A 37 base sequence in the leader region of human T-cell leukaemia virus type I is a high affinity dimerization site but is not essential for virus replication

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Mutagenesis has demonstrated a region in the human T-cell leukaemia virus type I (HTLV-I) 5' leader RNA which, when deleted, abolishes stable RNA dimer formation in vitro. We have further mapped, using both in vitro transcribed and synthesized RNA, this site to a 37 base region, which dimerizes with high affinity. When deleted from an HTLV-I Gag–Pol-expressing plasmid which was co-transfected with an envelope protein expression vector to produce virions capable of single round infection, the dimer linkage deletion did not affect viral protein production. In addition, virus infectivity was only slightly reduced, to approximately 75–80% of the wild-type.

Infection with human T-cell leukaemia virus type I (HTLV-I), the first pathogenic human retrovirus discovered, is usually asymptomatic but can be associated with a T-cell malignancy and an autoimmune demyelinating disease. Its genome is highly conserved. It is virtually always transmitted cell-to-cell, with little evidence of cell-free infectious virus.

Retroviruses carry two copies of their genome which, on electron microscopy studies, appear to be linked at the 5' end (Bender et al., 1978; Murthy et al., 1981). The conservation of the diploid genome, and of the dimer linkage site in retroviruses, suggests both are important, although it does not necessarily follow that they are functionally related. The dimer linkage has been postulated to contribute to the genomic RNA encapsidation signal (Kato et al., 1993; Torrent et al., 1994; Harrison et al., 1998; Bender et al., 1978). In addition, by physically linking the two RNA molecules, it may enhance the capacity for recombination between the two virus strands (Bender et al., 1978; Mikkelsen et al., 1996, 1998; Marquet et al., 1991; Wu & Temin, 1990). The molecular interactions involved in dimer linkage are, as yet, unknown. Purine tetrad structures have been invoked in structures analogous to telomeres, although this now seems less likely to be the major type of molecular interaction (Marquet et al., 1991). The interaction is too stable in vitro to be explained by Watson–Crick base pairs alone. Recently, it has been noted that palindromic sequences are commonly associated with dimer linkage sites (Haddrick et al., 1996; Laughrea & Jette, 1996; Muriaux et al., 1996; Paillart et al., 1996). In a nuclear magnetic resonance study of the dimer linkage structure of human immunodeficiency virus type 1 (HIV-1), distorted loops were proposed to join by Watson–Crick pairing at an autocomplementary loop in the 5’ leader upstream of the splice donor in what is known as the ‘kissing hairpin’ interaction (Mujeeb et al., 1998). Mutations of this region in HIV-1 have been performed by a number of groups. The conclusion from this work is that inhibiting dimerization can reduce virus encapsidation up to 5-fold and infectivity of the virions can be reduced up to 1000-fold compared to wild-type, although other data suggests that the defect is less severe (Berkhout & van Wamel, 1996; Haddrick et al., 1996; Harrison et al., 1996; Muriaux et al., 1996). We have previously published an extensive deletion and mutagenic analysis of the 5’ leader of HTLV-I in which we have identified a 32 base region, deletion of which leads to abrogation of dimerization of in vitro transcribed HTLV-I RNA (Greatorex et al., 1996; Delamarre et al., 1997). Dimeric HTLV-I RNA is extremely stable to 70 °C and above and remains dimeric, even in formaldehyde-containing gels. Thus, the RNA association is extremely stable. Although our previous mutagenesis identified a region necessary for dimerization, it was not clear whether the same region would be sufficient to form RNA dimers. We therefore proceeded with further 5’ and 3’ truncations of the HTLV-I leader sequence in the transcriptional unit previously described (Greatorex et al., 1996) (Fig. 1a). The end column (‘Dimer formation’) indicates whether or not a dimer was formed. Shown in Fig. 1b is dimer production of the truncated and full-length transcripts. Transcripts were unheated (−) and heated (+, 80 °C for 3 min) to produce monomer and then diluted in dimer buffer (250 mM cacodylic acid, 40 mM KCl and 5 mM MgCl) and run out on...
Fig. 1. (a) 5’ and 3’ truncations of the full length (531 bp) transcript. The end column indicates whether or not a dimer was formed. (b) Dimer production of the truncated and full-length transcripts. Transcripts were heated (+, 80 °C for 3 min) to produce monomer or unheated (−) and run on 2% (lanes 1–6) or 5% (lanes 7–10) Metaphor (Flowgen) agarose gels. Lanes 1 and 2, full length RNA transcript heated and unheated respectively; 3 and 4, pG11Δ561 heated and unheated; 5 and 6, pG11Δ735 heated and unheated; 7 and 8, pG11Δ735–Δ817 heated and unheated; 9 and 10, pG11Δ735–Δ772 heated and unheated. Markers are indicated by arrowheads. (c) Lanes 1, 22mer synthetic HTLV-I RNA, arrowheads denote dimer (D) and monomer (M); 2, 21mer RNA (KEN5, a hairpin representing the U1 SNRNA stem–loop II); 3, 100 bp RNA markers (Ambion). RNA was diluted as described in Methods and electrophoresed on a 5% Metaphor (Flowgen) gel.

