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An Antigen Encoded by the Latency-Associated Transcript in Neuronal Cell Cultures Latently Infected with Herpes Simplex Virus Type 1

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During latent infection of neurons with herpes simplex virus type 1, viral transcription is restricted to the latency-associated transcripts (LATs). These RNAs contain open reading frames, but detection of a protein encoded by the LATs has not been reported. We used immunocytochemical techniques to demonstrate that an antiserum directed against a bacterially expressed fusion protein containing part of a LAT-encoded polypeptide recognized an antigen present in primary neurons latently infected in vitro. This antigen (called LAA, for latency-associated antigen) was not detected in mock-infected neurons, in productively infected Vero cells, or in neurons latently infected with a mutant virus carrying a deletion in the LAT gene. By Western immunoblot analysis, we demonstrated the presence of a protein with an apparent molecular mass of 80 kDa recognized by the anti-LAA antiserum in latently infected neurons.

The ability to establish a latent infection is a property shared by several groups of viruses, including the herpesviruses. A latent infection is operationally defined as an infection during which viral antigens characteristic of a productive infection are not expressed although the viral genome is present in the infected cell. Under certain conditions the virus reactivates from the latent state and gives rise to a productive infection. Herpes simplex virus type 1 (HSV-1) establishes a latent infection in neurons in humans and in animal models. Following productive infection in the periphery, HSV-1 gains access to the nerve terminals and is transported by retrograde axonal transport to the cell body of sensory neurons in the ganglia, where a latent infection can be established. Certain stimuli, such as UV irradiation or damage to tissue at the site of inoculation, cause viral reactivation followed by anterograde transport of virus from the ganglion to the periphery. This leads to a secondary productive infection at the site of original inoculation. Recurrence can occur throughout the life of an infected individual (reviewed in references 8, 13, 14, and 18).

The molecular mechanisms involved in the establishment, maintenance, and reactivation of the latent virus are not understood. The only detectable viral RNAs in neurons of latently infected ganglia are transcribed from the region of the genome encoding the immediate-early gene ICP0 and have a polarity opposite to that of ICP0 mRNA (3, 4, 16, 19). The most abundant of these latency-associated transcripts (LATs) is 2 kb long, and its 3′ end overlaps with the 3′ end of ICP0 mRNA (Fig. 1). In addition to the major form of LAT, smaller species (1.5 and 1.45 kb) have been detected (16, 22). Most, if not all, of these molecules are not polyadenylated and are found in the nuclei of latently infected neurons (16, 22). It has been proposed that the three forms of LAT are derived by differential splicing from a larger precursor (6). The LAT has also been detected in the in vitro model of HSV-1 latency used for this study (6a), in which a latent infection is established in primary neuronal cultures prepared from fetal rat dorsal root ganglia (24–26).

A function has not yet been attributed to the LAT. The hypothesis that LAT could inhibit ICP0 expression through an antisense mechanism and thus prevent a productive infection was rejected after the finding that mutant viruses lacking the LAT are able to establish a latent infection in animal models (1, 2, 7, 9, 10, 12, 17). The LAT gene from the KOS strain of HSV-1 encodes two open reading frames (ORFs) (21). The larger of these ORFs (ORF 2) potentially encodes a 305-amino-acid polypeptide and is highly conserved among HSV-1 strains (21, 23).

We obtained antibodies against the potential polypeptide encoded by LAT ORF 2 by immunizing rabbits with a bacterially produced fusion protein containing part of this polypeptide. The vector system we used to express the LAT ORF 2 in *Escherichia coli* consists of a plasmid encoding approximately 37 kDa of the bacterial *trpE* ORF under the control of the inducible *trp* operon promoter (15). A polylinker at the 3′ end of the *trpE* ORF allows in-frame insertion of a foreign ORF. Using this system, we produced a chimeric protein containing the carboxy-terminal part of the LAT ORF 2 fused to the *trpE* ORF. The plasmid pATH-LAT2 encoding this chimeric gene is shown in Fig. 1. The plasmid pATH-ICP0 (Fig. 1) allowed the production of a fusion protein containing the carboxy-terminal part of the third exon of the immediate-early protein ICP0. The chimeric proteins present in bacterial extracts were gel purified and injected into rabbits, from which antisera were obtained. The anti-LAT2 antiserum was shown to contain antibodies against the LAT ORF 2; a polypeptide synthesized by in vitro transcription and translation from a plasmid containing part of the LAT ORF 2 was immunoprecipitated with this antiserum (data not shown).

