Journal of General Virology (2002), **83**, 2533–2542. Printed in Great Britain

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

Annette M. G. Dirac,[1,2] Hendrik Huthoff,[2] Jørgen Kjems[1] and Ben Berkhout[2]

[1] Department of Molecular and Structural Biology, Aarhus University, Denmark

[2] Department of Human Retrovirology, Academic Medical Center, University of Amsterdam, PO Box 22700, 1100 DE Amsterdam, The Netherlands

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.

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).

Author for correspondence: Ben Berkhout.
Fax +31 20 6916531. e-mail b.berkhout@amc.uva.nl

0001-8324 © 2002 SGM

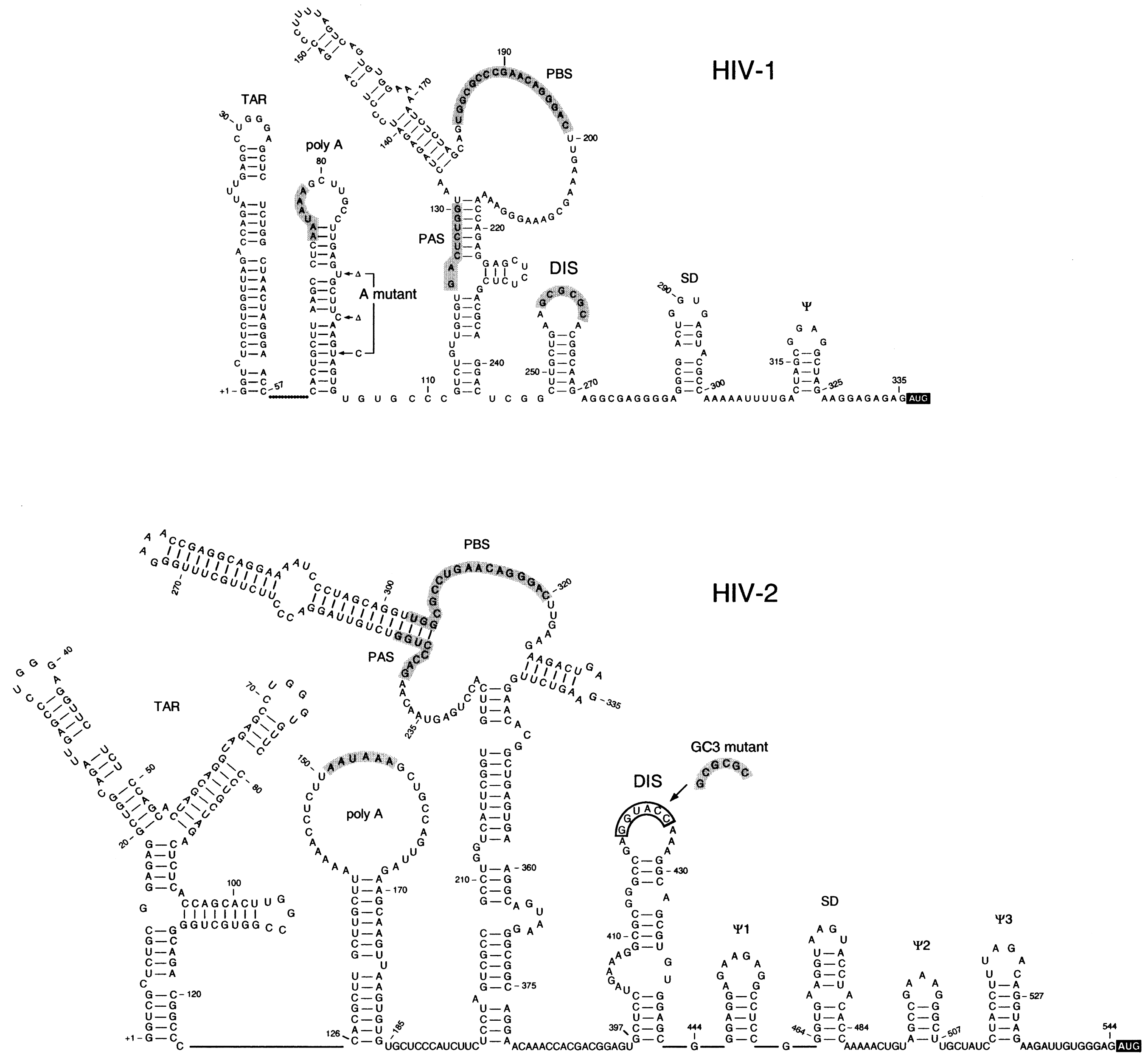

Fig. 1. Secondary structure models of the HIV-1 and HIV-2 leader RNA. The models of the 5′ untranslated leader RNA of the HIV-1 LAI isolate and HIV-2 ROD isolate are adapted from Berkhout (1996). Hairpin structures are named according to their putative function in HIV-1 replication and several critical 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 interstrand 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 & Panganiban, 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 & Panganiban, 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 Gotte *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-^{32}P]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, redissolved 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 Mg^{2+} 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 $MgCl_2$ 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 on 4% native polyacrylamide gels containing either 0·25 × TBE or 0·25 × Tris–borate–0·1 mM $MgCl_2$ (TBM). Gels were run at 150 V at room temperature, followed by drying and autoradiography. In the other experiments, approximately 2 pmol of radiolabelled RNA was first incubated, either separately or mixed, for 2 min at 85 °C in H_2O and immediately placed on ice. Dimerization buffer (10×) was added to make a final reaction mixture of 10 µl with 5 mM $MgCl_2$, 10 mM Tris–HCl pH 7·5 and 40 mM NaCl. For investigation of KL dimer formation, samples were incubated for 30 min at 37 °C. ED dimers were formed by incubation for 10 min at 65 °C and slow cooling to room temperature. Electrophoresis was performed in 0·25 × TBE and TBM gels followed by drying and autoradiography.

