Requirements for RNA heterodimerization of the human immunodeficiency virus type 1 (HIV-1) and HIV-2 genomes

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Introduction

Retroviruses are prone to recombination because they package two copies of the RNA genome. Whereas recombination is a frequent event within the human immunodeficiency virus type 1 (HIV-1) and HIV-2 groups, no HIV-1/HIV-2 recombinants have been reported thus far. The possibility of forming HIV-1/HIV-2 RNA heterodimers was studied in vitro. In both viruses, the dimer initiation site (DIS) hairpin is used to form dimers, but these motifs appear too dissimilar to allow RNA heterodimer formation. Multiple mutations were introduced into the HIV-2 DIS element to gradually mimic the HIV-1 hairpin. First, the loop-exposed palindrome of HIV-1 was inserted. This self-complementary sequence motif forms the base pair interactions of the kissing-loop (KL) dimer complex, but such a modification is not sufficient to permit RNA heterodimer formation. Next, the HIV-2 DIS loop size was shortened from 11 to 9 nucleotides, as in the HIV-1 DIS motif. This modification also results in the presentation of the palindromes in the same position within the hairpin loop. The change yielded a modest level of RNA heterodimers, which was not significantly improved by additional sequence changes in the loop and top base pair. No isomerization of the KL dimer to the extended duplex dimer form was observed for the heterodimers. These combined results indicate that recombination between HIV-1 and HIV-2 is severely restricted at the level of RNA dimerization.

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Introduction

Retroviral genomes are formed by two identical copies of unspliced viral RNA that are non-covalently linked near their 5’ ends in a region that is termed the dimer linkage structure (Bender et al., 1978; Coffin, 1990; Greatorex & Lever, 1998; Hoglund et al., 1997). Retroviral RNA dimerizes readily in vitro in the presence of cations (Awang & Sen, 1993; Clever & Parslow, 1997; Clever et al., 1996; Greatorex et al., 1996; Haddrick et al., 1996; Laughrea & Jette, 1994; Marquet et al., 1991; Paillart et al., 1994; Skripkin et al., 1994; Sundquist & Heaphy, 1993). Extensive mutational analysis of the human immunodeficiency virus type 1 (HIV-1) leader RNA revealed that sequences located immediately downstream of the primer-binding site (PBS) are essential for in vitro dimerization (Laughrea & Jette, 1994; Muriaux et al., 1995; Paillart et al., 1994; Skripkin et al., 1994). This so-called dimer initiation site (DIS) folds into a hairpin structure with a loop-exposed 6-mer palindrome (Fig. 1). Whereas different regions of the HIV-2 leader have been implicated in RNA dimerization (Berkhout et al., 1993; Jossinet et al., 2001), we demonstrated recently that the DIS hairpin of this virus is also both essential and sufficient for dimer formation (Dirac et al., 2001) (Fig. 1). Moreover, the requirements for DIS-mediated dimerization of HIV-1 and HIV-2 leader RNAs are similar. Dimerization is promoted by the presence of mono- and divalent cations and the palindrome is indispensable for dimer formation (Awang & Sen, 1993; Dirac et al., 2001; Laughrea & Jette, 1994; Lodmell et al., 2000; Marquet et al., 1991; Paillart et al., 1994; Skripkin et al., 1994; Sundquist & Heaphy, 1993). Furthermore, heterodimerization of transcripts with non-identical DIS loop sequences requires perfect complementarity between the central nucleotides in the palindrome region (Clever et al., 1996; Dirac et al., 2001; Paillart et al., 1997).
Fig. 1. Secondary structure models of the HIV-1 and HIV-2 leader RNA. The models of the 5′ untranslated leader RNA of the HIV-1 LAI isolate and HIV-2 ROD isolate are adapted from Berkhout (1996). Hairpin structures are named according to their putative function in HIV-1 replication and several critical sequence elements are highlighted (the polyadenylation signal, the primer activation signal (PAS), described recently by Beerens et al. (2001), the PBS, the palindrome in the DIS loop and the AUG start codon of the Gag open reading frame). In this study, we used the 1–290 HIV-1 transcript and the 1–444 HIV-2 transcript. In the HIV-1 structure, we indicated the two deletions and the one nucleotide substitution that stabilize the poly(A) hairpin in the A mutant. In the HIV-2 structure, we marked the wild-type DIS palindrome (GGUACC, white box) that was substituted for the HIV-1 palindrome (GCGCGC, grey boxes) in the GC3 mutant.

