The leader of the HIV-1 RNA genome forms a compactly folded tertiary structure

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ABSTRACT

The untranslated leader of the RNA genome of the human immunodeficiency virus type 1 (HIV-1) encodes multiple signals that regulate distinct steps of the viral replication cycle. The RNA secondary structure of several replicative signals in the HIV-1 leader is critical for function. Well-known examples include the TAR hairpin that forms the binding site for the viral Tat *trans*-activator protein and the DIS hairpin that is important for dimerization and subsequent packaging of the viral RNA into virion particles. In this study, we present evidence for the formation of a tertiary structure by the complete HIV-1 leader RNA. This conformer was recognized as a fast-migrating band on nondenaturing polyacrylamide gels, and such a migration effect is generally attributed to differences in compactness. Both the 5' and 3' domains of the 335-nt HIV-1 leader RNA are required for the formation of the compact RNA structure, and the presence of several putative interaction domains was revealed by an extensive analysis of the denaturing effect of antisense DNA oligonucleotides. The buffer conditions and sequence requirements for conformer formation are strikingly different from that of the RNA-dimerization reaction. In particular, the conformer was destabilized in the presence of Mg²⁺ ions and by the viral nucleocapsid (NC) protein. The presence of a stable RNA structure in the HIV-1 leader was also apparent when this RNA was used as template for reverse transcription, which yielded massive stops ahead of the structured leader domain. Formation of the conformer is a reversible event, suggesting that the HIV-1 leader is a dynamic molecule. The putative biological function of this conformational polymorphism as molecular RNA switch in the HIV-1 replication cycle is discussed.

Keywords: HIV-1 RNA genome; nondenaturing gel electrophoresis; retrovirus replication; RNA tertiary structure

INTRODUCTION

The untranslated leader region of a retroviral RNA genome contains multiple signals that control distinct steps of the viral life cycle. Motifs found in most retroviral species are signals that control mRNA splicing, dimerization and packaging, and reverse transcription (reviewed in Berkhout, 1996; Berkowitz et al., 1996). At the molecular level, these functions are executed by specific RNA–protein and RNA–RNA interactions of the leader RNA, for example, with the viral Gag precursor protein during packaging and with a specific tRNA molecule that acts as primer for reverse transcription.

The leader RNA of the human immunodeficiency viruses (HIV-1 and HIV-2) and the human T-cell leukemia viruses (HTLV-I and HTLV-II) seem particularly

complex because they encode additional regulatory motifs in the R (repeat) region that forms the 5' end of the leader, an exact copy of which is present at the extreme 3' end of the viral genome. The HTLV-I R region encodes the highly structured RexRE element that forms the binding site for the Rex regulator of splicing, and this structured RNA motif is also involved in regulated polyadenylation (Seiki et al., 1983; Ahmed et al., 1991). A secondary structure model of the HIV-1 leader RNA is shown in Figure 1. The HIV-1 RNA encodes two critical stem-loop structures in the R region (position +1/97). The TAR hairpin structure is essential for up-regulation of viral transcription by the Tat *trans*-activator protein (Muesing et al., 1985; Berkhout et al., 1989; Klaver & Berkhout, 1994a; Wei et al., 1998). The immediately adjacent poly(A) hairpin contains the AAUAAA polyadenylation signal. This structure is critical for repression of this signal in the 5' R of the leader, but the same signal is efficiently used in 3' R to provide HIV-1 RNAs with a regular poly(A) tail (Berkhout et al., 1995; Das et al., 1997, 1999; Klasens et al., 1998, 1999b).

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FIGURE 1. RNA secondary structure model of the untranslated leader RNA of HIV-1 isolate LAI and the ability of different leader fragments to form the aberrantly migrating conformer. The RNA secondary structure model was adapted from Berkhout (1996). Several regulatory RNA motifs shown in the RNA structure model are discussed in the text. All hairpin motifs have been named after their putative function in HIV-1 replication and/or the sequence elements encoded by them. Highlighted with a grey box are the following sequence motifs: the polyadenylation signal at position 73, the PBS at position 181, the DIS palindrome at position 257, and the Gag startcodon at position 336. The DIS hairpin with the palindromic loop sequence GCGCGC (or GC3) was changed into GC in the GC1 mutant, which is indicated in the insert above the structure model. The major splice donor site at position 290 is marked by a small arrow. The 5' end of the normal HIV-1 RNA is at position +1. Other start positions are circled (positions 105 and 240), and the 3' ends of the HIV-1 transcripts are marked with an open square (positions 134, 202, 245, 290, and 368). The bottom panel depicts the different HIV-1 transcripts used in this study and their capacity to form the fast-migrating conformer. The transcript lengths are not drawn to scale, but they align with the respective positions in the secondary structure model of the HIV-1 leader.

Sequences downstream of position 97 in the HIV-1 leader RNA are not part of the R region and are thus uniquely present at the 5' end of the HIV-1 genome (Fig. 1). The PBS region forms a complex RNA structure that influences the binding of the tRNA^{lys3} primer to the primer-binding site (PBS) and its subsequent elongation by the viral RT enzyme (Das et al., 1995; Isel et al., 1995; Wakefield et al., 1995; Whitcomb et al., 1995; Arts & Wainberg, 1996). The dimer initiation site (DIS) has been implicated both in RNA dimerization

through base pairing of the palindromic loop sequences and in RNA packaging (Skripkin et al., 1994; Berkhout & van Wamel, 1996b; Haddrick et al., 1996; Grootorex & Lever, 1998; Laughrea et al., 1999). The SD hairpin contains the major splice donor signal (Purcell & Martin, 1993), and the Ψ hairpin is a key component of the packaging signal (Lever et al., 1989; Aldovini & Young, 1990; Clavel & Orenstein, 1990), although several of the upstream leader motifs have been suggested to contribute to RNA packaging (Berkhout & van Wamel,

1996b; McBride & Panganiban, 1996; Clever & Parslow, 1997; Das et al., 1997, 1998; Clever et al., 1999; Helga-Maria et al., 1999).