MFOLD RNA secondary structure predictions. The sequences of the HIV-1 LAI and HIV-2 ROD isolates were downloaded from the HIV database (http://hiv-web.lanl.gov/). Secondary structure prediction was performed with the MFOLD algorithm, version 3.0 (Mathews *et al*., 1999), on the MBCMR MFOLD server (http://mfold.edu.burnet.au/) 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

Primers containing mutated HIV-2 DIS loop and stem regions are underlined.

Primer	Sequence 5′ to 3′
HIV-2 T7 +1	GAATTCTAATACGACTCACTATAG
HIV-2 444 (GC3 mutant)	CGCTCCACACGCTGCCTTTGCGCGCTCGGCC
HIV-2 444 (GC3 mutant) +A	CGCTCCACACGCTGCCTTTTGCGCGCTCGGCCCGCGCCTTT
HIV-2 444 (GC3 mutant) −A	CGCTCCACACGCTGCCTTGCGCGCTCGGCCCGCGCCTTT
HIV-2 444 (GC3 mutant) −2A	CGCTCCACACGCTGCCTGCGCGCTCGGCCCGCGCCTTT
HIV-2 444 (GC3 mutant) $-2A^{M}$	CGCTCCACACGCTGCCTGCGCGCTTGGCCCGCGCCTTT
HIV-2 444 (GC3 mutant) $-2A^{MM}$	CGCTCCACACGCTGCGTGCGCGCTTCGCCCGCGCCTTT
HIV-1 T7 +1	GAATTCTAATACGACTCACTATAGGGTCTCCTCTGGTTAGAC
HIV-1 R.G273	CAGTCGCCTCCCCTCGC

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

For both the KL and the ED dimer formation, the predicted thermodynamic stability (ΔG) is indicated in kcal/mol. The experimentally determined dimerization activity is indicated as active (+), intermediate (±) or inactive (−); ED structures that were not among the top 5% solutions are also indicated (<).

RNA	KL dimer			ED dimer		
	ΔG (kcal/mol)	bp	±	ΔG (kcal/mol)	bp	±
Homodimer						
HIV-1	−13·1	6	+	−40·3	28	+
HIV-2	−10·1	6	+	−34·4	20	+
HIV-2^{GC3}	−13·1	6	+	−37·3	20	+
Heterodimer						
1 and 2	−0·9	2	−	<	<	−
1 and 2^{GC3}	−13·1	6	−	−29·7	22	−
1 and 2 (+A)	−13·1	6	−	−29·2	22	−
1 and 2 (−A)	−13·1	6	−	−29·8	22	−
1 and 2 (−2A)	−13·1	6	±	−29·9	22	−
1 and 2 ($-2A^{M}$)	−13·1	6	±	−26·8	21	−
1 and 2 ($-2A^{MM}$)	−13·1	6	±	<	<	−

