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Elements located upstream and downstream of the major splice donor site influence the ability of HIV-2 leader RNA to dimerize *in vitro*[†]

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Abstract

An essential step in the replication cycle of all retroviruses is the dimerization of genomic RNA prior to or during budding and maturation of the viral particle. In HIV-1 a 5' leader region site referred to as stem-loop1 (SL1) promotes RNA dimerization *in vitro* and influences dimerization *in vivo*. In HIV-2, two sequences promote dimerization of RNA fragments *in vitro*: the 5'-end of the primer-binding site (PBS) and a stem loop homologous to the HIV-1 SL1 sequence. Because HIV-2 RNA constructs of different lengths use these two dimerization signals disproportionately, we hypothesized that other sequences could modulate their relative utilization. Here, we characterized the influence of sequences upstream and downstream of the major splice donor site on the formation of HIV-2 RNA dimers *in vitro* using a variety of RNA constructs and dimerization/electrophoresis protocols. We first assayed the formation of loose or tight dimers for 1-444 and 1-561 model RNAs. Although both RNAs could form PBS-dependent loose dimers, the 1-561 RNA was unable to make SL1-dependent tight dimers. Using RNAs truncated at their 5' and/or 3' ends and by making compensatory base substitutions we found that two elements interfere with the formation of SL1-dependent tight dimers. The cores of these elements are located at nucleotides 189-196 and 543-550. Our results suggest that base pairing between these sequences prevents the formation of SL1-

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¹Abbreviations:

HIV-1 & 2

human immunodeficiency virus types 1 & 2

SL1

stem-loop 1

PBS

primer binding site

DIS

dimerization initiation site

DLS

dimer linkage structure

TBE

Tris-Borate EDTA buffer

TBM

Tris-Borate magnesium buffer

NCp7

nucleocapsid protein

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dependent tight dimers, probably by sequestering SL1 in a stable intramolecular arrangement. Moreover we found that nucleotides downstream of SL1 decreased the rate of tight dimerization. Interestingly, dimerization at 37°C in presence of nucleocapsid protein increased the yield of SL1-mediated tight dimerization *in vitro*, even in presence of the two interfering elements, suggesting a relationship between the nucleocapsid protein and activation of SL1 dimerization signal *in vivo*.

Keywords

HIV-2; dimerization; molecular recognition; RNA structure

INTRODUCTION

Retroviruses encapsidate two copies of genomic single-stranded RNA in their infectious particles. Electron microscopy studies of the organization of genomic RNA isolated from retroviral particles revealed that the two strands are associated through a region located close to the 5' end, called the Dimer Linkage Structure (DLS) ((1) and for HIV-1 see (2)). Upon budding, retroviral particles undergo a maturation event that involves changes in the stability of the genomic RNA dimer (3,4) which may be reminiscent of the loose-tight dimer interconversion observed *in vitro* (described below).

Several model systems have been developed to study dimerization of the DLS. RNA fragments corresponding to the 5' end of the genomic RNA have been shown to dimerize *in vitro* in the absence of any viral or cellular protein (in the case of HIV-1, see (5)). Further analysis identified a short RNA sequence that promotes dimerization of HIV-1 transcripts *in vitro* and was named the dimerization initiation site (DIS) (6,7) or stem-loop 1 (SL1, (8)). Studies of the dimerization properties of HIV-2 leader RNA yielded results different from those obtained with similar HIV-1 fragments. First, an HIV-2 RNA fragment encompassing the complete leader region of the genomic RNA and the first several codons of the Gag protein (Figure 1A) was found to use the 5' end of the primer-binding site (PBS), and not the expected SL1, as a dimer-inducing element at physiological temperature (37°C) (9). Its properties were characteristic of a so-called loose dimer (10,11). In general, loose dimers can be observed on a native gel in Tris-borate magnesium (TBM) buffer at 4°C but are absent when assayed either on a semi-denaturing gel in Tris-borate EDTA (TBE) buffer or on a TBM gel at 24°C, presumably because the dimers dissociate under these electrophoresis conditions (12). Loose dimers of HIV-1 RNA are thought to be associated via an intermolecular kissing loop interaction (10,11,13-15).

A second way in which HIV-1 and HIV-2 RNAs behave differently is that the HIV-2 leader RNA fragment was unable to form tight dimers (16,17), contrary to HIV-1 RNA (10,11,18). These exceptionally stable dimers could withstand semi-denaturing electrophoresis in TBE buffer at 24°C. In HIV-2 only a shorter RNA (1-444), in which SL1 is located at the extreme 3'-end, was able to form a SL1-dependent tight dimer upon incubation at high temperature (16,17,19). Thus, the use of the SL1 dimer-inducing element depends on the size of the RNA fragment, as large RNAs (up to 950 nucleotides long (17)) are unable to form tight dimers *in vitro* despite the presence of the SL1 element in their sequences. This observation suggests the presence of elements which interfere with tight dimerization, especially downstream of SL1 and the major splice donor site, as was previously proposed (17).

In this study, we analyzed the influence of sequences upstream and downstream of the major splice donor site on the formation of HIV-2 leader RNA dimers *in vitro* using a variety of RNA constructs and dimerization/electrophoresis protocols. We tested the properties of 1-444 and 1-561 model RNAs formed at 37° or 55°C to assay for the formation of loose or tight dimers and to determine the relative use of the PBS region and SL1 dimer-inducing elements. Although

both RNAs could form PBS-dependent loose dimers, the 1-561 RNA was unable to form SL1-dependent tight dimers upon incubation at 55°C, contrary to the 1-444 RNA. Using RNAs truncated at their 5' or 3' ends we found that two elements prevent the formation of SL1-dependent tight dimers of the HIV-2 leader RNA. The cores of these interfering elements were localized between nucleotides 189-196 and 543-550, and verified using site-directed mutagenesis and compensatory mutations. Our dimerization results indicate that base pairing between these sequences prevents the use of SL1 in the formation of a tight dimer, probably by sequestering SL1 in a stable intramolecular base pairing arrangement. Furthermore, sequences between nucleotides 444 and 546 influenced the *rate* of tight dimerization. We also tested the influence of the nucleocapsid protein on the formation of tight dimer with 1-444 and 1-561 RNAs and observed that, similarly to the HIV-1 system, the yield of SL1-mediated tight dimerization increased in the presence of nucleocapsid protein, even with the large HIV-2 leader RNA fragments that were unable to form tight dimers without protein. Since the SL1 structure is very close to the core encapsidation signal of HIV-2 RNA (20) and one of the dimer-interfering elements encompasses the *gag* initiation codon, our results suggest a model of how the processes of translation, dimerization, and encapsidation of HIV-2 RNA and proteins could be interdependent *in vivo*.