There is ample evidence from biochemical, phylogenetic, and mutational studies for the folding of individual HIV-1 RNA hairpin motifs like TAR, poly(A), the PBS region, DIS, and the downstream hairpins (reviewed in Berkhout, 1996). Furthermore, some of the individual hairpins like TAR, DIS, and Ψ have been studied recently by biophysical methods like NMR (Calnan et al., 1991; Puglisi et al., 1992; Aboul-ela et al., 1995; Dardel et al., 1998; De Guzman et al., 1998; Mujeeb et al., 1998). A few models have been proposed for the overall secondary structure of the complete leader RNA of HIV-1 (Rizvi & Panganiban, 1993; Berkhout, 1996) and the related retrovirus HIV-2 (Berkhout, 1996), but it is likely that the specific interaction of the leader RNA with proteins and RNA is governed by the tertiary structure of this transcript, for which there is currently very little information available. One piece of evidence concerns the ultrastructure of the virion-associated RNA dimer as observed by electron microscopy (Hoglund et al., 1997), but this conformation may not reflect the structure of the intracellular

HIV-1 RNA transcript. In this study, we present evidence from in vitro studies that the leader RNA of HIV-1 adopts a compactly folded structure that can be identified as a fast-migrating band on nondenaturing RNA gels.

RESULTS

Unusual migration of HIV-1 leader RNA in nondenaturing gels

Transcripts mimicking the HIV-1 leader RNA were synthesized by T7 RNA polymerase. The set of RNAs used in this study is schematically depicted in Figure 1. Position +1 is the regular 5' end of HIV-1 transcripts, and 3' ends were chosen at positions 134, 202, 245, 290, and 368. This nested set of radiolabeled transcripts migrated at the expected position on a denaturing sequence gel (Fig. 2A, lanes 6–10). A progressive shift in gel mobility was also observed for a second set of HIV-1 transcripts that initiate at the upstream position –54, but with the same nested 3' ends (Fig. 2A, lanes 1–5). We next analyzed these two sets of HIV-1 transcripts on a nondenaturing polyacrylamide gel

FIGURE 2. Differential migration of HIV-1 leader RNA fragments on denaturing versus nondenaturing polyacrylamide gels. **A:** The ^{32}P -labeled T7 transcripts were denatured in formamide-containing sample buffer and separated on a 6% sequencing gel with urea. The transcripts are identified at the top of the lanes. **B,C:** The same set of RNAs analyzed on the denaturing gel in **A** were diluted in cacodylate buffer, mixed with nondenaturing sample buffer (glycerol and BFB dye) and split in three aliquots. The samples were either put on gel directly (lanes 1, 4, 7, 10, 13), heated for 5 min at 85°C and put on ice (lanes 2, 5, 8, 11, 14), or the same denaturing protocol was used, and then an incubation for 30 min at 37°C (lanes 3, 6, 9, 12, 15). We used a nondenaturing 4% polyacrylamide gel and electrophoresis was performed in 0.25× TBE. The transcripts with an unusually fast gel mobility are marked with an asterisk.

(Fig. 2B,C). Surprisingly, the 3'-extended transcripts ending at position 290 and 368 exhibited an unusually fast migration, suggesting the formation of a compactly folded conformer. The position of the fast-migrating RNAs is marked by an asterisk. This shift in gel migration appears rather dramatic. For instance, the +1/290 transcript runs much faster through the gel than the shorter +1/245 transcript, and its migration approaches that of the +1/202 transcript. This phenomenon would have been missed easily if we had not performed the analysis with the extended set of transcripts of increasing length, as there are no absolute markers for the migration of RNA transcripts on nondenaturing gels. The effect was observed with both sets of transcripts, starting either at -54 (Fig. 2B) or at +1 (Fig. 2C). The conformer was observed in the RNA samples that were loaded immediately on gel (Fig. 2B,C, lanes 10 and 13), but also in equivalent samples that were first denatured (5 min at 85 °C and quickly cooled on ice; Fig. 2B,C, lanes 11 and 14), or first denatured and subsequently incubated at 37 °C for 30 min (Fig. 2B,C, lanes 12 and 15). These results were obtained with purified HIV-1 transcripts and addition of 1% SDS did not affect the typical migration of the HIV-1 conformer (results not shown), thereby ruling out the involvement of any viral or cellular protein. In the subsequent experiments we arbitrarily used either the -54 or +1 transcripts because we did not observe any difference in the behavior of these RNAs.

Formation of the fast-migrating RNA conformer is a reversible event

We used the -54/290 and -54/368 transcripts for a more detailed analysis of the requirements for formation of the fast-migrating conformer. The *in vitro* transcribed RNAs were dissolved in TEN buffer, heat denatured, and allowed to renature at different temperatures. Samples were taken at all steps for subsequent analysis on a nondenaturing gel. We first discuss the results obtained for the -54/290 transcript (Fig. 3, lanes 1-5). The initial sample is shown in lane 1, and appears to be in the fast-migrating conformation that is marked *c**. Upon denaturation for 5 min at 85 °C, and then rapid cooling on ice, we observed the appearance of a slow-migrating form (Fig. 3, lane 2). This result confirms that the fast-migrating RNA represents a structured form that is sensitive to heat denaturation. The slow-migrating form is marked *m* for monomer, as we also detected RNA dimers in this particular experiment (marked *d*). Subsequent incubation of the denatured RNA sample for 30 min at 37 °C triggered the refolding of the fast-migrating, structured RNA form (Fig. 3, lane 3). This RNA conformer did not form efficiently at 50 °C and 60 °C (Fig. 3, lanes 4 and 5, respectively). When these same incubations were performed in cacodylate

