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A loop–loop "kissing" complex is the essential part of the dimer linkage of genomic HIV-1 RNA

(AIDS/dimerization/encapsidation/retrovirus/RNA–RNA interactions)

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ABSTRACT **RNA–RNA interactions govern a number of biological processes. Several RNAs, including natural sense and antisense RNAs, interact by means of a two-step mechanism: recognition is mediated by a loop–loop complex, which is then stabilized by formation of an extended intermolecular duplex. It was proposed that the same mechanism holds for dimerization of the genomic RNA of human immunodeficiency virus type 1 (HIV-1), an event thought to control crucial steps of HIV-1 replication. However, whereas interaction between the partially self-complementary loop of the dimerization initiation site (DIS) of each monomer is well established, formation of the extended duplex remained speculative. Here we first show that *in vitro* dimerization of HIV-1 RNA is a specific process, not resulting from simple annealing of denatured molecules. Next we used mutants of the DIS to test the formation of the extended duplex. Four pairs of trans-complementary mutants were designed in such a way that all pairs can form the loop–loop "kissing" complex, but only two of them can potentially form the extended duplex. All pairs of mutants form heterodimers whose thermal stability, dissociation constant, and dynamics were analyzed. Taken together, our results indicate that, in contrast with the interactions between natural sense and antisense RNAs, no extended duplex is formed during dimerization of HIV-1 RNA. We also showed that 55-mer sense RNAs containing the DIS are able to interfere with the preformed HIV-1 RNA dimer.**

Besides their central role in translation, RNA–RNA interactions regulate a number of biological processes—e.g., in *Bacillus subtilis* interaction of uncharged tRNAs with the mRNA leader region of the corresponding tRNA synthetase genes regulates the transcription antitermination of these genes (1). Natural antisense RNAs control various processes such as replication of plasmids, transposition, osmoregulation, conjugation, and cell division by regulating RNA processing, translation, or transcription (for reviews see refs. 2 and 3).

A particular case of RNA–RNA interactions is found in retroviruses whose genome consists of two homologous RNA molecules that are physically associated in the viral particles (4–6). The dimeric nature of the retroviral genome was proposed to regulate key steps of the viral replication cycle such as encapsidation (7–10), translation of the *gag* gene (7, 8), and recombination during reverse transcription of the genomic RNA (11). Thus, dimerization represents a promising target for antiviral agents such as sense or antisense oligonucleotides aimed at blocking retroviral replication, especially in the case of human immunodeficiency virus type 1 (HIV-1). However, the dimerization mechanism remained unclear until a few years ago (4–6, 12–17).

Recently, by combining chemical interference experiments with site-directed mutagenesis, we localized the dimerization initiation site (DIS) of HIV-1 genomic RNA between the $tRNA_3^{Lys}$ binding site and the 5′ splice donor site (Fig. 1*A*) (18). The DIS, which is the essential part of the dimer linkage structure, adopts a stem–loop structure (20). We proposed that dimerization may proceed through recognition of the self-complementary loop of the DIS present in each monomer, followed by formation of an extended duplex involving melting of the DIS stem (Fig. 1*B*) (18). The same dimerization mechanism was proposed for different HIV-1 isolates (18, 21, 22). The HIV-1 RNA dimer is further stabilized by interactions involving sequences located in the vicinity of the translation initiation site of the *gag* gene, downstream of the splice donor site (13, 19).

The proposed dimerization mechanism (Fig. 1*B*) presents strong similitudes with several natural sense–antisense RNA interactions. The sense–antisense RNA pairs that control the copy number of plasmids ColE1 and R1, RNA I–RNA II, and CopA–CopT, respectively, first form hairpin loop complexes, followed by propagation of the intermolecular base pairing (2, 3). Extended intermolecular base pairing is probably a general feature of all natural sense–antisense RNA pairs studied so far (2, 3). As in the RNA I–RNA II and CopA–CopT systems, point mutations in the palindromic sequence of the DIS loop alter specificity of recognition (19). However, dimerization of HIV-1 genomic RNA also presents specific features. (*i*) Retroviral genomic RNAs form homodimers, whereas RNA I and CopA are complementary but not identical to RNA II and CopT, respectively. (*ii*) Whereas natural antisense RNAs are small, HIV-1 genomic RNA is about 10,000 nt. (*iii*) The three loops of RNA I and the loop of CopA are fully complementary with the corresponding loops in RNA II and CopT, whereas only six of the nine nt of the DIS loop are self-complementary (18).

