. Probes were prepared as described below. Sections were hybridized with 1 ng of an individual ^3H -labeled probe per slide (approximately 10^5 cpm) for 72 h at 45°C in $2\times$ SSC–45% formamide–10% dextran sulfate–0.01 M Tris (pH 7.4)–0.001 M EDTA–0.02% bovine serum albumin–0.02% polyvinylpyrrolidone–0.02% Ficoll (Pharmacia Fine Chemicals)–0.5 mg of L-cell total nucleic acids per ml. As a control, hybridization with the same plasmid containing no viral insert or an adenovirus insert was done under identical conditions with 38% formamide. After hybridization, slides were washed in $2\times$ SSC–45% formamide–0.01 M Tris (pH 7.4)–0.001 M EDTA for 3 to 4 days at room temperature. After dehydration in graded ethanol solutions containing 0.3 M ammonium acetate, the slides were coated with NTB-2 emulsion (Kodak), exposed for 1 to 3 weeks at 4°C, developed, and stained with hematoxylin and eosin as described elsewhere (27). In general, backgrounds were relatively free of nonspecific autoradiographic grains. Cells were considered positive when they exhibited grains too numerous to count. The numbers of positive cells per 1,000 neurons were counted on a masked basis.

Northern blots. Trigeminal ganglia from latently infected rabbits were frozen in liquid nitrogen and ground into a fine powder with a mortar and pestle. RNA was isolated by guanidine isothiocyanate extraction followed by pelleting through 5.7 M CsCl (13). Control RNA was isolated from uninfected trigeminal ganglia, acutely infected trigeminal ganglia, acutely infected tissue culture cells, and uninfected tissue culture cells. The purified total RNA was denatured, electrophoresed on a formaldehyde submarine agarose gel, and transferred to Hybond-N nylon (Amersham Corp., Arlington Heights, Ill.) by blotting overnight (13). The blot was air dried, UV cross-linked, prehybridized in prehybridization buffer (50% formamide, $5\times$ SSC, 20% Denhardt solution, 2% sodium dodecyl sulfate [SDS], 200 μ g of calf thymus DNA per ml) at 42°C for 2 to 4 h, hybridized with ^{32}P -labeled randomly primed DNA probe in fresh prehybridization buffer for 16 h at 42°C, washed (5 min, $2\times$ SSC, 0.1%

SDS; 15 min, $2\times$ SSC, 0.5% SDS; 2 h, 68°C, $0.1\times$ SSC, 0.5% SDS; 30 min, 68°C, $0.1\times$ SSC, 0.5% SDS), air dried, and exposed to X-ray film (13). In different experiments, size markers included actin mRNA (1.8 kb), lipoprotein lipase mRNA (3.6 kb), and 18S and 28S ribosomal RNA.

Plasmids and probes. *Bam*HI fragments are in pBR322 (22). *Eco*RI fragments are in pBR325 (8). Probes 1, 2, 3, 4, and 5 were subcloned into pGEM-1.

Nick translations. Nick translations for in situ hybridizations were done on linearized plasmids containing the insert of interest by standard techniques (13).

Random priming. Random priming for Northern blots was done on linearized plasmids according to the instructions of the manufacturer (Bethesda Research Laboratories, Inc., Gaithersburg, Md.).

Riboprobes. Riboprobes labeled with ^3H for in situ hybridizations were generated from inserts in pGEM-1 according to the instructions of the supplier (Promega). Individual probes corresponding to both strands were made by using either SP6 (probe 3A) or T7 polymerase (probe 3B).

RESULTS

The HSV-1 genome is a linear, double-stranded DNA molecule consisting of two unique sequence regions (unique long and unique short) bounded by inverted terminal repeat sequences. The location of the *Eco*RI and *Bam*HI restriction fragments used in this study are shown in Fig. 1. Additional restriction fragments derived from *Bam*HI-B and *Bam*HI-SP are shown in the blowup in the lower portion of the figure. The restriction fragments shown in Fig. 1 were labeled with ^3H and used as probes for in situ hybridization of trigeminal ganglia from latently infected rabbits.