According to the current model of retroviral DIS-mediated dimer formation, the initial contact between two DIS hairpins occurs by base pairing of the palindromes, termed loop–loop kissing. Heat treatment or incubation with the nucleic acid chaperone viral nucleocapsid protein promotes melting of individual DIS stems and enables formation of extended intermolecular duplexes (Laughrea & Jette, 1996; Muriaux et al., 1996a). This transition is referred to as the conversion of the loose dimer (kissing-loop or KL dimer) to the tight dimer (extended-duplex or ED dimer). The structure of the HIV-1 KL dimer has been solved by nuclear magnetic resonance (NMR) spectroscopy (Dardel et al., 1998; Mujeeb et al., 1998). The KL
dimer interface extends beyond the 6-mer interaction of the palindromes and includes at least three flanking bases in each DIS molecule that stack to form a pair of interlocking triple-base arrays (Mujeeb et al., 1998). The two A loop residues immediately upstream of the HIV-1 palindrome and the C residue of the top base pair are involved in this stacking interaction (Fig. 1). The KL dimer complex gains stability by isomerization into the ED form, of which the NMR structure is also known (Girard et al., 1999; Mujeeb et al., 1999). Disruption of the top base pair of the DIS stems in the KL dimer, which was suggested to result from internucleotide base stacking, could serve to lower the energy requirements for the transition of the KL dimer into the ED dimer (Mujeeb et al., 1998).

Retroviral RNA dimerization affects the virus life cycle at several stages. Mutation of the DIS region results in reduced packaging of viral RNA (Berkhout & van Wamel, 1996; Haddrick et al., 1996; LaughrEA et al., 1997; Sakuragi & Panigan, 1997). Surprisingly, RNA isolated from these mutant virions is predominantly dimeric, indicating that there may be additional dimerization signals. These results may also indicate that RNA dimers are packaged preferentially over monomeric RNA and it has been proposed that the DIS functions directly as a packaging signal (Clever & Parslow, 1997; McBride & Panigan, 1996). Dimeric RNA allows for template switching during the process of reverse transcription, e.g. when the viral RNA template is damaged or when its secondary structure induces stalling of the reverse transcriptase enzyme (reviewed by Kotte et al., 1999; Negroni & Buc, 2001). Such recombinations promote genetic diversity and, in the event that different viral genomes are co-packaged, the formation of recombinant retroviruses with novel properties.

Mosaic retroviruses that result from recombination between different subtypes of HIV-1 have been reported and the same is true for HIV-2 subtypes (Bobkov et al., 1998; Carr et al., 1996, 1998; Gao et al., 1994; Piyasirisilp et al., 2000). Recombination can also take place between HIV-1 isolates from the distinct groups M and O, which have a limited sequence similarity of 65% (Peeters et al., 1999; Takehisa et al., 1999). Thus far, no recombinants between HIV-1 and HIV-2 have been reported. The creation of new recombinants is likely to depend, among other things, on the possibility of forming and packaging heterodimeric RNA genomes. In this study, we have examined the requirements for in vitro heterodimerization of the DIS-containing leader transcripts of HIV-1 and HIV-2. We report that an identical palindrome sequence, similar DIS loop size and/or palindrome orientation are essential requirements for KL heterodimerization, but formation of ED dimers is completely restricted.

Methods

- **Generation of PCR templates for in vitro transcription of HIV-2 DIS palindrome mutants.** The template that was used for transcription of the HIV-2 (GC3 mutant) 1–444 RNA was synthesized by PCR amplification of plasmid template HIV-2 ∆Ψ large (Oude Essink et al., 1996), which contains a T7 promoter directly upstream of the +1 position of the wild-type HIV-2 ROD sequence. Upstream T7 +1 primers and downstream HIV-2 444 (GC3 mutant) primers were used for PCR. All primer sequences are listed in Table 1. Templates for generation of the mutant transcripts, in which the HIV-2 DIS hairpin loop was changed in a stepwise manner to mimic that of HIV-1, were made with the HIV-2 (GC3 mutant) 1–444 PCR product as template. The standard T7 +1 upstream primer and the variable downstream primers, as indicated in Table 1, were used to generate the respective mutant templates. The wild-type and A mutant HIV-1 templates (1–290) were made by PCR amplification of pLAI and pLAI-R37-A (Das et al., 1997) with the HIV-1 primers listed in Table 1.

