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A 348-Base-Pair Region in the Latency-Associated Transcript Facilitates Herpes Simplex Virus Type 1 Reactivation

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Latency-associated transcript (LAT) promoter deletion mutants of herpes simplex virus type 1 have a reduced capacity to reactivate following adrenergic induction in the rabbit eye model. We have mapped a reactivation phenotype within LAT and describe the construction of recombinants in which poly(A) addition sites have been placed at intervals within the LAT region to form truncated LAT transcripts. These mutants localize the induced reactivation phenotype to the 5′ end of LAT. To further define this region, we constructed a recombinant containing a 348-bp deletion located 217 bp downstream of the transcription start site of the 8.5-kb LAT. This virus, 17Δ348, expresses LAT but exhibits a significantly reduced ability to reactivate following epinephrine iontophoresis into the cornea. Quantitative DNA PCR analysis reveals that 17Δ348 establishes a latent infection within rabbit trigeminal ganglia with the same efficiency as does either the rescuant or wild-type virus. The region deleted in 17Δ348 encodes three potential translational initiators (ATGs) which we have mutated and demonstrated to be dispensable for epinephrine-induced reactivation. In addition, three smaller deletions within this region have been constructed and were shown to reactivate at wild-type (parent) frequencies. These studies indicate that an undefined portion of the 348-bp region is required to facilitate induced reactivation. Sequence analysis of this 348-bp region revealed a CpG island which extends into the LAT promoter and which possesses homology to conserved elements within the mouse and human *XIST* transcript encoded on the X chromosome. Possible implications of these elements in the regulation of LAT expression are discussed.

Because of difficulties in manipulating in vitro neuronal systems of herpes simplex virus (HSV) latency, studies aimed at elucidation of the mechanism of HSV reactivation have been primarily performed with animals. In these animal models (for a review, see reference 45) latent virus has been reactivated by several methods, including UV irradiation (31) and treatment with hyperthermia (42, 43) or epinephrine (26, 29, 30). Induction of active infection in the rabbit cornea following iontophoresis of epinephrine is now a well-established and dependable method which has yielded valuable information concerning the reactivation process.

A hallmark of latent HSV infection is the transcription, in neurons, of a single transcription unit referred to as the latency-associated transcript (LAT). LAT is synthesized as a primary transcript of 8.5 kb and a 2-kb intron which localizes to the nucleus and has been demonstrated to be the predominant product detectable during the latent infection (for a review, see reference 39). While LAT has been shown to be dispensable for the establishment of latent infections, this transcription unit has been implicated in spontaneous and induced reactivation of the virus in a number of animal models (4, 5, 21, 24, 37, 49). Mechanistic studies of reactivation have been complicated by the fact that several important viral genes, including those for a major viral transactivator (ICP0) and a neurovirulence enhancer (ICP34.5), are found on the DNA strand opposite LAT, thus making difficult the evaluation of deletion mutants for establishment and reactivation effects specific to LAT.

In this study, a molecular genetic approach has been taken to map the reactivation phenotype within the primary LAT transcript. Using recombinants with an efficient polyadenylation transcript termination signal inserted at various sites along the primary transcript, we present evidence that the epinephrine-induced reactivation phenotype resides primarily within the 5′ region of the primary transcript. To further delineate the location of the reactivation phenotype, a recombinant with a deletion of 348 bp encompassing three ATGs at the 5′ end of the LAT primary transcript was constructed. Compared with the parent and rescuant, this recombinant was shown to reactivate at a much lower frequency from the rabbit eye following epinephrine induction. Oligonucleotide-directed mutagenesis of these ATGs demonstrated that they have no effect on induced reactivation, and additional recombinants with deletions within this 348-bp region have not permitted the identification of a specific element governing reactivation. This suggests that an undefined portion of the 348-bp region is required and that expression of a LAT-specific protein during the latent infection is not involved in the reactivation phenotype being studied. Sequence analysis of this region reveals numerous CpG islands which are conserved in analogous regions of the LAT of other members of the alphaherpesvirus group. In addition, this motif possesses significant homology to a region at the 5′ end of the X chromosome transcript *XIST*. Mechanisms by which this region could be governing herpesvirus reactivation will be discussed.

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MATERIALS AND METHODS

Cells and viruses. HSV type 1 (HSV-1) strain 17*syn*+ was used as the parental strain for all viruses constructed in this study and was propagated on rabbit skin cells as previously described (7, 48). Recombinants were generated by standard calcium phosphate transfection procedures and were characterized for growth characteristics and the ability to establish a latent infection as previously described (5, 13). Briefly, the viruses were assessed for their ability to replicate on mouse embryo fibroblasts and rabbit skin cells in vitro. In addition, the viruses were inoculated on the rear footpads of mice and evaluated for their ability to reactivate following explant cocultivation.

Construction of poly(A) viruses. A 600-bp fragment containing the rabbit β-globin poly(A) transcription termination signal was cloned into a plasmid containing the *Bam*HI S/Q region of HSV-1 strain 17*syn*+. This was placed at the sites *Hpa*I (nucleotide [nt] 120,299), *Bst*EII (nt 125,771), and *Rsr*II (nt 126,319) in the 5′-to-3′ orientation. These plasmids were used to make transfectant viruses as described above, and the recombinants were screened for the presence of the inserted fragment by hybridization with the β-globin fragment. The resulting viruses were assessed for their ability to express LAT by Northern (RNA) blot analysis of infected cell culture preparations, as well as screened for LAT expression by in situ hybridization in the dorsal root ganglia of mice at acute and latent times following footpad inoculation. The efficiency of termination of the poly(A) fragments was assessed by reverse transcription-PCR of acute and latently infected rabbit trigeminal ganglia using both the 5′ and 3′ PCR probes as previously described (5).

Construction of deletion constructs. The virus 17Δ348 was constructed with a bridge oligonucleotide to loop out a 348-bp region from a single-stranded phagmid containing a 4.4-kb region encompassing the LAT promoter. A 54-bp oligonucleotide (5′ GGT GTT TTT CGT TCC GTC GCC GGA AGA TCT TTC ACC CCC AGT ACC CTC CTC CCT 3′) was annealed to the single-stranded rescue template from plasmid DB26 (4.4-kb *Aat*II fragment extending from nt 115,950 to 119,293 of the HSV-1 genome). This primer was then extended with T7 DNA polymerase in a reaction mixture containing 10 mM Tris-HCl, pH 7.5; 2 mM dithiothreitol; 0.5 mM dATP; 0.5 mM dGTP; 0.5 mM dTTP; 0.5 mM 5-*methyl*-dCTP; 1 mM ATP; 2.5 U of T7 DNA polymerase; and 5 U of T4 DNA ligase. The resulting plasmids were transfected into *Escherichia coli* INV(F′), and replica nylon filter lifts were hybridized with a ^{32}P-labeled oligonucleotide (5′ GCA GAT CTC GGG GGC CGG CCG TTG GCG GTA ACC 3′) specific for the deleted region as well as DB26. Colonies containing the deletion (those hybridizing to DB26 and not the oligonucleotide corresponding to the deleted region) were grown up, further characterized by Southern blotting and DNA sequence analysis, and used to cotransfect HSV-1 DNA. Plaques were picked through agar, and replicate 96-well dishes were screened to identify wells harboring viruses containing the deletions.