Immunocytochemistry experiments showed that neurons latently infected in vitro were stained by the anti-LAT2 antiserum (Fig. 2A) but not by the anti-ICP0 antiserum (Fig. 2B). Essentially all the neurons present in a latently infected culture were stained with the anti-LAT2 antiserum. The anti-ICP0 antiserum provided a control to exclude the pos-

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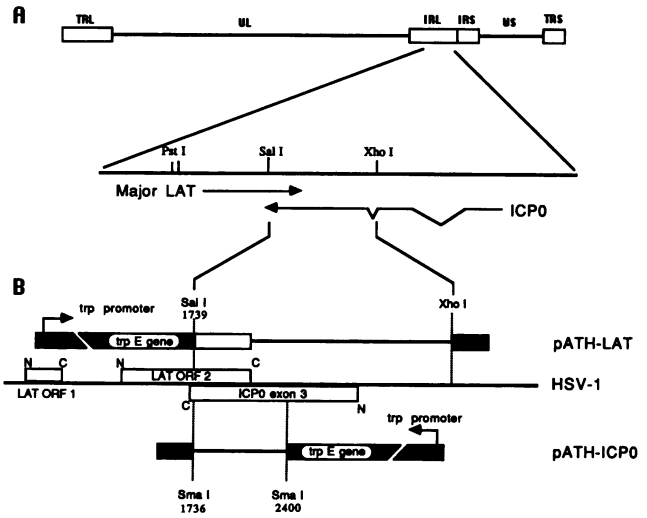

FIG. 1. Plasmids used in this study. (A) General structure of HSV-1 genome. UL, Unique long region; US, unique short region; TRL, long terminal repeat; IRL, long internal repeat; IRS, short internal repeat; TRS, short terminal repeat. The LAT and the ICP0 mRNAs are transcribed in opposite directions from the long repeat regions. (B) Enlargement of the region of overlap between LAT and ICP0 mRNA. Viral ORFs are represented as open boxes, with N referring to the amino and C to the carboxy terminus of each ORF. Numbering is from reference 21.

sibility that the signal given by the anti-LAT2 antiserum resulted from antibodies directed against the TrpE part of the fusion protein. To determine the specifity of the signal yielded by the anti-LAT2 antiserum, we used this antiserum to stain mock-infected neuronal cell cultures (Fig. 2C) and productively infected Vero cells (Fig. 2F). Similar cultures were stained with the anti-ICP0 antiserum (Fig. 2D and G). Mock-infected neurons did not react with either antiserum (Fig. 2C and D), whereas the productively infected Vero cells were stained only by the anti-ICP0 antiserum (Fig. 2F and G). Mock-infected Vero cells did not react with either antiserum (not shown). We repeated these experiments with neurons latently infected with the mutant virus *dl*1403 (20), in which both copies of the LAT gene have the *Sal*I-*Xho*I fragment deleted (Fig. 1B). Neurons infected with *dl*1403 were not stained by the anti-LAT2 antiserum (Fig. 2E). The mutant virus established a latent infection in vitro, as demonstrated by virus reactivation; 100% (12 of 12) of the cultures latently infected with *dl*1403 produced infectious virus after nerve growth factor deprivation, a stimulus previously shown to reactivate latent HSV-1 (24, 26).

Western immunoblot analysis of whole-cell extracts from latently infected neurons demonstrated the presence of a protein with an apparent molecular weight of 80 kDa recognized by the anti-LAT2 antiserum (Fig. 3A, lane 2). Whole-cell extracts from mock-infected neurons (Fig. 3A, lane 1), productively infected, or mock-infected Vero cells (data not shown) did not contain detectable levels of this protein. The anti-LAT2 antiserum also reacted with a 45-kDa polypeptide present in latently infected neurons. The intensity of the signal at 45 kDa varied from extract to extract, from barely detectable to an intensity similar to that of the 80-kDa signal. The 45-kDa band may represent a degradation product of the 80-kDa protein. More work is needed to clarify this point. The anti-ICP0 antiserum (Fig. 3A, lane 4) but not the anti-LAT2 antiserum (data not shown) allowed detection of ICP0 (110 kDa) in nuclear extracts from productively infected HeLa cells. To further examine the specificity of the

