Analysis of the expression directed by two HTLV-I promoters in transgenic mice

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We generated several lines of mice transgenic for the lacZ reporter gene under the control of an HTLV-I LTR. Two different LTR were used; one was isolated from a case of Adult T-cell Leukemia (ATL), the other from a case of Tropical Spastic Paraparesis (TSP/HAM). These LTR differed at 16 nucleotide positions. The pattern of expression of the transgene, studied at the RNA level by RT-PCR, was the same regardless of the origin of the promoter. The β-galactosidase activity was detected primarily in the central nervous system, in the parenchyma, the choroid plexus and the ependymal cells along the ventricles. In parenchyma, double labelling experiments showed that the cells expressing β-galactosidase were neurons. These results show that choroid plexus cells and ependymal cells, as well as some neurons, are permissive for the activity of the HTLV-I promoter. The origin of the LTR had no influence on the pattern of expression of the reporter gene.

Keywords: HTLV-I LTR; ATL; TSP/HAM; β-galactosidase; CNS

Introduction

Human T cell leukemia virus type I (HTLV-I) causes a variety of diseases, including adult T-cell leukemia/lymphoma (ATL) and an inflammatory neurological syndrome, HTLV-I associated myelopathy or tropical spastic paraparesis (HAM/TSP) (Gessain et al., 1985; Osame et al., 1986; Poiesz et al., 1980; Yoshida et al., 1982). Several other clinical conditions have been linked to HTLV-I infection, although in many cases no direct association with the virus has been demonstrated. These include uveitis (Mochizuki et al., 1992), Sjögren’s syndrome (Terada et al., 1994; Vernant et al., 1988), inflammatory arthropathies (Nishio et al., 1989), polymyositis (Morgan et al., 1989) and pneumopathies (Sugimoto et al., 1987). However, more than 90% of HTLV-I infected individuals remain symptom-free (Kaplan et al., 1990; Murphy et al., 1989).

A critical issue in the understanding of HTLV-I pathogenesis is the identity of the cells which are permissive for viral replication. The determinants of the cellular tropism of retroviruses are located either in the envelope glycoprotein, which interacts with the cellular receptor, or in the LTR, which influences cell type specific expression through interactions with cellular transcription factors (Paquette et al., 1990; Rassart et al., 1988). Since in vitro studies indicate that the HTLV-I receptor is broadly distributed (Nagy et al., 1984) the LTR could play a major role in the cell tropism of this virus.

HTLV-I infects mainly CD4+ lymphocytes (Richardson et al., 1990). In addition, proviral DNA has been found by PCR in the CNS of TSP/HAM patients (Bhigjee et al., 1991; Kira et al., 1992; Kubota et al., 1994) although it was impossible in these experiments to determine whether the viral DNA was present in contaminating blood cells or in the CNS parenchyma. Harai et al. (1994) using in situ PCR amplification, detected HTLV-I proviral DNA in the nucleus of infiltrating lymphocytes, but not in neural cells, in the spinal cord of TSP/HAM patients. In addition, Leon-Monzon et al. (1994) showed recently that rare endomysial macrophages, but not muscle fibres, harboured viral antigens in HTLV-I associated polymyositis. Lekhy et al. (1995) reported the presence of Tax mRNA in cells of the spinal cord of three TSP/HAM patients. Some of these cells were GFAP positive, indicating that astrocytes might be infected. One difficulty in studying the tropism of HTLV-I in vivo is the absence of a convenient animal model. Some years ago we showed, using a transgenic mouse model, that the LTR isolated from a HAM/TSP patient directed the expression of a reporter gene primarily in the CNS (Gonzalez-Dunia et al., 1992b).
In the study reported in this article we compared the expression pattern of two LTR promoters which differed by 18 point mutations and were isolated respectively from patients with ATL and HAM/TSP. We describe the tissues and the cell types which were permissive for the in vivo expression of these promoters.

**Results**

**Transgenic mice**

The two LTRs used (Figure 1) are described in Materials and methods. They come respectively from a case of ATL and a case of TSP/HAM. Their activities have been previously investigated in vitro by a transient transfection assay (González-Dunia et al., 1992a, 1993).

Two constructs described in Figure 1A were injected into one-cell mouse embryos. Three founder mice were obtained with the TSP LTR-nlsLacZ construct, and three lines of transgenic mice were derived from them by mating the founder animals with DBA/2 mice (lines G1, 1v and 2v). Likewise, three founders and three lines (Sie 2, Sie 5 and Sie 6) were obtained with the ATL LTR-nlsLacZ construct. Amplification by PCR of the LTR, nlsLacZ and splice site regions of the transgenes gave DNA products of the expected sizes. Southern blot analysis after BamHI digestion showed the expected pattern for all the founder mice (data not shown). Therefore, there was no indication of the existence of rearrangements within the transgenes. Furthermore, the sequence of the LTRs recovered from each line was identical to that of the original LTRs obtained from infected patients (Figure 1B).