In situ hybridization of DNA genomic probes to RNA from trigeminal ganglia of latently infected rabbits. ^3H -labeled cloned fragments representing over 99% of the HSV-1 genome were individually hybridized to sections of trigeminal ganglia taken from rabbits during the latent phase of infection with HSV-1 (McKrae). Representative photographs of in situ hybridizations are shown in Fig. 2. In all experiments, the treatment of latent trigeminal ganglionic tissue sections with RNase A before in situ hybridization resulted in the loss of detectable HSV-1 hybridization. Hybridization in latent tissue sections therefore detected HSV-1 RNA synthesized during latent infection and not genomic DNA. We have termed this RNA LR-RNA.

In situ hybridization in trigeminal ganglia from latently infected rabbits localized the LR-RNA in the nuclei of neurons (Fig. 2). This LR-RNA appears to be restricted to the nucleus, as previously reported (30, 31).

*Eco*RI fragments EK and JK and *Bam*HI fragments E, B, and SP all hybridized to trigeminal ganglia from latently infected rabbits (Table 1). None of the probes hybridized to trigeminal ganglia from uninfected rabbits. All probes hybridized to acutely infected tissue culture cells and to trigeminal ganglia from acutely infected rabbits (data not shown).

Although Table 1 and the upper portion of Fig. 1 show five restriction fragments that hybridized to latently infected trigeminal ganglia, the inverted repeats in the HSV-1 genome allow the number of restriction fragments to be effectively reduced to two. Most of the *Bam*HI E fragment and the *Eco*RI JK fragment are within the long terminal repeat. This region is repeated in the long internal inverted repeat, which is within the *Bam*HI-B and *Bam*HI-SP combination and also

FIG. 1. HSV-1 genomic structure and location of probes. The HSV-1 genome is a linear double-stranded DNA molecule consisting of two unique sequence regions (U_L and U_S) bounded by inverted repeat sequences (TR_L to IR_L and TR_S to IR_S). The long and the short regions exist in all four possible orientations generated by inversions about the L/S junction. The *Eco*RI and *Bam*HI restriction fragments used as probes are shown. Symbols: , areas that hybridized to RNA in latently infected trigeminal ganglia; , areas that hybridized to approximately 10 times more neurons from latently infected tissue than did stippled areas (see Table 3); , areas that did not hybridize during latency. The bottom portion of the figure represents a blowup of the *Bam*HI B and SP fragments. Additional probes made by additional restriction enzyme cuts are shown. Riboprobes 3A and 3B represent single-stranded RNA probes of opposite sense. The sense of probe 3A is complementary to that of IE110 mRNA. Probe 3B has the same sense as IE110 mRNA does and therefore would hybridize to RNA of opposite sense to that of IE110 mRNA. The position and direction of transcription of the ICP0 gene are also shown (21). Exons are shown by open rectangles. Introns are shown as solid lines. Restriction enzymes: S, *Sal*I; Sc, *Sac*I.

within *Eco*RI-EK. Most of *Bam*HI-E is therefore repeated within *Bam*HI-B, and most of *Eco*RI-JK is repeated within *Eco*RI-EK. In addition, except for a very short region at the left end (as shown in Fig. 1), the *Eco*RI EK fragment is completely contained within the combination of *Bam*HI fragments B, SP, Y, and N. The failure of *Bam*HI-Y and *Bam*HI-N to hybridize reduced the potential hybridizing region in *Eco*RI-EK to the area covered by *Bam*HI-B and *Bam*HI-SP. Thus, if we ignore the small regions of *Bam*HI-E and *Eco*RI-JK that are outside of the long terminal repeat and the short region of *Eco*RI-JK that is to the left of *Bam*HI-B in Fig. 1, then all of the HSV-1 sequences hybridizing to RNA from trigeminal ganglia from latently infected rabbits are contained in the combination of the *Bam*HI B and SP fragments. These two fragments were therefore chosen for more detailed analysis.