MATERIALS AND METHODS

Template construction for *in vitro* transcription

A sense primer containing a *Bam*HI site and the promoter for the T7 RNA polymerase and an antisense primer containing an *Eco*RI site (Table 1) were used to amplify the first 381, 444, 526, 541, 546, 550, 558, or 561 nucleotides of HIV-2 genomic RNA sequence, *ROD* isolate. The HIV-2 *ROD* DNA template (modified plasmid pROD10) was provided by the EU Programme EVA/MRC Centralised Facility for AIDS Reagents, NIBSC, UK (Grant Number QLK2-CT-1999-00609 and GP828102). The numbering is based on the genomic RNA sequence. The 5'-truncated fragments were made using a sense primer containing a *Bam*HI site, the promoter for the T7 RNA polymerase, and starting at different positions of the genomic RNA (128, 145, 164, 187, or 197), and an antisense primer containing an *Eco*RI site and a HIV-2 sequence ending at nucleotide 561. The plasmid containing the first 290 nucleotides of HIV-1 *Lai* genomic RNA was constructed using a similar strategy. The HIV-1 p83-2 template plasmid was obtained from Dr. Ronald Desrosiers through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (21). The digested PCR (Polymerase Chain Reaction) products were cloned in the *Bam*HI and *Eco*RI sites of the pUC18 plasmid. The base substitutions in the upstream and downstream elements were made using mutagenic primers sBAMT7GEE and asECOCEE to amplify the 187-550 construct. The 1-444 construct harboring a deleted loop in SL1 was described previously (22).

RNA synthesis and purification

The different plasmids were linearized with *Eco*RI prior to *in vitro* transcription. RNAs were synthesized by *in vitro* transcription of the *Eco*RI-digested plasmids with the AmpliScribe™ T7 transcription kit (Epicentre). After transcription, the DNA was digested with the supplied RNase-free DNase, and the RNA was purified by ammonium acetate precipitation followed by size exclusion chromatography (Bio-Gel® P-4, Bio-Rad).

In vitro dimerization of HIV-2 RNA

Five to seven picomoles of RNA were denatured in 8 µl water for 2 minutes at 90°C and quench cooled on ice for 2 minutes. After the addition of 2 µl monomer buffer (final concentrations: 50 mM Tris-HCl pH 7.5 at 37°C, 40 mM KCl, 0.1 mM MgCl₂) or dimer buffer (final concentrations: 50 mM Tris-HCl pH 7.5 at 37°C, 300 mM KCl, 5 mM MgCl₂), dimerization was allowed to proceed for 15 to 60 minutes at 37° or 55°C. We compared 37° to 55°C since

the incubation of the 1-444 RNA at 55°C allows a maximal yield of TBE-resistant SL1-dependent dimers (17). The samples were then cooled on ice to stabilize dimers formed during incubation and loaded on a 0.8% agarose gel with 2 µl of glycerol loading dye 6x (40% glycerol, Tris-borate 44 mM pH 8.3, 0.25% Bromophenol blue). Electrophoresis was carried out for 2 hours at 4°C and 5 V/cm in Tris-borate 44 mM, pH 8.3, 0.1 mM MgCl₂ (TBM) or at room temperature (24°C) in Tris-borate 44 mM pH 8.3, EDTA 1 mM (TBE). After electrophoresis, the ethidium bromide-stained gel was scanned on a Fluorescent Image Analyzer FLA-3000 (Fujifilm).

Kinetics of tight dimer formation

One hundred picomoles of RNA were denatured in 160 µl water for 2 minutes at 90°C and quench cooled on ice for 2 minutes. After the addition of 2 µl dimer buffer (final concentrations: 50 mM Tris-HCl pH 7.5 at 37°C, 40 mM NaCl, 5 mM MgCl₂) under the lid of 20 tubes, 8 µl of denatured RNA was aliquoted to each tube. The dimerization was then initiated by a 5 second spin in a bench top centrifuge and immediate loading of the tubes in a heating block at 50°, 55° or 60°C. Dimerization was allowed to proceed for 2 to 60 minutes or longer. At each time point a tube containing 10 µl reaction mixture was removed from the heating block, mixed with 2 µl of glycerol loading dye 6x and loaded on a 0.8% agarose TBE gel. Electrophoresis was carried out at room temperature (24°C) and 3 V/cm. After electrophoresis, the ethidium bromide-stained gel was scanned on a Fluorescent Image Analyzer FLA-3000 (Fujifilm). Quantification of the extent of dimerization was done using Fujifilm Image Gauge V3.3 software. The data were fitted using a second order conformation model (23):

$$1 / M_t = 1 / M_0 + 2k_{\text{dim}} t$$

where M_t is the concentration of monomer at time t , M_0 is the initial concentration of dimerization-competent monomer, and k_{dim} is the second order rate constant of dimerization ($\mu\text{M}^{-1} \cdot \text{min}^{-1}$).

NCp7-mediated tight dimerization assay

The HIV-1 nucleocapsid protein NCp7 was kindly provided by John Marino. This protein was expressed in *Escherichia coli* and purified by chromatography (for further details, see (24)). We based our NCp7-mediated dimerization assay on the protocol described by Muriaux *et al.* for an HIV-1 *Lai* isolate RNA fragment and nucleocapsid protein (25). Five picomoles of RNA were denatured in water for 2 minutes at 90°C and quench cooled on ice for 2 minutes. After the addition of monomer or dimer buffer, dimerization was allowed to proceed for 30 minutes at 37° or 55°C. Tubes containing 125 pmol of NCp7 in monomer buffer were incubated at 37°C for 30 minutes. The concentration of NCp7 (around one protein per 20 nucleotides) allows complete tight dimerization of the homologous HIV-1 RNA (25). Since NCp7 binding to the RNA changes the electrophoretic properties of the RNA and prevents the analysis of monomer and dimer levels, an SDS/phenol extraction step is necessary. Tubes containing the NCp7 and control tubes incubated at 37° or 55°C without NCp7 were incubated for 2 minutes at 24°C with 0.2% SDS and then extracted with phenol saturated with 0.2% SDS and monomer buffer 1x. All samples were then loaded on a 0.8% agarose TBE gel. Electrophoresis was carried out at room temperature (24°C) and 5 V/cm. After electrophoresis, the ethidium bromide-stained gel was scanned on a Fluorescent Image Analyzer FLA-3000 (Fuji Medical Systems).