- **In vitro transcription.** All transcripts were generated with the Ambion Megashortscript T7 Transcription kit, according to the accompanying protocol. A total of 1 µl of [α-32P]UTP (0.33 MBq/µl) was added to the transcription mixture to generate radioactively labelled transcripts. Transcripts were purified on 4% denaturing polyacrylamide gels and visualized by autoradiography. Upon overnight elution in TE buffer, the RNA was ethanol-precipitated, resuspended in water and quantified by scintillation counting. The transcript stock solutions were renatured by heating to 85 °C and slow cooling to room temperature. Aliquots of all transcripts were stored at −20 °C.

- **In vitro dimerization assays.** In the Mg2+ titration experiment, we used approximately 2 pmol of radiolabelled transcripts. The RNA was incubated for 10 min at 65 °C in dimerization buffer with 10 mM Tris–HCl pH 7.5, 40 mM NaCl and either 0.1, 1 or 5 mM MgCl2 and slowly cooled to room temperature. Each sample (10 µl) was mixed with 5 µl native gel loading buffer (30% glycerol with bromophenol blue dye) and analysed with standard settings. The thermodynamic stability values (ΔG, kcal/mol) presented in Table 2 are based on mfold analysis with model hairpin templates, of which the stem is composed of the two interacting sequences in the KL and ED dimers. We used the following model substrates: HIV-1 DIS as in Figs 1 and 4, with seven consecutive base pairs, and HIV-2 DIS as in Fig. 4 (not the extended format as shown in Fig. 1). It is obvious that this approach only provides an estimate of the thermodynamic stability of the KL and ED dimers. This method scores only the predicted Watson–Crick base pair interactions but will miss all non-canonical interactions that are likely to exist in both the KL and the ED dimer complexes.
Table 1. PCR primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5' to 3'</th>
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<tbody>
<tr>
<td>HIV-2 T7 +1</td>
<td>GAATTCTAAATACGACTCAGTATAG</td>
</tr>
<tr>
<td>HIV-2 444 (GC3 mutant)</td>
<td>CGCTACACAGCTCGCTTTAGCGGCCCTGCG</td>
</tr>
<tr>
<td>HIV-2 444 (GC3 mutant) + A</td>
<td>CGCTTACACAGCTCGCTTTAGCGGCCCTGCG</td>
</tr>
<tr>
<td>HIV-2 444 (GC3 mutant) - A</td>
<td>CGCTTACACAGCTCGCTTTAGCGGCCCTGCG</td>
</tr>
<tr>
<td>HIV-2 444 (GC3 mutant) - 2A</td>
<td>CGCTTACACAGCTCGCTTTAGCGGCCCTGCG</td>
</tr>
<tr>
<td>HIV-2 444 (GC3 mutant) - 2A(M)</td>
<td>CGCTTACACAGCTCGCTTTAGCGGCCCTGCG</td>
</tr>
<tr>
<td>HIV-1 T7 +1</td>
<td>GAATTCTAAATACGACTCAGTATAG</td>
</tr>
<tr>
<td>HIV-1 R.G273</td>
<td>CAGTGCCTCACCTGC</td>
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</table>

