The Herpes Simplex Virus Type 1 Reactivation Function Lies outside the Latency-Associated Transcript Open Reading Frame ORF-2

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The latency-associated transcription unit has been shown to be important for in vivo reactivation of herpes simplex virus from the latent state. A recombinant virus was constructed to alter the largest open reading frame in this region. This virus had a wild-type reactivation phenotype, suggesting that herpes simplex virus does not require a protein function from this reading frame for efficient reactivation from latency.

An important biological property of the herpesviruses is their ability to establish latent infections (7, 20, 21). Herpes simplex virus type 1 (HSV-1) establishes latent infections in the sensory ganglia of its host, where it is subject to periodic reactivations caused by a variety of stimuli. The specific mechanism that triggers a reactivation event is not known.

Viral transcription during latency is apparently limited to a single transcription unit (2, 4, 8, 16, 18, 22). Two abundant, collinear RNAs transcribed from this region, known as the latency associated transcripts (LATs), have been isolated from latently infected neurons. The most abundant species is a 2.0-kb transcript. The other transcript has an internal splice that removes about 600 bases from the 2.0-kb transcript (25–27). Results of experiments that employed recombinant viruses indicate that the abundant LAT RNAs are derived from a larger primary transcript and that the promoter for LAT lies nearly 700 bp upstream of the 5' end of the abundant LATs (4, 11, 12, 19). Latent transcripts were also detected by in situ hybridization several kilobases downstream of the 3' end of LAT. Detection of latent transcripts ends near a canonical polyadenylation signal (4, 15). These other RNAs, detectable by in situ hybridization of latently infected sensory ganglia, were presumably derived from the same primary transcript. Although structures for some of these RNAs have not yet been determined, recent studies have shown that the abundant 2.0-kb LAT RNA is actually a stable intron (3, 6).

Several animal models for studying the HSV-1 replicative cycle have been developed. The animals used in these studies are infected via the eye (mice and rabbits) or via the footpad (mice) (10, 21, 23). In some of these animal models, results of experiments employing recombinant viruses deleted or altered for LAT expression have implied a role for this transcription unit in reactivation (10, 12, 19). Thus far, only viruses that contain LAT promoter deletions have been

phenotypically altered. To date, no mRNA derived from the LAT primary transcript has been isolated.

The largest conserved open reading frame (ORF-2) found within the primary transcript lies within the 2.0-kb LAT intron (14, 24, 28) (Fig. 1). Ordinarily, an intron would not be expected to encode protein. However, since the primary transcript can also be detected by Northern (RNA) blot analysis, this unspliced transcript may contain the protein translation message. Alternatively, a potential splice acceptor signal located just 5' of this ORF may be utilized to produce an mRNA that has not yet been detected. In any case, immunohistochemistry has shown that cultured neurons infected with HSV-1 express a protein derived in part from this ORF (5).

One difficulty in studying the ORF located within the LAT intron is the possibility that the intron itself may also play a role in the viral replication cycle. Since the intron is antisense to the ICP0 mRNA (2, 14, 22), it may affect the viral replicative cycle by limiting the expression of ICP0, a gene that is essential for efficient viral reactivation (9, 13). Sawtell and Thompson have provided some evidence that the intron affects establishment of latency in the trigeminal ganglia of mice (17). Therefore, when altering the ORF, it is important that the structural integrity of the RNA be maintained so that phenotypic changes caused by loss of the ORF are not confused with or masked by changes caused by the loss of the intron.

To determine whether ORF-2 is necessary for efficient viral reactivation, a frameshift mutation near the 5' end of ORF-2 was made by the insertion of an 8-bp *Cla*I linker (5'-pdCdAdTdCdGdAdTdG-3'). Specifically, the *Cla*I linker was inserted 170 bp into the ORF and 50 bp downstream of the first ATG. To create this virus, a recombinant plasmid containing the *Cla*I linker near the 5' end of ORF-2 was constructed, sequenced, and then cotransfected with 17+ HSV-1 viral DNA that contains a small deletion in the LAT region. Homologous recombinants were identified by screening for the rescue of the small LAT deletion in the parent virus. The recombinant virus obtained (RP1, or reactivation positive 1) was plaque purified three times. Southern blot analysis showed that the viral DNA of this