RESULTS

The complete HIV-2 leader RNA cannot form tight dimers *in vitro*

We compared the effect of different incubation temperatures (37° or 55°C) and different electrophoretic conditions on the dimerization of 1-444 and 1-561 RNA fragments. Both RNA

constructs contain the PBS and SL1 sequences, with the SL1 at the extreme 3' end of the 1-444 fragment (Figure 1). The 55°C incubation temperature was chosen because it allows efficient formation of tight dimers *in vitro* for HIV-1 RNA (11), and HIV-2 1-444 RNA (17). Both RNAs were able to dimerize when assayed on a TBM/4°C gel (Figure 2A). We previously showed that both RNAs use the 5' end of the PBS as dimer-inducing element when incubated at 37°C (Figure 1A and (17)). However only the 1-444 dimerized when the RNAs were loaded on a TBE/24°C gel (Figure 2B). The 1-561 RNA did not dimerize significantly under these electrophoresis conditions after incubation at 37° or 55°C (Figure 2B, lanes 9 and 10). The formation of small amounts of 1-561 TBE-resistant dimers required the 55°C incubation step in monomer or dimer buffer (Figure 2B, lanes 8 and 10).

Thus, an RNA that encompasses the complete leader region of HIV-2 was not able to form a tight dimer *in vitro*, even though it contains two distinct dimer-inducing elements. Since we and others demonstrated that the tight dimer of 1-444 RNA occurs through the stem-loop 1 sequence (SL1) (17,19), this result suggested that, in the 1-561 RNA, all or part of the SL1 sequence may be involved in an intramolecular interaction that prevents formation of an extended intermolecular SL1-SL1 interaction (*i.e.*, a tight dimer). More specifically, the present results indicate that the formation of this intramolecular interaction depends on a structural element located between nucleotides 444 and 561.

Influence of the 3'-terminal sequence on tight dimer formation

To further define the element(s) that prevents the formation of SL1-mediated 1-561 RNA tight dimer, we assayed dimerization of RNA fragments of different lengths. An unconstrained M-fold software folding of the 1-561 RNA indicated a long-range interaction of sequences located between the poly (A) signal and PBS domains, and sequences located near the 3' end of the 1-561 RNA (Figures 1 and 7; (26,27)). We therefore synthesized HIV-2 RNAs comprising nucleotides 1-526, 1-541, 1-546, 1-550, and 1-558 to test the effects of these sequences (Figure 1B). After incubation in high potassium/high magnesium dimer buffer at 37° or 55°C, the RNAs were loaded either on a TBM/4°C (Figure 3A), or on a TBE/24°C gel (Figure 3B), respectively. All RNAs formed dimers at 37°C when observed on a TBM/4°C gel. Using antisense oligonucleotides we checked that the sequence engaged in the 37°C-dimerization involves the 5' end of the PBS for all RNAs tested (J.-M.L. & J.S.L., data not shown). When the 55°C-incubated RNAs were assayed for tight dimerization on a TBE/24°C gel, RNAs longer than 550 nucleotides formed very small amounts of dimer compared to RNAs smaller than 546 nucleotides (Figure 3B, compare lanes 1 to 4 to lanes 5 to 7). From this result we conclude that one interfering element is located upstream of nucleotide 550 and that the very different behaviors of 1-546 and 1-550 RNAs indicated that the core of this element may be located between nucleotides 546 and 550.

Influence of 5'-terminal truncations on the putative long-range interaction

Computer-assisted folding of the model 1-561 RNA (26,27) almost always suggested a long-range interaction between nucleotides 189 to 196 and 543 to 550 (Figures 1 and 7). Therefore we hypothesized that truncations at the 5'-end of RNAs terminating at nucleotide 561 should promote tight dimerization if the deletion included nucleotides involved in the putative long-range base pairing. We constructed and tested different RNAs for their ability to dimerize. The 5' ends of these truncated RNAs were at positions 128, 145, 164, 187, and 197, relative to the full length wild type RNA (Figure 1B). As shown in Figure 4A, these RNAs could form dimers when incubated at 37°C in a high potassium/high magnesium buffer and loaded onto a TBM gel run at 4°C. Using antisense oligonucleotides directed against either the PBS or the SL1 sequences, we determined that the 5' end of the PBS was responsible for the dimerization under these conditions (data not shown). However, when these RNAs were loaded onto a TBE/24°C gel, only the 197-561 RNA presented a high yield of dimer (very close to 100%; Figure 4B,

lane 6). Comparison of lanes 1 and 2 to lanes 3, 4, and 5 also indicated that a low level of *in vitro* dimerization/multimerization might be mediated by the TAR and poly (A) signal regions.

Thus, the 5' truncation of the upstream sequence had the same effect on dimerization as the truncation of the putative 3' partner sequence, which strongly suggests that the two sequences are involved in a long-range interaction.

Effects of simultaneous 5' and 3' truncations on dimerization

To ensure that the effect on dimerization of the 5' and 3' truncations was attributable to the loss of the long range interaction between the upstream and downstream RNA elements, we tested the dimerization properties of RNAs harboring truncations at both the 5' and 3' ends (Figure 5). In particular, we compared the dimerization of RNAs 187-550, 187-546, 197-550, and 197-546. As predicted by the truncation experiments described above, the only RNA that did not form TBE stable dimers was the 187-550 RNA which contained both elements involved in the long range interaction. Truncations of either the upstream or downstream element or deletion of both elements at the same time resulted in RNAs that efficiently formed TBE stable dimers, strongly implicating that the loss of the long range interaction, and not an independent effect of the truncations, was responsible for allowing formation of tight dimers.