FIGURE 3. Denaturation and refolding of the fast-migrating HIV-1 RNA conformer. The -54/290 transcript (lanes 1-5) and the -54/368 transcript (lanes 6-10) were diluted in TEN buffer and either analyzed directly on the nondenaturing gel (lanes 1 and 6), or treated as indicated below before loading onto the gel. The samples were denatured by heating (5 min, 85 °C) with subsequent rapid cooling on ice (lanes 2 and 7), and aliquots were subsequently incubated for 30 min at either 37 °C (lanes 3 and 8), 50 °C (lanes 4 and 9), or 60 °C (lanes 5 and 10). The samples were separated by electrophoresis on a nondenaturing polyacrylamide gel. The position of the conformer is marked *c**. We marked the position of the denatured transcript as *m* (for monomer) because a slow-migrating dimer form was also detectable in some samples (marked *d* in lane 1).

buffer, the formed conformer is much more stable (see, e.g., Fig. 2B,C; data not shown).

Similar results were obtained for the -54/368 RNA (Fig. 3, lanes 6-10), but the fast-migrating conformer formed by this 3'-extended transcript appeared more stable. For instance, it was more difficult to denature the conformer made by this transcript compared with that of the RNA that ends at position 290 (Fig. 3, lanes 7 and 2, respectively), and refolding of the -54/368 conformer at 50 °C was more efficient than formation of the -54/290 conformer (Fig. 3, lanes 9 and 4, respectively). Thus, the conformer formed by the -54/368 transcript is more stable and easier to form than the -54/290 transcript. These combined results confirm that the HIV-1 leader transcript can adopt a compactly folded conformation that runs unusually fast on nondenaturing gels, and formation of this conformer is a reversible event.

We next set out to determine in more detail the conditions that stabilize the fast-migrating conformer of HIV-1 leader RNA. The -54/290 and -54/368 transcripts were allowed to form the conformer and subsequently diluted in TEN or TE buffer because some level of denaturation was observed in that buffer system. The RNA samples were incubated for 10 min at the indicated temperature in the 50-90 °C range (Fig. 4A). The RNA samples were analyzed on a nondenaturing polyacrylamide gel and the percentage conformer that remained was calculated and these values are plotted in Figure 4. We observed melting of the

FIGURE 4. Stability of the HIV-1 leader RNA conformer. The HIV-1 RNA transcripts indicated at the top of the panels were either heat-denatured (**A** and **B**) or incubated at increasing levels of NaCl (**C**) and MgCl₂ (**D**). In **C** we used TE buffer that was supplemented with different NaCl concentrations. In **D**, we used TN buffer (with NaCl, but lacking EDTA) supplemented with increasing concentrations of MgCl₂. The amount of RNA conformer and denatured RNA was analyzed by quantitation of a non-denaturing gel, and the percentage conformer was calculated. Note that the Y-axis starts at 40%, indicating that only partial melting of the conformer was obtained.

–54/290 transcript at 60 °C and higher temperatures in TEN buffer with NaCl, but not in the equivalent TE buffer (Fig. 4A). The –54/368 transcript remained in the fast-migrating form upon heat treatment in both buffers (Fig. 4B), which is consistent with the previous experiment that indicated that the conformer of the 3'-extended transcript is more stable. This gel-based assay does not measure direct melting of the folded RNA molecule, so it is possible that the RNA was denatured and subsequently refolded during loading of the gel.

These results indicate that the HIV-1 RNA conformer is less stable in the presence of NaCl. This was analyzed more extensively in the 0–500 mM NaCl range for the semistable –54/290 transcript (Fig. 4C). In this experiment, the RNA was incubated for 30 min at 20 °C in TE buffer with the indicated NaCl concentration. Under these conditions, no melting of the conformer is observed

in buffers with 100 mM NaCl, but a gradual loss of the conformer is apparent at higher salt concentrations. We also tested the effect of Mg²⁺ on the stability of the RNA conformer in TN buffer lacking EDTA (Fig. 4D). The RNA conformer was destabilized by MgCl₂. Please note that complete unfolding of the RNA was not observed in these experiments. This could be because of refolding of the conformer during electrophoresis on the gel that lacks Na⁺ and Mg²⁺ ions. Indeed, complete unfolding was observed with RNA samples that were analyzed on gels with MgCl₂ (results not shown).

Formation of the RNA conformer is not dependent on the DIS motif and the NC protein

HIV-1 leader transcripts that include sequences up to position 245 do not form the fast-migrating conformer,

whereas this alternative structure is efficiently formed with 3'-extended transcripts that include the 245–290 domain. This finding raised the possibility that the DIS hairpin, which is encoded by this domain of the HIV-1 leader RNA (Fig. 1), is required for conformer formation. This DIS hairpin plays an important role in the formation of HIV-1 RNA dimers through base pairing of the palindromic loop sequence. To test whether the DIS loop palindrome is also required for the folding of the conformer, we made the GC1 mutant in which the wild-type GCGCGC (GC3) palindrome is truncated to GC (see insert in Fig. 1). Both the mutant and wild-type –54/290 transcripts were able to form the fast-migrating conformer (marked *c** in Fig. 5A, lanes 1 and 6), which could be melted in TEN buffer at 85 °C (Fig. 5A, lanes 2 and 7), and subsequently refolded in a 30-min incubation at 37 °C (Fig. 5A, lanes 3 and 8), but not at 60 °C (Fig. 5A, lanes 4 and 9). The effect of the GC1 deletion on the process of HIV-1 RNA dimerization cannot be studied under these experimental conditions (TEN buffer and a gel lacking Mg^{2+}), but we observed a defect in appropriate *in vitro* dimerization assays (J.L.B. van Wamel & B. Berkhout, unpubl. results). These results

indicate that the sequences required for folding of the leader RNA conformer differ from the RNA signals that mediate RNA dimerization.