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^{GC3}) 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 °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^{GC3} 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+}

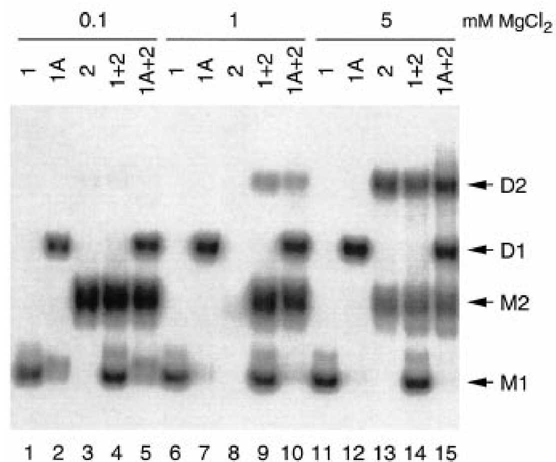

Fig. 2. Mg^{2+}-dependent dimerization of HIV-1 and HIV-2 transcripts. Dimerization of the wild-type and A mutant HIV-1 transcripts and the HIV-2^{GC3} mutant transcript was investigated at increasing $MgCl_2$ concentrations. Transcripts were incubated individually or as HIV-1/HIV-2 combinations, as 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 $MgCl_2$ (lanes 1–5), 1 mM $MgCl_2$ (lanes 6–10) or 5 mM $MgCl_2$ (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.

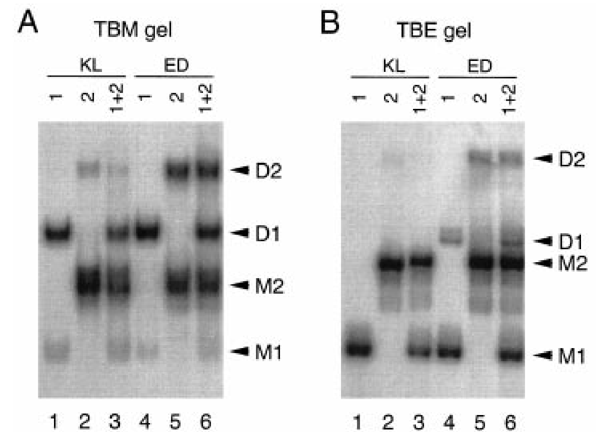

Fig. 3. Different dimerization conditions to probe for heterodimers between HIV-1 and HIV-2 RNA. The HIV-1 A mutant (labelled 1) and the HIV-2^{GC3} mutant (labelled 2) were incubated individually or in combination, as indicated on top. Samples were first denatured by incubation for 2 min at 85 °C and then incubated in dimerization buffer (5 mM $MgCl_2$ 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.

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 $MgCl_2$ (Fig. 1, lane 2) and 100% dimer was obtained at 1 and 5 mM $MgCl_2$ (Fig. 1, lanes 7 and 12).

Having established the conditions that yield fair amounts of the HIV-1 and HIV-2^{GC3} homodimers, we next tested whether heterodimers can be formed. The HIV-2^{GC3} 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 $MgCl_2$. 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-2^{GC3} 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 $MgCl_2$ 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 Mg^{2+} ions prevents disruption of these relatively instable 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

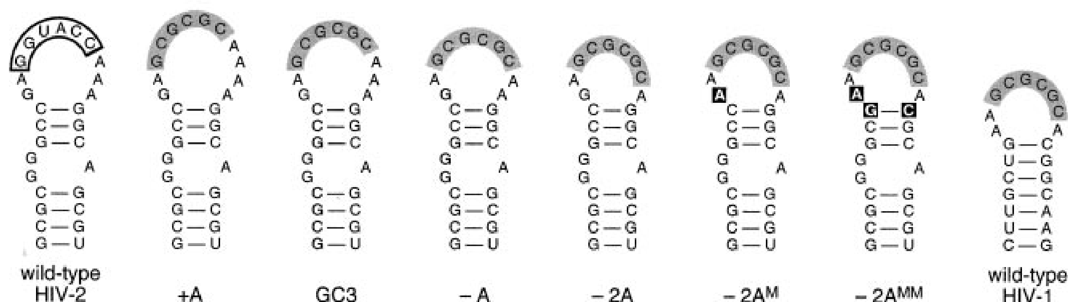

Fig. 4. Overview of the wild-type and mutant DIS hairpins tested in this study. The wild-type HIV-2 DIS hairpin is shown on the left with the GGUACC palindrome (white box). All other HIV-2 transcripts and the wild-type HIV-1 DIS (on the right) have the GC3-type palindrome (grey boxes). The HIV-2 DIS 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 $-2A^{M}$ and, in addition, the top base pair in $-2A^{MM}$ (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).