Although interaction of the self-complementary sequences of the DIS loop during dimerization of HIV-1 RNA is well established (18, 19), formation of the extended duplex remains speculative. In this paper, we studied the importance of the DIS stem on the formation, the stability, and the dynamics of the RNA dimer. Our results suggest that in contrast with the natural sense–antisense RNA pairs, hybridization of the loop does not propagate to the DIS stem to form an extended duplex.

MATERIALS AND METHODS

Plasmid Construction and RNA Synthesis. Plasmid construction and cleavage by restriction enzymes were conducted according to published procedures (23). Plasmid pJCB con-

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Abbreviations: HIV-1, human immunodeficiency virus type 1; DIS, dimerization initiation site.

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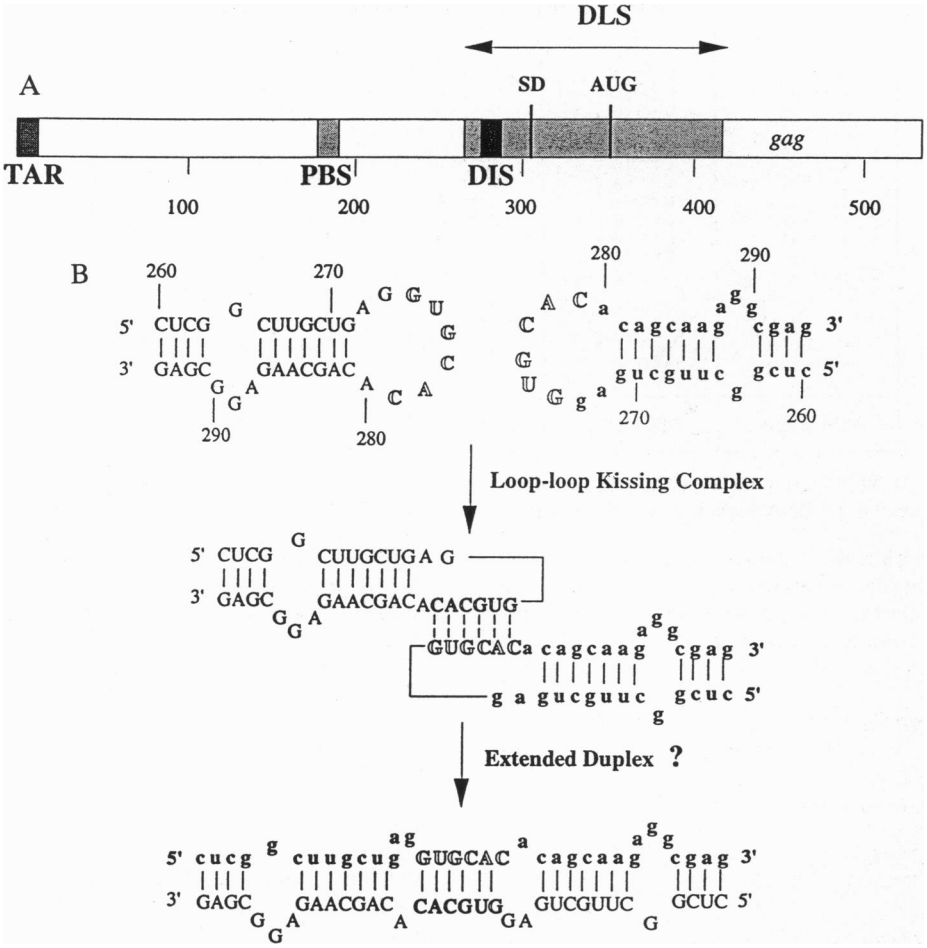

FIG. 1. Sequences involved in the dimerization of the HIV-1 genomic RNA. (*A*) Schematic representation of the 500 5′ nt of HIV-1 RNA. The transactivation responsive element (TAR), the primer binding site (PBS), the DIS, the dimer linkage structure (DLS), the 5′ splice donor site (SD), and the initiation codon of the *gag* gene are indicated. (*B*) The secondary structure of the DIS and the two steps of the previously proposed dimerization mechanism are shown. The loop–loop interaction was evidenced in refs. 18 and 19. Formation of the proposed extended duplex is tested in this paper.

tains nt 1–615 of the HIV-1 genome (Mal isolate, ref. 24; +1 being the first nucleotide of the genomic RNA) under the control of the promoter of RNA polymerase from phage T7, followed by a *Pvu*II restriction site (19). Plasmids pDISC275, pDISG278, pDISHxC275, and pDISHxG278 were obtained by inverse PCR on pJCB. The PCR products were phosphorylated, purified on agarose gels, and used to transform JM 109 *Escherichia coli* cells after ligation.