Additional deletion viruses were constructed by PCR amplification of the three subfragments within the 348-bp region deleted in virus 17Δ348. The first, a 110-bp fragment, was synthesized by using the primer pair 5′ GCA GAT CTA TGT TTC GTT CGT CTG TCC CCT CAC GG 3′ and 5′ GCA GAT CTC GCG TGC CGG CGA GCG GGT CCG GA 3′; the second, a 91-bp fragment, was synthesized by using the primer pair 5′ GCA GAT CTA CGC GAA AAA GGC CCC CCG GAG GCT T 3′ and 5′ GCA GAT CTC GGG GGC CGG CCG TTG GCG GTA ACC 3′; and the third, a 116-bp fragment, was synthesized by using the primer pair 5′ GCA GAT CTT GGC GGC CCG GCC CGG GGC CCC GG 3′ and 5′ GCA GAT CTC GAT GGA GCC CGG CAG AAC ACC GAG G 3′. The PCR fragments generated by these primers contained terminal *Bam*HI sites which were used to subclone the respective fragments into a unique *Bam*HI site in the parent deletion plasmid. The resulting fragments were then used to rescue the 17Δ348 virus with the recombinants being screened by probing with the respective labeled PCR product.

Site-directed mutagenesis. Disruption of the ATG initiators was carried out by *dut-ung* selection of oligonucleotide-directed templates (25). Three separate oligonucleotides were employed: ATG-1 (5′ CCG TCG CCC GGA ATT TTT CGT TCG T 3′), ATG-2 (5′ GGG GCC TGA GAT TAA CAC TCG GGG T 3′), and ATG-3 (5′ AAC GGC CGG CGC ATT CGC TGT GGT T 3′). These three oligonucleotides were used as described above to mutagenize the plasmid DB26 by *dut-ung* selection. Mutagenized plasmids and the resulting viruses were sequenced with the Sequenase sequencing kit version 2.0 (U.S. Biochemicals). Viruses were screened by performing blots of 96-well dishes and hybridizing with end-labeled oligonucleotides specific for the mutated gene by TMAC hybridization (3).

Reactivation in rabbits. Infections in rabbits latently infected with HSV were reactivated by methods previously described (5, 19, 20). Briefly, each eye of 2-kg New Zealand White rabbits was inoculated with 1×10^5 to 2×10^5 PFU of virus. The eyes were examined for corneal epithelial lesions as evidence of acute infection, and rabbits were monitored for 40 days prior to induction. Shedding was assessed by daily swabbing of the eyes after day 35 and before epinephrine iontophoresis to assay for recovery of infectious virus prior to treatment. There was no shedding observed 3 to 5 days prior to epinephrine induction.

The rabbits were induced to reactivate by transcorneal iontophoresis of 0.01% epinephrine (0.8 mA for 8 min) once a day for three consecutive days. Eye swabs were performed daily for up to 7 days postinduction and assayed on primary rabbit kidney cell monolayers for the detection of infectious virus.

In vitro cocultivation of rabbit trigeminal ganglia. Rabbit trigeminal ganglia were removed at latent times and placed in culture as previously described (21). The cultures were scored for cytopathic effects as evidence of reactivated virus.

In situ hybridization of rabbit trigeminal ganglia. Latently infected rabbit trigeminal ganglia were sectioned and prepared for in situ hybridization by standard techniques. Sections were hybridized with a LAT-specific probe as previously described (5).

Extraction of RNA and DNA from rabbit trigeminal ganglia. DNA was extracted from rabbit trigeminal ganglia by digesting tissue with 250 μg of proteinase K (Sigma) in 1% sodium dodecyl sulfate-containing buffer consisting of 150 mM NaCl, 10 mM Tris, and 25 mM EDTA (pH 7.4) as described previously (21). RNA was isolated by extraction of tissue with guanidinium isothiocyanate and purified by centrifugation for 16 h at 36,000 rpm in a Beckman SW41 rotor through a 5.7 M CsCl cushion, and the pelleted RNA was then extensively digested with RNase-free DNase (46).

Quantitation of viral DNA and RNA by PCR. The primer pairs used for PCR amplification of viral DNA and cDNA were described previously (13). For DNA PCR, 100-μl reaction mixtures containing the DNA extracted from tissues were used directly. RNA extracted from the tissues was reverse transcribed by oligo(dT) priming using the reverse transcription kit from Invitrogen (San Diego, Calif.). PCR was performed in a Thermalcycler (M. J. Research, Cambridge, Mass.). Cycles consisted of denaturation at 94°C for 30 s, annealing at 5°C below the melting temperatures of the primers, and extension for 60 s at 72°C. The final cycle of each reaction was terminated with an extension time of 10 min. Reaction products were labeled by incorporation of 0.2 μCi of [α-^{32}P]dCTP, resolved by electrophoresis on 6% polyacrylamide gels, and visualized by autoradiography. Quantitation of bands was performed on a Bio-Rad model 620 video densitometer.

DNA sequence analysis. Sequence analyses were performed using TBLASTN for analyses of open reading frames (ORFs) and BLASTN for nucleic acid homology searches against sequences contained within GenBank, versions 81 to 91. The searches were performed through the National Center for Biotechnology Information using the GENINFO Experimental BLAST Network service (1). Default settings were utilized for most searches; however, *S* values were lowered in certain cases (see Results).

RESULTS

Poly(A) insertions generate nested truncations of the primary LAT transcript and indicate that the reactivation phenotype resides at the 5′ end. Since previous studies using LAT promoter mutants indicated that transcription of LAT was required for induced reactivation, we sought to identify the genetic element(s) responsible for this phenotype. Accordingly, several recombinant viruses which had the rabbit β-globin poly(A) region inserted at intervals along the length of the LAT region were constructed (Fig. 1). These poly(A) insertions allowed us to truncate the LAT transcript at the site of the poly(A) insertion and to determine whether the region downstream of each insertion has a role in reactivation. This approach was chosen over the alternative approach of making nested deletions since it allowed us to truncate LAT without deleting or interrupting the ICP0 and ICP34.5 transcripts encoded on the opposite strand of the genome. This is critical since these genes have been demonstrated to have important roles in the replication of virus, and interfering with their transcription would make the assessment of the contribution of the deleted region of LAT impossible. We used PCR analysis of oligo(dT)-primed cDNA generated from RNA extracted from latently infected rabbit ganglia to confirm that the poly(A) transcription termination signals inserted into the latency transcription unit (i) efficiently terminated transcription upstream of the normal LAT polyadenylation site and (ii) did not interfere with transcription from the LAT promoter. We used PCR primer sets described previously (13) which generate products from the 5′ and 3′ ends of the primary LAT transcript to examine the general extent of the truncated LAT expressed by the recombinant viruses. As shown in Fig. 2A, primer sets specific for both the 5′ and 3′ ends generate the appropriately sized product with wild-type (wt) virus, but neither set generated a signal in the LAT-negative 17ΔPst mutant.