Site directed mutagenesis and compensatory mutations confirm the long range interaction

The truncation analysis suggested that base pairing between the upstream and downstream elements of the long range interaction inhibited the capacity to form tight dimers. To explicitly test this, we introduced mutations into both sequences, either independently or together (Figure 5). The results clearly demonstrated that interfering with base pairing between the upstream and downstream elements allowed formation of tight dimers, while restoration of base pairing by swapping the 5' and 3' sequences resulted in RNAs that were compromised in their ability to form tight dimers.

Kinetic influence of the 3'-terminal sequence

Even though all RNAs smaller than 546 nucleotides were able to form a high level of tight dimer after an hour incubation at 55°C, the quantification of two to three independent experiments indicated that their behavior was different. The yield of tight dimerization decreased when more sequence was present at the 3' end of the RNA beyond position 444 (Figure 3B, lanes 1 to 4, and Figure 3C). We therefore investigated whether the presence of nucleotides downstream of SL1 influenced the rate of tight dimerization.

We performed kinetic analysis of tight dimerization at different incubation temperatures (50°, 55°, or 60°C) for 1-444, 1-526, 1-541, and 1-546 RNAs, assayed on a TBE/24°C gel. To aid in the direct comparison of this experiment with results of a previous study (16) we used a low sodium/high magnesium dimerization buffer. Results obtained using high potassium/high magnesium dimerization buffer were very similar (data not shown). A typical experiment is shown in Figure 6A (1-526 RNA at 55°C) and in Figure 6B, the dimerization kinetics at 55°C of 1-444, 1-526, 1-541 and 1-546 RNAs are represented. To determine the dimerization rate constant, the data were fitted using a second order reaction model (see Materials and Methods). At 55°C the dimerization rate of the 1-444 RNA was two to three-fold faster than the other RNAs (Figure 6B and 6C)). Analysis of the dimerization rates at different temperatures revealed two interesting results. First, the 1-444 RNA dimerized more rapidly at every temperature tested (50, 55, and 60°C, Figure 6C) than any other RNA. Second, the dimerization rates of all RNAs increased with temperature, approximately doubling with each 5°C increase in temperature (Figure 6C).

Thus, the 3'-terminal location of the SL1 structure in the 1-444 RNA corresponded to a positive kinetic effect on the rate of tight dimer formation. The presence of nucleotides downstream of the SL1 structure negatively affected the ability of large RNAs to form tight dimers *in vitro*, probably by forcing/sequestering all or part of SL1 into a more stable intramolecular structure. This inhibition of the use of SL1 for tight dimerization was maximal when the RNA was 550 nucleotides or longer.

Effect of nucleocapsid protein on dimerization

Since the genomic RNA interacts with the Gag precursor polyprotein predominantly through its nucleocapsid domain during encapsidation and later steps, we wished to examine the effects of nucleocapsid protein (NCp7) on the dimerization of various RNA constructs. We used HIV-1 NCp7 protein which was kindly provided by J. Marino and purified as described (24). First, we verified that the incubation at 37°C of HIV-1 1-290 RNA with NCp7 yielded the same amount of TBE-resistant dimer as incubation at 55°C without NCp7 (Figure 7A, and (25)). Second, 1-444 and 1-561 HIV-2 RNAs were incubated with or without NCp7. Since there is an obligatory SDS/phenol extraction to remove NCp7 from the RNA before loading on the gel, we also included controls to show that this extraction step did not influence RNA dimerization (Figure 7B, compare lanes 2, 3, 10, and 11, to 4, 5, 12, and 13, respectively). There was a low yield of dimer of 1-561 RNA with a 55°C incubation temperature. We showed previously that these dimers were not sensitive to the antisense oligonucleotides directed against PBS or SL1 dimer-inducing elements (17), and they appear from results in Figure 4B to be TAR-TAR dimers (compare lanes 1 and 2 to 3, 4, and 5).

When the 1-444 RNA was incubated with NCp7 at 37°C, and SDS/phenol extracted, the level of tight dimer was almost as high as when this RNA was incubated in dimer buffer at 55°C (Figure 7B, compare lane 6 to 8, and Figure 7C). The level of 1-561 tight dimer reached 45% in presence of NCp7 (Figure 7B, lane 13, and Figure 7C), which is three-fold higher than a protein-free dimerization at 37°C (Figure 7B, lane 12) or two-fold higher than a 55°C dimerization (Figure 7B, lane 16). Thus, incubation of the 1-561 RNA with the mature form of the nucleocapsid protein promotes the use of the SL1 and allows the formation of stable tight dimer, although the presence of sequence downstream of the SL1 structure may reduce the yield *in vitro*.

To verify that NCp catalyzed the formation of tight dimers through SL1 and not through another point of contact, we repeated the dimerization assay using NCp with wild type 1-444 RNA and 1-444 RNA harboring a deletion in the loop of SL1 (22). The results of this experiment are presented in Figure 7C. Nucleocapsid protein efficiently converted wild type 1-444 RNA to tight dimers at 37°C (lanes 1-3), but failed to convert the SL1 mutant 1-444 RNA to tight dimers (lanes 4-6). This result strongly suggests that the principal tight dimerization site is at SL1.

Computer-assisted folding of the 1-561 HIV-2 RNA

The secondary structure model of the 1-561 RNA in Figure 8 shows a possible secondary structure arrangement that maintains the long range interaction and simultaneously makes the SL1 sequence unavailable for intermolecular base pairing. The model was constructed using Mfold 3.0 software with two local constraints (26,27). First, nucleotides 34 to 39 were kept single-stranded, so that the TAR domain would adopt the empirically determined bifurcated conformation and not the thermodynamically more stable conformation (28,29). Second, preliminary results of RNA chemical probing experiments indicated that nucleotides A423 and A426 located in the loop of SL1 are not accessible to chemical probes specific for single-stranded nucleotides when the 1-561 RNA is incubated at 55°C, but are susceptible to chemical modification when mutations are present in the RNA that allow SL1-mediated dimerization (J.D.I. & J.S.L., data not shown). Thus, for this model we constrained the nucleotides A423

and A426 to be double-stranded, although strictly speaking the loss of reactivity could also be due to base stacking. The resulting most stable structure is shown in Figure 8. Although this model was constructed using additional constraints based on structural data for the TAR and the 423+426 regions, the long-range interaction we propose is also found when the 1-561 RNA is folded without constraint. It is notable that other nearly isoenergetic Mfold structures depict SL1 in its 'classic' stem-loop structure, suggesting that this region may be able to change conformations without a large thermodynamic cost.