Plasmids cut with *Pvu*II or *Rsa*I were used for transcription with the RNA polymerase from phage T7 to synthesize RNAs containing the first 615 or 311 nt of the HIV-1 genomic RNA. *In vitro* transcription and RNA purification were as described (12). The DIS region of the wild-type and mutant RNAs is shown in Fig. 2. Fifty-five-mer RNAs corresponding to nt 249–302 of pDISG278 and pDISHxG278 were obtained by *in vitro* transcription of PCR products obtained using these plasmids as template and the appropriate primers. In addition to the viral sequence, the sense primer contained the promoter of RNA polymerase from phage T7, followed by two G residues that allow efficient transcription. Internally labeled RNAs were synthesized using [α^{32}P]ATP (Amersham) during transcription (37.5 μM of ATP/50 μCi/μg of DNA template). Alternatively, RNAs (2 μg) were labeled at their 3′ end with 50 μCi of [5′^{32}P]pCp (Amersham) and RNA ligase from phage T4.

***In Vitro* Dimerization of HIV-1 RNAs.** In a typical experiment unlabeled wild-type RNA or a mixture of two unlabeled RNAs with complementary mutations in the DIS loop were diluted in Milli-Q (Millipore) water at a final strand concentration of 400 nM of each RNA species, together with a corresponding labeled RNA (3–5 nCi/10–40 ng). When pairs of complementary mutants were used, only one labeled RNA species was added to the reaction mixture. The sample was heated for 2 min at 90°C, chilled for 2 min on ice, and dimerization was initiated by addition of 2 μl of 5-fold concentrated dimerization buffer (final concentration = 50 mM sodium cacodylate, pH 7.5/300 mM KCl/5 mM $MgCl_2$). Dimerization was for 30 min at 37°C.

Samples were analyzed on 1.1% agarose gels at 4°C. Electrophoresis buffer and gels contained 45 mM Tris borate (pH 8.3) and 0.1 mM $MgCl_2$. Gels were fixed, dried, and analyzed using a BAS 2000 BIO-Imager (Fuji) as described (13, 22) using either the Fuji or the Whole Band Analyzer (BioImage, Ann Arbor, MI) softwares. The fraction of RNA dimer, $f_D^{(w/w)}$, was defined as the weight-by-weight ratio of the dimer to the total RNA species.

For the RNA dimer stability experiments, samples were incubated for 30 min at 30°C, and then the temperature was gradually increased by 7°C steps. After a 5-min incubation at the appropriate temperature, an aliquot was loaded on gel after addition of glycerol (20% final concentration). The melting temperature of the dimer, T_m, was defined as the temperature at which $f_D^{(w/w)}$ is reduced by 2-fold compared with the maximal $f_D^{(w/w)}$ observed at low temperature.

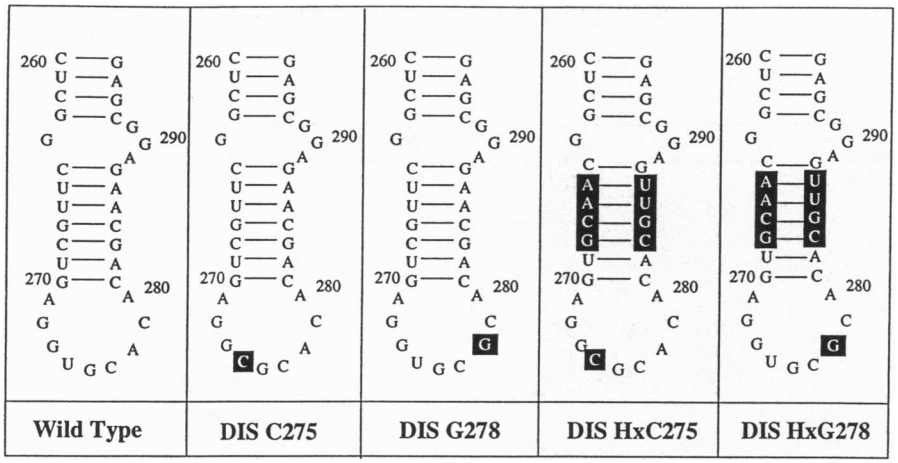

FIG. 2. Mutant RNAs. Mutations in the palindromic loop and in the stem of the DIS are highlighted on the seconary structure of the DIS. All mutations were introduced in an RNA spanning nt 1–615 of HIV-1 RNA (MAL isolate).