**Expression of the transgene at the RNA level**

The expression of the transgene was studied at the RNA level in brain, spinal cord, lung, kidney, heart, gonads, gut, salivary glands, liver, lymph nodes, Peyers patches, spleen and thymus. Several mice (2–3 months old) from each transgenic line were examined (Table 2). The transgene mRNA was detected by RT–PCR using the LacZ and splice site primers (Table 1). The GAPDH mRNA was also amplified, as a control. Samples in which this housekeeping mRNA could not be amplified were discarded. The amplification with primers for the splice site showed that there was no contamination of the RNA samples by genomic DNA (see Materials and methods).

![Figure 1](image)

**Figure 1** (A) Structure of the transgenes. nls stands for the nuclear localization sequence which was derived from the SV40 T antigen. In all constructs, the splice site and polyadenylation signal is brought in by the same BamHI fragment derived from the pSVK3 vector. (B) Comparison of the two LTR sequences. ATL LTR came from a case of ATL from Ivory Coast (Komurian-Pradel et al., 1991). TSP LTR has been isolated from proviral DNA of a TSP patient (Jacobson et al., 1988).

<table>
<thead>
<tr>
<th>Table 1 Primers used in this study</th>
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<tr>
<td><strong>Region</strong></td>
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<td>LTR&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>LacZ&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>targaLacZ&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>splice site&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>GAPDH</td>
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<sup>a</sup>Primers used for screening transgenic mice as well as for RT-PCR analysis of the RNA extracted from several organs. The size expected after amplification with RTTG1 and RTTG2 primers, that flank the splice site present in all transgenes, is 168 bp for the amplification of DNA and 100 bp for the amplification of RNA.

<sup>b</sup>Primers used as internal probes (after terminal transferring labelling) for Southern blot analysis of the PCR products.
Table 2 Expression of the transgenes in the different lines of transgenic mice

<table>
<thead>
<tr>
<th>Transgene Origin of LTR</th>
<th>N. of mice tested</th>
<th>Gli</th>
<th>HAM/TSP</th>
<th>LTR-fgal</th>
<th>ATL-fgal</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>2v</td>
<td>4</td>
<td>2</td>
<td>4</td>
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<tr>
<td>Spinal cord</td>
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<tr>
<td>Brain</td>
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<td>Lung</td>
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<td>Kidney</td>
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<td>Heart</td>
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<td>Liver</td>
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<tr>
<td>Lymph nodes</td>
<td>+</td>
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<tr>
<td>Peyer's patches</td>
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<tr>
<td>Spleen</td>
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<tr>
<td>Thymus</td>
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</table>

++: The band of amplified DNA was visible in every mouse analyzed, after ethidium bromide staining of the agarose gel.
+: The band was visible in some of the mice analyzed, only after Southern blotting of the gel and hybridization with a 32P-labelled probe.
-: No band was detected.

Table 2 summarizes the results obtained with all transgenic lines. Expression of the transgene was not detected in two ATL-nlsLacZ lines (Sie 2 and Sie 5). In all other transgenic lines, the pattern of expression was remarkably similar, suggesting that it was due to determinants present in the LTRs and not to other factors such as the integration site. The transgene was expressed mainly in the CNS (brain and spinal cord) as well as in testis. In addition, the lymphoid organs, i.e. lymph nodes, Peyer patches and spleen, showed varying low levels of expression. No difference was observed in the overall pattern of expression according to the origin of the LTR (HAM/TSP or ATL).

**LTR methylation analysis**

The reporter gene was not expressed in the Sie 2 and Sie 5 lines. This could have been due to methylation of the ATL LTR in these mice (Figure 2). In order to examine the extent of this methylation, brain and kidney DNA were digested with Xmal and HindIII. The 718 bp LTR fragment produced by this digestion contains nine HpaII sites. As shown in Figure 2, this fragment could not be digested with HpaII in the case of Sie 2 and Sie 5 mice, indicating methylation of the HpaII sites. In contrast, the LTR fragment prepared from the Sie 6 mice was digested by HpaII, indicating that the LTR was not methylated in the Sie 6 mice. This difference of LTR methylation may explain the lack of expression in the Sie 2 and Sie 5 lines.