DISCUSSION

In this study we analyzed the *in vitro* dimerization properties of HIV-2 leader RNA fragments, focusing especially on the use of two previously described dimer-inducing elements: the 5' end of the primer-binding site (PBS) and the stem-loop 1 structure (SL1) (Figure 1) (9,17,19). We showed that an HIV-2 leader RNA could use the 5' end of the PBS as a loose dimer-inducing element at physiological (37°C) or high (55°C) incubation temperature. This dimerization is a salt-dependent process: the optimal dimerization buffer contains 300 mM potassium and 5 mM magnesium, while our monomer buffer contains 40 mM potassium and 0.1 mM magnesium. It is interesting to note that the concentration of intracellular potassium was found to be higher in HIV-1 infected T cells than in non-infected cells (200-400 and 100-130 mM, respectively (30)). This difference may positively influence HIV-2 RNA dimerization *in vivo*. The PBS-PBS interaction in 1-444 and 1-561 RNA dimers is characteristic of a loose dimer, as described for the HIV-1 *Lai* SL1-SL1 interaction (10,11) and may indicate the involvement of a magnesium-dependent tertiary structure element(s). A similar feature has been described for HIV-1 *Mal* RNA dimerization (31,32).

In a previous paper we showed that the nature and position of the 3' end of the construct and, to a lesser degree the dimerization protocol, influenced the use of the SL1 dimer-inducing element in the HIV-2 leader sequence (17). The structure and the location of this element are homologous to those of the HIV-1 SL1, which is the element that mediates dimerization of HIV-1 RNA fragments *in vitro* (5-7,13,14,33,34). However the HIV-2 SL1 sequence does not mediate the formation of loose dimers *in vitro*, either as an isolated structure (9) or as a structural element in the 1-561 RNA (17). In contrast, the 1-444 RNA was uniquely able to form SL1-dependent tight dimers upon incubation at high temperature (17,19). These tight dimers are defined by their resistance to dissociation during electrophoresis at room temperature and in the presence of EDTA. The tight dimer behavior of small HIV-1 or HIV-2 RNAs is thought to be caused by an extended base pairing arrangement of SL1 (first proposed in (6,33,34) and further analyzed in (10,11,15)). The 1-444 RNA is well suited to forming a tight dimer probably because the SL1 dimer-inducing element is located at the very 3' end of this fragment (Figure 1).

Here we demonstrate that large HIV-2 RNAs encompassing the complete leader region cannot efficiently form tight dimers *in vitro*. Indeed, the low level of tight dimer we observed with the 1-561 RNA is probably not mediated by SL1 based on two lines of evidence. First, these dimers are not inhibited by an antisense oligonucleotide directed against the SL1, to which the 1-444 RNA tight dimers are very sensitive (17). Second, as pointed out by Laughrea *et al.*, some stem-loops may have a melting temperature close to the 55°C incubation temperature and could occasionally switch from intrastrand to interstrand base pairing (35). This might also be the case for HIV-2, since for example, the level of tight dimer was lower when the TAR and poly (A) signal structures were missing (Figure 4B, compare lanes 1 and 2 to 3, 4, and 5).

Using RNAs truncated at their 5' or 3' ends and using compensatory site-directed mutagenesis, we found that two elements, between nucleotides 189-196 and 543-550, prevent the formation of SL1-dependent tight dimers of HIV-2 leader RNA. A similar study by Dirac *et al.*

approximated the border of an upstream dimer-interfering element to between nucleotides 507 and 527, since they did not observe tight dimerization for RNAs longer than 527 nucleotides (16). There are several experimental differences that could explain the discrepancy between their results and our own. First, the dimerization protocols are somewhat different (a thorough comparison was published in (17)). Briefly, in the present study the denatured RNA was incubated at a constant temperature of 55°C to induce tight dimerization; the Dirac *et al.* study used a slow-cooling protocol from 65°C to room temperature. Second, in our hands, the optimal dimerization yield with 1-444 RNA was higher (compare (16,19) and this manuscript). In order to resolve this discrepancy we analyzed the kinetics of tight dimer formation for the RNAs that were able to form a substantial level of tight dimer at 50°, 55°, or 60°C in Dirac dimer buffer (*i.e.* RNAs 1-444, 1-526, 1-541, and 1-546). We observed that sequences downstream of SL1 influence the tight dimerization through a decrease of the dimerization rate. Thus we think the difference in tight dimerization yield between the 1-507 and 1-527 RNAs seen by Dirac *et al.* is due to the additive effects of differences in the overall dimerization levels and the kinetic influence of sequences upstream of nucleotide 546 on the SL1-dependent dimerization.

Effects of sequences upstream and downstream of the major splice donor site on the dimerization process have been demonstrated in HIV-1 as well. The HIV-1 *Mal* isolate SL1-dependent dimerization is influenced by sequences located downstream of SL1. The thermal stability and yield of the dimers for a large RNA in presence of the downstream sequences were increased by 10°C and 18%, respectively (34). A similar region in HIV-1 *Lai* isolate increased the tight dimerization rate by a factor of 2 to 3 but did not change the thermal stability of the SL1-dependent dimers (36). Thus, the downstream sequences of the leader RNA in HIV-1 and HIV-2 have opposite effects on SL1-mediated dimerization. There is a strong negative influence of sequences downstream (and upstream) of the major splice donor site on the SL1-mediated HIV-2 RNA dimerization. This is reminiscent of the interference between non-native SL1 loop sequence and 3'-Dimer Linkage Structure in the *in vivo* replication and *in vitro* dimerization of HIV-1 *Lai* RNA (35,37).