2% (lanes 1–6) or 5% (lanes 7–10) Metaphor (Flowgen) agarose gels. Transcripts were labelled with [32P]UTP (ICN) incorporated during transcription. Lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10 are the full-length (531 bp) RNA transcript or pG11Δ561, pG11Δ735, pG11Δ735–Δ817 and pG11Δ735–Δ772 transcripts, heated and unheated respectively. We were able to demonstrate that an in vitro transcript of only 37 bases encompassing the 32 bp DM2 deletion formed a predominantly dimeric molecule (Fig. 1b, lane 10). To ensure this was not an artefact of the transcriptional process and to attempt finer mapping, RNA was chemically synthesized using an ABI 394 DNA/RNA synthesizer that corresponded to a 22 bp region capable by MFOLD analysis of forming secondary structure (CUAUAGCACUAUCCAGGAGA) contained within the 37 bp sequence (Fig. 1c). RNA was diluted (1 µg/µl) in dimer buffer as before, and run out on 5% Metaphor agarose (Fig. 1c, lane 1). The chemically synthesized RNA was dimeric under the same conditions as those used for in vitro transcription. The RNA dimer formed by this oligomer was extremely stable, failing to dissociate even when heated at 80 °C for 3 min. KEN5, a control RNA, was a hairpin representing the U1 SNRNA stem–loop II (CUACCAUUG-CACUCCGAUGU, a kind gift from C. Oubridge) diluted 1 µg/µl and used as a size marker which does not dimerize (Fig. 1c, lane 2).

The 32 bp deletion which abrogated RNA dimerization in vitro was introduced into a provirus subclone of HTLV-I in several steps. Initially a subclone containing the 1340 bp EcoRV to PstI fragment of the pCS-HTLV-I provirus clone positions 30–1370 was constructed. Site-directed mutagenesis was performed using the Kunkel method. This gave rise to a 32 bp deletion KS-EP–ΔDM2. The EcoRV–PstI fragment was then subcloned back into pCS-HTLV-neo, a provirus clone in which the env gene has been replaced by the neomycin resistance gene under the control of the SV40 promoter. The resulting construct was named pCS–ΔDM2-neo.