Latency-specific RNA expressed by the three poly(A) mu-

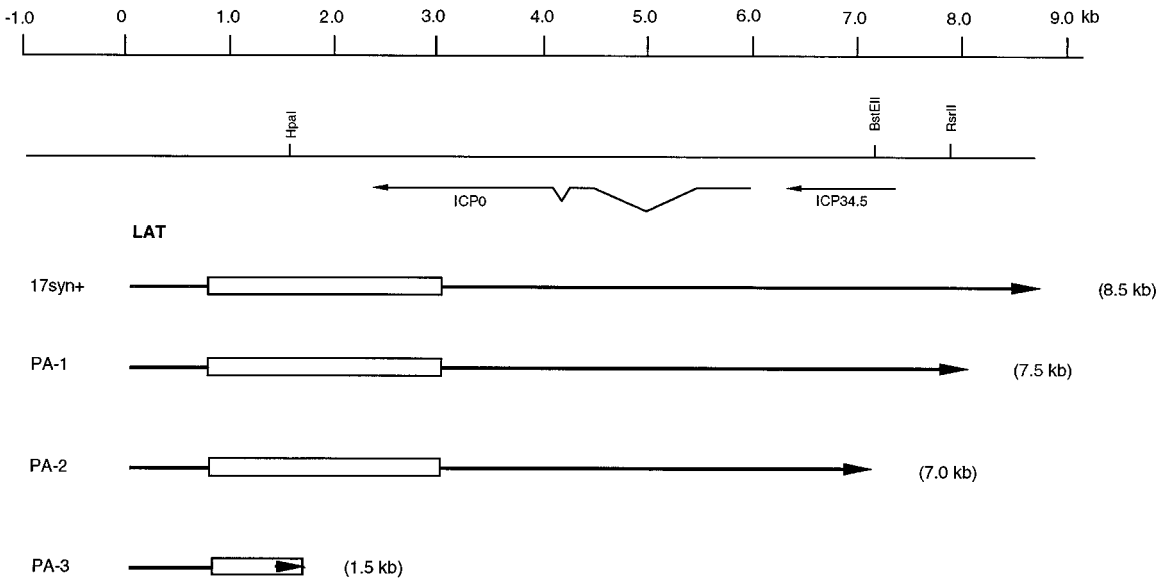

FIG. 1. Sites of poly(A) insertion into the LAT region. The primary LAT transcript is indicated by a solid line, and the location of the 2.0-kb stable intron is indicated by the open box. The sites where the rabbit β-globin poly(A) was inserted to construct viruses PA-1, PA-2, and PA-3 are depicted by arrowheads. The size of the resulting primary transcript for each virus is shown in parentheses. Construction of these recombinants is described in Materials and Methods.

tants provided positive signals with the LAT 5′ (PCR11) primer set but not with the primer set specific for the 3′ end of the transcript. Since the PA-3 recombinant virus potentially expressed the most truncated LAT derivative, it was analyzed in the greatest detail. There, as shown in Fig. 2B, when oligo(dT)-primed cDNA was prepared from ganglia latently infected with this virus, the LAT 5′ primer set generated an appropriately sized product. In addition, the PCR11 primer set, which is specific for transcription upstream of the inserted polyadenylation-termination signal, generates the expected 130-base product with this cDNA. In contrast, a LAT 3′ primer set (PCR9), which is downstream of the inserted polyadenylation-termination signal and which generates a 244-base product with cDNA synthesized from wt LAT (data not shown), generates no product with cDNA synthesized from the truncated LAT expressed in neurons latently infected with the PA-3 virus (Fig. 2B). Although the actin control signal was not maximal in all instances, identical results were obtained with 5′ and 3′ LAT primer sets used on RNA from ganglia latently infected with the PA-1 and PA-2 recombinants and two independent isolates of the PA-3 virus (panel C).

We also utilized in situ hybridization with the stable 2-kb LAT-intron probe to confirm the fact that latent infections with recombinant viruses PA-1 and PA-2 yielded wt levels of this product, while infections with PA-3 virus, in which this intron is truncated, result in no readily detectable signal (data not shown).

The poly(A) insertion viruses were next tested for their response to induced reactivation in rabbits in vivo (Table 1). In distinct contrast to the results with latent infections with 17ΔPst virus, all reactivated with efficiencies and kinetics equivalent to those seen with the wt. The most direct interpretation of these results is that the reactivation phenotype is dependent upon wt levels of transcription of DNA sequences within the first 1,500 bp of the primary LAT transcription unit.

A recombinant with a deletion of a 348-bp region within the 5′ end of the 8.5-kb LAT is impaired in epinephrine-induced reactivation. Since the poly(A) viruses focused attention on the 5′ end of the LAT transcript, we constructed additional recombinants to further localize the active region, and since it did not overlap with other viral transcripts, deletion mutagenesis could be employed. Initially, a virus (17Δ348) with 348 bp deleted from the 5′ region of the LAT transcript was constructed (Fig. 3). This region was deleted since it contains three ATGs which could act as translational initiators for three LAT-encoded ORFs with predicted coding capacities of 54, 60, and 116 amino acids. Additional ORFs of greater sizes can be constructed if splicing is considered.

The recombinant 17Δ348 showed no obvious general defects; compared with its rescuant (17Δ348R), it replicated equivalently in mouse embryo fibroblasts and rabbit skin cells in vitro, established latent infections within spinal ganglia of mice, and reactivated following explant cocultivation with the same efficiency (data not shown). Of importance here is that 17Δ348 and its rescuant established equivalent latent infections within the trigeminal ganglia of rabbits as determined by quantitative PCR assays for viral DNA (Fig. 4; Table 2). We also showed that these viruses could be reactivated from latently infected rabbit trigeminal ganglia by explant cocultivation with the same efficiency as the wt (Table 3).

Despite its ability to establish latent infections with normal efficiency, 17Δ348 was not induced to reactivate at high frequencies from rabbits following epinephrine induction. As shown in Table 4, the percentage of eyes in which 17Δ348 was induced to reactivate (28%) is comparable to values observed with 17ΔPst (33%), a promoter deletion of LAT, while wt 17*syn*+ and the rescuant (17Δ348R) were seen to reactivate in 77 and 89% of the eyes, respectively. Note also that the 17Δ348 rabbits had only 7% total positive swabs compared to 17*syn*+ (48%) and the rescuant, 17Δ348R (45%). Fisher's exact test (two-tailed analysis) of the 17Δ348-positive rabbit/total rabbit and 17Δ348-positive eye/total eye ratios yields P values of <0.001 and <0.0001, respectively, compared with 17*syn*+ results. What is most striking is that while 17Δ348 exhibited a significant reduction in the induced reactivation, comparable to that of LAT promoter deletion viruses, it produced levels of