Our data suggest that a long-range base pairing interaction prevents the use of the SL1 sequence for tight dimerization. We believe there is competition between intramolecular and intermolecular interactions of the SL1 structure of HIV-2 leader RNA. An example of an energetically favorable secondary structure in which SL1 is sequestered intramolecularly is shown in Figure 8. Base pairing between nucleotides 189-196 and 543-550 favors an intramolecular folding of the SL1 sequence, preventing its use in an intermolecular SL1-SL1 interaction. We cannot formally rule out the possibility of a direct interaction between the upstream and downstream interfering elements and the SL1 sequence. However, computer-assisted folding of the 1-561 RNA could not demonstrate such an interaction ((26,27); J.-M.L. & J.S.L., data not shown). Moreover, analysis of HIV-2 and SIV aligned sequences in the HIV Sequence Compendium 2001 showed a perfect conservation of all nucleotides involved in the long-range base pairing (38). Although the *gag* initiation and subsequent codons are highly conserved because of the selective pressure on translation and post-translational modifications of the Gag protein, it is interesting to find 100% conservation in the 189-196 region. The only role so far suggested for this sequence may be as part of the downstream GU/U-rich element cis-acting signal required for mammalian polyadenylation (39).

It appears significant that one of the sequences involved in the long-range interaction proposed here includes the *gag* initiation codon. Experiments from the Lever group showed that HIV-2 selects its genomic RNA for encapsidation cotranslationally (20). Two elements seem to mediate this activity: the nucleocapsid domain of the Gag protein and the 380-408 sequence located just upstream of the SL1 sequence in the leader RNA region, called the encapsidation signal or Ψ (Figure 8) (20). Mutagenesis studies have shown that retroviral encapsidation and dimerization elements overlap *in vivo* (for a review, see (40)). This functional overlap may be

explained by a structural overlap. Computer-assisted folding of the 1-561 RNA without any constraint showed two main foldings for the SL1 domain: either as an individual stem-loop structure, as described in Berkhout *et al.* (29), or involved in a secondary structure with the core HIV-2 RNA encapsidation signal Ψ (Figure 8). Since the initiating ribosome complex reads the initiation codon of *gag* and surrounding nucleotides, it may disrupt the long-range interaction and allow conformational changes of the leader RNA region. After translation of the nucleocapsid domain, this domain may preferentially interact with its own messenger RNA and promote more extensive conformational changes thus modulating dimerization and encapsidation. In fact, evidence along these lines has been presented *in vitro* for the Gag precursor (41-43) and for the mature nucleocapsid protein (5,18,24,25,41,44). We showed in this study that the nucleocapsid protein is able to promote the formation of SL1-dependent HIV-2 RNA tight dimers *in vitro*. It would be of interest to determine the conformation of the core encapsidation and dimerization sequences prior to and after translation or encapsidation *in vivo*. A similar relationship between translation and encapsidation has been recently shown in HIV-1, even though the cis-preference of the Gag protein for encapsidation of its own messenger RNA was not absolute and could be overcome (42,43).

Although there are many questions that remain to be answered, it is already clear that the 5' leader region of HIV-2 and other retroviral RNAs is replete with functionality. Furthermore, the emerging view of the complex and switchable structure variations possible *in vitro* in the 5' leader region strongly implicates it as a key *regulatory* element *in vivo*. The complete characterization of dimerization, encapsidation as well as other regulatory roles for this region will require continued dissection of these closely linked processes using a variety of *in vitro* and *in vivo* techniques.

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References

1. Bender W, Davidson N. Cell 1976;7:595–607. [PubMed: 182376]
2. Hoglund S, Ohagen A, Goncalves J, Panganiban AT, Gabuzda D. Virology 1997;233:271–9. [PubMed: 9217051]
3. Laughrea M, Shen N, Jette L, Darlix JL, Kleiman L, Wainberg MA. Virology 2001;281:109–16. [PubMed: 11222101]
4. Fu W, Gorelick RJ, Rein A. Journal of Virology 1994;68:5013–5018. [PubMed: 8035501]
5. Darlix JL, Gabus C, Nugeyre MT, Clavel F, Barre-Sinoussi F. J Mol Biol 1990;216:689–99. [PubMed: 2124274]
6. Laughrea M, Jette L. Biochemistry 1994;33:13464–74. [PubMed: 7947755]
7. Skripkin E, Paillart JC, Marquet R, Ehresmann B, Ehresmann C. Proc Natl Acad Sci U S A 1994;91:4945–9. [PubMed: 8197162]
8. McBride MS, Panganiban AT. J Virol 1996;70:2963–73. [PubMed: 8627772]
9. Jossinet F, Lodmell JS, Ehresmann C, Ehresmann B, Marquet R. J Biol Chem 2001;276:5598–604. [PubMed: 11092889]
10. Laughrea M, Jette L. Biochemistry 1996;35:1589–98. [PubMed: 8634290]
11. Muriaux D, Fosse P, Paoletti J. Biochemistry 1996;35:5075–82. [PubMed: 8664300]
12. Shubsda MF, McPike MP, Goodisman J, Dabrowiak JC. Biochemistry 1999;38:10147–57. [PubMed: 10433723]