The previous experiments were performed without any protein cofactor, but we were interested in studying the influence of the HIV-1 NC protein on formation of the RNA conformer. This small viral protein has been demonstrated to stimulate a variety of reactions that are performed by the leader RNA, including RNA dimerization (Prats et al., 1988; Bieth et al., 1990) and annealing of the tRNA primer (Prats et al., 1988; You & McHenry, 1994). The NC protein was included in the 37 °C incubation step (Fig. 5A, lanes 5 and 10), which should be compared with the control incubation without NC protein (Fig. 5A, lanes 3 and 8). Despite the stimulatory effect of NC protein on a variety of leader RNA functions, we found that RNA conformer formation was inhibited efficiently by NC protein. In addition, a preformed conformer could be unfolded by addition of NC protein (results not shown). It is possible that coating of the transcript by NC protein masks the sequences that are required for conformer formation.

FIGURE 5. Mapping of leader RNA domains that are required for conformer formation. **A:** The GC1 mutant contains a 4-nt deletion within the palindromic loop of the DIS hairpin (indicated in the insert of Fig. 1). Both this GC1 mutant (lanes 1–5) and the GC3 wild-type transcript (lanes 6–10) were synthesized as –54/290 transcript and dissolved in TEN-buffer. Aliquots of the RNA samples were analyzed directly on the nondenaturing polyacrylamide gel (lanes 1 and 6). Multiple bands are visible in these lanes. The slow-migrating species is not an RNA dimer, but we do not know the identity of this band. The other samples were heat denatured at 85 °C (lanes 2 and 7), and subsequently incubated at 37 °C (lanes 3 and 8) to allow refolding of the conformer. As a negative control, the latter incubation was also performed at 60 °C (lanes 4 and 9). In addition, we tested the effect of the NC protein on the ability to form the conformer in the 37 °C incubation (lanes 5 and 10). The position of the fast-migrating conformer (*c**) and the denatured form (*m* for monomer) is indicated. **B:** The transcripts indicated at the top of the panel were dissolved in TEN buffer and analyzed on the nondenaturing polyacrylamide gel. The samples were either heat denatured (5 min at 85 °C, lanes 1, 3, 5, 7, 9), or heat denatured with subsequent incubation for 30 min at 37 °C (lanes 2, 4, 6, 8, 10). The fast-migrating conformer of the positive control transcript –54/290 is marked with an asterisk. A significant portion of the 240/290 transcript, which encompasses the DIS hairpin and flanking sequences, formed an insoluble aggregate that did not leave the slot of the gel (lanes 5 and 6). Interestingly, all other transcripts contain this 240/290 domain, but do not form the precipitate, and the reason for this RNA aggregation is currently unknown.

Both 5' and 3' domains of the leader are required for conformer formation

The results described above indicate that inclusion of the 245–290 leader region triggers the formation of the conformer, even though the DIS loop is not critically involved. This may suggest that both upstream and downstream leader domains are required for the folding of this special conformation, perhaps through a long-distance base pairing interaction. To test this idea, we synthesized 5'-truncated transcripts that start at position 105 or 240, and with the 3' end at position 290 or 368. These transcripts are included in the scheme of Figure 1 and their gel mobility is shown in Figure 5B. We included the –54/290 transcript as a positive control, which shows both RNA conformations upon heat denaturation in TEN buffer (Fig. 5B, lane 9), and the fast-migrating conformer after a subsequent 30-min, 37 °C incubation (Fig. 5B, lane 10). Interestingly, none of the 5'-truncated transcripts were able to form a fast-migrating RNA species. These combined results indicate that both the 5' and 3' domains of the HIV-1 leader RNA (region 1–105 and 245–290) are required for folding of the conformer, which is consistent with the involvement of a long-distance RNA–RNA interaction.

Antisense oligonucleotides that target different leader domains can denature the conformer

Probing with antisense DNA oligonucleotides can provide information on the structure of a target RNA, and we used this method to verify whether multiple leader domains are required to form the conformer. The +1/290 transcript forms the conformer (marked * in Fig. 6, lane 10) and the different antisense DNA oligonucleotides were heat annealed in cacodylate buffer. The antisense DNAs, the target sequence in the leader of which is indicated at the top of the panel, produced quite diverse effects on the gel migration of the transcript. Some DNAs had little or no effect on the folding of the fast-migrating conformer (Fig. 6, lanes 1, 3, and 4). Annealing of other DNAs produced a partial shift in the migration of the RNA conformer (Fig. 6, lanes 2 and 5), and some DNAs were able to change the migration of this RNA to the approximate position of the denatured RNA molecule (Fig. 6, lanes 6 and 7). To complicate matters further, the 93/63 oligonucleotide caused rather complete unfolding of the RNA, but only for 50% of the molecules (Fig. 6, lane 8). All these oligonucleotide-mediated effects are specific for antisense molecules because several control DNAs (results not shown), including a sense molecule (Fig. 6, lane 9), have no effect on the migration of the HIV-1 RNA conformer. We think that these DNA-induced migration shifts are caused by binding and (partial) unfolding of the RNA conformer, but it is also possible that the formed RNA–DNA duplexes migrate with decreased mobility in this

FIGURE 6. Unfolding of the HIV-1 RNA conformer by antisense DNA oligonucleotides. The +1/290 transcript (lanes 1–10) was annealed in cacodylate buffer with two antisense oligonucleotides (indicated at the top of the lanes is the targeted HIV-1 leader RNA region) and analyzed on a nondenaturing 4% polyacrylamide gel. We used a molar excess of antisense DNA to target RNA to allow all transcripts to form hybrids. Control reactions were performed with a sense oligonucleotide (lane 9) and a mock incubation (lane 10). The latter sample shows the conformer, marked with an asterisk. A similar set of DNA oligonucleotides was incubated with the control transcript 105/290 that is unable to form a fast-migrating conformer (lanes 11–18).

nondenaturing gel. To rule out the possibility of such a band-shift effect, we incubated several of the antisense DNAs with the shorter 105/290 transcript (Fig. 6, lanes 11–18). The antisense DNAs do anneal to this transcript, as was verified in experiments with labeled DNA instead of labeled RNA (results not shown). However, this 105/290 does not form the fast-migrating RNA conformer, and oligo binding does not have an effect on the gel migration. These combined results indicate that the oligo-mediated effects observed with the +1/290 transcript are caused by (partial) unfolding of the conformer.