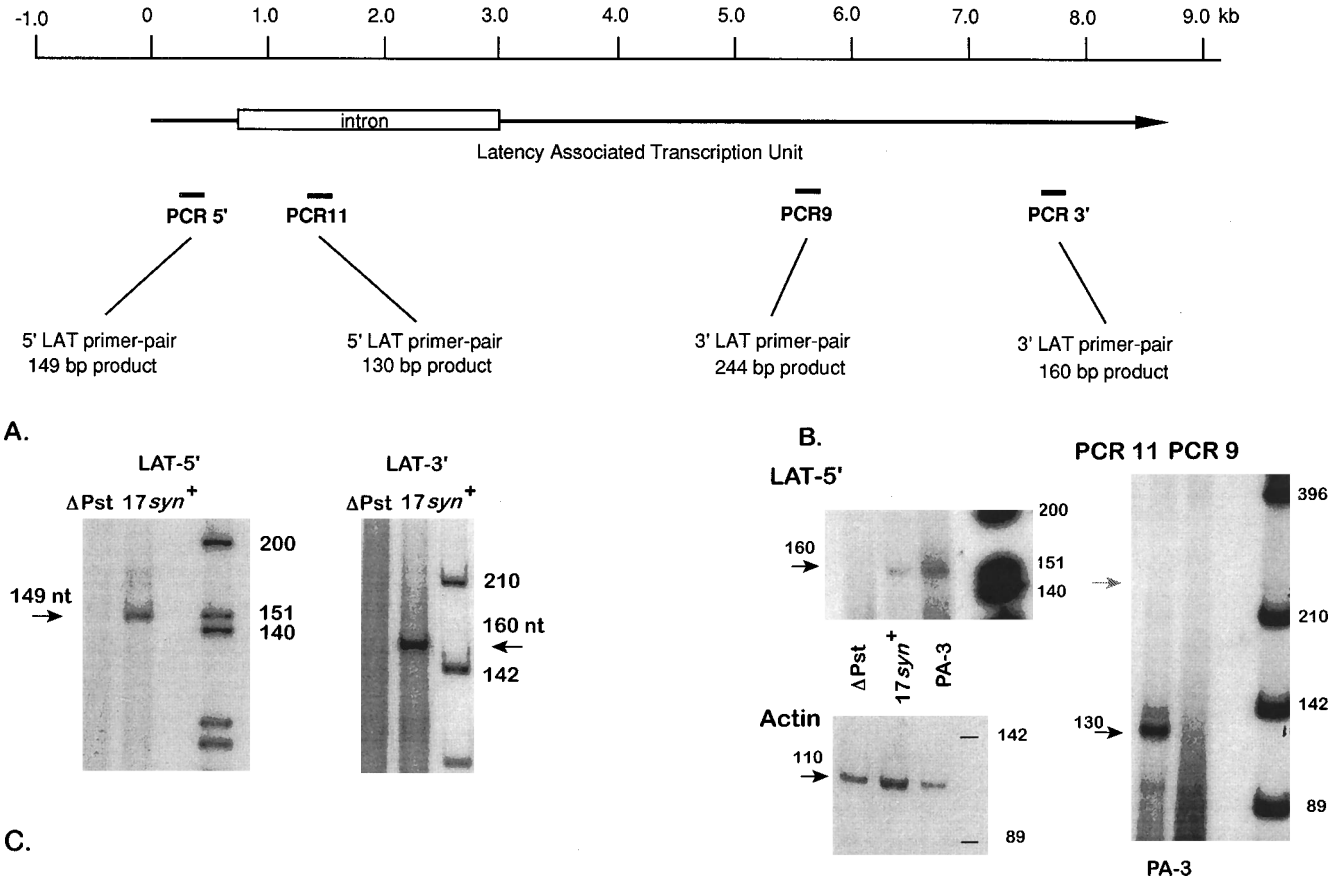

FIG. 2. PCR analysis of oligo(dT)-primed cDNA generated from rabbit trigeminal ganglia latently infected with recombinant HSV-1 expressing truncated LAT. The rabbit globin transcript polyadenylation-termination signal was inserted into three locations within HSV-1 DNA transcribed by LAT as shown in Fig. 1. RNA was isolated from latently infected ganglia and used as a template for oligo(dT)-primed cDNA synthesis. Following this, aliquots of the cDNA were subjected to PCR with various primer sets specific for LAT (13). In all experiments exposure was overnight without intensifying screens. (Top) Locations of the PCR primer pairs within the LAT region, as well as the sizes of the predicted products. (A) Demonstration that a positive PCR signal requires LAT expression. In the experiments shown, cDNA generated from RNA from neurons latently infected with the LAT-negative 17ΔPst mutant (ΔPst) provides a negative control. (B) Analysis of cDNA generated from ganglia latently infected with the PA-3 recombinant virus. This virus contains the globin polyadenylation-transcription termination signal 1,500 bases 3′ of the LAT cap site. The cDNA generates the appropriate signal with PCR primer sets situated 88 to 237 (LAT-5′) and 1,200 to 1,330 (PCR11) bases 3′ of the cap site but no signal with a primer set situated 5,172 to 5,416 (PCR9) bases 3′ of this location. The unlabeled arrow indicates where this missing product would migrate on the gel shown. Results of amplification of cDNA from LAT-negative and wt latent infections are included as controls. The actin primer set was included as a control for tissue recovery. (C) Analysis of cDNA generated from ganglia latently infected with PA-1, PA-2, and PA-3 virus. Ganglia from rabbits infected with two independent isolates of PA-3 virus (PA-3.1 and PA-3.2) are shown. Details are as described for panel B.

the primary LAT transcript which are similar to that produced by wt virus on the basis of the PCR signal obtained with the 3′ LAT primer set (Fig. 5). It should be noted that while the amount of LAT produced by 17Δ348 as shown in Fig. 5 is less than that produced by the wt, this reflects experimental variation and is not significantly reduced when multiple samples are analyzed. In addition, when analyzed by in situ hybridization methods, no differences in the number of cells expressing the 2-kb LAT intron or the density of grains per nucleus were observed (data not shown). These data reconfirm our conclusions based on the data obtained with the poly(A) viruses; namely, expression of RNA within the region encompassed by LAT is not sufficient for reactivation. In contrast, our data suggested that we had altered an element encoded by the LAT RNA which mediates the process of reactivation. This led us to investigate whether the deletion functionally perturbed poten-

TABLE 1. Epinephrine-induced reactivation of poly(A) viruses

Virus	No. positive/total no. (%) of:		
	Rabbits	Eyes	Swabs
17*syn*+	7/8 (88)	13/15 (87)	50/105 (48)
17ΔPst	2/8 (25)	2/13 (15)	5/91 (5.5)
PA-1 [*Rsr*II-poly(A)]	7/8 (88)	12/15 (80)	51/105 (49)
PA-2 [*Bst*EII-poly(A)]	5/6 (83)	8/12 (66)	32/84 (38)
PA-3 [*Hpa*I-poly(A)]	5/6 (83)	7/11 (64)	33/77 (43)

tial peptides emanating from the ATG(s) contained within this 348-bp region.

Mutagenesis of three ATGs at the 5′ end of the LAT transcript has no effect on reactivation. The three ATGs located within the 348-bp region reside in different translational reading frames (Fig. 6a). Therefore, to determine if a LAT-encoded peptide initiating from one of these ATGs conferred the ability to reactivate, each ATG was mutagenized separately. Three viruses illustrated in Fig. 6 were constructed as described in Materials and Methods. When tested in the rabbit for epinephrine-induced reactivation, all three viruses reactivated at the same efficiency as did the wt (Table 5). These data indicated that the three ATGs are dispensable for reactivation.

Subdeletions within the 348-bp region reactivate normally. Since the ATGs residing within the 348-bp region have no apparent role in reactivation, we constructed viruses with smaller deletions within this region in order to delimit the reactivation phenotype (Fig. 6b). These recombinants were constructed by PCR to generate nonoverlapping subfragments of 110, 91, and 116 bp from within the 348-bp region. These cloned fragments were then used to rescue the 17Δ348 parent as described in Materials and Methods. When tested in the rabbit for their ability to reactivate following epinephrine induction, these recombinants all reactivated with high efficiency (Table 6). These results indicate then that these smaller deletions within the 348-bp region did not individually remove the element(s) within this region that is required for reactivation.