Table 2. Watson–Crick base-pairing capacity of RNA dimers

<table>
<thead>
<tr>
<th>RNA</th>
<th>KL dimer</th>
<th>ED dimer</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(\Delta G) (kcal/mol)</td>
<td>bp</td>
</tr>
<tr>
<td>Homodimer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-1</td>
<td>-13.1</td>
<td>6</td>
</tr>
<tr>
<td>HIV-2</td>
<td>-10.1</td>
<td>6</td>
</tr>
<tr>
<td>HIV-2(CG)</td>
<td>-13.1</td>
<td>6</td>
</tr>
<tr>
<td>Heterodimer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 and 2</td>
<td>-9.9</td>
<td>2</td>
</tr>
<tr>
<td>1 and 2(CG)</td>
<td>-13.1</td>
<td>6</td>
</tr>
<tr>
<td>1 and 2 (+ A)</td>
<td>-13.1</td>
<td>6</td>
</tr>
<tr>
<td>1 and 2 (− A)</td>
<td>-13.1</td>
<td>6</td>
</tr>
<tr>
<td>1 and 2 (− 2A(M))</td>
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<td>1 and 2 (− 2A(MM))</td>
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<tr>
<td>1 and 2 (− 2A(MM))</td>
<td>-13.1</td>
<td>6</td>
</tr>
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</table>

Results

Heterodimers are not formed between the leader RNA of HIV-1 and HIV-2

Complementarity between the central DIS palindrome nucleotides is essential for in vitro dimerization of both HIV-1 and HIV-2 RNA (Clever et al., 1996; Dirac et al., 2001). The DIS palindromes of HIV-1 and HIV-2 differ at several positions (Fig. 1) and are therefore not expected to form heterodimers. To meet the minimal requirements for heterodimer formation, we synthesized a mutant HIV-2 transcript encompassing nucleotides 1–444 in which the wild-type GGUACC palindrome was substituted for the HIV-1 palindrome GCGCGC (Fig. 1, GC3 mutant). This transcript has been shown previously to form homodimers at wild-type levels (Dirac et al., 2001, 2002). A DIS-containing HIV-1 transcript covering the leader region from position 1–290 was also synthesized by T7 RNA polymerase (Fig. 1).

The MgCl\(_2\) requirement for dimerization of the HIV-1 and the GC3 mutant of HIV-2 (HIV-2\(CG\)) transcripts was investigated. Approximately 2 pmol of each radiolabelled transcript was incubated in dimerization buffer with either 0.1, 1 or 5 mM MgCl\(_2\) for 10 min at 65 \(\degree\)C, followed by slow cooling of the samples to room temperature and analysis on a native TBM gel (Fig. 2). Dimerization of the HIV-2\(CG\) transcript is barely detectable at 0.1 mM MgCl\(_2\) (Fig. 2, lane 3) and approximately 50% dimerization is observed at 5 mM MgCl\(_2\) (Fig. 2, lane 13). The HIV-1 transcript remains dimerization-inactive at all Mg\(^{2+}\)
concentrations tested (Fig. 2, lanes 1, 6 and 11): this transcript is dimerization-impaired because it folds into an RNA conformation that masks the DIS motif (Huthoff & Berkhout, 2001). Therefore, we also synthesized the A mutant transcript (Das et al., 1997), which contains a stabilized poly(A) hairpin that favours folding of a dimerization-competent leader RNA conformation that exposes the DIS hairpin (Fig. 1). This A mutant displays efficient dimerization at 0.1 mM MgCl₂ (Fig. 1, lane 2) and 100% dimer was obtained at 1 and 5 mM MgCl₂ (Fig. 1, lanes 7 and 12).

Having established the conditions that yield fair amounts of the HIV-1 and HIV-2GC3 homodimers, we next tested whether heterodimers can be formed. The HIV-2GC3 transcript was incubated either with the wild-type HIV-1 transcript (Fig. 2, lanes 4, 9 and 14) or with the A mutant (Fig. 2, lanes 5, 10 and 15) in dimerization buffer with 0.1, 1 or 5 mM MgCl₂. Heterodimers are expected to have an electrophoretic mobility that is intermediate to that of the two homodimers D1 and D2. We did not observe a band with this migration property, indicating that no heterodimers were formed at these conditions.