13. Clever JL, Wong ML, Parslow TG. *J Virol* 1996;70:5902–8. [PubMed: 8709210]
14. Haddrick M, Lear AL, Cann AJ, Heaphy S. *J Mol Biol* 1996;259:58–68. [PubMed: 8648648]
15. Paillart JC, Skripkin E, Ehresmann B, Ehresmann C, Marquet R. *Proc Natl Acad Sci U S A* 1996;93:5572–7. [PubMed: 8643617]
16. Dirac AM, Huthoff H, Kjems J, Berkhout B. *Nucleic Acids Res* 2002;30:2647–55. [PubMed: 12060681]
17. Lanchy JM, Lodmell JS. *J Mol Biol* 2002;319:637–48. [PubMed: 12054860]
18. Takahashi K, Baba S, Koyanagi Y, Yamamoto N, Takaku H, Kawai G. *J Biol Chem* 2001;276:31274–8. [PubMed: 11418609]
19. Dirac AM, Huthoff H, Kjems J, Berkhout B. *J Biol Chem* 2001;276:32345–52. [PubMed: 11397802]
20. Griffin SD, Allen JF, Lever AM. *J Virol* 2001;75:12058–69. [PubMed: 11711596]
21. Gibbs JS, Regier DA, Desrosiers RC. *AIDS Res Hum Retroviruses* 1994;10:343–50. [PubMed: 8068414]
22. Lanchy JM, Lodmell JS. *J Mol Biol* 2002;319:637–648. [PubMed: 12054860]
23. Marquet R, Paillart JC, Skripkin E, Ehresmann C, Ehresmann B. *Nucleic Acids Res* 1994;22:145–51. [PubMed: 8121797]
24. Rist MJ, Marino JP. *Biochemistry* 2002;41:14762–14770. [PubMed: 12475224]
25. Muriaux D, De Rocquigny H, Roques BP, Paoletti J. *J Biol Chem* 1996;271:33686–92. [PubMed: 8969239]
26. Mathews DH, Sabina J, Zuker M, Turner DH. *J Mol Biol* 1999;288:911–40. [PubMed: 10329189]
27. Zuker, M.; Mathews, DH.; Turner, DH. *RNA Biochemistry and Biotechnology*. Barciszewski, J.; Clark, BFC., editors. Kluwer Academic Publishers; 1999. p. 11–43.
28. Rhim H, Rice AP. *Virology* 1994;202:202–11. [PubMed: 8009832]
29. Berkhout B, Schoneveld I. *Nucleic Acids Res* 1993;21:1171–8. [PubMed: 8464701]
30. Voss TG, Fermin CD, Levy JA, Vigh S, Choi B, Garry RF. *J Virol* 1996;70:5447–54. [PubMed: 8764056]
31. Jossinet F, Paillart JC, Westhof E, Hermann T, Skripkin E, Lodmell JS, Ehresmann C, Ehresmann B, Marquet R. *RNA* 1999;5:1222–34. [PubMed: 10496223]
32. Paillart JC, Westhof E, Ehresmann C, Ehresmann B, Marquet R. *J Mol Biol* 1997;270:36–49. [PubMed: 9231899]
33. Muriaux D, Girard PM, Bonnet-Mathoniere B, Paoletti J. *J Biol Chem* 1995;270:8209–16. [PubMed: 7713927]
34. Paillart JC, Marquet R, Skripkin E, Ehresmann B, Ehresmann C. *J Biol Chem* 1994;269:27486–93. [PubMed: 7961663]
35. Laughrea M, Jette L. *Biochemistry* 1997;36:9501–8. [PubMed: 9235995]
36. Laughrea M, Jette L. *Biochemistry* 1996;35:9366–74. [PubMed: 8755714]
37. Laughrea M, Shen N, Jette L, Wainberg MA. *Biochemistry* 1999;38:226–34. [PubMed: 9890902]
38. Kuiken, C.; Foley, B.; Hahn, B.; Marx, P.; McCutchan, F.; Mellors, J.; Wolinsky, S.; Korber, B. *HIV Sequence Compendium 2001*. Theoretical Biology and Biophysics Group, Los Alamos National Laboratory; 2001.
39. Levitt N, Briggs D, Gil A, Proudfoot NJ. *Genes Dev* 1989;3:1019–25. [PubMed: 2570734]
40. Greutorex J, Lever A. *J Gen Virol* 1998;79:2877–82. [PubMed: 9880000]
41. Damgaard CK, Dyhr-Mikkelsen H, Kjems J. *Nucleic Acids Res* 1998;26:3667–76. [PubMed: 9685481]
42. Feng YX, Campbell S, Harvin D, Ehresmann B, Ehresmann C, Rein A. *J Virol* 1999;73:4251–6. [PubMed: 10196321]
43. Zeffman A, Hassard S, Varani G, Lever A. *J Mol Biol* 2000;297:877–93. [PubMed: 10736224]
44. Lapadat-Tapolsky M, Pernelle C, Borie C, Darlix JL. *Nucleic Acids Res* 1995;23:2434–41. [PubMed: 7543198]

Figure 1.

The 5' leader region of HIV-2 ROD genomic RNA. (A) The landmark sequences with known functions are indicated by boxes. TAR, polyA signal, PBS, SL1, SD, and *gag* represent the trans-activation region, the poly (A) signal domain, the primer-binding site, the stem loop 1, the major splice donor site, and the 5' end of the Gag protein coding region, respectively. (B) RNAs used in this study. The closed boxes represent the two dimer-inducing elements, namely the 5' end of the PBS and SL1. The open boxes represent the cores of the two dimer-interfering elements characterized in this study, 189-196 and 543-550. The numbers to the left and right indicate the 5' and 3' termini of the various RNAs used here, respectively.

Figure 2.

Differential dimerization properties of 1-444 and 1-561 RNAs. 1-444 and 1-561 RNAs were assayed for dimerization with monomer (M) or dimer (D) buffer at 37° or 55°C. After 30 minutes incubation, duplicates were subjected to electrophoresis on agarose gels. (A) Electrophoresis was carried out in TBM buffer at 4°C. (B) Electrophoresis was carried out in TBE buffer at 24°C. The two different electrophoresis conditions allow comparison of the stability of the dimers. Loose and tight dimers are observed on TBM gels, but only tight dimers withstand the TBE electrophoresis.

Figure 3.

Dimerization of RNAs harboring 3' truncations. RNAs were incubated in high potassium/high magnesium dimer buffer and duplicates were subjected to electrophoresis on agarose gels. The number below each lane indicates the final 3' nucleotide. (A) Electrophoresis was carried out in TBM buffer at 4°C. (B) Electrophoresis was carried out in TBE buffer at 24°C. (C) Percent dimerization for each RNA when assayed on a TBM/4°C gel (stippled bars) or TBE/24°C gel (filled bars). The y-axis error bars represent the standard deviation of replicate experiments.

Figure 4.
Dimerization of RNAs harboring 5' truncations. The number under each lane indicates the 5' terminus for each RNA; the 3' terminus of each RNA was position 561. RNAs were incubated in high potassium/high magnesium dimer buffer and duplicates were subjected to agarose electrophoresis. (A) Electrophoresis was carried out in TBM buffer at 4°C. (B) Electrophoresis was carried out in TBE buffer at 24°C.