Nucleotide sequence analysis of conserved DNA elements within the 348-bp region. Since our deletion and mutagenesis analyses of the 348-bp region did not allow us to further define the functional region, we examined the entire 348-bp region by sequence analysis and compared it with other members of the alphaherpesviruses. We reasoned that a region having a central role in reactivation might contain conserved elements which could provide a clue as to its function in reactivation. Although sequence comparison of the 348-bp region with HSV-1, HSV-2, pseudorabies virus, and bovine herpesvirus did not demonstrate significant homology, all exhibited a high abundance of the dinucleotide 5′CpG3′. While the entire LAT promoter region, as well as the 5′ region of the LAT transcript, is GC rich (6, 12), the 348-bp regions of HSV-1 and -2 are particularly saturated with the CpGs, with 31% of the nucleotides being CpGs compared with a predicted value of 18%, based upon the GC content of this region alone. In addition, a comparison of CpGs to GpCs reveals a ratio of 1.5, which is a diagnostic feature of the regulatory CpG islands present in the promoters of many cellular housekeeping genes (2). The abundance of CpGs is highly conserved between HSV-1 and HSV-2, while the ATGs are not (Fig. 7A). Our analysis here also demonstrates extensive CpG clusters within the promoter and intron of ICP0, as well as a major cluster within the ICP4 promoter (Fig. 7B).

In addition to the CpGs, a search of GenBank with the 5′ region of LAT using BLASTN (default settings) identified similarity with human sequences located on the X chromosome. Specifically the homology was to the human transcription unit termed *XIST* located near the inactive center of the X chromosome (9, 10) and produced a BLASTN score of 128, with a *P* value of 0.9999. Further analysis revealed that regions of similarity extended along the length of the LAT transcript, with the greatest homology being observed in the intron and the 3′ region. Segments of the 348-bp region were seen to have similarity with the first block of the *XIST* repeats. Specifically, this region contains six smaller reiterated elements with the core sequence GGCCCC which shares homology with the first of five blocks of reiterated elements found near the 5′ end of *XIST* (Fig. 7C). This homology with this first block begins with the T-rich region of the mouse and human repeats at LAT nt 273 and extends to nt 484 within the 348-bp region. The con-

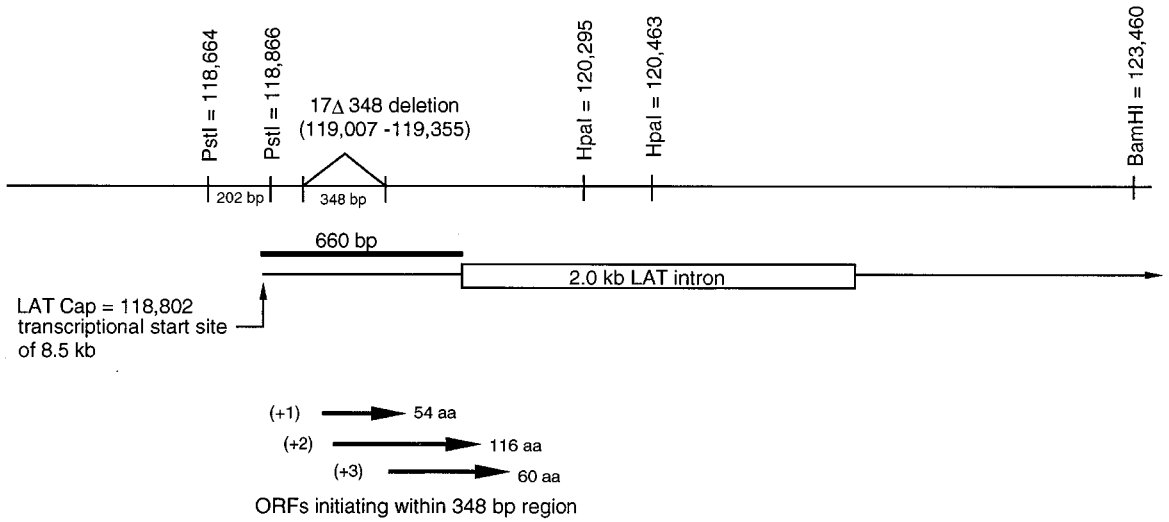

FIG. 3. The 348-bp deletion in LAT. The LAT primary transcript, intron, and location of the LAT transcriptional start site are illustrated (top). ORFs originating within this region, starting with a translational initiator, and possessing a coding capacity of greater than 50 amino acids are shown below. The sizes of the predicted proteins encoded by these ORFs are 54, 116, and 60 amino acids (aa), respectively.

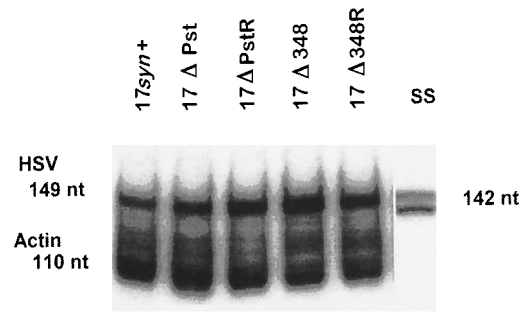

FIG. 4. Amounts of latent viral DNA in rabbit trigeminal ganglia infected with HSV strains 17*syn*+, 17ΔPst, 17ΔPstR, 17Δ348, and 17Δ348R. DNA was extracted from latently infected (40 days postinfection) trigeminal ganglia and subjected to PCR with primers specific for viral HSV DNA (VP5) and cellular DNA (actin). The PCR products were resolved on acrylamide gels as described in Materials and Methods. A 142-bp size standard (SS) is included. Comparisons of VP5/actin ratios from three separate rabbit trigeminal ganglia are shown in Table 2.

servation of four of the other (different) blocks of repeats present within the *XIST* transcript is illustrated in Fig. 7D. One of the proposed functions of *XIST* is the regulation of expression of genes on the X chromosome through methylation repression, though the roles of the individual blocks of repeats are unknown. Potential implications of *XIST* as a model for LAT reactivation are discussed in the next section.

DISCUSSION

Mutants of HSV-1 and -2 with deletions in the LAT promoter do not reactivate efficiently in several in vivo models (4, 5, 21, 24, 37, 49), yet the biological mechanism for this apparent linkage between LAT expression and efficient reactivation is unknown. Characterization of several precisely defined mutants has eliminated reasonable mechanisms involving any role for the translation of the major translational reading frames contained within the major LAT introns (15, 16); however, mechanisms involving the latent-phase expression of other peptides whose coding sequences are contained within the primary LAT transcript remain a possibility. Also formally consistent with available data in rabbits are other mechanisms requiring the presence of high levels of LAT introns and/or the transcription of the region of the HSV-1 genome encompassed by LAT, either to facilitate the expression of another transcript or to maintain a transcriptionally active viral template. The primary aim of the current study was to further characterize the role of LAT expression in reactivation and to localize any genetic elements within this genomic region involved in this process.

Our results allow us to make two specific conclusions involving the role of latent-phase transcription within the region of the HSV genome expressed as LAT and its relation to the efficient reactivation phenotype: (i) efficient reactivation requires readily measurable levels of transcription of no more than the immediate 1.5 kb of the 5′ region of LAT (Fig. 1) during the latent phase of infection, and (ii) there is no LAT-encoded reactivation peptide expressed in detectable amounts during the latent infection.