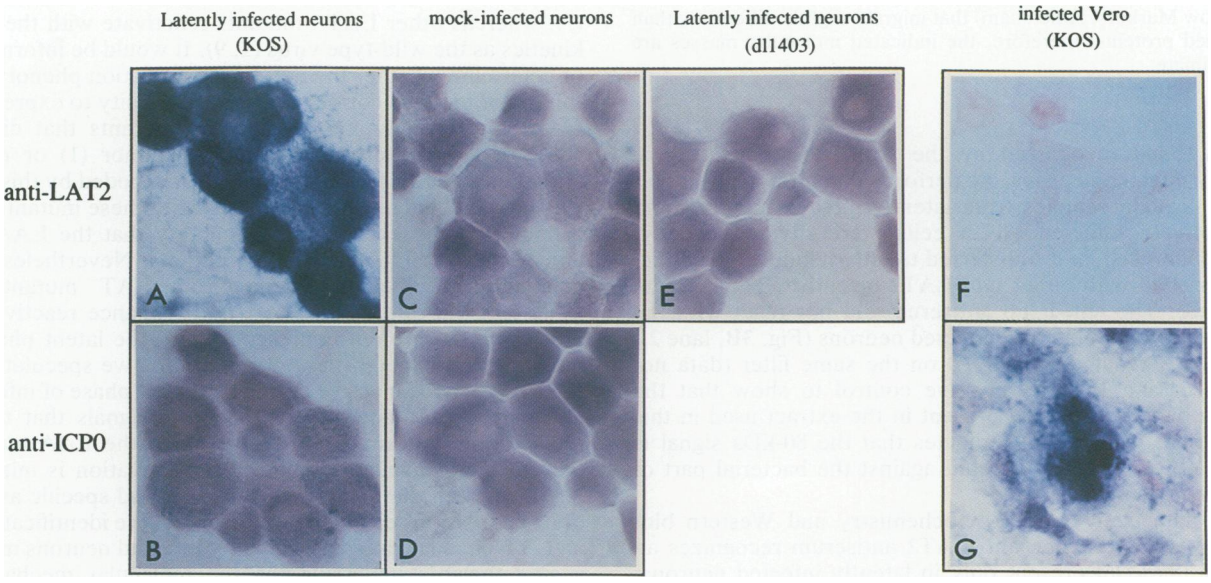

FIG. 2. Detection of the latency-associated antigen (LAA) by immunocytochemistry. Neuronal cultures and establishment of latent infection in vitro with the KOS strain of HSV-1 were done as described before (24–26). Indirect immunocytochemistry was done by the avidin-biotin-glucose oxidase complex method according to the manufacturer's instructions (Vectastain; Vector Laboratories). Nitro Blue Tetrazolium was used for the substrate, producing a purple-blue color, and the cells were counterstained with eosin. Cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). Immunoglobulins were obtained from serum by ammonium sulfate precipitation, dissolved in PBS (half the original volume of serum), and used at a 1:500 dilution. The antisera used are indicated to the left. (A and B) Latently infected neurons (14 days postinfection); (C and D) mock-infected neurons; (E) neurons infected with the LAT$^-$ mutant *dl*1403 (20) (14 days postinfection); (F and G) infected Vero cells (12 h postinfection).

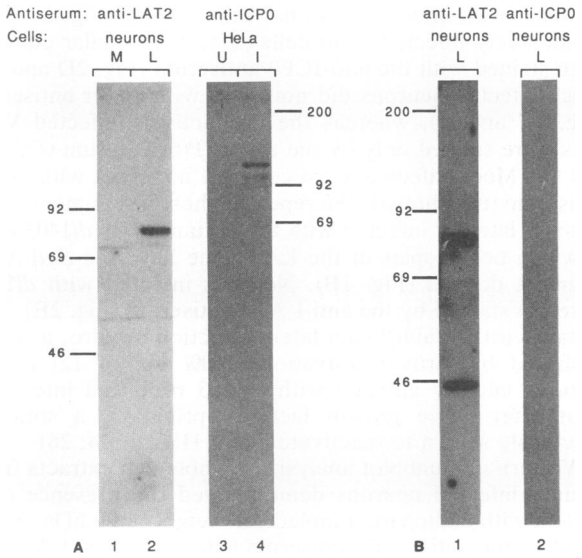

FIG. 3. Western blot analysis. Cells from neuronal cultures were washed with an isotonic buffer, concentrated by centrifugation, and lysed in buffer C (5). Cell debris was removed by centrifugation prior to electrophoresis. HeLa cell nuclear extracts were obtained by the method of Dignam et al. (5). Whole-cell extracts from neuronal cell cultures (70 μg) or nuclear extracts from HeLa cells (25 μg) were electrophoresed on polyacrylamide gels (11) and transferred to nylon-supported nitrocellulose filters (Hybond Extra-C; Amersham). The filters were incubated with the antisera (dilution, 1:300) as indicated at the top of the figure, washed, incubated with ^{125}I-protein A (0.1 μCi/ml; specific activity, >70 μCi/μg; New England Nuclear), washed, and exposed for autoradiography. Extracts were from: M (lane A1), mock-infected neuronal cell cultures; L (lanes A2, B1, and B2), latently infected neuronal cultures (14 days postinfection); U (lane A3), uninfected HeLa cells; and I (lane A4), productively infected HeLa cells. Molecular masses are indicated to the left (in kilodaltons) and are from prestained proteins (Rainbow Markers; Amersham) that migrate slightly differently than unstained proteins; therefore, the indicated molecular masses are approximate.