Figure 5.
Dimerization of RNAs harboring simultaneous 5' and 3' deletions or mutations in the sequences involved in the long range interaction. (A) Schematic of the sequences involved in the long range interaction for all of the RNAs used in panel B. In the left column the 5' and 3' truncations used for lanes 1-8 of panel B are shown. The right column shows the strategy employed to disrupt and restore base pairing between the upstream and downstream long range interaction elements. 187-550 Cg shows the wild type interaction; in the 187-550 gg mutant, the wild type upstream element has been replaced with an inverted copy of the downstream element, which destroys base pairing; in the 187-550 CC mutant, the downstream element has been replaced with an inverted copy of the upstream element, again destroying base pairing between the two elements; in the 187-550 gC compensatory mutant, the upstream and downstream inverted elements were swapped, effectively restoring potential base pairing between the elements. (B) Results of dimerization experiments conducted with the RNAs described in panel A. Lanes 1-4 and 9-13 show denatured monomeric RNA. On the left side of the gel, lanes 5-8 demonstrate that only RNA 187-550 (lane 5), containing both the upstream and downstream element is not capable of efficient tight dimerization. The combinations of truncated RNAs that lack either or both long range interaction elements dimerize very efficiently (lanes 6-8). On the right side of the gel, the wild type and 187-550gC RNAs, which are capable of forming base pairs between the upstream and downstream elements, do not

dimerize efficiently (lanes 13 and 16), while the 187-550 gg and CC mutants dimerize completely (lanes 14 and 15).

Figure 6.
Kinetics of tight dimerization of 1-444, 1-526, 1-541, and 1-546 RNAs at different temperatures. (A) The time dependent yield of dimerization of 1-526 RNA at 55°C was monitored on a TBE/24°C agarose gel. Lanes 1 to 16 represent incubation times of 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 minutes, respectively. (B) Plots of the kinetic data for RNAs 1-444, 1-526, 1-541, and 1-546. $[M]_t$ is the concentration of monomer at time t and $[M]_0$ is the initial concentration of dimerization-competent monomer. (C) Kinetic constants, k_{dim} , were obtained by fitting the data to a second order reaction model (23). The resulting dimerization rates are represented for the different RNAs and incubation temperatures. Open, stippled, and filled bars correspond to the 50°, 55°, and 60°C incubation temperatures, respectively. The y-axis error bars represent the standard deviation of replicate experiments.

Figure 7.
Influence of nucleocapsid protein on the dimerization of 1-444 and 1-561 RNAs. (A) HIV-1 *Lai* 1-290 RNA was incubated for 30 minutes at 37° or 55°C with or without HIV-1 nucleocapsid protein NCp7 in monomer buffer (50 mM Tris-HCl pH7.5, 40 mM potassium and 0.1 mM magnesium). After SDS/phenol extraction, the samples were loaded on a TBE/

24°C gel. (B) HIV-2 *ROD* 1-444 (left panel) and 1-561 (right panel) RNAs were incubated as in (A). Two supplementary lanes showed incubation of the RNAs in dimer buffer (high potassium/high magnesium) without NCp7 at 37°C (lanes 7 and 15) or 55°C (lanes 8 and 16). SDS/phenol extracted samples are boxed. (C) 1-444 wild type (lanes 1-3) or 1-444 with the GUAC sequence deleted from the apical SL1 loop (lanes 4-6) were dimerized at 37°C with or without NCp, or at 55°C without NCp, as indicated. The RNAs lacking an intact SL1 were incapable of forming a TBE stable tight dimer. (D) A plot of dimerization yield as a function of SDS/phenol extraction and presence of NCp7 at 37°C for the 1-444 (open bars) and 1-561 (filled bars) RNAs. The y-axis error bars represent the standard deviation of two experiments.

Figure 8.

Secondary structure model of the HIV-2 ROD leader RNA. The model was generated using M-fold (26,27) and represents the most stable secondary structure returned for the 1-561 RNA that also agrees with the biochemical analysis (see Results). The proposed long-range base pairing between nucleotides 189-196 and 543-550 is indicated at the left of the figure. The *gag* initiation codon is indicated by the solid horizontal bar below the sequence. ψ represents the core encapsidation signal of HIV-2 genomic RNA (nucleotides 380 to 408) defined by deletion mutagenesis (20). Thick lines indicate the PBS and the entire SL1 sequence (nucleotides 409 to 439; (29)).

Table 1.

Oligonucleotides used in this study. The 5' to 3' sequence is indicated from left to right.

sBAMT7LAI	TAG GAT CCT AAT ACG ACT CAC TAT AGG TCT CTC TGG TTA GAC C
sBAMT7R	TAG GAT CCT AAT ACG ACT CAC TAT AGG TCG CTC TGC GGA GAG
sBAMT7128	TAG GAT CCT AAT ACG ACT CAC TAT AGG CTT GCT TGC TTA AAA ACC TC
sBAMT7145	TAG GAT CCT AAT ACG ACT CAC TAT AGG CCT CTT AAT AAA GCT GCC AG
sBAMT7164	TAG GAT CCT AAT ACG ACT CAC TAT AGG TTA GAA GCA AGT TAA GTG
sBAMT7187	TAG GAT CCT AAT ACG ACT CAC TAT AGG CTC CCA TCT CTC CTA GTC
sBAMT7197	TAG GAT CCT AAT ACG ACT CAC TAT AGG TCC TAG TCG CCG CC
sBAMT7GEE	ATG GAT CCT AAT ACG ACT CAC TAT AGG CTG GGT AGA GTC CTA GTC GCC GCC TGG
asECOCEE	TTG AAT TCG GGT AGA GCC ACA ATC TTC TAC CTG TC
asECOLAI	AAG AAT TCC AGT CGC CGC CCC
asECO444	AAG AAT TCG CTC CAC ACG CTG CCT TTG
asECO526	AAG AAT TCG TCT AAA GGT AGG ATA G
asECO541	AAG AAT TCA CAA TCT TCT ACC TG
asECO546	AAG AAT TCT CCC ACA ATC TTC TAC C
asECO550	AAG AAT TCC CAT CTC CCA CAA TC
asECO558	AAG AAT TCT CGC GCC CAT CTC CC
asECO561	AAG AAT TCA GTT TCT CGC GCC CAT CTC CC