These promising results prompted us to test a larger set of antisense DNAs that target different regions of the +1/290 leader RNA. The length of the antisense DNAs and their hybridizing position on the HIV-1 RNA target are schematically depicted in Figure 7. A representative nondenaturing gel is shown in the top panel, but the scheme includes data obtained with additional antisense DNAs (results not shown). The shift in migration of the HIV-1 RNA conformer was arbitrarily scored on a 0–5 scale, with 0 representing the position of the fast-migrating conformer and 5 representing the position of the denatured transcript. We mentioned earlier that some antisense DNAs shift only a fraction of the RNA transcript, and we identified these DNAs by an open bar (less than 30% of the RNA is shifted) or hatched bar (between 40 and 85% of the RNA is shifted).

FIGURE 7. Scanning across the HIV-1 leader for antisense DNA that interfere with the RNA conformer. The conditions of antisense DNA annealing and nondenaturing gel electrophoresis are as in Figure 6. The mock incubation of the +1/290 transcript is in the last lane (—), showing the conformer marked with an asterisk. The antisense oligonucleotides are indicated at the top of the lanes. An arbitrary 0–5 scale was used to score the effect of the DNA oligonucleotide on the migration of HIV-1 RNA, and this is plotted in the bottom panel as a function of their annealing position on the HIV-1 RNA (the position of some leader motifs such as the DIS hairpin are indicated). Bars represent the antisense DNA oligonucleotides, and their respective length is indicated. DNA oligonucleotides that shift only a fraction of the transcript are marked as hatched bars, and the 134/115 DNA that shifts only a minority of the RNA is marked as an open bar.

It is probable that such a partial effect reflects binding of the DNA to a subset of the transcripts. Consistent with this idea is the fact that some of the shortest antisense DNAs did cause a partial shift. Similar results were obtained with the +1/368 transcript as target RNA, although the DNA-induced migration shifts were less severe with this transcript (experiment not shown). We mentioned earlier that this 3'-extended transcript folds a more stable conformer, which may therefore be more resistant to the effect of antisense DNAs. We did not analyze this possibility in more quantitative detail.

The results summarized in Figure 7 indicate that two domains of the HIV-1 RNA conformer are exquisitely sensitive to antisense DNA-mediated melting. Unfolding of the RNA conformer was most effective with antisense molecules that bind the 70–150 and 220–270 regions. However, these two regions are not solely responsible for the formation of the conformer, because partial denaturation was observed with several anti-

sense DNAs that target other parts of the leader RNA. The only region that was relatively insensitive to melting by antisense DNA is the extreme 5' end of the transcript (region +1–40), which encodes part of the TAR hairpin structure. Thus, multiple domains along the leader RNA seem important for conformer formation, which is consistent with the idea that this structure is held together by multiple (long-distance) RNA–RNA interactions. This result also confirms the result of the deletion mapping (summarized in Fig. 1), which indicated the involvement of the nearly complete leader region.

Our interpretation of the minor band shift caused by some antisense DNAs is that these reagents unfold the conformer structure only partially. This hypothesis predicts that progressive unfolding may take place by annealing of multiple antisense DNAs. Indeed, this was observed for several, but not all, DNA combinations. One positive and one negative example are shown in

Figure 8. The migration of the +1/290 transcript is shifted partially by the 245/216 antisense DNA (Fig. 8, lane 3), and progressive unfolding is apparent by addition of the 151/123 DNA (Fig. 8, lane 4), and the 199/182 DNA (Fig. 8, lane 5). The 290/270 and 245/216 DNAs also produce a minor band shift when provided individually (Fig. 8, lanes 12 and 13), but we measured no additive effect when both oligonucleotides were annealed onto the same target RNA (Fig. 8, lane 14). Two control experiments were performed. The three antisense DNAs that produce a progressive band shift with the +1/290 transcript (Fig. 8, lane 5) were also incubated with the 105/290 transcript that cannot form the conformer. As expected, no effect on the gel migration of this RNA was observed (Fig. 8, lanes 6 and 7). In the standard assay, we preincubate the transcript and the antisense DNA at 85 °C, with subsequent slow cooling of the sample. Thus, it remains possible that the antisense DNAs preclude the formation of the conformer instead of disrupting the preexisting conformer. However, incubation of the 245/216 antisense DNA with the preformed RNA conformer at 37 °C also resulted in complete conversion into the slow-migrating RNA form (Fig. 8, lane 10).

The compactly folded HIV-1 leader RNA can block an elongating RT enzyme

In an attempt to map in more detail the 3' border of the leader domain that forms the compactly folded RNA structure, we performed reverse transcription on the HIV-1 leader RNA template. It is well known that structured regions in the RNA template can induce stops or pauses during elongation of reverse transcription (Harrison et al., 1998; Klasens et al., 1999a). A DNA oligonucleotide primer was annealed to the 348–368 region of two templates that differ in their ability to form the conformer; transcripts +1/368 and 105/368. The DNA primer was extended by addition of RT enzyme and dNTPs, and the radiolabeled cDNA products of reverse transcription were analyzed on a denaturing sequencing gel. Two pronounced stops were encountered in the DIS region on the +1/368 template (Fig. 9, lanes 9–12). These stops are marked S1 and S2 and were mapped to positions 277/280 and 265/266 by comparison with the sequence ladder (Fig. 9, lanes 1–4). In addition to these two major stops, several minor stops were observed, but their intensity was approximately the same on the two templates. Most importantly, the

FIGURE 8. Progressive unfolding of the HIV-1 RNA conformer with multiple antisense DNAs. Transcripts indicated at the top of the panels were annealed with mixtures of antisense DNAs and analyzed on a nondenaturing 4% polyacrylamide gel. Control reactions include a mock incubation (lane 1) and incubation with a sense oligonucleotide (lane 2). The antisense DNA(s) used to melt the HIV-1 leader conformer are indicated above the lanes. The position of the fast-migrating conformer is marked with an asterisk. The 105/290 transcript (lanes 6 and 7) does not form the fast-migrating conformation.