For the determination of the dissociation constant (K_d), increasing concentrations of the wild-type or mutant RNAs, ranging from 2 to 400 nM, were incubated at 37°C for 30 min. K_d was determined from a nonlinear fit of the experimental data to equation:

$$f_D^{(w/w)} = \frac{(4\beta M_0 + K_d) - \sqrt{8M_0\beta K_d + K_d^2}}{4M_0}$$

for homodimers, or to equation:

$$f_D^{(w/w)} = \frac{(2\beta M_0 + K_d) - \sqrt{4M_0\beta K_d + K_d^2}}{2M_0}$$

for hetero dimers. M_0 is the strand concentration of the wild-type RNA (homodimers) or of each mutant species (heterodimers). β is the maximum fraction of RNA that was able to dimerize. β was smaller than unity owing to the presence of RNA conformers that were unable to dimerize (7, 13). K_d and β were allowed to vary during curve fitting of $f_D^{(w/w)}$ versus M_0. Comparison of the β value obtained from curve fitting with the experimental values of $f_D^{(w/w)}$ obtained at high RNA concentration indicated that saturation of the dimerization process was obtained in all cases. K_d values were estimated to be significantly different if they differed by at least a 4- to 5-fold factor. The variability of the measurements of the K_d values was primarily due to the rather high background of the native gels that was differently appreciated by different softwares and for each software from one experiment to another.

RESULTS AND DISCUSSION

To test the proposed dimerization mechanism of HIV-1 RNA, we introduced mutations in the loop and stem of the previously identified DIS (18, 19) (Fig. 2). Because previous experiments showed that truncation of the 5′ part of the genomic RNA may induce dimerization artefacts such as G-quartets (13–15), we introduced all mutations in the context of an RNA encompassing the complete 5′ untranslated region and the first 265 coding nt of the HIV-1 genomic RNA (RNA 1–615).

Specificity of the Dimerization Process. We previously used mutant RNAs DISC275 and DISG278 to evidence the intermolecular interaction of the DIS loop during dimerization of HIV-1 RNA (22). We showed that mutation U275 to C (DISC275) totally abolishes dimerization and that mutant DISG278 (A278 to G) forms low levels of unstable dimers. However, these mutants are able to complement each other in trans by forming a heterodimer (22).

Here we took advantage of these mutants to test whether dimerization is the result of an unspecific hydridization of unfolded molecules or whether the folded RNA monomers are able to dimerize. Because *in vitro* dimerization of retroviral RNA is induced by monovalent and bivalent cations (8, 9, 12, 13), renaturation and dimerization of the wild-type RNA take place simultaneously. However, these two processes can be uncoupled when using DISC275 and DISG278. We initiated heterodimerization of these mutants either by adding cations to the unfolded RNA mixture or by mixing the prerenaturated monomers. In the latter case, the mutants were separately denaturated at 90°C and renaturated in the dimer buffer at 37°C for 15 min; then they were mixed and incubated together for 20 min. Separation of the monomer and dimer species on agarose gels followed by autoradiography indicates that similar dimerization yields are obtained regardless of the dimerization protocol (data not shown). Moreover, the dimers formed under these two different experimental conditions have identical thermal stability (Fig. 3). The results of this experiment indicate that *in vitro* dimerization of HIV-1 RNA does not simply result from unspecific hybridization of the denaturated molecules. Furthermore, the thermal stability of the heterodimers is the same, within the experimental errors, as that of the wild-type homodimer (Fig. 3*B*). Thus, the variations of standard free energy associated with dissociation of homo- and heterodimers differ by not more than 5%.

Influence of the DIS Stem on the Dimer Stability. Because intermolecular base pairing is much more extended in the potential extended duplex than in the loop–loop "kissing" complex (Fig. 1*B*) (18, 19), it must be possible to discriminate between these complexes on the basis of their thermal stability. Indeed, these two kinds of complexes were identified during thermal denaturation of complexes formed by RNA hairpin loops derived from *E. coli* RNA I and RNA II (25).

