Virus inactivation in a proportion of human T-cell leukaemia virus type I-infected T-cell clones arises through naturally occurring mutations

Nicola J. Rose,1 Jennifer H. Richardson,1† Ulrich Desselberger2 and Andrew M. L. Lever1

Human T-cell leukaemia virus type I (HTLV-I) is the aetiological agent of adult T-cell leukaemia/lymphoma and tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP/HAM). The transcriptional activating protein (Tax) of HTLV-I is strongly implicated in cellular proliferation. We examined the tax gene and 5’ long terminal repeat (LTR) sequences in eight naturally infected T-cell clones derived from TSP/HAM-affected individuals who were either productively (proliferate spontaneously) or silently (do not proliferate spontaneously) infected. In two silently infected clones point mutations within the proviruses resulted in truncation of the Tax protein. One clone harboured both a deleterious tax gene mutation and a point mutation in an enhancer element of the 5’ LTR. Sequence changes, immunological escape mutation, integration site context and host cell phenotype may all contribute to the high proportion of latently or silently infected T-cells found in vivo in virus carriers.

Despite possessing oncogenic properties, no part of the HTLV-I genome shows identity to known oncogenes. As the site of HTLV-I provirus integration into the host cell genome is generally regarded as random, oncogenesis by insertional mutagenesis is probably extremely infrequent. Despite this, a predilection for transcriptionally active proviruses to be found in G+ C-rich chromatin was previously noted (Zoubak et al., 1994). With only one report to date of two individuals sharing a common HTLV-I integration site (Macerca et al., 1992), the involvement of viral gene products in cellular transformation is more likely. The progression to leukaemia is probably a multistep process (Okamoto et al., 1989) with Tax involvement likely occurring early in virus pathogenesis.

HTLV-I has been divided into genetic groups [reviewed in Slattery et al. (1999)]. HTLV-I sequences, including tax, differ between strain types (Ratner et al., 1991). There is a greater degree of sequence conservation within TSP/HAM patients than within or between asymptomatic carriers (Niewiesk et al., 1994, 1995). No specifically neuropathic strain of HTLV-I has been identified.

We have studied a series of HTLV-I-infected T-cell clones generated from the blood of infected individuals, Du, Mu and Ph1C, as previously described (Wucherpfennig et al.,...
Table 1. Sequence variants identified in the pX(tax) region

Nucleotide changes within the tax gene are illustrated alongside the corresponding amino acid changes. Nucleotide numbering follows that of Seiki et al. (1983). Amino acids are abbreviated according to the standard single-letter code. Also shown are residues in Rex and Tof overlapping those in Tax: some are altered by the tax gene variants.

<table>
<thead>
<tr>
<th>Amino acid change in Tax*</th>
<th>Nucleotide changes within the tax gene and predicted amino acid changes†</th>
<th>Amino acid changes in Rex and Tof</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Du4 (P) Du20 (NP) Du26 (P) Du34 (NP) Du43 (NP) Mu16 (P) Mu40 (NP) Ph1C (NP)</td>
<td>Rex Tof</td>
</tr>
<tr>
<td>G14R</td>
<td>GGA → AGA</td>
<td>S32S R171Q</td>
</tr>
<tr>
<td>W28STOP</td>
<td>TGG → TAG</td>
<td>G47S L185L</td>
</tr>
<tr>
<td>Q53Q</td>
<td>CAG → CAA CAG → CAA TGG → TAG</td>
<td>R72K D211N</td>
</tr>
<tr>
<td>W56STOP</td>
<td>CAG → CAA</td>
<td>G75R L213L</td>
</tr>
<tr>
<td>L87L</td>
<td>TCC → TCT</td>
<td>L106S E167E</td>
</tr>
<tr>
<td>G149S</td>
<td>TCC → TCT</td>
<td>P169Q P180L</td>
</tr>
<tr>
<td>S150S</td>
<td>TCC → TCT</td>
<td></td>
</tr>
<tr>
<td>S160S</td>
<td>GGC → AGC GGC → AGC</td>
<td></td>
</tr>
<tr>
<td>E192E</td>
<td>E192E</td>
<td></td>
</tr>
<tr>
<td>E210E</td>
<td>E192E</td>
<td></td>
</tr>
<tr>
<td>A221V#</td>
<td>GCT → GGT GCT → GGT GCT → GGT GCT → GGT GCT → GGT GCT → GGT GCT → GGT</td>
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</tr>
<tr>
<td>L227L</td>
<td>CTA → CTG CTA → CTG</td>
<td></td>
</tr>
<tr>
<td>S268S</td>
<td>TCT → TCC</td>
<td></td>
</tr>
<tr>
<td>H279R</td>
<td>CAC → CGC CAC → CGC CAC → CGC CAC → CGC CAC → CGC CAC → CGC</td>
<td></td>
</tr>
<tr>
<td>H279Q</td>
<td>CAC → CAT TTT → TTC</td>
<td></td>
</tr>
<tr>
<td>F290F</td>
<td>CAG → AAA GAA → GAG GAA → GAG</td>
<td></td>
</tr>
<tr>
<td>Q298Q</td>
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<tr>
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</tr>
<tr>
<td>E310E</td>
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<tr>
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</tr>
<tr>
<td>G339S</td>
<td>G339S</td>
<td></td>
</tr>
<tr>
<td>R349R</td>
<td>G339S</td>
<td></td>
</tr>
</tbody>
</table>

