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A 371-Nucleotide Region between the Herpes Simplex Virus Type 1 (HSV-1) LAT Promoter and the 2-Kilobase LAT Is Not Essential for Efficient Spontaneous Reactivation of Latent HSV-1

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The herpes simplex virus type 1 (HSV-1) latency-associated transcript (LAT) gene is essential for efficient spontaneous reactivation of HSV-1 from latency. However, neither the mechanism by which LAT carries out this function nor the region of LAT responsible for this function is known. LAT is transcribed as an unstable 8.3-kb RNA that gives rise to a very stable 2-kb LAT RNA that is readily detected in latently infected sensory neurons. We show here that 371 of the 662 nucleotides located between the start of LAT transcription and the 5′ end of the 2-kb LAT RNA do not appear to be essential for wild-type levels of spontaneous reactivation in the rabbit ocular model of HSV-1 latency. We deleted LAT nucleotides 76 to 447 from both copies of the LAT gene (one in each viral long repeat) to produce the mutant *d*LAT371. Rabbits were ocularly infected with *d*LAT371, and spontaneous reactivation was measured in comparison with the marker-rescued virus *d*LAT371R. Both *d*LAT371 and *d*LAT371R had spontaneous reactivation rates of approximately 13 to 14%. This was consistent with the parental McKrae wild-type virus (11.7%; $P = 0.49$) and significantly higher than the LAT transcription-negative mutant *d*LAT2903 (2.4%; $P < 0.0001$). Southern analysis confirmed that the spontaneously reactivated *d*LAT371 virus retained the deletion in both copies of LAT. Therefore, it appeared that the function of LAT involved in efficient spontaneous reactivation mapped outside the 371-nucleotide region deleted from the LAT gene of *d*LAT371.

Following primary herpes simplex virus type 1 (HSV-1) infection of the eye, the virus travels up nerves to the trigeminal ganglia (TG), where it establishes a latent infection in the nuclei of sensory neurons. At various times throughout the life of the latently infected individual, the virus may reactivate, travel back to the eye, and produce recurrent disease. This in turn can produce scarring of the cornea and loss of vision, making recurrent HSV-1 the most common cause of infectious blindness in the developed world (13). A similar HSV latency reactivation-recurrent disease cycle produces recurrent cold sores in and around the mouth and recurrent genital lesions.

During latency, LAT is the only viral gene that is abundantly transcribed (21, 26). LAT transcription-negative mutants reactivate poorly by explant or induced reactivation in mice (1, 6, 8–10, 22, 24, 27) and by induced (2, 16) and spontaneous (16) reactivation in rabbits. Thus, LAT is essential for efficient reactivation of HSV-1 from sensory neurons. The primary LAT transcript is 8.3 kb in size and gives rise to a family of LAT RNAs (LATs), including a very stable 2-kb LAT and a stable 1.5-kb LAT that appears to be derived from the 2-kb LAT (3, 4, 21, 23, 25, 26, 28–33). Although the mechanism by which LAT enhances spontaneous reactivation remains unknown, we recently were able to show that the region of the primary LAT transcript downstream of nucleotide (nt) 1499 is not required for this function (18). Here, we show that LAT nt 76 to 447 are also not required.

Structure of *d*LAT371. McKrae was used as the parental virus because its high spontaneous-reactivation rate in rabbits allows changes in the spontaneous-reactivation rates of mutants to be detected and analyzed statistically (16, 19). A *Swa*I-*Bam*HI fragment corresponding to nt −798 to 4658 relative to the start of LAT transcription was subcloned from the previously cloned *Eco*RI EK fragment of HSV-1 strain McKrae (17). It was then digested with *Sty*I, religated to produce a 371-nt deletion corresponding to LAT nt 76 to 447, and cotransfected with infectious McKrae DNA as we previously described to generate mutants by homologous recombination (16, 19). A recombinant virus from the cotransfection (*d*LAT371) was triple plaque purified and grown up into a stock, and the presence of the 371-nt deletion in both copies of LAT (one in each viral long repeat) was confirmed by restriction digestion and Southern analysis. A marker rescued virus (*d*LAT371R) was then made by analogous methods.

The genomic structures of wild-type HSV-1 McKrae and *d*LAT371R are shown in Fig. 1A. The long repeats are expanded (dashed lines) to show the relative locations and statuses of the LAT, ICP0, and ICP34.5 genes (Fig. 1B). The primary 8.3-kb LAT transcript is unstable and difficult to detect. It gives rise to the very stable and easily detected 2-kb LAT transcript. *d*LAT371 contains a 371-nt deletion of LAT nt 76 to 447 (Fig. 1C; dashed rectangle), prior to the start of the stable 2-kb LAT at nt 662.