FIGURE 9. The HIV-1 leader RNA conformation blocks an elongating RT enzyme. The +1/368 transcript (lanes 9–12) and control RNA 105/368 (lanes 5–8) were used as template in a primer-extension reaction with the HIV-1 RT enzyme. The antisense DNA oligonucleotide 348/368 was used as primer for reverse transcription, and the same DNA primer was used in a sequencing reaction on an HIV-1 plasmid (lanes 1–4). The primer was first bound onto the transcripts by heat annealing (2 min at 85 °C), and subsequent slow cooling to allow refolding of the RNA conformer by the +1/368 transcript. Reverse transcription was performed at 37 °C for 60 min and the sample was subsequently denatured in formamide buffer and analyzed on a denaturing sequencing gel. Side-by-side comparison of the cDNA products on the two templates indicates two major stops (marked S1 and S2) that are specific for the +1/368 template. These stops map to the G-rich sequences near the DIS region (S1 = GGGG 277/280, S2 = GG 265/266). The reactions were performed in quadruplo with minor differences between the samples. We added 200 ng of the following recombinant proteins to some reverse transcription mixtures: helicase (lanes 6 and 10), maltose-binding protein (lanes 7 and 11), and purified bovine serum albumin (lanes 8 and 12).

S1 and S2 stops/pauses were not observed in the reverse transcription reaction on the 105/368 template (Fig. 9, lanes 5–8), which is striking because the identical nucleotide sequence is encountered during reverse transcription on these RNA templates. Thus, it is very likely that the two stops are induced by a stable secondary and/or tertiary RNA structure that is specific

for the +1/368 template. Even though the two stops coincide with the DIS dimerization signal, we do not think that the loop-loop-kissing interaction is responsible for the observed effects. First, although both RNAs can form dimers under the appropriate conditions (results not shown), these transcripts are in the monomer form under the experimental conditions of Figure 9, which makes it unlikely that the massive RT stop is caused by the dimer interaction. Second, we observed similarly profound stops during reverse transcription on the GC1 mutant template with a 4-nt deletion in the DIS loop (results not shown). These combined results indicate that the 3' border of the compactly folded HIV-1 leader RNA domain is located near position 280, which correlates nicely with the results of the deletion mapping (summarized in Fig. 1).

DISCUSSION

We produced a set of transcripts that encode different parts of the HIV-1 leader RNA and their mobilities were compared under denaturing and nondenaturing electrophoresis conditions. Transcripts encompassing the nearly complete leader region (+1/290) exhibited an unusually fast migration compared with shorter transcripts that lack either the 5' or 3' end sequences. These results indicate that the nearly complete leader region has the capacity to fold a compact tertiary structure. This is the first evidence for a higher-order structure of the HIV-1 leader RNA, and we will first discuss the potential biological functions of such a conformational polymorphism, which may act as a regulatory RNA switch in the viral replication cycle.

It is currently unknown whether the compactly folded leader RNA structure represents a biologically relevant structure of the HIV-1 genome. It is also unknown whether this structure is adopted by the viral RNA when present in infected cells or within virion particles, although there are several findings that argue against the latter possibility. In particular, we observed major differences in the optimal conditions for conformer formation versus the formation of HIV-1 RNA dimers, which is the intravirion form of the HIV-1 genome. Whereas RNA dimerization is stimulated by the *trans*-acting NC protein that is abundantly present in virion particles, this RNA chaperone was found to interfere with formation of the RNA conformer. The ionic requirements for the two RNA reactions also appear rather different. In particular, we measured a negative effect of Mg^{2+} on the conformer stability, whereas this ion has a positive effect on dimer formation and stability. However, without data on the intracellular versus intravirion concentration of magnesium, this finding does not present direct evidence against a role of the conformer in the virion particle. Furthermore, RNA sequence requirements appear quite different for the processes of RNA dimerization and conformer formation. Whereas the palindromic

loop of the DIS hairpin is critical for dimerization, deletion of this sequence did not affect folding of the fast-migrating conformer. Consistent with the idea that different sequences are involved is the finding that both RNA interactions can occur simultaneously, as we have observed that the RNA dimer can also adopt a fast-migrating conformation at the appropriate buffer conditions (J.L.B. van Wamel and B. Berkhout, unpubl. results).

It remains possible that the dimeric RNA genome within virion particles adopts this conformation, which may cause problems during the process of reverse transcription, because we observed massive RT stops on the leader RNA template in the region just ahead of the conformer. Interestingly, pauses during reverse transcription can induce recombination events (Klaver & Berkhout, 1994b; Kulpa et al., 1997), and a recombination hot spot was identified in this part of the leader region of the murine leukemia virus RNA genome (Mikkelsen et al., 1998). Although a reverse transcription stop would seem detrimental to virus replication, it is possible that conditions within the virion particle, for example, the presence of NC protein, overcome this structure-induced blockade (Peliska et al., 1994; Ji et al., 1996). It also remains possible that this tertiary template structure plays a regulatory role during the complex reverse transcription reaction (Telesnitsky & Goff, 1997).