* Changes altering the residue are underlined. Bold type indicate Tax residue changes likely to have an effect on abrogation of provirus proliferation.
† NP, non-proliferative; P, proliferative.
‡ Residue 221 is altered at nucleotide 7959. This was published as a TSP/HAM variant but found by another group to be a natural variant of the Cosmopolitan strain (see text).
1989; Hyer et al., 1991; Hölsgberg et al., 1994; Zoubak et al., 1997). Several of these clones, despite being isolated from a single TSP/HAM-affected individual, differ in their proliferative phenotypes: some exhibiting relatively IL-2-independent proliferation while others do not. An identical host background highlights the relative contribution of viral sequence changes to cellular dysfunction. Since the tax gene and its product are likely to be involved in cell proliferation (Richardson et al., 1997), we characterized the tax gene sequence of each of these clones to look for mutations. PCR amplification of tax genes was effected using overlapping primer pairs spanning nucleotides 7195 and 8474 [ATK sequence; Seiki et al. (1983); tax1s 5’-bio-TTCTC TTCACACAGGTCTCTT, tax1a 5’-TGGTAGGATT AGTTCCTTA 3’, tax2s 5’-bio-GCTGTCAGCGTGACG 3’]. The antisense oligonucleotide is complementary to sequences in the 5′ region of gag. The additional sequencing of clones Mu16 and Du26 proviral LTRs identified patient-specific variation. An internal control for Ph1C was unavailable. Sequencing of two independent PCR reactions was performed using an ABI 310 automatic sequencer employing dichlororhodamine terminator chemistry and oligonucleotides LTR1.seq 5’-TCCCGGAACAGAAGGTCT 3’ and LTR2.seq 5’-ATCCACACGGCGTTAGTCG 3’ (Fig. 2). Alignment and screening of HTLV-I strains highlighted naturally occurring variants.

The LTR changes were predominantly naturally occurring strain variants or changes occurring in transcriptionally active proviruses or in both active and silent proviruses. The Du34 provirus harboured a G(nt174)A change in the first conserved element of the second TRE and a C(nt229)A change in a region possessing transcription-factor binding sites. The Mu40 provirus also possessed the C(nt229)A variant as well as one change in each of the R [G(nt505)C] and U5 [C(nt662)T] regions. The U5 variant was also found in the Ph1C provirus. Nucleotide 662 is in a region not present in CR-CAT; as this construct is functional (see below) this change is unlikely to explain provirus silencing in these two clones. As the R variant falls within the Rex-binding site sequence (Hanly et al., 1989; Toyoshima et al., 1990; Ratner et al., 1991) Rex function may be affected by this mutation in the context of the 3′ LTR. A Y323G change in the Du34 provirus does not occur within a functionally significant region of the Rex-responsive element (RxE) structure. Since it is likely that Tax is responsible for the cellular proliferation seen in the clones, RxE changes would not influence this as Tax expression is Rex-independent.

Mutagenesis of plasmids pcDNA3Tax/Rex (Tax expression vector, Tax gene driven by cytomegalovirus promoter) and CR-CAT [pU3R-1 (Sodroski et al., 1984); Cat gene driven by HTLV-I LTR] was effected via site-directed mutagenesis (Kunkel et al., 1987) of a pBluescript KSII(+)-Stratagene) intermediate containing either the 2047 bp HindIII–EcoRI fragment overlapping the tax region from pcDNA3Tax/Rex or the 716 bp Xhol–HindIII LTR-containing fragment from CR-CAT. Construct pcDNA3Tax(Du34)Rex produces the G14R mutant; pcDNA3Tax(Ph1C)Rex produces the H279Q mutant; CR(Du34)-CAT harbours the TRE II mutation and CR(Mu40)-CAT harbours the RBS mutation. The function of these mutants was assessed. DEAE-dextran (Sambrook et al., 1989) transient cotransfection of 5 μg of CR-CAT and an equal amount of either pcDNA3Tax(Du34)Rex or pcDNA3Tax(Ph1C)Rex, or 5 μg of either CR(Du34)-CAT or CR(Mu40)-CAT and 5 μg of pcDNA3Tax/Rex into COS-1 fibroblasts was performed. The combined effect of the Tax and LTR mutations from clone Du34 was analysed by cotransfecting 5 μg CR(Du34)-CAT and 5 μg pcDNA3Tax(Du34)/Rex. CAT acetylation was assessed by thin layer chromatography and quantified on an Instant Imager (Canberra Packard).
The pcDNA3Tax(Ph1C)Rex and CR(Mu40)-CAT constructs function at a level comparable to wild-type (Fig. 1b); however, the pcDNA3Tax(Du34)Rex and CR(Du34)-CAT constructs show acetylation levels approximately fivefold and twofold lower than the wild-type. In this context the effects of the Du34 Tax and enhancer mutations were not additive. A Tax allele containing the G14R substitution has been shown to be functionally compromised (Niewiesk et al., 1995): our data support this observation. This change falls within a defined CTL epitope and results in loss of CTL recognition in vitro and trans-activation pertaining to the in vitro up-regulation of the viral LTR or the IL-2Rα chain promoter (Parker et al., 1994; Niewiesk et al., 1995). A high level of Tax11–19-specific CD8+ T-cells in the peripheral blood of TSP/HAM-affected individuals with enrichment occurring in the cerebrospinal fluid of these patients was demonstrated (Greten et al., 1998).
HTLV-I transcriptional inactivity