Replication and virulence of *d*LAT371. Rabbit skin cells were infected with 0.01 PFU of *d*LAT371, *d*LAT371R, or wild-

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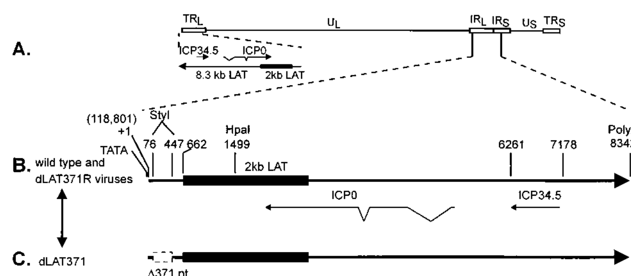

FIG. 1. Structure of *d*LAT371. (A) Schematic representation of the prototypic orientation of wild-type HSV-1. HSV-1 contains a unique long (U_L) region and a unique short (U_S) region, each bounded by inverted repeats. The unique regions are indicated by solid lines. The repeats are indicated by open rectangles. TR_L, long terminal repeat; IR_L, long internal repeat; TR_S, short terminal repeat; IR_S, short internal repeat. The long repeat regions containing the LAT gene are exploded, as indicated by the dashed lines. (B) Detailed blowup of the region from wild-type virus and the marker-rescued *d*LAT371R virus that contains the 8.3-kb primary LAT transcript. The direction of transcription is indicated by the arrowhead. TATA indicates the location (in the genomic DNA) of the LAT promoter TATA box. The start of LAT transcription is indicated by +1, corresponding to nt 118801 of the genome. The solid rectangle within the primary 8.3-kb LAT transcript indicates the location of the stable 2-kb LAT which starts at LAT nt 662. The locations and directions of the ICP34.5 and ICP0 transcripts are shown for convenience. (C) The *d*LAT371 mutant. The 371-nt deletion is indicated by the dashed rectangle and is bounded by the *Sty*I restriction sites shown in panel B. This deletion is present in both copies of LAT (one in each long repeat).

type virus per cell. All viruses replicated with the same kinetics (12, 24, and 48 h postinfection), producing the same final titer of approximately 3×10^8 PFU/ml at 72 h postinfection (data not shown). Groups of 15 rabbits were infected with 2×10^5 PFU of *d*LAT371 or *d*LAT371R per eye. Rabbit survival for both groups was similar to that of wild-type virus (15, 16, 18, 19) for *d*LAT371, 6 of 15 survivors; for *d*LAT371R, 7 of 15 survivors [P = 1.0]). Thus, the 371-nt deletion did not alter replication of the virus in tissue culture or virulence of the virus in rabbits.

LAT transcription occurs in the TG of rabbits latently infected with *d*LAT371. TG were removed from latently infected rabbits 60 days postinfection (>30 days after latency had been established). RNA was isolated from individual TG and subjected to first-strand cDNA synthesis, and the cDNA product was amplified by PCR as we previously described (18) (Fig. 2). One set of primers generated a 160-bp product specific for a region of the primary LAT transcript in front of the 2-kb LAT. A second set generated a 170-bp product specific for a region within the 2-kb LAT (Fig. 2B). Southern analysis indicated that TG latently infected with either *d*LAT371 or *d*LAT371R transcribed LAT RNA corresponding to both regions while no reverse transcription (RT)-PCR products were detected in uninfected TG (not shown) or TG latently infected with the LAT deletion mutant *d*LAT2903 (Fig. 2, lanes 5 and 6). Thus, *d*LAT371 appeared capable of transcribing LAT during latency.

Spontaneous reactivation of *d*LAT371. Rabbit eyes were infected with 2×10^5 PFU of *d*LAT371 or *d*LAT371R per eye. Starting 30 days postinfection, at which time latency had already been established, tear films were individually collected from all eyes and plated on indicator cells to detect spontaneously reactivated virus as we previously described (15, 18). The cumulative numbers of virus-positive tear film cultures per eye during 26 days for each virus are shown in Fig. 3A. *d*LAT371 (open squares) and *d*LAT371R (solid squares) appeared to have very similar cumulative spontaneous reactivation rates of over 3.5 positive cultures per eye on day 26 of collection. For comparative purposes, Fig. 3B shows a compilation of our results with wild-type virus (solid circles) and *d*LAT2903 (open circles), a LAT promoter deletion mutant that makes no LAT RNA. The cumulative spontaneous reactivation rate for wild-type virus was similar to those of *d*LAT371 and *d*LAT371R, reaching over 3.5 positive cultures per eye by the 26th day of collection. In sharp contrast, *d*LAT2903, appears to have a much lower spontaneous reactivation rate (open circles) of less than 1 positive culture per eye.