Our analysis of RNA expressed by the poly(A) insertion mutants employed demonstrates that the insertion of the rabbit β-globin transcript polyadenylation-termination signal efficiently truncates the primary LAT transcript. Although we cannot rule out low levels of transcription downstream of the sites of insertion, our data clearly demonstrate that appreciable levels of transcription more than 1,500 bases downstream of the transcript start site are not required for efficient reactivation. Thus, expression of only the most 5′ region of this 8.5-kb transcript during latency appears to be involved in induced reactivation. This clearly excludes any role for the stable LAT intron and the downstream region of the primary LAT transcription unit in the reactivation phenotype that we are studying. This conclusion is reinforced by our determination that a specific 348-bp region situated between 217 and 565 bases 3′ of the LAT cap site is critical for efficient reactivation. To reiterate, a detectable level of transcription downstream of the 5′ portion of LAT during latent infection is irrelevant to the efficient reactivation phenotype. This conclusion is consistent with the finding of smaller latent-phase transcription units expressed by other alphaherpesviruses (38, 40).

Our results also demonstrate that readily measurable levels of independent transcription mediated by the so-called LAP2 promoter (17), even in conjunction with the major LAT promoter, do not occur during latent infection and that such transcription is not essential for efficient reactivation. This is apparent because the deletion in 17Δ348 is within the region described as the LAP2 promoter, yet we see no major effects on the amount of downstream transcription of LAT during latent infection, as evidenced by the strong reverse transcription-PCR signal generated by oligo(dT)-primed cDNA from RNA isolated from 17Δ348-infected ganglia (Fig. 5B). In addition, the poly(A) insertion viruses preclude downstream transcripts from having a role in reactivation. These results also show that measurable levels of expression of transcripts including downstream translational reading frames, including the recently identified ORF-P (15, 27, 28, 39), controlled by other promoters are not involved with reactivation.

We observed normal levels of induced reactivation by HSV-1 mutants in which each of the ATGs in the 348-bp region of LAT was either modified or deleted. This and the exclusion of downstream regions of LAT make it highly unlikely that a peptide encoded by LAT expressed during latent infection is involved in the process of efficient reactivation. This evidence against a peptide having a role in the phenotype directs us to focus on models of reactivation in which LAT may act either as a structural RNA or as models in which LAT influences transcription through the critical region itself. The

TABLE 2. Quantitative PCR analysis of latent viral DNA in rabbit trigeminal ganglia

Virus	Densitometry of VP5/actin band intensity[a]			
	Expt 1	Expt 2	Expt 3	Avg
17*syn*+	0.64/0.7 (0.91)	1.12/0.98 (1.14)	0.87/0.75 (1.16)	0.88/0.81 (1.08)
17ΔPst	0.60/0.62 (0.97)	1.11/0.90 (1.23)	0.91/0.71 (1.28)	0.87/0.74 (1.17)
17Δ348	0.98/0.62 (1.58)	1.25/0.92 (1.35)	1.12/0.71 (1.57)	1.12/0.75 (1.49)
17Δ348R	0.83/0.61 (1.36)	1.23/0.92 (1.33)	1.02/0.7 (1.45)	1.03/0.74 (1.39)

[a] In experiments 1 to 3, each ratio represents the results obtained from one trigeminal ganglion.

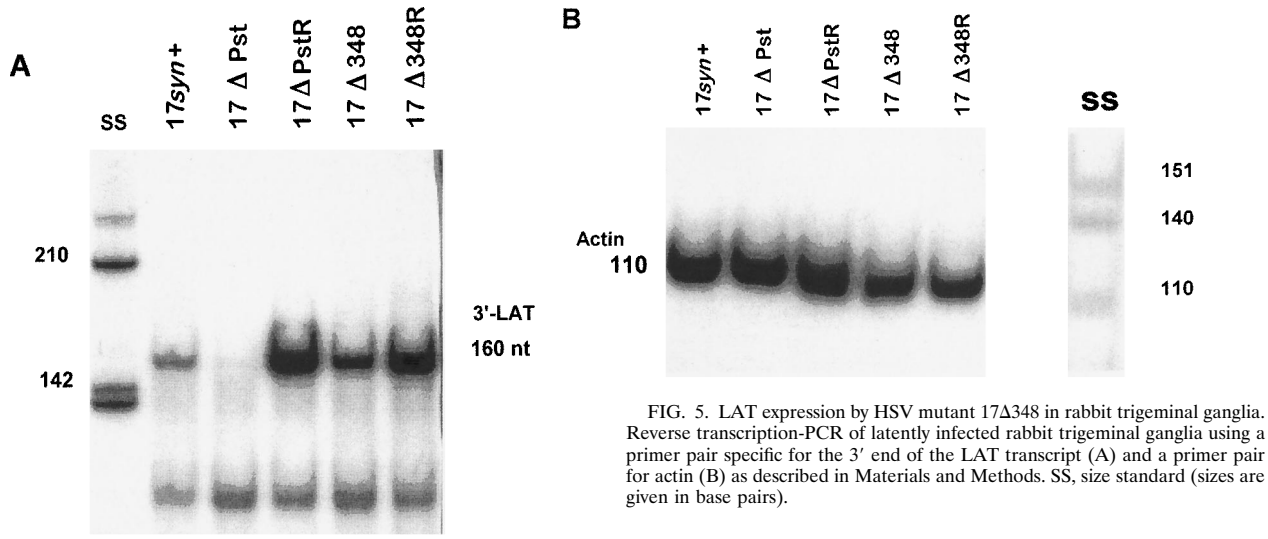

FIG. 5. LAT expression by HSV mutant 17Δ348 in rabbit trigeminal ganglia. Reverse transcription-PCR of latently infected rabbit trigeminal ganglia using a primer pair specific for the 3′ end of the LAT transcript (A) and a primer pair for actin (B) as described in Materials and Methods. SS, size standard (sizes are given in base pairs).

high GC contents of the genomes of many alphaherpesviruses, along with differences in the distribution of CpG dinucleotides throughout each genome, have led to postulates that methylation of CpGs plays a role in latency (11, 22, 50). The conservation of CpGs within the 5′ end regions of LATs encoded by HSV-1, HSV-2, pseudorabies virus, and bovine herpesvirus lends support to such views. It is notable that the high relative abundance of CpGs within the 348-base region in the 5′ region of LAT is reminiscent of situations in the 5′ ends of a number of cellular housekeeping genes (47). Such CpG "islands" (or *Hpa*II tiny fragments) are usually not methylated and may have a role in maintaining an open structure of the DNA in these areas of such genes by a process which displaces nucleosomes and facilitates transcription (2). Reasoning by analogy, we propose that the CpG islands within the 5′ portion of LAT are involved, perhaps through differential methylation during the latent phase, in maintaining the availability of the LAT promoter. Deletion of a large block of these islands would result in the disruption of this functional structure and thus impair reactivation. The redundancy of the islands, however, militates against smaller deletions having a similar effect. Because methylation of these CpGs may have a positive or negative regulatory effect on gene expression (2), it is impossible to predict a priori which role these islands may play with respect to LAT.

A major conundrum in attempts to link LAT expression with reactivation is the fact that although the LAT intron is quite stable, the actual amount of active latent-phase transcription is drastically decreased over that seen during the acute infection (34); further, many latently infected neurons if not the majority of them, do not express LAT or, at least, the LAT intron (18, 36, 41). However, induction of LAT and/or other genes within the same general portion of the genome in nonexpressing neurons could be responsible for initiating reactivation. There are a number of candidate regulatory elements located in the LAT promoter which could act as a switch in such a model; two obvious ones are ATF/CREB sites, which have been implicated in some reactivation models (23, 32). It is possible, then, that differential methylation of the region around the LAT promoter alters its structure in such a way as to make it accessible to the binding of these transcriptional factors. In such a model, some or all neurons which do not express LAT may possess a promoter region that is structurally primed for a reactivation signal. To summarize, the identification of CpGs within the 348-bp region has the implication that these elements could regulate LAT expression, and their deletion in 17Δ348 may be a factor in this virus's inability to reactivate. The methylation status of these CpGs and their contribution to differential LAT expression during latency are currently being investigated.