In situ detection of β-galactosidase activity

The activity of both LTRs was examined by histochemical detection of β-galactosidase activity in several organs. Normal DBA/2 mice were included in the experiments as controls. Despite paraformaldehyde fixation, gonads, kidney and salivary glands showed some cytoplasmic endogenous β-galactosidase activity. However, background
activity was never detected in the CNS. X-Gal staining was faint for the TSP lines (Gri, 1v and 2v). It has been suggested that the activity of HTLV-I LTR might be higher in outbred animals (M Nerenberg, personal communication). Therefore we crossed the Gri, 1v and 2v lines with CD1 Swiss mice. In some of these hybrid animals, X-Gal staining was detected in discrete areas of the brain, mostly the hippocampus (Figure 3A) and the choroid plexus (Figure 3B).

The Sie 6 line exhibited a much higher level of β-galactosidase activity in the CNS than the other lines and therefore allowed a more detailed description of cells permissive for HTLV-I LTR expression. Microscopic analysis showed that the ependymal cells were stained strongly, particularly in the cerebral ventricles (Figure 3F). X-Gal staining was also observed in many cells in the grey matter of the brain, mostly in the cortex and ventral part of striatum (Figure 3C). The staining was often peri-

Figure 3 β-Galactosidase activity in transgenic mice. (A, B): Staining obtained with brain slices from a mouse of the Gri transgenic line. Blue X-gal staining is visible in hippocampus (A) and in choroid plexus (arrow in B). Similar results were observed with animals from the other TSP-nLoxZ lines. (C, D): Staining obtained with brain slices from a mouse of the Sie 6 transgenic line. Blue reaction product is observed in the grey matter, in the lateral grey nuclei (putamen, pallidum and striatum) (C). Staining was also detected in other grey nuclei and in choroid plexus (arrow in D). In 7 μm thick paraffin sections, the X-gal staining is localized at the nuclear membrane in parenchyma (E) and ependymal cells (F).
nuclear (Figure 3E), as already observed when a nls-LacZ construct is expressed in neurons; nls-β-gal protein seems too large to migrate through the nuclear pores and remains mainly located at the nuclear periphery. (Bonnerot et al., 1987).

**Identification of the CNS cells expressing β-galactosidase**

The SIE 6 line was used to characterize the cells expressing β-galactosidase using CNS cells specific markers. Immunocytochemistry was used to detect

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**Figure 4** Identification of CNS cells expressing β-galactosidase activity. (A) Immunoperoxidase detection of neurons using an anti-NF monoclonal antibody (brown). Blue X-gal nuclear staining reveals β-galactosidase activity. Arrows point to examples of double-labelled cells × 500. (B) Immunoperoxidase detection of astrocytes with an anti-GFAP antibody. Arrows point to GFAP positive astrocytes. Blue nuclear staining shows cells positive for β-galactosidase activity × 500. (C) Identification of oligodendrocytes by in situ hybridization with an anti-sense PLP-specific riboprobe (arrows). × 500. (D) Immunoperoxidase detection of microglial cells with an anti-F4/80 serum. Arrows point to brown microglial cells. Blue nuclei indicate β-galactosidase positive cells × 312.
antigenic markers specific for neurons, astrocytes and microglial cells. In situ hybridization was used to identify oligodendrocytes. Figure 4A shows that the cells expressing β-galactosidase in the brain were located in the grey matter and had the morphology of neurons. Furthermore, the majority of them could be labelled with an anti-neurofilament monoclonal antibody. Immunostaining with an anti-GFAP serum, a marker for astrocytes, or with an F4/80 serum, which stains macrophage-microglial cells, never coincided with the X-Gal staining (Figure 4B and D). Likewise, in situ hybridization with a probe specific for PLP mRNA, a marker of oligodendrocytes, did not coincide with the X-Gal staining (Figure 4C). These results indicate that the transgene is expressed in a subset of neurons. No expression was detected in glial cells.

Discussion

The low level of expression of HTLV-I in vivo makes the detection of infected cells in human tissue difficult. This may explain why contradictory results have been obtained concerning the neurotropism of this virus. Since HTLV-I can infect a large range of cells grown in vitro, it is assumed that the viral receptor is widely distributed throughout the organism. Therefore, transcriptional regulation controlled by the viral LTR could be a major determinant of cell tropism. We tested this hypothesis using transgenic mice.