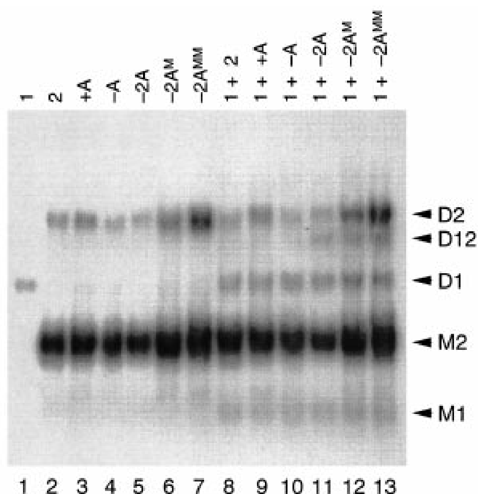

Fig. 5. Homo- and heterodimerization properties of the mutant HIV-2 DIS transcripts. The HIV-1 A mutant (labelled 1) and the series of HIV-2 DIS mutant transcripts were incubated either individually (lanes 1–7) or mixed in HIV-1/HIV-2 combinations (lanes 8–13). Samples were first denatured by incubation for 2 min at 85 °C and then incubated in dimerization buffer (5 mM $MgCl_2$ 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 11–13) is marked D12.

to mimic that of HIV-1 (Fig. 4). The HIV-1 and HIV-2 DIS loops differ in size and the palindrome is oriented differently in the two elements. The NMR structure of the HIV-1 KL dimer complex proposes a U-like bending of the backbone of the DIS loop to optimize dimer interaction (Mujeeb *et al.*, 1998). 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 palindrome 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 A^{256} of one DIS hairpin and $A^{255'}/C^{264'}$ 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 $-2A^{M}$ mutant, a G to A substitution was therefore introduced, creating a DIS loop that is identical to that of HIV-1. In the $-2A^{MM}$ mutant, the top C–G base pair of HIV-2 was substituted for G–C, as in HIV-1. The $-2A^{MM}$ 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 $MgCl_2$ 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, $-2A^{M}$ and $-2A^{MM}$ (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 $-2A^{MM}$ 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 more intermediate thermodynamic stability of approximately −30·0 kcal/mol, which apparently is not sufficient to drive the KL to ED conversion.

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 $-2A^{MM}$) 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.

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 & van Wamel, 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

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*., 1996). This raises the possibility of interaction between the gene therapy vector and a wild-type lentivirus obtained through natural infection. Upon co-infection 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).

We thank Wim van Est for the artwork. This work was funded by the European Molecular Biology Organization, the Netherlands Organization for Scientific Research (NWO-CW), the Norfa Foundation, Plasmid Fondet, Aarhus University and SSVF (Danish National Foundation of Health).

References

Awang, G. & Sen, D. (1993). Mode of dimerization of HIV-1 genomic RNA. *Biochemistry* **32**, 11453–11457.

Balakrishnan, M., Fay, P. J. & Bambara, R. A. (2001). The kissing hairpin sequence promotes recombination within the HIV-1 5′ leader region. *Journal of Biological Chemistry* **276**, 36482–36492.

Beerens, N., Groot, F. & Berkhout, B. (2001). Initiation of HIV-1 reverse transcription is regulated by a primer activation signal. *Journal of Biological Chemistry* **276**, 31247–31256.

Bender, W., Chien, Y. H., Chattopadhyay, S., Vogt, P. K., Gardner, M. B. & Davidson, N. (1978). High-molecular-weight RNAs of AKR, NZB, and wild mouse viruses and avian reticuloendotheliosis virus all have similar dimer structures. *Journal of Virology* **25**, 888–896.

Berkhout, B. (1996). Structure and function of the human immunodeficiency virus leader RNA. *Progress in Nucleic Acid Research and Molecular Biology* **54**, 1–34.