To test the formation of the extended duplex, we constructed mutants DISHxC275 and DISHxG278 by replacing nt 266–269 and 283–286 by their Watson–Crick complement (Fig. 2). Thus, the stem of mutant DISC275 is identical (compatible) to that of DISG278, but not to that of DISHxG278. Similarly, the stem of DISHxC275 is compatible with that of mutant DISHxG278, but not of DISG278. The base pairs at both ends of the helix were not mutated in order to avoid structural perturbations in the terminal and internal loops (Fig. 2). We tested the four pairs of RNAs with complementary mutations in the DIS loop for their ability to form heterodimers, and compared the thermal stability of these dimers with that of the wild-type RNA dimer (Fig. 4). Each of the four pairs of mutant RNAs is able to form the loop–loop kissing complex, but only two pairs, DISC275 + DISG278 and DISHxC275 +

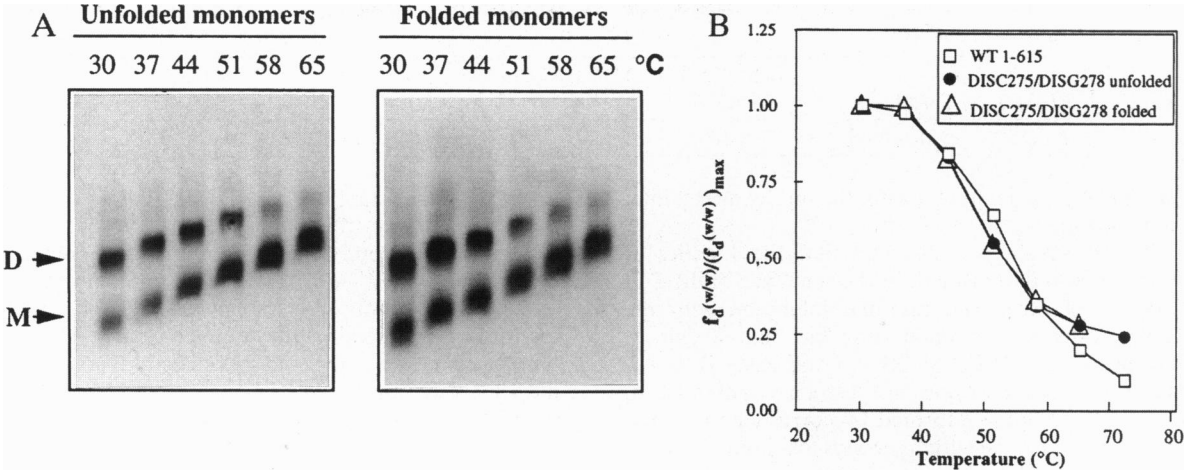

FIG. 3. Specificity of the dimerization process. (*A*) Autoradiography of the agarose gels showing the thermal stability of the RNA dimers. Denaturated monomers (left part of the gel) or renaturated monomers (right part of the gel) of RNAs DISC275 and DISG278 were mixed and allowed to form heterodimers at 30°C; than the temperature was gradually increased as described. The final temperature is indicated at the top of each lane. The monomeric (M) and dimeric (D) forms of the RNAs are indicated. The difference in migration from lane to lane is due to sequencial loading of the samples on running gels. RNA DISG278 is the labeled species. (*B*) Quantification of the melting experiments. The dimer fraction $f_D^{(w/w)}$ divided by its value at 30°C is plotted versus temperature. The thermal stability of the heterodimers formed by the denaturated (●) or the renaturated (△) mutant RNAs is compared to the stability of the wild-type RNA dimer (□).

DISHxG275, fulfill the sequence requirements to form extended duplexes (Figs. 1 and 2). It turns out that all pairs of mutant RNAs give similar amounts of dimer whose thermal stability is indistinguishable from that of the wild-type dimer (Fig. 4). Thus, in the case of dimerization of HIV-1 RNA, only one kind of complex is detected irrespective of the DIS stem sequence. We previously showed that interactions involving sequences downstream of the splice donor site account for 10°C in the thermal stability of the wild-type RNA corresponding to nt 1–615 of the HIV-1 genomic RNA (13, 19). Thus, the thermal stability of the HIV-1 loop–loop kissing complex is in the same range as that of the kissing complexes formed by RNA hairpin loops derived from *E. coli* RNA I and RNA II (15°C to 65°C, depending on the loop sequences) that are associated through seven Watson–Crick base pairs (25).