In a search for conditions that would allow RNA heterodimerization, we incubated the transcripts at conditions that promote the formation of the KL or ED dimer (Laughrea & Jette, 1996; Muriaux et al., 1996b). We have used the HIV-1 A mutant and HIV-2GC3 transcripts, which homodimerize efficiently in all subsequent experiments; these transcripts are denoted HIV-1 and HIV-2 for simplicity. The HIV-1 and HIV-2 transcripts were incubated individually or in combination. To disrupt any homodimers that might have formed during the initial RNA renaturation step of purified T7 transcripts, we first mixed the two RNAs and denatured them by incubation in water for 2 min at 85 °C, followed by immediate chilling on ice. Transcripts were then incubated in dimerization buffer (5 mM MgCl₂ and 40 mM NaCl), either for 30 min at 37 °C to promote KL dimer formation or for 10 min at 65 °C and slow cooling to room temperature to promote ED dimer formation. Samples were subjected to electrophoresis on TBM and TBE gels (Fig. 3A, B, results are summarized in Table 2). The TBM gel allows the detection of KL dimers because the presence of Mg²⁺ ions prevents disruption of these relatively unstable dimers during electrophoresis. The efficiency of KL homodimerization is approximately 80 and 20% for HIV-1 and HIV-2 RNA, respectively (Fig. 3A, lanes 1 and 2), whereas both transcripts form ED homodimers with an efficiency of approximately 20% (Fig. 3B, lanes 4 and 5). Most importantly, it is apparent that neither KL nor ED heterodimer formation occurs under the assay conditions used. These results indicate that the sequence or structural differences between the HIV-1 and the HIV-2 DIS elements are too severe to allow heterodimerization, even though we have inserted the homologous GC3 palindrome in HIV-2.

The minimal DIS requirements for RNA heterodimer formation

To force the formation of heterodimers between HIV-1 and HIV-2 RNA, we created a nested set of HIV-2 mutants in which the DIS hairpin loop was changed in a stepwise manner.

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**Fig. 2.** Mg²⁺-dependent dimerization of HIV-1 and HIV-2 transcripts. Dimerization of the wild-type and A mutant HIV-1 transcripts and the HIV-2GC3 mutant transcript was investigated at increasing MgCl₂ concentrations. Transcripts were incubated individually or as HIV-1/HIV-2 combinations, as indicated on top. Samples were treated for 10 min at 65 °C and slowly cooled to room temperature in dimerization buffer with 0.1 mM MgCl₂ (lanes 1–5), 1 mM MgCl₂ (lanes 6–10) or 5 mM MgCl₂ (lanes 11–15). Samples were analysed on a TBM gel followed by autoradiography. The monomer (M) and dimer (D) bands of HIV-1 and HIV-2 transcripts are marked 1 and 2, respectively.

**Fig. 3.** Different dimerization conditions to probe for heterodimers between HIV-1 and HIV-2 RNA. The HIV-1 A mutant (labelled 1) and the HIV-2GC3 mutant (labelled 2) were incubated individually or in combination, as indicated on top. Samples were first denatured by incubation for 2 min at 85 °C and then incubated in dimerization buffer (5 mM MgCl₂ and 40 mM NaCl), respective to either the KL protocol [30 min at 37 °C (lanes 1–3)] or the ED protocol [10 min at 65 °C and slowly cooled to room temperature (lanes 4–6)]. Samples were split and analysed on a TBM (A) and a TBE (B) gel, followed by autoradiography. The monomer (M) and dimer (D) bands of HIV-1 and HIV-2 transcripts are marked 1 and 2, respectively.
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Fig. 4. Overview of the wild-type and mutant DIS hairpins tested in this study. The wild-type HIV-2 DIS hairpin is shown on the left with the GGUACC palindrome (white box). All other HIV-2 transcripts and the wild-type HIV-1 DIS (on the right) have the GC3-type palindrome (grey boxes). The HIV-2 DIS was mutated to deviate further from HIV-1 by the inclusion of an additional loop nucleotide (+A mutant). The other mutants were designed to gradually resemble the HIV-1 DIS loop. The −A and −2A mutations decrease the loop size and tilt the palindrome towards the right, as in HIV-1. To further mimic the HIV-1 DIS element, we mutated a DIS loop nucleotide in −2AM and, in addition, the top base pair in −2AMM (mutations are indicated in black). These latter changes facilitate a triple-base stacking interaction that was described for the HIV-1 KL dimer (Mujeeb et al., 1998).