A statistical analysis of positive (spontaneously reactivated) tear film cultures versus total cultures is shown in Table 1. Approximately 13.1% (38 of 290) of the tear films from rabbits latently infected with *d*LAT371 contained spontaneously reactivated virus. This was similar to the results for *d*LAT371R (13.8%; 52 of 378 [P = 0.82 by Fisher's exact test]) but significantly higher than the low level of spontaneous reactivation seen with the LAT-negative virus *d*LAT2903 (2.4%; 37 of 1,516 [P = < 0.0001]). Because the above analysis does not take into account the numbers of eyes in each group, we calculated the fraction of virus-positive cultures for each eye in each group (i.e., the fraction of time each eye was virus positive) and analyzed these individual fractions using the Mann-Whitney rank sum test. By this analysis (Table 1), the spontaneous-reactivation rate for *d*LAT371 was also significantly higher than that of *d*LAT2903 (P < 0.0001) and indistinguishable from that of *d*LAT371R (P = 0.50) or wild-type virus (P = 0.68).

The numbers of eyes in each group that reactivated at least once were also analyzed (Table 1). Again, the result for *d*LAT371 was similar to that for *d*LAT371R (P = 0.31) and significantly higher than that for *d*LAT2903 (P = 0.002). Likewise, the numbers of rabbits that had at least one spontaneous reactivation (Table 1) were similar for *d*LAT371 and *d*LAT371R (P = 1.0) and significantly higher than that for *d*LAT2903 (P = 0.006). All of the above analyses indicate that *d*LAT371 reactivated spontaneously at a rate indistinguishable

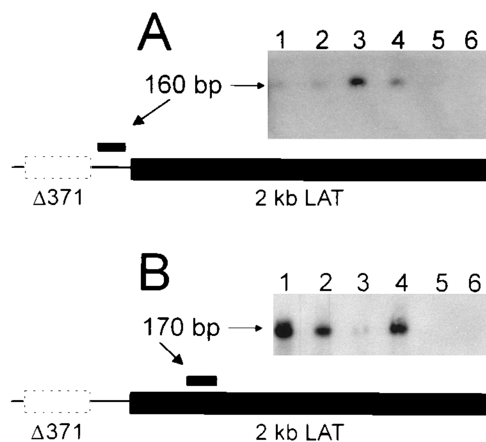

FIG. 2. Transcription of LAT in rabbits latently infected with *d*LAT371. RT-PCR of total RNA isolated from individual TG from latently infected rabbits was done as we previously described (18). The schematic under each autoradiogram indicates the location in the LAT region of the expected RT-PCR products (small black rectangles). (A) Primers corresponding to LAT nt 471 to 500 and nt 602 to 631 between the 371-nt deletion and the start of the 2-kb LAT. The internal ^{32}P-labeled probe corresponds to LAT nt 550 to 579. (B) Primers corresponding to LAT nt 960 to 989 and nt 1100 to 1129 within the 2-kb LAT. The internal ^{32}P-labeled probe corresponds to LAT nt 1070 to 1099. Each lane in panel A shows the RT-PCR product from RNA isolated from one TG, each from a different rabbit. The RT-PCRs in panel B were done with the same RNAs. Lanes: 1 and 2, *d*LAT371; 3 and 4, *d*LAT371R; 5 and 6, *d*LAT2903 (LAT-negative control).