In situ hybridization with restriction fragments subcloned from *Bam*HI-B. The *Bam*HI B fragment was digested with *Sal*I, and the three resultant fragments were cloned into pGEM-1. The results of in situ hybridizations with these probes are shown in Fig. 1 and Table 2. As expected, all three probes hybridized to tissue from acutely infected sections but not from uninfected controls. In latent tissue sections, the left end (as shown in Fig. 1) of *Bam*HI-B (probe 1) did not hybridize, while probes 2 and 3 did, thus mapping the LR-RNA to the region of the genome represented by the latter two probes. As indicated in Fig. 1, most of the immediate-early gene ICP0 is contained in probe 3, while a small portion of the 3' end of ICP0 extends into probe 2. This suggests that the LR-RNA may be ICP0 mRNA. However,

this is incorrect, since we show below that the sense of this LR-RNA is opposite to that of ICP0 mRNA and that this LR-RNA maps to a region overlapping the 3' end of ICP0.

In situ hybridization with restriction fragments subcloned from *Bam*HI-SP. *Bam*HI-SP was digested with *Sac*I. Two of the resulting fragments (probes 4 and 5, Fig. 1) were cloned into pGEM-1. Both probes hybridized to acutely infected tissue but not to uninfected controls (Table 2). Both probes also hybridized to tissue from latently infected rabbits, indicating the presence of an LR-RNA coded for by this genomic region. Although ICP0 extends slightly into probe 4, probe 5 does not contain any of the ICP0 gene. Thus, the LR-RNA detected by probe 5 is not ICP0 mRNA.

Quantitation of the number of neurons containing LR-RNA.

The percentages of neurons from latently infected rabbits that hybridized with probes 2, 3, 4, and 5 were determined by using five rabbits for each probe (Table 3). The percentages of neurons to which probes 2 and 3 (derived from *Bam*HI-B) hybridized (and which therefore contained LR-RNA) were similar and ranged from approximately 1 to 10%, with a mean of 3.2%. In contrast, the percentages of neurons containing LR-RNA that hybridized to probes 3 and 4 derived from *Bam*HI-SP were approximately 10-fold lower, ranging from only 0.02 to 0.93%, with a mean of 0.31%. This result suggests that there may be two distinct LR-RNAs, a major LR-RNA detected by probes 2 and 3 and a less abundant, minor LR-RNA detected by probes 4 and 5.

Direction of synthesis of the major LR-RNA. The sense of the major LR-RNA was determined by in situ hybridiza-

FIG. 2. In situ hybridization of trigeminal ganglia from latently infected rabbits. Trigeminal ganglia were removed, fixed, sectioned, and hybridized with the ^3H -labeled, nick-translated, 2.6-kb *Sall*-*Bam*HI fragment shown in Fig. 1 (probe 3). Representative tissue sections are shown. Dense black grains indicate positive hybridization. (A) Trigeminal ganglia from a latently infected rabbit. Magnification, $\times 364$. (B) Higher magnification ($\times 910$) of panel A. (C) Trigeminal ganglia from an acutely infected rabbit. Magnification, $\times 364$. (D) Trigeminal ganglia from an uninfected rabbit. Magnification, $\times 364$.

tions, with single-stranded RNA as a probe (riboprobe). Individual riboprobes were made from both strands of probe 3 (which contains the majority of the ICP0 gene) by in vitro transcription with either SP6 or T7 polymerase. The rightward synthesized probe (3A) that is complementary to ICP0 mRNA did not hybridize with trigeminal ganglionic tissue from any of 22 latently infected rabbits, indicating that the major LR-RNA is not ICP0 mRNA (Table 2 and Fig. 1). In contrast, the leftward synthesized probe (3B) that has the same sense as ICP0 mRNA hybridized to 25 of 26 specimens, indicating that the sense of LR-RNA is opposite to that of ICP0 mRNA. These results make it tempting to speculate that the major LR-RNA is involved with antisense control of ICP0 transcription.

Northern blot analysis of LR-RNA. Total RNA was isolated from latently infected rabbit trigeminal ganglia, resolved on formaldehyde gels, blotted to nylon filters, and hybridized with ^{32}P -labeled randomly primed DNA probes 2 and 3 (Fig. 3). These probes detected a distinct LR-RNA band of approximately 1.8 to 2.2 kb (lanes 2 and 4). This band did not appear in uninfected trigeminal ganglionic

controls (lane 1) or uninfected tissue culture controls (lane 5). The major LR-RNA was also not readily detected in RNA from trigeminal ganglia of acutely infected rabbits (lane 3) or in RNA from acutely infected tissue culture cells (lane 6). As expected, probes 2 and 3 hybridized to ICP0 mRNA (approximately 2.7 kb) from acutely infected tissue culture cells (lane 6). Interestingly, in many preparations of RNA from acutely infected rabbit trigeminal ganglia, ICP0 mRNA was not detected. This correlates with the in situ hybridization data presented in Table 2, in which probe 3 (ICP0 only) hybridized to only about half of the acutely infected rabbit samples (see Discussion).