Fig. 5. Homo- and heterodimerization properties of the mutant HIV-2 DIS transcripts. The HIV-1 A mutant (labelled 1) and the series of HIV-2 DIS mutant transcripts were incubated either individually (lanes 1–7) or mixed in HIV-1/HIV-2 combinations (lanes 8–13). Samples were first denatured by incubation for 2 min at 85 °C and then incubated in dimerization buffer (5 mM MgCl₂ and 40 mM NaCl) for 30 min at 37 °C, according to the KL protocol. Samples were analysed on a TBM gel. The monomer (M) and dimer (D) bands of HIV-1 and HIV-2 transcripts are marked 1 and 2, respectively. The heterodimer formed between HIV-1 and some HIV-2 transcripts (the DIS mutants in lanes 8–13) is marked D12.

The degree of loop bending may be influenced by the loop size; alteration of the HIV-1 DIS loop size has been reported to cause a loss of virus infectivity (Berkhout & van Wamel, 1996). Therefore, we systematically changed the size of the HIV-2 DIS loop in the context of the GC3 mutant. The DIS loop size is decreased in the −A and −2A mutants that gradually ‘tilt’ the palindrome towards the right, as in HIV-1. In the +A mutant, an additional A is inserted on the 3′ side of the palindrome to tilt the HIV-2 palindromic further to the left. This mutant deviates even more from the HIV-1 DIS structure. The identity of the palindrome-flanking loop nucleotides and the top base pair of the HIV-1 and HIV-2 DIS elements are also different. The NMR structure of the HIV-1 KL dimer complex proposes an interstrand triple-base stacking interaction between the second loop nucleotide A²⁵₆ of one DIS hairpin and A²⁶₅/C²₆₄ of the second DIS hairpin (Fig. 1) and this interaction may strengthen the dimer interface (Mujeeb et al., 1998). The first DIS loop nucleotide in HIV-2 is G, which may be unable to participate in the triple-base stacking with the HIV-1 DIS element. In the −2AM mutant, a G to A substitution was therefore introduced, creating a DIS loop that is identical to that of HIV-1. In the −2AMM mutant, the top C–G base pair of HIV-2 was substituted for G–C, as in HIV-1. The −2AMM mutant contains all DIS nucleotides involved in the triple-base stacking interaction described for HIV-1.

This set of HIV-2 DIS mutants was tested in dimerization assays with or without HIV-1 RNA (Fig. 5, results are summarized in Table 2). After heat denaturation, approximately 2 pmol of the HIV-1 transcript and the mutant HIV-2 transcripts were incubated individually (Fig. 5, lanes 1–7) or in HIV-1/HIV-2 combinations (Fig. 5, lanes 8–13). Samples were incubated for 30 min at 37 °C in dimerization buffer (5 mM MgCl₂ and 40 mM NaCl) and subsequently analysed by electrophoresis on a TBM gel to detect KL-type dimers. As expected, all transcripts can form KL homodimers (Fig. 5, lanes 1–7). No KL heterodimers were formed between HIV-1 RNA and the HIV-2 mutants +A and −A (Fig. 5, lanes 9 and 10) but a faint heterodimer band is apparent in combination with the HIV-2 mutants −2A, −2AM and −2AMM (Fig. 5, lanes 11–13, marked D12). This result indicates that an identical DIS...
loop size and/or palindrome orientation is required for KL heterodimer formation. We also analysed these samples with the ED dimer protocol on a TBE gel and, whereas ED homodimer formation occurs with similar efficiencies for all HIV-2 mutants, we did not observe any ED heterodimers (results not shown).

**Modelling of the base pair interaction of the RNA homo- and heterodimers**

We used the mfold algorithm to evaluate the hypothetical base-pairing capacity of all the possible KL and ED homo- and heterodimer combinations of the HIV-1 and HIV-2 DIS transcripts (Mathews et al., 1999). The results are summarized in Table 2. Introduction of the GC3 palindrome in HIV-2 increases the thermodynamic stability of the KL homodimer to that of the HIV-1 DIS element (−13.1 kcal/mol). Our experiments revealed that the HIV-1 transcript is superior to the HIV-2 GC3 mutant in KL homodimerization. This finding is consistent with the existence of additional non-canonical interactions in the KL dimer complex, for instance the proposed triple-base stacking interaction (Mujeeb et al., 1998).