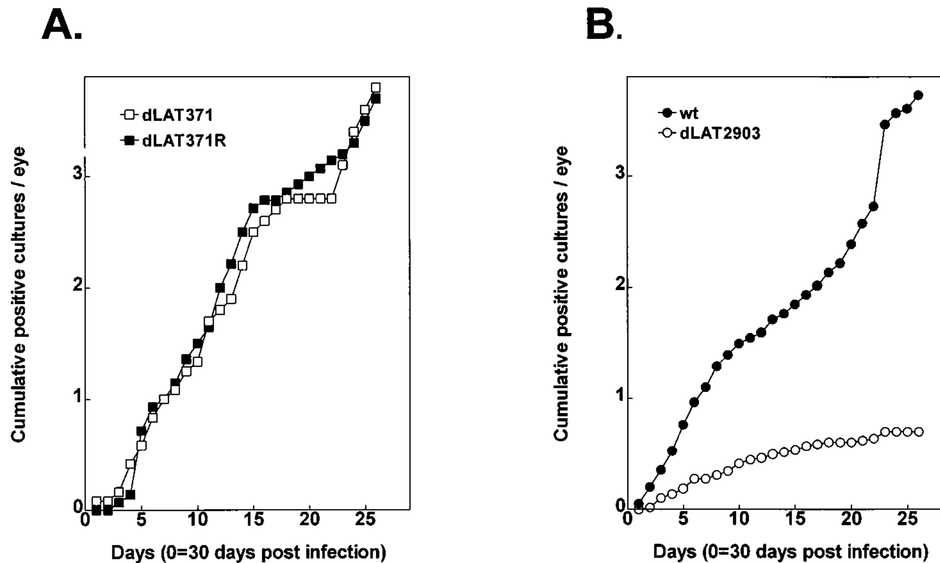

FIG. 3. Spontaneous reactivation in rabbits latently infected with *d*LAT371. Rabbits were ocularly infected and latency was established as we previously described (14). Beginning on day 30 postinfection (day 0), at which time latency had been established, tear films were collected daily, plated on primary rabbit kidney cells, and observed for up to 30 days for the presence of cytopathic effect. All positive cultures were confirmed by passage and Southern analysis. The *y* axis represents the cumulative number of HSV-1-positive cultures for each virus group divided by the number of eyes in the group. Statistical analyses are shown in Table 1. (A) Current results obtained with *d*LAT371 and *d*LAT371R; (B) results compiled from previous studies with wild-type (wt) viruses and the LAT promoter deletion virus *d*LAT2903 (16).

from those of the marker-rescued *d*LAT371R virus and wild-type virus and that this rate was significantly higher than that seen with the LAT-negative virus *d*LAT2903. Southern analysis of randomly selected spontaneously reactivated viruses grown from tear films confirmed that the spontaneously reactivated *d*LAT371 virus retained the 371-nt deletion in both copies of LAT (data not shown).

Many LAT-negative mutants have reduced reactivation frequencies in mice or rabbits (1, 2, 6–10, 16, 22, 24, 27). Mutations of other HSV-1 genes, such as the thymidine kinase gene and the ICP34.5 gene, also appear to reduce HSV-1 reactivation (5, 19). However, these mutants all impair the ability of the virus to replicate either in tissue culture or in the infected animal, particularly in neurons. The impact of these mutants on reactivation is therefore likely to be due to these more general deficits rather than to an alteration specific for the latency reactivation cycle. In contrast, LAT mutants appear to be completely wild type except for reactivation (possibly because of reduced establishment of latency). Thus, of the HSV-1 genes so far examined, LAT alone appears to have a specific latency reactivation function that does not involve other viral functions. Even so, it is not yet clear whether LAT enhances spontaneous reactivation by enhancing establishment of latency or if LAT has a function directly involved in the reactivation stage. Regardless, it is still appropriate to state that LAT is required for efficient spontaneous reactivation.

Speculation as to how LAT enhances reactivation includes models of (i) an antisense mechanism (21, 26) by which production of ICP0 and perhaps ICP34.5 is prevented, thereby increasing the initial rate at which the virus establishes latency; (ii) a (presumably regulatory) LAT-encoded protein; and (iii) a non-antisense interaction between LAT RNA and cell or viral factors. Our strategy to help distinguish among these possibilities has been to genetically map the essential func-

TABLE 1. Spontaneous reactivation and statistical analysis of *d*LAT371[a]

Virus or statistical correlation[b]	No. of positive tear film cultures/total (%)[c]	Fraction positive cultures/eye[d]	No. of eyes that reactivated/total (%)	No. of rabbits that reactivated/total (%)
*d*LAT371	38/290 (13.1)	0.127	9/12 (75)	5/6 (83)
*d*LAT371R	52/378 (13.8)	0.137	13/14 (93)	6/7 (86)
Wild type	183/1,570 (11.7)	0.112	44/60 (73)	25/30 (83)
*d*LAT2903	37/1,516 (2.4)	0.024	15/58 (26)	13/28 (46)
P (*d*LAT371 vs *d*LAT371R)	0.82[e]	0.50[f]	0.31[e]	1.0[e]
P (*d*LAT371 vs wild type)	0.49[e]	0.68[f]	1.0[e]	1.0[e]
P (*d*LAT371 vs *d*LAT2903)	<0.0001[e]	<0.0001[f]	0.002[e]	0.006[e]