We examined whether deletion of the 32 bp dimerization sequence had an effect on HTLV-I protein expression and virus particle production. To do so, COS-1 cells were transiently transfected with either wild-type pCS-HTLV-neo or the pCS-
ADM2-neo, and HTLV proteins were sought in the transfected cell lysates and supernatants. Lysates and virus pellet samples were collected, fractionated by SDS–PAGE and immunoblotted to detect Gag proteins. Immunoblotting was carried out using sera from HTLV-I-infected individuals as primary antibodies and 125I-labelled protein A as the visualization step. C91PL HTLV-I-infected cells were used as a positive control, and transfection with a Tax-only expressing plasmid (Fig. 2, lane 2) used to standardize protein loading. The amounts of Gag proteins produced by pCS-ADM2-neo were similar to those produced by the wild-type construct (Fig. 2a, b). Fig. 2(a) shows the cell lysates. In the first lane is a positive control, C91PL HTLV-I-infected cells. The second lane contains cell lysate from a mock control and the third and fourth lanes contain lysates from wild-type and the provirus with the dimerization site removed respectively. The Gag protein precursor, a 55 kDa protein from HTLV-I, underwent normal intracellular maturation and was normally cleaved into mature Gag proteins, as shown by the normal amounts of capsid (CAp24) and matrix (MA-p19) proteins obtained. Analysis of the proteins in the cell supernatant showed that the deletion had no effect on virus particle production (Fig. 2b, lanes as in a).

We then investigated whether deletion of the dimerization site affected HTLV-I transmission in a single round infection assay. This is a quantitative assay, which has been described previously (Delamarre et al., 1997), based on trans-complementation by an env expression plasmid (CMV-ENV1) of the above-mentioned HTLV-I provirus clone in which the env gene has been replaced by a selectable marker. Forty-eight hours following co-transfection of the COS cells with either the mutant or the wild-type Gag–Pol expressor (0 hours following co-transfection of the COS cells with either gene has been replaced by a selectable marker. Forty-eight

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gene). G418 sulfate-resistant colonies were counted after 2 to 3 weeks. The infectivity index was calculated as described previously. The level of background transfer was evaluated by co-transfection using pCS-DM2-neo with a plasmid having a nonsense codon at the beginning of the envelope gene. Two independent experiments were performed. In each experiment, the number of G418-resistant clones obtained with the deletion mutant was slightly lower than that obtained with the wild-type construct. Overall, the transduction efficiency was about 20–25% lower than the wild-type sequence (Table 1). To ensure that the deletion mutation had not been altered or re-arranged during the single round of transfection, the leader region of the integrated provirus in the DM2-containing clones was amplified by PCR. In order to do this, resistant clones were grown to confluence in 75 cm² tissue culture flasks. The DNA from both wild-type and DM2-infected cells was extracted using a Nucleon DNA prep (Nucleon Biosciences). To PCR amplify the region spanning the deletion, three primers were used. One was an antisense biotinylated primer, LTRgagBIO (CTCATCCCGATCGGGATGC), and the other two were sense primers used separately, LTR354 (GGCTGC- GACATCTTCCTTACGC) and LTR2F (ATCCACGCGGTTGAGTGC). The products were run out on 0.8% TBE–agarose gels and the PCR product was extracted from the gel. Primer LTR2F was then used as an internal primer to sequence the products and check for the presence of the deletion. This showed that the 32 bp deletion was intact in the B5 indicator cells which had been transduced (data not shown).

These results confirm the ability of a small region of the HTLV-I leader sequence to form stable dimers in vitro and also demonstrate that a slight replication defect is noted when this deletion is introduced into an in vitro infection assay. They do not exclude the possibility that other regions of the genome are involved in this process. Ideally, one would like to examine the monomeric or dimeric state of RNA in virion particles in experiments such as these. However, the nature of HTLV-I does not facilitate such analyses, since the virus is virtually entirely transmitted from cell to cell. These experiments do allow the observation that deletion of a dimer linkage site does not abrogate cell-to-cell transmission of HTLV-I. Dimerization is, therefore, not an absolute requisite for infection in this virus,

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<th>Table 1. Cell-to-cell transmission of the DM2 HTLV-I dimerization site mutant</th>
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<td>Experiment 1</td>
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<td>No. of clones</td>
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<td>Wild-type</td>
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* DM2 mean is 80.5% of wild-type.
although it may contribute to efficient infection. A 20–25% decrease in infectivity, although insignificant in vitro, over the course of the prolonged in vivo infection which occurs in carriers of HTLV-I would, in fact, provide a significant selective disadvantage to HTLV-I.

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References


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