In addition to the abundance of CpGs, we also identified homology between this region of LAT and a conserved set of repeats found within the *XIST* transcript produced by the inactive X chromosome. Some interesting parallels between these two transcription units are that both transcripts are quite large (17 kb in humans; 15 kb in mice) and localize to the nucleus and attempts to identify functional ORFs have been unsuccessful (for a review of *XIST*, see reference 44). *XIST* is found on the X chromosome, near the inactive center. In cells containing two X chromosomes, one of the chromosomes becomes silent (inactive X). The *XIST* transcript is produced only from the inactive X chromosome, not from the active one. On the silent chromosome, the CpGs in the *XIST* promoter are unmethylated, whereas the promoters of the other genes on

TABLE 3. Recovery of infectious virus from rabbit trigeminal ganglia by explant cocultivation

Virus	No. of positive trigeminal ganglia/ total no.	Avg no. of days until positive
17Δ348	6/7	15.5
17*syn*+	3/4	17.3
17Δ348R	4/4	16.0

TABLE 4. Epinephrine-induced reactivation of 17Δ348 virus

Virus	No. positive/total no. (%) of:		
	Rabbits	Eyes	Swabs
17*syn*+	9/9 (100)	14/18 (77)	61/126 (48)
17ΔPst	4/9 (44)	6/18 (33)	12/126 (10)
17Δ348	3/9 (33)	5/18 (28)	9/126 (7)
17Δ348R	9/9 (100)	16/18 (89)	57/126 (45)

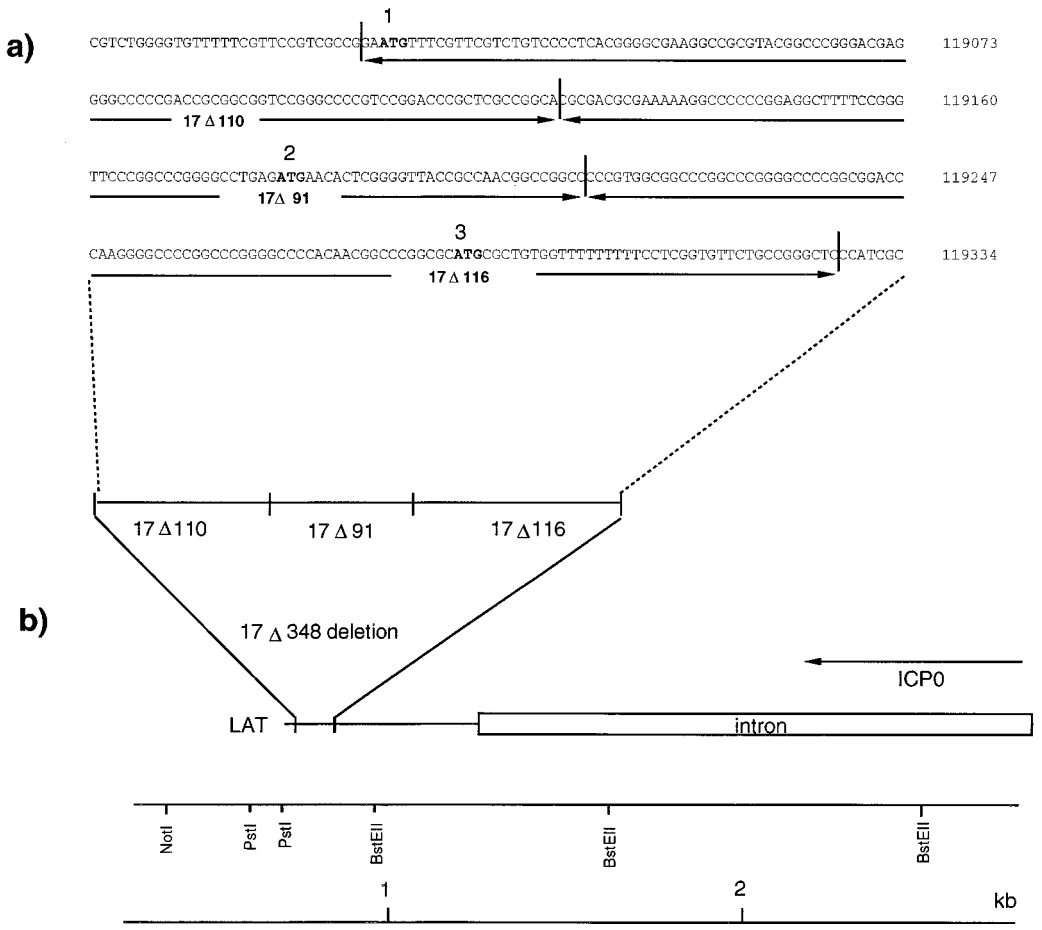

FIG. 6. Genomic structure of HSV-1-derived viruses. (a) ATG-1, ATG-2, and ATG-3. Oligonucleotide-directed mutagenesis was used to construct the viruses, and each contains a single mutated ATG. (b) 17Δ110, 17Δ91, and 17Δ116. Deletions were made within the 348-bp region originally deleted in 17Δ348. The extent of each smaller deletion is shown in the expanded map at the bottom. The oligonucleotides and the procedure used to mutagenize these sequences and to screen for recombinants are as described in Materials and Methods.

this chromosome are methylated and silent. On the active X chromosome, the *XIST* promoter is methylated and silent, whereas the other promoters are unmethylated and active. It has been proposed that *XIST* plays a role in silencing the X-chromosome genes through methylation of CpG islands within the promoters of other genes on the inactive X chromosome, though it is not clear whether it is the *XIST* transcript per se or structural changes dictated by the repeats within the DNA which mediate this process (8). It is also not clear whether *XIST*'s role in this shutdown is in initiation, maintenance, or both (44). Since there is general, but not complete (42), agreement that LAT expression plays no role in the of the establishment of latency, future investigations based on the *XIST* model should involve structural studies of the DNA. It can be suggested that HSV is employing a mechanism in which the LAT region acts as *XIST* and plays a role in silencing the rest of the HSV genome, perhaps through the directed methylation of CpGs contained in the immediate-early promoters, specifically ICP4 and ICP0. A conserved methylated DNA-binding protein binding site has been identified in the ICP4 promoter (51), and if the ICP4 promoter were methylated during latency, one could postulate that the binding of a methylated DNA-binding protein could modulate ICP4 expression or function during reactivation. Overall then, as discussed earlier, the 348-bp region of LAT may play a structural role in

TABLE 5. Epinephrine-induced reactivation of ATG mutation viruses

Virus	No. positive/total no. (%) of:		
	Rabbits	Eyes	Swabs
ATG-1	6/7 (62)	8/13 (62)	30/91 (33)
ATG-2	7/8 (88)	10/15 (67)	45/105 (43)
ATG-3	7/9 (78)	9/18 (50)	36/126 (29)
17Δ348	3/9 (33)	3/18 (17)	13/126 (10)
17Δ348R	5/6 (83)	8/12 (67)	35/74 (47)