[a] Statistical analysis of the data presented in Fig. 3.
[b] Rabbits were infected with *d*LAT371 or marker-rescued LAT371R as described in Materials and Methods. The results were compared between these groups and with a compilation of our previous results with wild-type virus and the LAT-negative virus *d*LAT2903.
[c] Spontaneous reactivation was assessed by culturing tear films as described in the legend to Fig. 3.
[d] For each eye within each group, the fraction of days on which positive cultures were obtained was calculated (total number of HSV-1-positive cultures for each eye/total number of cultures for each eye). The average is shown.
[e] Fisher's exact two-sided test. The groups are considered significantly different when $P < 0.05$.
[f] Mann-Whitney rank sum two-sided test. The groups are considered significantly different when $P < 0.05$.

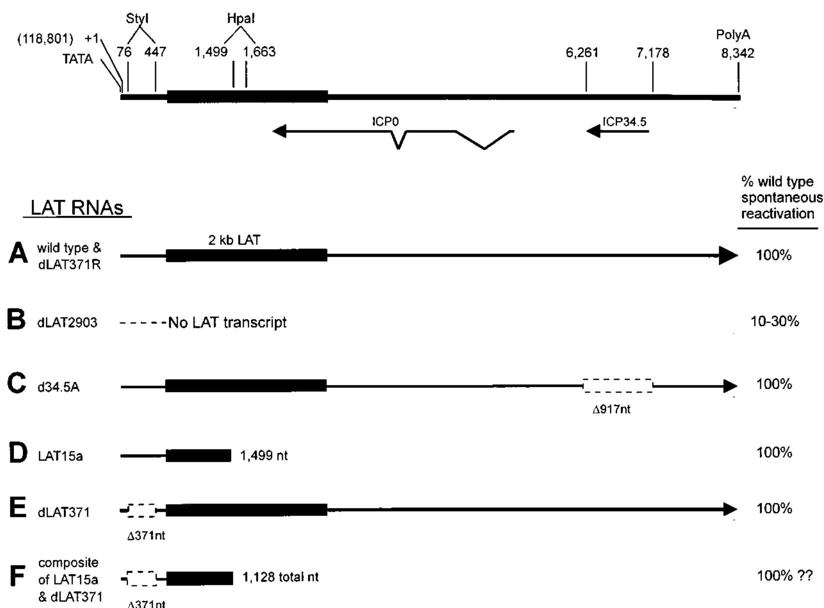

FIG. 4. LAT RNAs transcribed by various LAT mutants. The top of the schematic summarizes the genomic region containing LAT, including relevant restriction sites and the relative locations of the ICP0 and ICP34.5 RNAs. (A) Wild-type and *d*LAT371R primary LAT RNA structure; (B) *d*LAT2903, an *Eco*RV-*Hpa*I deletion from LAT nt −161 to +1673 that makes no LAT RNA (16); (C) *d*34.5A (15) containing a functional copy of the ICP34.5 gene inserted into the unique long region to provide the virus with the ICP34.5 function that was removed by deletion of LAT nt 6261 to 7178 (19) (which overlaps in an antisense direction the normal location of the ICP34.5 gene); (D) LAT15a (18), in which the LAT promoter and the first 1.5 kb of LAT were inserted into the unique long region of *d*LAT2903 (see panel B); (E) *d*LAT371, the mutant presented in this report; (F) Composite of *d*LAT371 and LAT15A illustrating the 1,128 nt expected to be sufficient for efficient spontaneous reactivation. The question marks in the reactivation column indicate the predicted but untested reactivation phenotype of the composite.

tional portion(s) of the 8.3-kb LAT. In this report, we have continued this strategy. We deleted LAT nt 76 to 447 from both copies of LAT in HSV-1 strain McKrae and determined that the resulting virus, *d*LAT371, reactivated spontaneously in the rabbit ocular model with wild-type virus efficiency, thus demonstrating that this region of LAT was not essential for wild-type levels of spontaneous reactivation.