We measured no ED heterodimer formation between HIV-1 RNA and any of the HIV-2 mutants (results not shown). It is possible that the remaining sequence differences in the DIS hairpin impose restrictions on the formation of ED heterodimers. This idea is substantiated by mfold modelling of the thermodynamic stability of the predicted RNA complexes. A thermodynamic stability of approximately −30.0 kcal/mol is predicted for ED heterodimers between HIV-1 RNA and the set of HIV-2 transcripts containing the GC3 palindrome, except for the −2A M and −2A M M mutants (Table 2). However, the homodimers can form much more stable ED complexes (−40.3 and −34.7 kcal/mol for HIV-1 and HIV-2, respectively). Conversion of KL dimers into ED dimers requires melting of the stem region of two DIS hairpins, corresponding to a thermodynamic stability of −13.3 and −11.9 kcal/mol per hairpin for HIV-1 and HIV-2, respectively. For the ED homodimers, there will be a significant gain of thermodynamic stability, which will shift the KL–ED equilibrium to the right. The ED heterodimers are predicted to achieve a stability, which will shift the KL–ED equilibrium to the homodimers, there will be a significant gain of thermodynamic energy gain may simply not be sufficient to drive the isomerization of the KL dimer into the ED dimer. For comparison, the ED homodimers of HIV-1 and HIV-2 exhibit a thermodynamic stability of −40.3 and −34.4 kcal/mol, respectively.

This *in vitro* study indicates that heterodimerization of wild-type HIV-1 and HIV-2 RNA genomes does not readily occur. We have observed that DIS-mediated homodimerization is highly favoured over heterodimerization. However, these results do not preclude that heterodimerization may take place by a DIS-independent mechanism. For instance, dimerization of the HIV-1 genome has been reported to occur *in vivo* in the absence of a functional DIS element, suggesting that additional dimerization signals exist within retroviral genomes (Berkhout et al., 1996; Haddrick et al., 1996; Laughrea et al., 1997). Consistent with this idea, it was described that the presence of non-homologous DIS elements from different HIV-1 subtypes is not a principal obstacle to intersubtype recombination (St Louis et al., 1998). If the additional dimerization signals are compatible between the genomes of HIV-1 and HIV-2, the occurrence of heterodimerization *in vivo* may not be as rare as predicted by our *in vitro* experiments.

Despite extensive recombination within the HIV-1 group and within the HIV-2 group, no HIV-1/HIV-2 recombinants have been described thus far: this is not simply due to the lack of HIV-1/HIV-2 co-infections, for which there are multiple reports (Evans et al., 1988; George et al., 1992; Peeters et al., 1992). The non-appearance of HIV-1/HIV-2 recombinants may be related to the apparent inability to make RNA heterodimers but incompatibility problems may also arise at other levels. Packaging of the heterodimeric RNA genome into virions may present a bottleneck. Primate lentiviruses can