Berkhout, B. & van Wamel, J. L. (1996). Role of the DIS hairpin in replication of human immunodeficiency virus type 1. *Journal of Virology* **70**, 6723–6732.

Berkhout, B., Gatignol, A., Silver, J. & Jeang, K. T. (1990). Efficient trans-activation by the HIV-2 Tat protein requires a duplicated TAR RNA structure. *Nucleic Acids Research* **18**, 1839–1846.

Berkhout, B., Essink, B. B. & Schoneveld, I. (1993). *In vitro* dimerization of HIV-2 leader RNA in the absence of PuGGAPuA motifs. *FASEB Journal* **7**, 181–187.

Bobkov, A., Kazennova, E., Selimova, L., Bobkova, M., Khanina, T., Ladnaya, N., Kravchenko, A., Pokrovsky, V., Cheingsong-Popov, R. & Weber, J. (1998). A sudden epidemic of HIV type 1 among injecting drug users in the former Soviet Union: identification of subtype A,

subtype B, and novel *gag*A/*env*B recombinants. *AIDS Research and Human Retroviruses* **14**, 669–676.

Bukovsky, A. A., Song, J. P. & Naldini, L. (1999). Interaction of human immunodeficiency virus-derived vectors with wild-type virus in transduced cells. *Journal of Virology* **73**, 7087–7092.

Carr, J. K., Salminen, M. O., Koch, C., Gotte, D., Artenstein, A. W., Hegerich, P. A., St Louis, D., Burke, D. S. & McCutchan, F. E. (1996). Full-length sequence and mosaic structure of a human immunodeficiency virus type 1 isolate from Thailand. *Journal of Virology* **70**, 5935–5943.

Carr, J. K., Salminen, M. O., Albert, J., Sanders-Buell, E., Gotte, D., Birx, D. L. & McCutchan, F. E. (1998). Full genome sequences of human immunodeficiency virus type 1 subtypes G and A/G intersubtype recombinants. *Virology* **247**, 22–31.

Clever, J. L. & Parslow, T. G. (1997). Mutant human immunodeficiency virus type 1 genomes with defects in RNA dimerization or encapsidation. *Journal of Virology* **71**, 3407–3414.

Clever, J. L., Wong, M. L. & Parslow, T. G. (1996). Requirements for kissing-loop-mediated dimerization of human immunodeficiency virus RNA. *Journal of Virology* **70**, 5902–5908.

Coffin, J. M. (1990). *Retroviridae* and their replication. In *Fields Virology*, 2nd edn. Edited by B. N. Fields & D. M. Knipe. New York: Raven Press.

Dardel, F., Marquet, R., Ehresmann, C., Ehresmann, B. & Blanquet, S. (1998). Solution studies of the dimerization initiation site of HIV-1 genomic RNA. *Nucleic Acids Research* **26**, 3567–3571.

Das, A. T., Klaver, B., Klasens, B. I., van Wamel, J. L. & Berkhout, B. (1997). A conserved hairpin motif in the R-U5 region of the human immunodeficiency virus type 1 RNA genome is essential for replication. *Journal of Virology* **71**, 2346–2356.

DiFronzo, N. L. & Holland, C. A. (1993). A direct demonstration of recombination between an injected virus and endogenous viral sequences, resulting in the generation of mink cell focus-inducing viruses in AKR mice. *Journal of Virology* **67**, 3763–3770.

Dillon, P. J., Nelbock, P., Perkins, A. & Rosen, C. A. (1990). Function of the human immunodeficiency virus types 1 and 2 Rev proteins is dependent on their ability to interact with a structured region present in *env* gene mRNA. *Journal of Virology* **64**, 4428–4437.

Dirac, A. M., Huthoff, H., Kjems, J. & Berkhout, B. (2001). The dimer initiation site hairpin mediates dimerization of the human immunodeficiency virus type 2 RNA genome. *Journal of Biological Chemistry* **276**, 32345–32352.

Dirac, A. M., Huthoff, H., Kjems, J. & Berkhout, B. (2002). Regulated HIV-2 RNA dimerization by means of alternative RNA conformations. *Nucleic Acids Research* **30**, 2647–2655.

Emerman, M., Guyader, M., Montagnier, L., Baltimore, D. & Muesing, M. A. (1987). The specificity of the human immunodeficiency virus type 2 transactivator is different from that of human immunodeficiency virus type 1. *EMBO Journal* **6**, 3755–3760.