While this work was in progress, there was a report that a similar mutant made in HSV-1 strain 17 syn$^+$ (17ΔSty) was not impaired for in vitro explant reactivation in the mouse model (11). Our findings in the present report extend this result to include in vivo spontaneous reactivation. This is important, because the mechanisms and the viral genes or regions of LAT involved in in vitro explant reactivation in mice and in vivo spontaneous reactivation in rabbits may differ. The authors of the report on 17ΔSty stated the following: "given the different behaviors of the same mutants in different models of latency and reactivation, it will be important to study 17ΔSty in the in vivo rabbit eye system." For example, the mutant X10-13, a LAT promoter deletion mutant, was reported to reactivate normally in in vitro explant assays from latently infected mice (8) but to reactivate poorly in induced in vivo assays by the same investigators (6). Furthermore, many HSV-1 strains, such as KOS and F, reactivate efficiently in the mouse in vitro explant assay but reactivate very poorly, if at all, in the in vivo spontaneous-reactivation rabbit eye system. Thus, the finding that deletion of LAT nt 76 to 447 in strain 17 syn$^+$ did not alter explant reactivation in the mouse model did not exclude this region of LAT from being essential for spontaneous reactivation in vivo.

We recently showed that deletion of the LAT promoter and the first 1,673 nt of LAT resulted in a virus with severely impaired spontaneous reactivation (Fig. 4B), indicating that LAT was essential for efficient spontaneous reactivation (16). We then found that although deletion of the ICP34.5 gene, which overlaps LAT nt 6200 to 7100, reduces spontaneous reactivation (19), wild-type reactivation can be restored by insertion of the ICP34.5 gene at a distant location, while still retaining the 6.2- to 7.1-kb deletion in LAT (15). Thus, the primary LAT transcript does not require the 6.2- to 7.1-kb region of LAT for wild-type reactivation (Fig. 4C). We then found that inserting the LAT promoter and the first 1,499 nt of LAT into the unique long region of a LAT-negative mutant produced a virus capable of transcribing only the first 1.5 kb of LAT. This virus reactivated spontaneously with wild-type efficiency (Fig. 4D), thus mapping the LAT function involved in spontaneous reactivation to within the first 1,499 nt of the 8.3-kb LAT (18). In the present report, we have shown that the region of LAT from nt 76 to 447 is not essential for wild-type spontaneous reactivation (Fig. 4E). Assuming that the results of different mutants can be combined without concern for possible harmful interactions, a compilation of our recent findings with the results reported here produces the conclusion that the functional portion of LAT resides within nt 1 to 76 and/or nt 447 to 1499 (Fig. 4F). This is a total of only 1,128 nt, compared with the 8,342 nt that compose the primary LAT transcript. Our results have, therefore, eliminated approximately 86% of the 8.3-kb LAT from being essential for the spontaneous reactivation function of LAT and have mapped this function to within no more than 14% of the LAT gene. Of course, our results have not formally eliminated the possibility that LAT's spontaneous reactivation function may reside somewhere within the 1,792 nt upstream of the start of LAT transcription.

If there is a LAT protein that is essential for wild-type levels of spontaneous reactivation, it (or at least its 5′ end and its functional domains) must map within the 1,128 nt determined by the above genetic analyses. Analysis of the published se-

quence for strains 17 syn$^+$ (12, 20) reveals four potential open reading frames (ORFs) within this region that start with an ATG and end with a typical termination codon (ORF-1, LAT nt 488 to 667; ORF-2, nt 915 to 1118; ORF-3, nt 979 to 1062; and ORF-4, nt 1163 to 1354). Examination of the published sequence for strain KOS indicates that ORF-1, ORF-2, and ORF-3 of strain 17 syn$^+$ have counterparts in KOS. Because of sequence differences, the KOS ORFs have some internal variations, but they start and stop at the same locations. Examination of the published sequence for strain F (32) indicates that the starts of ORF-2 and ORF-3 are in common with 17 syn$^+$ but that in F both ORFs are significantly longer. F does not have an ORF that corresponds to ORF-1. Neither KOS nor F has an ORF that corresponds to ORF-4. Thus, of these three HSV-1 strains, ORF-4 is unique to strain 17. The fact that KOS and F reactivate poorly compared with 17 syn$^+$ suggests that the unique ORF-4 of 17 syn$^+$ may be involved in the more efficient reactivation of 17 syn$^+$. We are currently sequencing this region from strain McKrae, which has a spontaneous-reactivation rate higher than that of syn 17$^+$ and which is the parental virus for all of our mutants. If ORF-4 is conserved between the two strains with high rates of reactivation, i.e., McKrae and syn 17$^+$ (while not being present in the strains with low rates of reactivation [F and KOS]), the likely importance of ORF-4 would be greatly strengthened.

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