Besides their thermal stability, extended duplexes are also expected to differ from loop–loop complexes by their dissociation constant (26, 27). We studied the concentration dependence of dimerization for each pair of trans-complementary mutants and for wild-type RNA, and determined the equilibrium dissociation constants (K_d) (Table 1). In these experiments, mutant RNAs were in stoichiometric amounts at all concentrations. The K_d values of the two pairs of mutants that can form either the kissing complex or the extended duplex, and those of the pairs of mutant RNAs that can only form the loop–loop kissing complex are all the same within the experimental errors. In addition, the K_d values of the four heterodimers are not significantly different from the K_d of the wild-type homodimer. These K_d values are in the same range as the dissociation constants of kissing complexes derived from *E. coli* RNA I–RNA II (26, 27). In all cases, the maximum dimerization yield at high RNA concentration was comprised between 80 and 100% (data not shown).

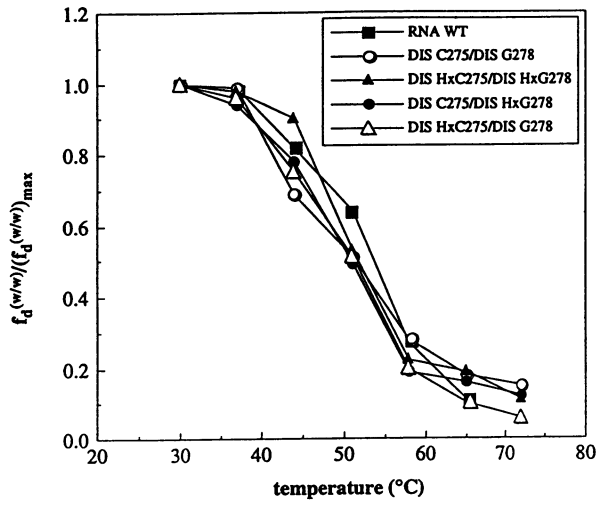

FIG. 4. Effect of mutations in the DIS stem on the thermal stability of the RNA dimers. Pairs of mutant RNAs with either compatible (identical) or incompatible DIS stems were mixed and allowed to dimerize. The thermal stability of the dimers was determined as described. For each heterodimer, $f_D^{(w/w)}$ divided by its value at 30°C is plotted as a function of temperature. The stability of the wild-type RNA dimer is indicated for comparison.

Dynamics of the RNA Dimer. To test the dynamic behavior of the dimer, we preformed the DISC275/DISG278 dimer, and then added increasing concentrations of competitor RNAs having either a wild-type or mutant DIS stem. Results using truncated forms of DISG278 (1–311G278) and DISHxG278 (1–311HxG278) as competitors are shown in Fig. 5. These two RNAs efficiently interfere with the preformed dimer independently of the sequence of the DIS stem. These experiments indicate that the RNA dimer dissociates within minutes, and that an RNA that can only form a kissing complex with the target RNA compete the preformed dimer as efficiently as an RNA that can potentially form an extended duplex.

Similar results were obtained when 55-mer RNAs containing the DIS and bearing the G278 or HxG278 mutations were used as competitors (Fig. 5). This result confirms that the DIS is the essential part of the dimer linkage structure of HIV-1 RNA 1–615. In addition, the interactions at the DIS are not only required for initiation of dimerization (18, 19), but are maintained after completion of the dimerization process. Our results also indicate that the interactions involving sequences downstream of the splice donor site that stabilize the dimer (19) either are not strong enough to maintain the dimer structure in the absence of the loop–loop interaction at the DIS or cannot exist in the absence of the kissing complex. Accordingly, RNAs corresponding to nt 1–178 or nt 311–615 of the HIV-1 genomic RNA, which do not contain an intact DIS

Table 1. Equilibrium dissociation constants (K_ds) of the dimers formed by wild-type RNA or by pairs of mutant RNAs

RNA(s)	wild type	DISC275 + DISG278	DISH×C275 + DISH×G278	DISC275 + DISH×G278	DISH×C275 + DISG278
K_d (M)	21×10^{-9}	7×10^{-9}	32×10^{-9}	17×10^{-9}	11×10^{-9}

loop, were unable to compete with the preformed dimer (results not shown).