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FIG. 1. Structure of recombinant HSV-1 RP1. The map shows the location of ORF-2 contained in the latency-associated transcription unit. An arrow denotes the location of an 8-bp insertion. This virus was created by cotransfecting plasmid DNA containing a *Cla*I 8-bp linker at a *Hpa*I site 50 bp into ORF-2 with a derivative of HSV-1 strain 17+ containing a small deletion. Recombinant virus was identified and isolated as described previously (4). Probes used in Northern and Southern blot analyses are indicated. IR_L, long inverted repeat; IR_S, short inverted repeat.

recombinant contains the entire LAT region and the *Cla*I site of the recombinant plasmid (Fig. 2a). Northern blot analysis showed that this virus produces the abundant 2.0-kb LAT; i.e., the insertion of 8 bp does not alter the stability or size of the transcript (Fig. 2b). In mice latently infected with RP1, RNA from the 2.0-kb LAT region was found by in situ hybridization at an intensity and in a number of sites similar to those of the wild-type virus (data not shown). This indicates that RP1 is not impaired in the synthesis and stability of the 2.0-kb LAT in neurons or in cell culture and is not altered in its ability to establish a latent infection.

To determine the importance of ORF-2 with respect to reactivation, trigeminal ganglia that innervated rabbit eyes

were infected with either wild-type 17+ virus or an equivalent amount of RP1. Induction of reactivation by iontophoresis of epinephrine into the rabbit eyes resulted in recovery of RP1 from 14 of 16 eyes, a proportion equivalent to that induced from eyes latently infected with the wild-type virus (Table 1). Southern blot analyses of the DNA of the reactivated RP1 viruses showed that they all contained the *Cla*I linker insertion (data not shown). Since insertion of this linker causes a frameshift in ORF-2 without affecting other aspects of the LAT transcript and the virus is not altered phenotypically, we conclude that ORF-2 does not encode a function essential for reactivation of the virus from the latent state.

Recently, Block et al. (1) constructed a viral mutant, TB1, that has a 440-bp insertion in the LAT intron. This virus was reactivated efficiently from the trigeminal ganglia of mice in a cocultivation assay. RP1 is reactivated efficiently from the latently infected trigeminal ganglia of rabbits whose eyes have been iontophoresed with epinephrine. There are important differences between these viruses. In TB1, the insertion of 440 bp into the LAT intron disrupts the structure of the 2.0-kb LAT either by diminishing its stability or by causing transcription termination. In RP1, the 8-bp insertion into the LAT intron does not alter this transcript.

Since the stable LAT intron may act as an antisense inhibitor of ICP0 mRNA production during reactivation, it is important that this characteristic of the LAT not be altered when attempting to determine the effect of the large ORF on reactivation. The structure of the LAT stable intron pro-

FIG. 2. Southern and Northern blot analyses of RP1. (a) Viral DNA prepared from RP1-infected rabbit skin cells (lane B) or 17+-infected rabbit skin cells (lane C) was digested with restriction enzymes *Cla*I and *Bst*EII, electrophoresed through a 0.7% agarose gel, blotted, and probed with ³²P-labelled JB153. Lane A contains markers. Southern blot hybridization and analysis was performed as described previously (4). (b) Total RNA prepared from uninfected rabbit skin cells (lane A), RP1-infected rabbit skin cells (lane B), and 17+-infected rabbit skin cells (lane C) was electrophoresed through a formaldehyde-agarose gel, blotted, and probed with ³²P-labelled pATD19 as described previously (4). Northern blot hybridization and analysis was performed as described previously (4).

TABLE 1. Induction of reactivation

Reactivation	Virus	No. of reactivations/total no. (%)	
		Rabbits	Eyes
Induced	RP1 (<i>Cla</i> I)	8/8 (100)	14/16 (87)
	17+	7/9 (78)	11/18 (61)
Spontaneous	RP1 (<i>Cla</i> I)	3/8 (37)	3/16 (19)
	17+	8/17 (47)	8/34 (23)

duced from RP1 contains the portion of the transcript that is antisense to ICP0. Therefore, any effect upon reactivation caused by elimination of ORF-2 would not be confused with or masked by the loss of antisense inhibition of ICP0 mRNA production. Any effects related to elimination of this ORF would have been detected in our reactivation assay.

Clearly, to determine which regions of the LAT transcription unit are required for efficient HSV-1 reactivation, other recombinant viruses must be constructed and analyzed. This study decisively demonstrates that the largest ORF located within the LAT transcription unit has no effect on reactivation of the virus from latency.

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