Evans, L. A., Moreau, J., Odehouri, K., Seto, D., Thomson-Honnebier, G., Legg, H., Barboza, A., Cheng-Mayer, C. & Levy, J. A. (1988). Simultaneous isolation of HIV-1 and HIV-2 from an AIDS patient. *Lancet* ii, 1389–1391.

Gao, F., Yue, L., Robertson, D. L., Hill, S. C., Hui, H., Biggar, R. J., Neequaye, A. E., Whelan, T. M., Ho, D. D., Shaw, G. M. and others (1994). Genetic diversity of human immunodeficiency virus type 2: evidence for distinct sequence subtypes with differences in virus biology. *Journal of Virology* **68**, 7433–7447.

George, J. R., Ou, C. Y., Parekh, B., Brattegaard, K., Brown, V., Boateng, E. & De Cock, K. M. (1992). Prevalence of HIV-1 and HIV-2 mixed infections in Cote d'Ivoire. *Lancet* **340**, 337–339.

Girard, F., Barbault, F., Gouyette, C., Huynh-Dinh, T., Paoletti, J. & Lancelot, G. (1999). Dimer initiation sequence of HIV-1Lai genomic RNA: NMR solution structure of the extended duplex. *Journal of Biomolecular Structure and Dynamics* **16**, 1145–1157.

Gotte, M., Li, X. & Wainberg, M. A. (1999). HIV-1 reverse transcription: a brief overview focused on structure–function relationships among molecules involved in initiation of the reaction. *Archives of Biochemistry and Biophysics* **365**, 199–210.

Greatorex, J. & Lever, A. (1998). Retroviral RNA dimer linkage. *Journal of General Virology* **79**, 2877–2882.

Greatorex, J. S., Laisse, V., Dockhelar, M. C. & Lever, A. M. (1996). Sequences involved in the dimerisation of human T cell leukaemia virus type-1 RNA. *Nucleic Acids Research* **24**, 2919–2923.

Haddrick, M., Lear, A. L., Cann, A. J. & Heaphy, S. (1996). Evidence that a kissing loop structure facilitates genomic RNA dimerisation in HIV-1. *Journal of Molecular Biology* **259**, 58–68.

Hoglund, S., Ohagen, A., Goncalves, J., Panganiban, A. T. & Gabuzda, D. (1997). Ultrastructure of HIV-1 genomic RNA. *Virology* **233**, 271–279.

Huthoff, H. & Berkhout, B. (2001). Two alternating structures of the HIV-1 leader RNA. *RNA* **7**, 143–157.

Jossinet, F., Lodmell, J. S., Ehresmann, C., Ehresmann, B. & Marquet, R. (2001). Identification of the *in vitro* HIV-2/SIV RNA dimerization site reveals striking differences with HIV-1. *Journal of Biological Chemistry* **276**, 5598–5604.

Kaye, J. F. & Lever, A. M. (1998). Nonreciprocal packaging of human immunodeficiency virus type 1 and type 2 RNA: a possible role for the p2 domain of Gag in RNA encapsidation. *Journal of Virology* **72**, 5877–5885.

Laughrea, M. & Jette, L. (1994). A 19-nucleotide sequence upstream of the 5′ major splice donor is part of the dimerization domain of human immunodeficiency virus 1 genomic RNA. *Biochemistry* **33**, 13464–13474.

Laughrea, M. & Jette, L. (1996). Kissing-loop model of HIV-1 genome dimerization: HIV-1 RNAs can assume alternative dimeric forms, and all sequences upstream or downstream of hairpin 248–271 are dispensable for dimer formation. *Biochemistry* **35**, 1589–1598.

Laughrea, M., Jette, L., Mak, J., Kleiman, L., Liang, C. & Wainberg, M. A. (1997). Mutations in the kissing-loop hairpin of human immunodeficiency virus type 1 reduce viral infectivity as well as genomic RNA packaging and dimerization. *Journal of Virology* **71**, 3397–3406.

Lever, A. M. (2000). HIV RNA packaging and lentivirus-based vectors. *Advances in Pharmacology* **48**, 1–28.

Lodmell, J. S., Ehresmann, C., Ehresmann, B. & Marquet, R. (2000). Convergence of natural and artificial evolution on an RNA loop–loop interaction: the HIV-1 dimerization initiation site. *RNA* **6**, 1267–1276.