80-kDa band recognized by the anti-LAT2 antiserum in latently infected neurons, we performed the following experiment. Protein samples from latently infected neurons and productively infected HeLa cells were divided equally, electrophoresed, and transferred to nitrocellulose; the filters were probed with either anti-LAT2 or anti-ICP0 antiserum (Fig. 3B). The anti-ICP0 antiserum did not react with the 80-kDa protein in latently infected neurons (Fig. 3B, lane 2), although it reacted with ICP0 on the same filter (data not shown). Lane 1 is the positive control to show that the 80-kDa band was indeed present in the extract used in this experiment. This result indicates that the 80-kDa signal is not due to antibodies directed against the bacterial part of the fusion protein.

The data from immunocytochemistry and Western blot analysis show that the anti-LAT2 antiserum recognizes an antigen (80 kDa) present only in latently infected neurons. Our interpretation of these results is that the LAT-encoded ORF 2 is translated in latently infected neurons. We have called this protein latency-associated antigen (LAA). We are currently investigating the status of this antigen in latently infected ganglia.

The transcription pattern of the region encoding the LAT is complex. Because of lack of polyadenylation and nuclear localization, the 2-kb, 1.5-kb, and 1.45-kb LAT species may not function as LAA mRNA. A series of low-abundance LAT-related transcripts that extend several kilobases downstream from the major LAT 3′ end to a polyadenylation signal located 8.5 kb from the LAT TATA box have been identified in productively infected cell cultures (6, 18). RNA hybridizing to probes extending 5 kb downstream of the 3′ end of the major LAT has also been detected in latently infected ganglia by in situ hybridization (6, 18). These RNA molecules have been shown to be polyadenylated in productively infected cells (6, 18) and are presumably polyadenylated in neurons as well. The current thinking is that the 2-kb LAT is a stable intron from a larger transcript. Alternative splicing resulting in polyadenylated transcripts containing ORF 2 has not been ruled out, and such transcripts may function as LAA mRNAs.

From its size (305 codons), the LAT ORF 2 would be expected to yield a protein of about 33 kDa. There are several possible explanations for the observed size of the LAA. First, the polypeptide has a high proline content (59 residues, or about 19% of the amino acids encoded by the ORF, are prolines) that could result in aberrant electrophoretic mobility. Second, the primary translation product could be modified by phosphorylation, glycosylation, or other processes. Third, larger ORFs could be generated by RNA splicing. Since the difference between the observed and calculated sizes of the LAA is very large (about 50 kDa), the third possibility is the most likely.

The function of this protein remains to be determined. Despite the fact that the LAA is present in neurons during the latent phase of infection, the ability of LAT$^-$ mutants to establish latent infections (1, 2, 7, 9, 10, 12, 17) suggests that LAT gene products are not necessary for the establishment or maintenance of the latent state. Some reports indicate that LAT$^-$ viruses (including the *dl*1403 mutant used in this study) show delayed reactivation kinetics in explantation assays (2, 12, 17) or reduced efficiency of in vivo reactivation (7), whereas other LAT$^-$ mutants reactivate with the same kinetics as the wild-type virus (1, 9). It would be informative to determine whether the delayed-reactivation phenotype in this set of mutants correlates with an inability to express the LAA. Interestingly, the two LAT$^-$ mutants that did not show delayed reactivation had very minor (1) or no (9) alteration in ORF 2; even though RNA encoded by this ORF was not detected in ganglia infected with these mutants, one cannot formally exclude the possibility that the LAA was indeed produced under these conditions. Nevertheless, the delayed-reactivation phenotype of some LAT$^-$ mutants suggests that the role of the LAA is to enhance reactivation. However, the protein is present during the latent phase of infection. To explain these observations, we speculate that the LAA may be inactive during the latent phase of infection and may be activated by extracellular signals that trigger reactivation. Alternatively, to be active the LAA may require a factor available only after reactivation is initiated. Although both the function of the LAA and specific aspects of its expression remain to be elucidated, the identification of an LAT-encoded antigen in latently infected neurons may be a step towards understanding the molecular mechanisms involved in the control of HSV latency.

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