Alternatively, the compactly folded leader RNA structure may exist in the intracellular phase of the HIV-1 replication cycle. The leader of the viral genome is recognized by the cellular machinery to control the processes of polyadenylation, splicing, and mRNA translation. The RNA conformation can theoretically affect any of these steps, and we will briefly mention several possibilities. For polyadenylation, there is some recent evidence that the downstream part of the leader RNA can inhibit the use of the upstream polyadenylation signal (Ashe et al., 1997; Klasens et al., 1999b), and RNA folding may bring these two RNA domains in proximity to each other. The higher-order leader RNA structure may also influence the process of splicing at the major splice donor site. Likewise, the conformer may interfere with the scanning of ribosomes, thereby affecting the translational efficiency of HIV-1 mRNAs. Although several reports indicated a posttranscriptional role of the HIV-1 leader RNA (SenGupta et al., 1990; Lenz et al., 1997), additional experiments are required to test some of these possibilities.

Another possibility is that the tertiary leader RNA structure is recognized by the viral Gag protein during specific encapsidation in virion particles. Retroviral packaging signals are notoriously difficult to map, which may be due to the recognition of a complex tertiary structure instead of a simple, discrete sequence motif (Berkowitz et al., 1996; Banks et al., 1998). Although the core packaging signal of HIV-1 RNA is located near

the Ψ hairpin in the untranslated leader (Fig. 1), the upstream TAR, poly(A) and DIS hairpins have also been reported to contribute to optimal packaging (Berkhout & van Wamel, 1996b; McBride & Panganiban, 1996, 1997; Clever & Parslow, 1997; Das et al., 1997, 1998; Grotorex & Lever, 1998; Clever et al., 1999; Helga-Maria et al., 1999). These combined results are consistent with the recognition of a tertiary leader RNA fold by the retroviral Gag protein during packaging of the RNA genome. In addition, there is evidence that the viral Gag protein can induce a structural rearrangement in the HIV-1 leader RNA (Damgaard et al., 1998).

The presence of higher-order structure in the leader of a retroviral RNA genome may be a more general phenomenon. For instance, a conformational transition was recently reported for the leader RNA of the cauliflower mosaic virus (CaMV) of the pararetrovirus family (Hemmings-Mieszczak et al., 1998). The leader RNA of retroviral genomes may represent a dynamic molecule that forms a compact tertiary structure under specific conditions in the cell and/or virion to execute a particular replication step. In other words, the HIV-1 leader may represent an "RNA switch" by folding of alternative secondary and/or tertiary structures. For instance, full-length HIV-1 transcripts may undergo a transition from acting as messenger RNA to genomic RNA that is packaged in virions, and the conformational RNA switch may control this event.

Future studies should reveal details of the compactly folded structure that is adopted by the HIV-1 leader RNA. This research should explain the profound shift in gel migration and the exquisite sensitivity of the conformer towards Mg^{2+} ions. Nondenaturing gel electrophoresis is extensively used for studying conformational differences between RNA molecules, and some of these conformational changes can be regulated with Mg^{2+} (Bassi et al., 1995, 1996; Friederich & Hagerman, 1997; Hammann et al., 1997; Jabri & Cech, 1998; Wu & Tinoco, 1999). Gel migration has been reported to be sensitive to the global structure of four-way RNA junctions, in particular the rotational angle between the helical regions, and Mg^{2+} can influence this conformation (Walter et al., 1998). Strikingly, the +1/290 HIV-1 RNA also consists of four stem regions (Fig. 1), and coaxial stacking has been proposed for the TAR and poly(A) hairpins because an unpaired nucleotide is never found in between the two stems in a wide variety of virus isolates (Berkhout et al., 1995; Berkhout & Das, 1999). Further biochemical and biophysical studies may provide clues on the global orientation of the stems in the HIV-1 leader RNA. The HIV-1 conformer has an unusually low Mg^{2+} requirement for folding. One other example of a higher-order RNA structure that is biologically active at an extremely low Mg^{2+} concentration is the group II self-splicing intron (Costa et al., 1997, 1998). Interestingly, higher Mg^{2+} concentrations have a negative effect on the stability of the HIV-1 RNA conformer,

and it is possible that raising the concentration of MgCl_2 results in an increased number of secondary structure base pairings, thereby melting the tertiary structure (Draper, 1996). Please note that the common assumption that RNA folds by first forming secondary structure and then forming tertiary interactions from the unpaired bases is not always correct. A detailed study of the group I intron ribozyme of *Tetrahymena thermophila* indicated that folding of tertiary structure causes rearrangement of the secondary structure, with the formation of some new base pairs, but also the breakage of others (Wu & Tinoco, 1999).

A more complete analysis of antisense DNA-mediated denaturation of the RNA conformer may yield important insight into the RNA domains involved, and the effect of different combinations of antisense DNAs may reveal regions of the HIV-1 transcript that interact. Of note, the power of RNA probing with antisense DNA has increased dramatically with the introduction of the array technology (Sohail et al., 1999). Despite these recent developments, traditional approaches will remain important to further dissect the HIV-1 RNA conformer structure. These approaches include the analysis of transcripts with mutations in particular domains of the leader and RNA structure probing with transcripts that differ in their ability to form the conformer (e.g., +1/368 versus 105–368). The leader RNA function should eventually be studied in the context of the replicating virus, but this route has proven to be particularly difficult for the HIV-1 leader because several of the regulatory signals overlap, thereby causing a multitude of replication defects upon mutational inactivation (Berkhout, 1999).