Using 55-mer RNAs, we observed that the kinetics of interference are also independent of the sequence of the DIS stem (data not shown). The maximal inhibition with the 55-mer competitors was obtained after less than 10 min of incubation. In the case of *E. coli* RNA I and RNA II, it was shown that extended duplex does not dissociate within 24 hr, while the kissing complexes formed by corresponding truncated stem–loops dissociate within seconds to minutes (26, 27).

CONCLUSIONS

In this study, we first showed that *in vitro* dimerization does not simply result from unspecific hybridization of the denaturated molecules. Next we showed that melting of the DIS stem and formation of extended intermolecular base pairing does not take place during dimerization of HIV-1 RNA. Thus, the essential part of the HIV-1 dimer linkage structure is a loop–loop kissing complex. This situation strongly differs from the natural sense–antisense RNA systems in which extended intermolecular base pairing is observed (2, 3). In these systems, interaction is limited to the loop–loop kissing complex stage only when using isolated RNA hairpins, but proceeds to an extended duplex in the natural RNA context (27, 28). In the HIV-1 RNA dimer, we observed no extended duplex even though we used RNAs that correspond to the complete 5′ untranslated region and part of the *gag* coding region of the HIV-1 genomic RNA. This finding does not preclude the existence of other interactions that contribute to the overall stability of the HIV-1 RNA dimer. Indeed, sequences located downstream of the major 5′ splice donor site were found to affect the dimer stability (13, 22). The interactions involved in the stabilization of the dimer remain unkown.

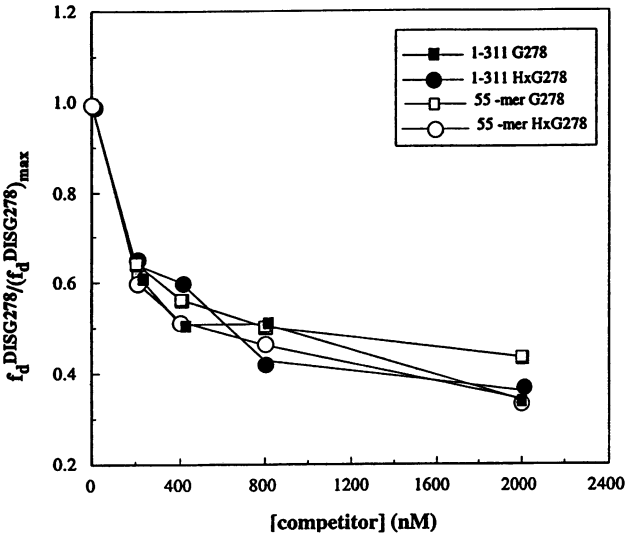

FIG. 5. Dynamics of the RNA dimer. Mutants DISC275 and DISG278 (400 nM each) were mixed and allowed to dimerize for 30 min at 37°C. Competitor RNAs, either 311 or 55 nt in length, with the wild-type or mutated DIS stem were added at various concentrations and the samples were further incubated for 30 min. For each competitor RNA, the fraction of labeled RNA DISG278 in the dimeric form in the presence of competitor ($f_D^{DISG278}$) relative to its value in the absence of competitor is plotted versus the competitor concentration.

Several parameters may explain the difference between dimerization of HIV-1 RNA and the sense–antisense systems. (*i*) In the sense–antisense systems studied so far, the interacting loops are generally fully complementary. Thus, complete base pairing of the loops induces structural constraints on the loop–loop kissing complex (29) that may favor subsequent melting of the stems. (*ii*) Sense and antisense RNAs contain sequences that are single-stranded in the transient loop–loop kissing complex and form intermolecular base pairs in the extended duplex. Because the dimer of the HIV-1 genomic RNA is a homodimer, the base pairing possibilities are the same in the kissing complex and in the putative extended duplex, and formation of an extended duplex may not be energetically favorable. (*iii*) Due to the large size of the genomic RNA compared to antisense RNAs, formation of an extended duplex may have been evolutionary disfavored because formation of an extended helix requires the molecules to rotate around each other, potentially causing topological problems.

The finding that the essential part of the HIV-1 DLS is a loop–loop kissing complex may be of importance for the inhibition of HIV-1 replication by sense or antisense oligonucleotides. Our results with the 55-mer RNA competitors indicate that sense and antisense oligonucleotides should be able to prevent dimerization of the genomic RNA, but also to interfere with genomes that are already in the dimeric form. Existence of a kissing complex rather than an extended duplex may also be crucial for efficient reverse transcription of the dimeric RNA template. A very stable intermolecular structure would probably dramatically reduce the efficiency of this process.

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