A second, fainter band was also detected in lanes 2 and 4 from latently infected rabbits. This RNA band had an approximate size of 1.2 to 1.5 kb. It was detected in all 11 latently infected rabbits examined, suggesting that it was not an artifact. In addition, in some experiments the smaller band had the same intensity as the larger band did. The origin of this band is not known. Possibilities include its being a specific breakdown product of the larger RNA, a spliced product of the larger RNA, a result of an alternative

TABLE 1. Detection of LR-RNA by in situ hybridization^a

HSV-1 probe ^b	Hybridization ^c detected in:	
	Uninfected controls	Latently infected rabbits
EcoRI fragments^d		
JK	0/5	11/11
D	0/4	1/19
G	0/6	0/20
N	0/6	0/9
F	0/4	0/4
M	0/4	0/4
O ^e		
L	0/4	0/4
A	0/11	2/19
I	0/8	0/32
EK	0/3	16/16
H	0/7	0/35
BamHI fragments^f		
E	0/5	12/13
B	0/3	6/6
SP	0/6	16/18
Y	0/11	0/23
N	0/11	0/23

^a Trigeminal ganglia from latently infected rabbits were hybridized with individual ³H-labeled, nick-translated *Eco*RI and *Bam*HI probes representing over 99% of the HSV-1 genome.

^b All probes hybridized to HSV-1-infected tissue culture cells.

^c Ratio of the number of rabbits showing positive hybridization to the total number of rabbits tested.

^d *Eco*RI fragments were cloned in pBR325 (8).

^e Not done. *Eco*RI-O represents less than 1% of the HSV-1 genome. Northern blots with this fragment were negative (data not shown). A probe covering this region has been reported to be negative by in situ hybridization (30).

^f *Bam*HI fragments were cloned in pBR322 (22).

splicing site, a truncated transcript from the same initiation site as the larger RNA, or a separate transcript from the same general region but with a different initiation site.

Both LR-RNA bands detected here were also detected with probes 2 and 3 individually (data not shown). In addition, with probes that corresponded to probe 1 (the left side of *Bam*HI-B in Fig. 1) or probes 4 and 5 (*Bam*HI-SP, to the right of *Bam*HI-B in Fig. 1), neither of the LR-RNA bands nor any other bands could be detected on Northern blots of RNA from latent rabbits (data not shown).

DISCUSSION

The results presented here indicate that during HSV-1 latency, significant viral RNA transcription (i.e., transcription detectable with current technology) is limited to the vicinity of the ICP0 gene. Other reports (4, 23, 30) have also concluded that transcription is limited to this region. However, these reports have not rigorously eliminated all other regions of the HSV-1 genome. Nearly 40% of the genome was untested by Deatly et al. (4). A crude mixture of cDNA clones made from poly(A)⁺ mRNA from trigeminal ganglia of latently infected mice was used by Puga and Notkins (23) to probe Southern blots of HSV-1 DNA fragments. Poly(A)⁺ RNAs were therefore specifically excluded. Stevens et al. (30) detected LR-RNA by using mixtures of probes covering the long and short repeats and the entire short unique region. Probes specific for three immediate-early genes from these regions were then used. No information was presented for those portions of the original positive regions not covered by

FIG. 3. Northern blot of RNA isolated from trigeminal ganglia of rabbits latently infected with HSV-1. Total RNA was isolated, run on a gel, and blotted to a nylon filter. The blot was dried, UV cross-linked, prehybridized, probed with a mixture of ³²P-labeled randomly primed probes 2 and 3, washed, and exposed to X-ray film for 1 week. Each lane represents total RNA recovered from either two trigeminal ganglia (from the same rabbit) or one 175-cm² tissue culture flask monolayer of Vero cells. Lanes: 1, uninfected ganglia; 2, latently infected ganglia (rabbit 1); 3, acutely infected ganglia; 4, latently infected ganglia (rabbit 2); 5, uninfected Vero cells; 6, acutely infected Vero cells. Similar results have been obtained with RNA from 11 latently infected rabbits and by using probes 2 and 3 individually.

these specific gene probes. Thus, large regions were left untested for the possibility of additional LR-RNAs. In contrast, we eliminated virtually all of the genome except for the region in the immediate vicinity of ICP0.