Lund, A. H., Mikkelsen, J. G., Schmidt, J., Duch, M. & Pedersen, F. S. (1999). The kissing-loop motif is a preferred site of 5′ leader recombination during replication of SL3-3 murine leukemia viruses in mice. *Journal of Virology* **73**, 9614–9618.

McBride, M. S. & Panganiban, A. T. (1996). The human immunodeficiency virus type 1 encapsidation site is a multipartite RNA element composed of functional hairpin structures. *Journal of Virology* **70**, 2963–2973.

Malim, M. H., Bohnlein, S., Fenrick, R., Le, S. Y., Maizel, J. V. & Cullen, B. R. (1989). Functional comparison of the Rev trans-activators encoded by different primate immunodeficiency virus species. *Proceedings of the National Academy of Sciences, USA* **86**, 8222–8226.

Marquet, R., Baudin, F., Gabus, C., Darlix, J. L., Mougel, M., Ehresmann, C. & Ehresmann, B. (1991). Dimerization of human

immunodeficiency virus (type 1) RNA: stimulation by cations and possible mechanism. *Nucleic Acids Research* **19**, 2349–2357.

Mathews, D. H., Sabina, J., Zuker, M. & Turner, D. H. (1999). Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *Journal of Molecular Biology* **288**, 911–940.

Mikkelsen, J. G., Lund, A. H., Kristensen, K. D., Duch, M., Sorensen, M. S., Jorgensen, P. & Pedersen, F. S. (1996). A preferred region for recombinational patch repair in the 5′ untranslated region of primer binding site-impaired murine leukemia virus vectors. *Journal of Virology* **70**, 1439–1447.

Mujeeb, A., Clever, J. L., Billeci, T. M., James, T. L. & Parslow, T. G. (1998). Structure of the dimer initiation complex of HIV-1 genomic RNA. *Nature Structural Biology* **5**, 432–436.

Mujeeb, A., Parslow, T. G., Zarrinpar, A., Das, C. & James, T. L. (1999). NMR structure of the mature dimer initiation complex of HIV-1 genomic RNA. *FEBS Letters* **458**, 387–392.

Muriaux, D., Girard, P. M., Bonnet-Mathoniere, B. & Paoletti, J. (1995). Dimerization of HIV-1Lai RNA at low ionic strength. An autocomplementary sequence in the 5′ leader region is evidenced by an antisense oligonucleotide. *Journal of Biological Chemistry* **270**, 8209–8216.

Muriaux, D., De Rocquigny, H., Roques, B. P. & Paoletti, J. (1996a). NCp7 activates HIV-1Lai RNA dimerization by converting a transient loop–loop complex into a stable dimer. *Journal of Biological Chemistry* **271**, 33686–33692.

Muriaux, D., Fosse, P. & Paoletti, J. (1996b). A kissing complex together with a stable dimer is involved in the HIV-1Lai RNA dimerization process *in vitro*. *Biochemistry* **35**, 5075–5082.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., Verma, I. M. & Trono, D. (1996). *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272**, 263–267.

Negroni, M. & Buc, H. (2001). Retroviral recombination: what drives the switch? *Nature Reviews in Molecular and Cellular Biology* **2**, 151–155.

Oude Essink, B. B., Das, A. T. & Berkhout, B. (1996). HIV-1 reverse transcriptase discriminates against non-self tRNA primers. *Journal of Molecular Biology* **264**, 243–254.

Paillart, J. C., Marquet, R., Skripkin, E., Ehresmann, B. & Ehresmann, C. (1994). Mutational analysis of the bipartite dimer linkage structure of human immunodeficiency virus type 1 genomic RNA. *Journal of Biological Chemistry* **269**, 27486–27493.

Paillart, J. C., Westhof, E., Ehresmann, C., Ehresmann, B. & Marquet, R. (1997). Non-canonical interactions in a kissing loop complex: the dimerization initiation site of HIV-1 genomic RNA. *Journal of Molecular Biology* **270**, 36–49.

Peeters, M., Gershy-Damet, G. M., Fransen, K., Koffi, K., Coulibaly, M., Delaporte, E., Piot, P. & van der Groen, G. (1992). Virological and polymerase chain reaction studies of HIV-1/HIV-2 dual infection in Cote d'Ivoire. *Lancet* **340**, 339–340.