**Discussion**

The possible dimer formation between the HIV-1 and the HIV-2 RNA genomes has been investigated in this study. Mutations were introduced in HIV-2 RNA providing both viral transcripts with identical GCGCGC palindromes that form a 6-mer interaction in the hypothetical KL dimer complex (Table 2). Nevertheless, we did not observe formation of heterodimers between HIV-1 and this GC3 mutant of HIV-2. To investigate the degree of DIS loop similarity that is required for heterodimer formation, we created a nested set of HIV-2 DIS mutants in which the loop sequence was gradually changed to mimic that of the HIV-1 DIS hairpin. These results indicate that an identical DIS loop size and palindrome orientation is required to attain even a low level of KL heterodimer formation. However, we observed no formation of ED heterodimers. This was somewhat unexpected because the KL dimer complex of the wild-type HIV-1 DIS has a marked tendency to isomerize into the ED dimer form. Specifically, this dimer gains stability through reformation of a ruptured base pair, through increased base stacking, and through a decrease in mechanical strain (Girard et al., 1999; Mujeeb et al., 1999). We cannot accurately predict these parameters for the putative ED heterodimer between HIV-1 and HIV-2 RNA. Modelling of the base pair interactions in the ED heterodimer complex provides a likely explanation for the lack of KL–ED isomerization. The introduction of the HIV-1-like GC3 palindrome allows the formation of an ED heterodimer with a thermodynamic stability of approximately −30.0 kcal/mol (Table 2). This value does not improve for the HIV-2 mutants containing additional mutations (in fact, a much less stable ED heterodimer is predicted for −2A M and −2A M M) and the energy gain may simply not be sufficient to drive the isomerization of the KL dimer into the ED dimer. For comparison, the ED homodimers of HIV-1 and HIV-2 exhibit a thermodynamic stability of −40.3 and −34.4 kcal/mol, respectively.
cross-package each other’s RNA genome, e.g. HIV-1 is capable of packaging HIV-2 RNA (Kaye & Lever, 1998), although the reverse is not possible, and there is reciprocal cross-packaging between HIV-1 and simian immunodeficiency virus (Rizvi & Panganiban, 1993; White et al., 1999). Although efficient cross-packaging of genomic RNA occurs between different lentiviruses, this is not necessarily identical to the packaging of a heterodimeric RNA genome, which is the key event that must take place prior to recombination. Because dimerization of retroviral RNA may be closely linked to the encapsidation process (Berkhout & van Wamel, 1996; Clever & Parslow, 1997; Lever, 2000), it is quite possible that the inability to form heterodimers between HIV-1 and HIV-2 RNA will also inhibit co-packaging. Even if a heterodimeric RNA genome is packaged successfully, there may be additional hurdles on the way to a recombinant virus. For instance, reverse transcription may be aborted because it requires identical repeat sequences in the long terminal repeat elements. Furthermore, the recombination rate may be affected directly for co-packaged retroviral RNA dimers with a suboptimal DIS interaction (Balakrishnan et al., 2001; Lund et al., 1999). The likelihood of creating a viable recombinant virus is further restricted by the complexity of the lentiviral genomes. The lentiviral genome encodes certain combinations of genetic elements that may loose their function when detached by recombination. For instance, the Tat–TAR and Rev–RRE interactions (Berkhout et al., 1990; Dillon et al., 1990; Emerman et al., 1987; Malim et al., 1989) may hinder the generation of viable HIV-1/HIV-2 recombinants.

It has been suggested that at least 10% of the HIV variants currently isolated may have mosaic genomes due to recombination (Robertson et al., 1995, 2000). Sequential recombination events, in which an early recombinant serves as a parental strain for subsequent recombinations, have also been described (Salminen et al., 1997). Two distinct DIS palindromes are present among the different HIV-1 subtypes of the major group M: GCGCGC is present in subtypes B and D and GUGCAC in subtypes A, C, E, G, H and J. Both DIS palindromes have been described for subtype F viruses but GCGCGC is found more frequently. Interestingly, we identified both palindrome sequences in an individual infected with a subtype F virus (E. Andersen, R. Jeeninga, B. Berkhout and J. Kjems, unpublished results). The GUGCAC palindrome is also present in viruses of the outlier group O and the new group N. Because the sequence of the palindrome may influence RNA heterodimerization, we investigated whether the known recombination events occurred preferentially between HIV-1 subtypes with the same DIS palindrome. Inspection of the HIV sequence database (http://hiv-web.lanl.gov/CRFs/CRFs.html) indicates that most of the circulating recombinants have putative parents with an identical DIS palindrome. It will be of interest to perform a more in-depth study once more recombinant viruses have been sequenced. We realize that these recombination patterns may have been strongly influenced by other factors, most notably the requirement for the two parental viruses to share a habitat.

The results presented in this study are also relevant for safety issues concerning the use of retrovirus vectors for gene therapy. Besides HIV-1, other lentiviruses, including HIV-2, have been proposed as gene therapy vectors (Lever, 2000; Naldini et al., 1999). This raises the possibility of interaction between the gene therapy vector and a wild-type lentivirus obtained through natural infection. Upon coinfection of the same cell, RNA heterodimerization and co-packaging, chimeric retroviruses with unknown pathogenic potential may be generated by recombination. We already mentioned that the likelihood of recombination between the genomes of different retrovirus species is minute. Safety concerns are more serious in situations where the same retrovirus is encountered as gene therapy vector and as infectious agent. In this case, vector mobilization and recombination are a much more likely scenario (Bukovsky et al., 1999). Extensive studies with animal retroviruses provide ample support for this scenario (DiFronzo & Holland, 1993; Mikkelsen et al., 1996; Schwartzberg et al., 1985).

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References


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