In the present work, we studied the expression of a reporter gene, nlsLacZ, controlled by HTLV-I LTR isolated from patients with either ATL or HAM/TSP. The expression of the transgene could be detected by RT-PCR in the three lines obtained with the TSP LTR and in one of the lines obtained with the ATL LTR. The absence of expression in two other lines obtained with the ATL LTR (Sie 2 and Sie 5), cannot be explained by differences in the number of copies of the transgene, because these mice have 10 times more copies than the Sie 6 mice (data not shown). On the other hand, methylation of the viral promoter seems to correlate with the absence of expression (Figure 2). As shown recently (Cañens et al., 1994), the average CpG density in the HTLV-I LTR (5.7%) is considerably higher than in eukaryotic cellular DNA (about 1%). Hence the HTLV-I promoter may be particularly sensitive to inactivation by methylation. In this context, it has been proposed that the LTR methylation is one of the factors leading to HTLV-I latency (Cañens et al., 1994; Kitamura et al., 1985; Saggioro et al., 1990, 1991). Others have already demonstrated that the methylation of the promoter and non-promoter sequences correlates with inactivation and silencing of various genes (Graessmann et al., 1994; Ottaviano et al., 1994).

We considered the possibility that the differences in transgene expression between the Sie lines were due to differences in the level of CREB and AP-2 transcription factors in the CNS of each line. Therefore we examined the levels of these factors, using specific sera and Western blots, in brain extract from the Sie 2, Sie 5 and Sie 6 lines. No differences were observed (not shown).

The pattern of expression of the transgene, detected by RT-PCR, was the same regardless of the origin of the LTR. This is consistent with our previous observations with cell lines transfected in vitro (Gonzalez-Dunia et al., 1993). In all the transgenic lines, the CNS was the organ which expressed the transgene at the highest level. This raises interesting issues for the pathogenesis of HAM/TSP. In particular, we showed that the choroid plexus and ependymal cells express high levels of the reporter gene. If these cells are indeed permissive in man, they could represent one way of entry of the virus into the cerebrospinal fluid and from there into CNS parenchyma. Interestingly, we observed that sheep choroid plexus cells grown in vitro are permissive for HTLV-I replication (data not shown). We showed also that, besides choroid plexus and ependymal cells, a subset of neurons located mainly in cortex, striatum, and hippocampus expressed the reporter gene.

Bieberich et al (1993) showed that the HTLV-I LTR could direct the expression of LacZ in the cortex and hippocampus of transgenic mice. The expression of the transgene was minimal in the absence of transactivation by Tax. Interestingly, the only organ which expressed the transgene in the absence of transactivation was the brain. Transactivation, obtained by mating LTR lacZ transgenic mice with tax transgenic mice, increased the expression in brain. Muscle, salivary glands and thymus expressed the transgene only after transactivation. On the whole, the results obtained in our studies and in those of Bieberich et al (1993) are congruent.

Therefore, there is now accumulating evidence, from the study of several lines of transgenic mice obtained in different laboratories, that the HTLV-I promoter can be active in neurons. Furthermore, neuronal cell lines of nontumorigenic origin can be infected with HTLV-I (Lehky et al., 1995). Obviously, this does not demonstrate that neurons are infected in HAM/TSP patients. Nevertheless, this possibility should be examined carefully. The lesions of HAM/TSP are located mainly in the white matter and consist in inflammation and demyelination. This is not inconsistent with the fact that the neurons might be infected. The infection of neurons could eventually result in secondary demyelination with the activation of
macrophages and astrocytes and the induction of an inflammatory response.

In conclusion, our results show that ependymal cells and neurons are permissive for the expression of the HTLV-I LTR. The infection of these cells could play a role in the entry of the virus in the CNS and in the demyelination process observed in TSP/HAM.

Materials and methods

Construction of transgenes

The transgenes are described in Figure 1. The ATL LTR came from a 19 year old female patient (Sle) from Ivory Coast (Komurian-Pradel et al., 1991). It was amplified by PCR from DNA extracted from Ficoll-Plaque separated peripheral blood lymphocytes, using specific primers with flanking restriction sites (5’ Sall and 3’ HindIII) as described previously (Gonzalez-Dunia et al., 1993). This LTR was cloned in a Bluescript SK vector. The E. coli LacZ gene, fused to the SV40 nuclear localization signal (nlsLacZ) was inserted downstream from the LTR. The 3’ end of the nlsLacZ gene was fused to a 1 Kb BamHI fragment derived from the pSVK3 vector. This fragment contains the SV40 splice site and polyadenylation signal. The TSP LTR- nlsLacZ construct was derived from that previously described (Gonzalez-Dunia et al., 1992a). The 3’ extremity of this previous vector was modified by adding the pSVK3-derived BamHI fragment described above. Hence the two LTR-nlsLacZ constructs were identical, except for the LTR region.