TABLE 6. Epinephrine-induced reactivation of subdeletion viruses

Virus	No. positive/total no. (%) of:		
	Rabbits	Eyes	Swabs
17Δ110	5/5 (100)	8/10 (80)	31/70 (44)
17Δ91	5/5 (100)	8/10 (80)	32/70 (46)
17Δ116	7/7 (100)	12/13 (92)	57/91 (63)
17Δ348	3/9 (33)	3/18 (17)	13/126 (10)
17Δ348R	5/6 (83)	8/12 (67)	35/74 (47)

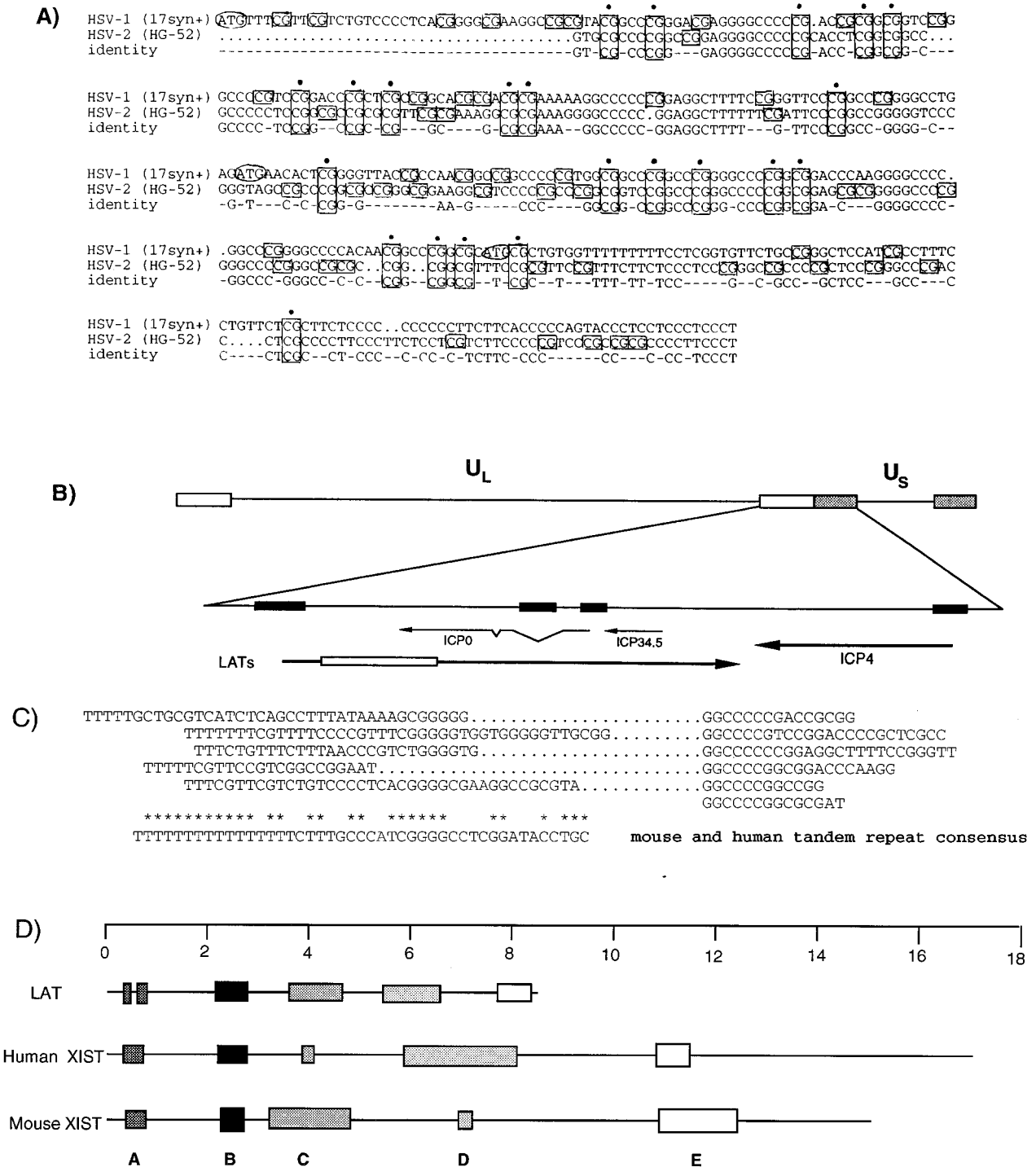

FIG. 7. Identification of CpG islands and homology with *XIST* repeats within the 348-bp fragment. (A) CpG islands in the LAT promoter, ICP0 promoter and intron, and ICP4 promoter and island in the 348-bp deletion in virus 17Δ348. Alignment of HSV-1 strain 17*syn*+ (35), HSV-2 strain HG-52, and consensus sequences. All CpG residues are boxed, and residues conserved between HSV-1 and -2 are indicated with dots. ATGs present in HSV-1 are circled. (B) Locations of four CpG islands (solid boxes) over the LAT promoter and 348-bp region, ICP0 promoter and intron, and ICP4 promoter. (C) Alignment of the A repeats of *XIST* (nt 292 to 713 of the mouse sequence) with the repeats extending into the 348-bp region of LAT (nt 276 to 484 from the cap site of the 8.5-kb LAT). Shown below the alignment of the four reiterated HSV repeats is the alignment with mouse and human consensus sequence. Identity with 50% or greater of the residues in the HSV repeats is represented by asterisks. Note that the first block of repeats in LAT differs from the *XIST* repeats in that the duplicated block is split into two segments. The two blocks are reiterated independently and separated by approximately 100 bp. (D) Alignment of HSV LAT with *XIST*-related repeats on the mouse and human X chromosomes. The five sets of repeats found in *XIST* are shown by boxes, and each repeat block labeled A to E is unrelated to the others. The LAT repeats that have composition and spacing similar to those of the corresponding *XIST* repeats are shown with the same shading.

facilitating reactivation and in addition, through the conserved elements that the LAT region shares with *XIST*, could also play a role in the regulation of the expression of other genes. Indeed, the LAT region may be multifunctional, with the 5′ end playing a major role in reactivation but other elements influencing establishment or even modulating reactivation.

Finally, as we have previously noted, there are number of differences in the effect of LAT expression in various animal models. One demonstrated by the present work is that, unlike the situation with mutants containing deletions of the LAT promoter, mutant 17Δ348 was recovered with efficiencies equivalent to that of its rescuant by explant cocultivation of murine spinal ganglia. This finding could be attributable to differences in establishment of a latent infection by LAT promoter deletions (but not 17Δ348) in mouse spinal ganglia which are not seen in the rabbit trigeminal ganglia (5, 13). The observation that 17Δ348 reactivates efficiently from murine spinal ganglia may also be consistent with recently reported findings on a recombinant that contains a deletion partially overlapping with 17Δ348 which also reactivates from mouse ganglia in vitro (33), though the region deleted in 17Δ348 extends 103 bp 3′ of the *Sty*I deletion mutant studied by those workers.

As to the future, two specific areas are of immediate interest: (i) a further localization of the 5′ region essential for induced reactivation and (ii) an analysis of the structural and regulatory contributions of the 348 bp to latent transcription within the LAT region. While the methylation status of the CG-rich HSV genome has been previously investigated (14), the investigation has not been conducted at a level of resolution to specifically evaluate the regions within the HSV repeats implicated by the location of the CpG islands. To study this, PCR-directed methylation assays of the LAT and immediate-early promoters in latently infected ganglia are currently under way. This work, as well as the construction of additional HSV deletion recombinants, will allow us to address the role of LAT in the reactivation process and assess the possibility that LAT plays a role similar to that of *XIST*.

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