Fig. 2. A consensus sequence for the 5' HTLV-I LTR is given. Nucleotide numbering follows that of Seiki et al. (1983). The consensus sequence (con) was derived from available database sequences [accession nos: M69044 (Paine et al., 1991); M86840 (Evangelista et al., 1990); J02029 (Seiki et al., 1983); D00294 (Malik et al., 1988); L02534 (Gessain et al., 1993)]. Zairean sequences were not included as they were incomplete. Standard codes for nucleotide/nucleotide combinations have been used. Gaps in the consensus indicate regions where a single isolate possessed additional sequence. The points at which the sequence of the analysed proviral LTRs differ are indicated below the consensus sequence. Changes which are natural variants are also shown. Functionally important regions are identified with boxes: an asterisk denotes Ets-binding (Bosselut et al., 1990; Gitlin et al., 1991) and a dagger denotes SP1-binding motifs (Gégonne et al., 1993); SD, splice donor; RBS (Rex-binding sequences), nucleotides proposed to form a contact structure for Rex protein (see text); DLS, dimer linkage site (Greatestre et al., 1996); PBS, primer-binding site. The boundaries of the U3, R, U5 and the RxRE sequences are indicated by arrows as are the end of LTR sequences and the start of the gag gene.

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These data highlight how escape mutations at immunologically important epitopes may compromise virus protein function.

The central conserved domain of the TRE is crucial for CREB-binding. When the Du34 Tax and LTR mutants were cotransfected, the resulting level of acetylation mirrored that of the Tax mutant alone, suggesting that the Tax change is the predominant silencing mutation in the in vitro system.

The nucleotide changes in tax might also affect the overlapping rex and tof genes (Fig. 1, Table 1). The rex ORF overlaps the tax gene as far as tax codons 171/172 whereas tof only overlaps as far as tax codons 84/85 (Ciminale et al., 1992; Koralnik et al., 1992). Changes in Rex that abrogate its function may result in altered post-translational splicing control of the mRNA species. An arginine-rich N-terminal region of Rex is involved in its nuclear localization and binding to the RxRE (Hammes & Greene, 1993), and an activation domain thought to be a target for a cellular factor spans residues 79 and 99 (Weichselbraun et al., 1992). The R72K and P169Q variants do not affect Rex function as these occur in the productively infected clone Du4, which expresses all of the structural viral proteins. The functional significance of the G47S, G75R, and P180L and L106S changes has yet to be evaluated as they were identified in silently infected clones, the first three occurring in the proviruses from either Du20 or Du43, which have premature stop codons in tax.

The Tof D211N substitution appears to be functionally insignificant as it occurs within the Du4 provirus. The significance of a R171Q change in the clone Du34 is unclear. Given the lack of evidence that Tof is essential it seems unlikely that this change in Du34 is central to the ablation of provirus activity.

The cause of the transcriptional inactivity of the three proviruses harbourd by clone Ph1C may relate to the CD8 phenotype of this clone. Much higher levels of transcription from the HTLV-I LTR in primary CD4+ T-cells, compared with primary CD8 cells, have been demonstrated, suggesting that cellular factors within CD8 T-cells may be insufficient to support efficient use of the HTLV-I promoter (Newbound et al., 1996). Alternatively active suppression of transcription by CREB family members or other cellular factors might contribute (Xu et al., 1990).

As well as contributing to the functional mapping of Tax, Rex and Tof and the 5’ LTR our results illustrate the diversity of proviral sequence within an individual and show that transcriptional inactivity of the HTLV-I provirus in naturally infected T-cell clones can be caused by naturally occurring mutations, suggesting interplay with a variety of factors. Defective tax genes causing truncated proteins, or proteins with reduced trans-activation activity are associated with the non-virus-expressing and non-proliferative cellular phenotype. LTR defects may also contribute to virus silencing. A CD8 cell phenotype, integration site context and mutations resulting in abnormal mRNA production (Major et al., 1995) may also cause provirus inactivation. In some clones, however, none of these factors pertain, the LTR is functionally intact and the reasons for transcriptional inactivity are unclear. Further work to investigate the causes of virus silencing in these clones is ongoing.

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References


activated in peripheral blood and accumulate in cerebrospinal fluid from HAM/TSP patients. *Proceedings of the National Academy of Sciences, USA* 95, 7568–7573.


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