MATERIALS AND METHODS

HIV-1 DNA templates for T7 transcription

The HIV-1 transcripts used in this study are all derived from the LAI isolate. The LAI infectious molecular clone was a kind gift of Keith Peden (Food and Drug Administration, USA) (Peden et al., 1991). All nucleotide positions refer to the transcription start site of HIV-1 RNAs at position +1. The 4-nt deletion within the DIS hairpin loop of the GC1 mutant was introduced as described previously (Berkhout & van Wamel, 1996a). The wild-type and mutant plasmids were used as template for PCR amplification and subsequent in vitro transcription. PCR primers used to introduce a T7 promoter immediately upstream of position +1 and –54 have been described previously (Klasens et al., 1999b), and we used a similar strategy to fuse the T7 promoter sequence upstream of leader positions 105 and 240. All downstream PCR primers are antisense DNA oligonucleotides that are complementary to the HIV-1 leader. The 5' end of these antisense DNA primers will determine the 3' end of the run-off transcripts (positions 134, 202, 245, 290, and 368). The PCR reactions were performed in PCR buffer (20 mM Tris-HCl, pH 8.3, 2 mM MgCl_2 , 50 mM KCl, 0.1 mg/mL BSA) containing 100 ng of

each primer, 10 mM of each dNTP, 1.5 U *Taq* polymerase, and 10 ng plasmid. The HIV-1 PCR fragments were phenol extracted, precipitated, and dissolved in water.

T7 transcription

The in vitro transcription reaction was performed in 10 μL transcription buffer (40 mM Tris, pH 7.5, 2 mM spermidine, 10 mM DTT, and 12 mM MgCl_2) containing approximately 0.5 μg DNA template, 20 μCi [α - ^{32}P]UTP (3,000 Ci/mmol), 0.06 μmol ATP, 0.06 μmol GTP, 0.06 μmol CTP, 0.02 μmol UTP, 10 U T7 RNA polymerase (Boehringer Mannheim) and 20 U RNase inhibitor (Boehringer Mannheim). RNA synthesis was continued for 2 h at 37 °C, and the template was subsequently removed by addition of 1 μL (2 U) DNase I (Boehringer Mannheim) for 30 min at 37 °C. The samples were denatured by addition of 2 μL formamide-loading buffer and separated on a 6% denaturing sequencing gel. The gel was exposed and the full-length transcripts were eluted from the gel piece in an overnight incubation at 37 °C in 400 μL elution buffer (0.3 M Na acetate, pH 5.2). The RNA was precipitated with 1 mL 96% ethanol, washed with 500 μL 70% ethanol, and resuspended in 20–100 μL TE to make the RNA stock solutions for subsequent studies.

Unlabeled T7 transcripts were synthesized as templates for reverse transcription assays. Following the DNase I treatment, the samples were phenol extracted, and 2 vol (900 μL) ethanol (96%) was added for precipitation of the RNA transcripts. The transcripts were dissolved in renaturation buffer (10 mM Tris, pH 7.5, 100 mM NaCl), heated for two min at 85 °C, and then cooled slowly to room temperature, and stored at –20 °C. The transcripts were analyzed on agarose gels and quantitated with UV spectroscopy.

In vitro RNA studies

The RNA samples were diluted 5- or 10-fold in the buffers indicated in the individual experiments. We used either TEN buffer (50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 100 mM NaCl), TEN buffer with 1% SDS, TE buffer without NaCl, or cacodylate buffer (50 mM sodium-cacodylate, pH 7.5, 300 mM NaCl, 5 mM MgCl_2). The samples were treated as indicated for the individual experiments. The radiolabeled transcripts (approximately 0.2 pmol) were either mixed with formamide sample buffer, heated, and separated on a denaturing 6% acryl/bisacrylamide (19/1)/7 M urea gel, or the samples were mixed with nondenaturing glycerol-based sample buffer and analyzed on a nondenaturing 4% acryl/bisacrylamide (19/1) gel in 0.25 $\times$ TBE buffer. When the HIV-1 transcript was incubated with one or multiple antisense DNA oligonucleotides, the RNA was diluted in cacodylate buffer with an approximate 100-fold excess DNA oligonucleotide (15 pmol), heated for 2 min at 85 °C, and slowly cooled to room temperature before gel analysis. We also performed an RNA analysis in the presence of the NCp7 protein (MN isolate, obtained from Dr. Louis Henderson, National Cancer Institute, USA). We used 100 ng NC protein, which results in complete coating of the RNA transcript. For this calculation, we assumed binding of 1 NC protein per 7 nt of the template RNA (You & McHenry, 1994). The gels were dried and signals were quantitated with a PhosphorImager (Molecular Dynamics).

Reverse transcription assays

Reverse transcription was performed with the antisense DNA primer 368/348 (2.9 pmol) that was annealed to the RNA template (0.014 pmol) in 12 μ L annealing buffer (83 mM Tris-HCl, pH 7.5, 125 mM KCl) by heating for 2 min at 85 °C and subsequent gradual cooling to room temperature. This primer is complementary to position 348/368 of the HIV-1 genome. Reverse transcription on the template primer RNA-DNA hybrid was initiated by addition of 12 μ L of 2 $\times$ RT-buffer containing 6 mM MgCl₂; 20 mM DTT; 0.5 pmol of HIV-1 p66/p66 RT (2.5 U); dGTP, dATP, and dTTP, each at 20 μ M; dCTP at 10 μ M; and 0.3 μ L of [α -³²P]dCTP (10 mCi/mL). The final reaction mixture (24 μ L end volume; 44 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, 10 mM DTT, 62.5 mM KCl, 20 mM NaCl) was incubated at 37 °C for 1 h. Reactions were terminated with 1 μ L of 0.5 M EDTA, pH 8.0. The cDNA products were ethanol precipitated and resuspended in formamide-loading buffer and heated for 2 min at 85 °C. Samples were analyzed on a denaturing 6% polyacrylamide/urea sequencing gel. The length of the partial reverse transcription products was determined by comparison with a ³⁵S-labeled sequence reaction on the HIV-1 plasmid with the same DNA primer. Sequencing was performed according to the Sequenase kit 2.0 (Amersham).

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