We demonstrated the presence of one and possibly two distinct LR-RNAs in trigeminal ganglia of rabbits latently infected with HSV-1. The more abundant (major) LR-RNA was detected in approximately 3% of the neurons from latent rabbit trigeminal ganglia. The less abundant (minor) LR-RNA was detected in less than 0.3% of the neurons from latent rabbit trigeminal ganglia. The minor LR-RNA has not previously been reported. This may be because probes for the minor LR-RNA region that lacked the major LR-RNA region were not used (30) (hybridization with the major

TABLE 2. In situ hybridization of trigeminal ganglia from rabbits with subclones from positively hybridizing *Bam*HI B and SP fragments

Probe	Hybridization ^a detected in:		
	Uninfected controls	Acutely infected rabbits	Latently infected rabbits
1	0/11	4/11	1/34
2	0/6	7/8	15/15
3	0/9	7/13	32/32
3A ^b	0/7	ND ^c	0/22
3B ^d	0/8	ND	25/26
4	0/8	4/9	14/17
5	0/5	2/3	12/15

^a Ratio of the number of rabbits showing positive hybridization to the total number of rabbits tested.

^b Riboprobe made from probe 3 and synthesized from left to right. This probe would hybridize to ICP0 mRNA, which has the opposite sense.

^c ND, Not done.

^d Riboprobe made from probe 3 and synthesized from right to left. This probe is of the same sense as ICP0 and would hybridize to RNA that is complementary to ICP0 mRNA.

TABLE 3. Percentage of neurons in latently infected trigeminal ganglia hybridizing with probes derived from *Bam*HI-B and *Bam*HI-SP probes

Source of neurons	No. (%) of neurons that hybridized with probe:			
	2 (from <i>Bam</i> HI-B)	3 (from <i>Bam</i> HI-B)	4 (from <i>Bam</i> HI-SP)	5 (from <i>Bam</i> HI-SP)
Rabbit no.				
06-4	108/4,534 (2.4)	63/3,916 (1.6)	8/3,036 (0.26)	8/2,301 (0.35)
06-5	104/3,741 (2.8)	74/3,325 (2.2)	2/2,234 (0.09)	1/1,540 (0.02)
06-7	162/4,978 (3.3)	401/3,952 (10)	3/3,971 (0.08)	1/4,594 (0.02)
06-17	51/5,890 (0.9)	85/4,383 (1.9)	12/2,748 (0.25)	3/2,116 (0.14)
06-19	159/3,437 (4.6)	77/3,194 (2.4)	17/3,813 (0.45)	39/4,200 (0.93)
Total ^a	584/22,580 (2.59)	700/18,770 (3.73)	42/15,802 (0.27)	52/14,751 (0.35)
Positive controls ^b	1,175/3,054 (38.5)	653/3,046 (21.4)	1,098/3,080 (35.6)	1,376/3,081 (44.7)

^a A Student *t* test comparing the extent of hybridization of *Bam*HI-B probes 2 and 3 (1,284/41,350 [3.11%]) with that of *Bam*HI-SP probes 4 and 5 (94/30,553 [0.31%]) indicated that the two groups were significantly different ($P < 0.01$).

^b Tissue culture controls were acutely infected with HSV-1.

LR-RNA would mask any in situ hybridization with the minor LR-RNA) or because of insufficient sensitivity (4, 23).

Northern blot analysis detected two LR-RNA bands of 1.8 to 2.2 kb and 1.2 to 1.5 kb in latently infected rabbits that both mapped to probes 2 and 3 (toward the left end of ICP0 in Fig. 1). It is not known whether the smaller LR-RNA band seen in lanes 2 and 4 in Fig. 3 is a unique transcript representing an additional LR-RNA gene or whether it is a breakdown product.