The activity of each construct was tested in vitro using a  β-galactosidase assay. After transfection in C33 cells, the basal activity of both LTRs was detected. Enhanced expression was observed following co-transfection with a Tax expression vector (data not shown).

The constructs were digested with KpnI and SpeI enzymes. The inserts were purified by agarose gel electrophoresis, NaI/glass beads extraction (Genclean, Bio 101), Elutip purification and injected in fertilised eggs.

Production and screening of transgenic mice

Transgenic mice were obtained on a (C57BL/6j x DBA/2) background. Transgenic animals ( founders and progeny) were screened by PCR analysis of DNA from tail biopsies. The primers are described in Table 1. The integrity of the transgene in founder animals was checked by Southern blot analysis (data not shown). The F1 progeny was derived by back crossing to DBA/2 mice. In some instances, crosses were also performed with CD1 Swiss mice.

Methylation of the LTR

DNA extracted from brain and kidney of transgenic animals was digested with Xmal and HindIII in order to generate the 718 bp fragment correspond-

ing to the LTR. HpaII and MspI restriction enzymes, which are respectively sensitive and insensitive to methylation, were used to study the methylation of the viral promoter. After digestion (3 units of enzyme/μg of DNA) the DNA fragments were separated on agarose gel, transferred onto nylon membrane and hybridized at 65°C, in 0.5 M phosphate buffer containing 7% SDS, with a 32P-labelled random-primed probe corresponding to the LTR.

Isolation and analysis of RNA

Mice were anesthetized and perfused with PBS. Total RNA was prepared from different organs by the GACP method (Chomczynski and Sacchi, 1987). The RNA samples were treated with RQ1 DNase, phenol/chloroform extracted, and ethanol precipitated. Complementary DNA was produced by reverse transcription with random hexamers. LacZ sequence (see Table 1 for the primers used) was PCR amplified (35 cycles: 94°C/30 s, 55°C/30 s, 72°C/30 s) and detected by Southern blot hybridization with a [32P] labelled oligonucleotide internal probe. Each RNA sample was also incubated in the absence of reverse transcriptase to provide a control. The RTTG1 and RTTG2 primers, which flank the splice site in both transgenes, were used to check for DNA contamination of the RNA (after splicing, the size of the amplified fragment is reduced from 168 bp to 100 bp). The amplification conditions used for the RTTG1 and RTTG2 primers were: 35 cycles: 94°C/30 s, 55°C/30 s, 72°C/30 s. In order to check for the integrity of mRNA and the efficacy of reverse transcription, the glyceraldehyde phosphate dehydrogenase (see Table 1 for the GADPH primers used) mRNA was also amplified in each cDNA sample (25 cycles: 94°C/30 s, 50°C/30 s, 72°C/30 s). All PCR reactions were performed in 100 μl reactions with 2.5 U of Taq polymerase (Amersham), 200 μM dNTPs, 20 pmoles of each primer in the supplied buffer.

Histological detection of β-galactosidase

Mice were anesthetized and perfused with 25 ml of PBS followed by 25 ml of 4% buffered parafomaldehyde. The organs were removed and incubated in fixative for 15 min at 4°C. The tissues were cut into 200 μm slices and β-galactosidase activity was detected by overnight staining at 30°C with 5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside (X-gal) (Sanes et al., 1986). The tissues were then extensively post fixed in 4% buffered paraformaldehyde, embedded in paraffin and processed using standard histological procedures.

Characterization of cells expressing β-galactosidase in the CNS

CNS cells were identified on 7 μm thick sections with specific antigenic markers and an immuno- peroxidase assay with diaminobenzidine (DAB) as
a substrate. Astrocytes were identified with an anti-glial fibrillary acid protein (GFAP) rabbit serum (Dakopatts) diluted 1/450. Microglial cells were identified with an hyperimmune anti F4/80 rabbit serum (kindly provided by H Perry), diluted 1/4000. Neurons were identified with a mouse monoclonal anti neurofilament (NF) antibody (provided by D Paulin, Institut Pasteur, Paris) diluted 1/200. These primary antibodies were detected with a biotinylated secondary antibody (diluted 1/200) and an avidin-biotin-peroxidase complex (ABC Vectorstain, Vector Laboratories). For the F4/80 staining, detection of peroxidase with DAB was performed with imidazole enhancement as described (Straus, 1982).

Oligodendrocytes were identified by the detection of the proteolipid (PLP) mRNA by in situ hybridization (ISH) as previously described (Brahic and Ozden, 1992; Ozden et al., 1991). We used an 35S-labelled antisense riboprobe (492 bp), corresponding to the Apal-Pstl fragment of the rat PLP gene. Exposure time was 48 h at 4°C.

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References