Peeters, M., Liegeois, F., Torimiro, N., Bourgeois, A., Mpoudi, E., Vergne, L., Saman, E., Delaporte, E. & Saragosti, S. (1999). Characterization of a highly replicative intergroup M/O human immunodeficiency virus type 1 recombinant isolated from a Cameroonian patient. *Journal of Virology* **73**, 7368–7375.

Piyasirisilp, S., McCutchan, F. E., Carr, J. K., Sanders-Buell, E., Liu, W., Chen, J., Wagner, R., Wolf, H., Shao, Y., Lai, S., Beyrer, C. & Yu, X. F. (2000). A recent outbreak of human immunodeficiency virus type 1 infection in southern China was initiated by two highly homogeneous, geographically separated strains, circulating recombinant form AE and a novel BC recombinant. *Journal of Virology* **74**, 11286–11295.

Rizvi, T. A. & Panganiban, A. T. (1993). Simian immunodeficiency virus RNA is efficiently encapsidated by human immunodeficiency virus type 1 particles. *Journal of Virology* **67**, 2681–2688.

Robertson, D. L., Sharp, P. M., McCutchan, F. E. & Hahn, B. H. (1995). Recombination in HIV-1. *Nature* **374**, 124–126.

Robertson, D. L., Anderson, J. P., Bradac, J. A., Carr, J. K., Foley, B., Funkhouser, R. K., Gao, F., Hahn, B. H., Kalish, M. L., Kuiken, C., Learn, G. H., Leitner, T., McCutchan, F., Osmanov, S., Peeters, M., Pieniazek, D., Salminen, M., Sharp, P. M., Wolinsky, S. & Korber, B. (2000). HIV-1 nomenclature proposal. *Science* **288**, 55–56.

St Louis, D. C., Gotte, D., Sanders-Buell, E., Ritchey, D. W., Salminen, M. O., Carr, J. K. & McCutchan, F. E. (1998). Infectious molecular clones with the nonhomologous dimer initiation sequences found in different subtypes of human immunodeficiency virus type 1 can recombine and initiate a spreading infection *in vitro*. *Journal of Virology* **72**, 3991–3998.

Sakuragi, J. I. & Panganiban, A. T. (1997). Human immunodeficiency virus type 1 RNA outside the primary encapsidation and dimer linkage region affects RNA dimer stability *in vivo*. *Journal of Virology* **71**, 3250–3254.

Salminen, M. O., Carr, J. K., Robertson, D. L., Hegerich, P., Gotte, D., Koch, C., Sanders-Buell, E., Gao, F., Sharp, P. M., Hahn, B. H., Burke, D. S. & McCutchan, F. E. (1997). Evolution and probable transmission of intersubtype recombinant human immunodeficiency virus type 1 in a Zambian couple. *Journal of Virology* **71**, 2647–2655.

Schwartzberg, P., Colicelli, J. & Goff, S. P. (1985). Recombination between a defective retrovirus and homologous sequences in host DNA: reversion by patch repair. *Journal of Virology* **53**, 719–726.

Skripkin, E., Paillart, J. C., Marquet, R., Ehresmann, B. & Ehresmann, C. (1994). Identification of the primary site of the human immunodeficiency virus type 1 RNA dimerization *in vitro*. *Proceedings of the National Academy of Sciences, USA* **91**, 4945–4949.

Sundquist, W. I. & Heaphy, S. (1993). Evidence for interstrand quadruplex formation in the dimerization of human immunodeficiency virus 1 genomic RNA. *Proceedings of the National Academy of Sciences, USA* **90**, 3393–3397.

Takehisa, J., Zekeng, L., Ido, E., Yamaguchi-Kabata, Y., Mboudjeka, I., Harada, Y., Miura, T., Kaptu, L. & Hayami, M. (1999). Human immunodeficiency virus type 1 intergroup (M/O) recombination in Cameroon. *Journal of Virology* **73**, 6810–6820.

White, S. M., Renda, M., Nam, N. Y., Klimatcheva, E., Zhu, Y., Fisk, J., Halterman, M., Rimel, B. J., Federoff, H., Pandya, S., Rosenblatt, J. D. & Planelles, V. (1999). Lentivirus vectors using human and simian immunodeficiency virus elements. *Journal of Virology* **73**, 2832–2840.

Received 8 January 2002; Accepted 5 June 2002