It is interesting that probes 1 and 3 hybridized to tissue from only about half of the acutely infected rabbits, while probe 2 hybridized to almost all acutely infected tissues. This result may reflect the fact that the areas of the genome represented by probes 1 and 3 contain only immediate-early genes (ICP27 and ICP0, respectively), while the region represented by probe 2 may contain non-immediate-early genes in addition to part of ICP27 and part of ICP0. Thus, at the time the rabbits were sacrificed (4 to 7 days postinfection), the immediate-early genes may be shutting off while later genes continue to be expressed. This would also explain why Northern blots detected ICP0 mRNA in acutely infected cells (5 h postinfection) but only occasionally detected ICP0 mRNA in trigeminal ganglia (5 to 7 days postinfection).

The data presented in this report mapped the major LR-RNA in rabbits to the vicinity of the ICP0 gene, as previously reported for the mouse (4, 23), but it had an orientation (sense) opposite that of ICP0 (30; D. L. Rock and A. B. Nesburn, 11th International Herpesvirus Workshop, 21 to 26 July 1986). In addition, the use of individual smaller probes combined with our Northern blot data allowed us to further deduce a more exact map location for the major LR-RNA. First, the major LR-RNA did not map to the extreme right (5') end of ICP0, since probe 4, which covers that region, hybridized only to the minor LR-RNA. Second, probe 2, which covers only a small stretch of the left (3') end of ICP0, hybridized to the major LR-RNA very efficiently. This short stretch of ICP0 in probe 2 might not be expected to account for the strong hybridization by itself. Third, Northern blot analysis indicated an approximate size of 1.8 to 2.2 kb for the major LR-RNA, compared with a size of 3.7 kb for the entire ICP0 gene. Thus, rather than covering the entire ICP0 gene, the major LR-RNA more likely begins to the left of ICP0 and partially overlaps the left (3') end of ICP0. We have not definitively shown in this report that the major LR-RNA begins to the left of ICP0. However, work currently under way in our laboratory (by S.L.W., H.G.,

and A.B.N.) to map the ends of the major LR-RNA with more precision indicates that several probes just to the left of ICP0, but not overlapping ICP0 (*Hpa*I-*Hpa*I and *Sph*I-*Hpa*I fragments), do hybridize to the major LR-RNA. This finding supports the notion that the LR-RNA begins to the left of ICP0.

Less can be deduced about the potential minor LR-RNA. Because of the decreased quantity of this LR-RNA, we have not yet determined its size by Northern blot analysis. On the basis of hybridization with probe 5, at least part of the minor LR-RNA maps outside (to the right) of ICP0. On the basis of positive hybridization with probe 4, the minor LR-RNA may or may not extend into the 5' (right) end of ICP0. Whether the minor LR-RNA hybridizes with probe 3 has not been determined, since the hybridization of probe 3 with the major LR-RNA would mask any in situ hybridization with the minor LR-RNA.

The presence of LR-RNAs during HSV-1 latency suggests that these RNAs may play a functional role in the initiation or maintenance of latency. On the basis of what is known about the major LR-RNA, several possible functions for this LR-RNA can be envisaged. (i) The LR-RNA may function as an antisense regulatory factor for ICP0. Although the data presented here show that the major LR-RNA is complementary to the 3' end of ICP0 rather than the 5' end, it is possible to obtain effective antisense suppression by 3' complementarity (9, 11). (ii) The LR-RNA may code for a (regulatory) protein. This is supported by published sequence data of the partially overlapping ICP0 gene (21), from which we have deduced that the LR-RNA contains at least one long open reading frame. (iii) The LR-RNA may function directly as a *trans*-acting factor. This is supported by localization of the LR-RNA to the nucleus during latency. (iv) Transcription of the LR-RNA may have a *cis*-acting effect that physically prevents transcription of ICP0 mRNA on the same stretch of DNA.

Evaluating potential functions of the minor LR-RNA is extremely difficult because of the lack of information on its identity. However, the minor LR-RNA may partially overlap the 5' end of ICP0. Although we have not yet determined the orientation (sense) of the minor LR-RNA, its position relative to ICP0 makes it a potential candidate for antisense control of ICP0.

Much work remains to characterize and decipher the possible roles of the major and minor LR-RNAs. We are currently completing the detailed fine mapping of the genes for these RNAs, which should provide valuable insight into the roles of these latency-related viral transcripts.

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