**In Vitro** Evidence That the Untranslated Leader of the HIV-1 Genome Is an RNA Checkpoint That Regulates Multiple Functions through Conformational Changes*

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The HIV-1 RNA genome forms dimers through base pairing of a palindromic 6-mer sequence that is exposed in the loop of the dimer initiation signal (DIS) hairpin structure (loop-loop kissing). The HIV-1 leader RNA can adopt a secondary structure conformation that is not able to dimerize because the DIS hairpin is not folded. Instead, this DIS motif is base-paired in a long distance interaction (LDI) that extends the stem of the primer-binding site domain. In this study, we show that targeting of the LDI by either antisense oligonucleotides or specific mutations can induce the conformational switch to a branched multiple hairpin (BMH) structure, and this LDI-to-BMH switch coincides with increased RNA dimerization. Another interesting finding is that the extended LDI stem can resist a certain level of destabilization, indicating that a buffer is created to prevent a premature conformational switch and early dimerization. Because the tRNA\(^{Lys}\) primer for reverse transcription anneals to multiple sequence elements of the HIV-1 leader RNA, including sequences in the LDI stem, we tested whether tRNA-annealing can destabilize the LDI stem such that RNA dimerization is triggered. Using a combination of stem-destructabilizing approaches, we indeed measured a small but significant effect of tRNA-annealing on the ability of the RNA template to form dimers. This observation suggests that HIV-1 RNA can act as a checkpoint to control and coordinate different leader functions through conformational switches. This *in vitro* result should be verified in subsequent *in vivo* studies with HIV-infected cells.

The untranslated leader region of the HIV-1\(^1\) RNA genome contains multiple regulatory motifs that play important roles in the viral replication cycle (1, 2). Distinct functions have been assigned to individual sequence and/or structure motifs (see Fig. 1A). The 5’-terminal TAR hairpin forms the binding site for the viral Tat protein and the cellular cyclin T protein (3, 4). The formation of this RNA-protein complex on the nascent transcript is essential for high level transcription from the LTR promoter (5). The poly(A) hairpin also functions as part of the nascent transcript. The structure inhibits polyadenylation through the masking of the AUAUU polyadenylation signal (6, 7). Both the TAR and poly(A) sequences are part of the terminal repeat (R), and a second copy is present at the extreme 3’-end of the viral RNA genome. At this position, inhibition of polyadenylation by the suppressive RNA structure is overcome by an upstream enhancer element that facilitates the binding of the CPSF polyadenylation factor. Thus, the upstream TAR and poly(A) hairpins play pivotal roles during the early phase of viral gene expression. In addition, these structure motifs in the R region also participate in later phases of the viral replication cycle (2), e.g. in the strand transfer reaction during the process of reverse transcription (8).

Several RNA motifs are located further downstream in the untranslated leader RNA. An extended structure domain encompasses the primer-binding site (PBS) that binds the tRNA\(^{Lys}\) primer for reverse transcription. Whereas the PBS motif is largely exposed within this structured RNA domain, there is some recent evidence for an additional upstream tRNA interaction site that is actively masked by base pairing in the extended PBS stem. This primer activation signal (PAS) is not involved in tRNA-annealing, but is essential for efficient initiation and elongation of reverse transcription (9, 10). Further downstream we find structure motifs that have been implicated in dimerization and packaging of the HIV-1 RNA into virion particles. The dimer initiation signal (DIS) folds a hairpin structure that exposes a 6-mer palindromic sequence within the loop, and two DIS motifs can form a “kissing-loop” complex by base pairing (11–16). This RNA dimer is further stabilized by melting of the stem regions and subsequent formation of a more extended intermolecular duplex (17). The PSI region binds the viral nucleocapsid (NC) protein and has been implicated in the selective packaging of the HIV-1 RNA into virion particles (18, 19). Thus, a multitude of critical RNA signals are clustered in the 336 nucleotides of the untranslated leader. The functional analysis of these motifs in replication studies with mutant viruses is seriously complicated by the overlap of some of these RNA replication signals. This problem is less severe in defined biochemical assays that focus on a particular RNA function, e.g. RNA dimerization with *in vitro* synthesized transcripts.
A static secondary structure model of the HIV-1 leader RNA has been proposed (1). However, we recently presented evidence that the HIV-1 leader does not constitutively adopt the conformation that is characterized by the multiple hairpins as shown in Fig. 1A. In fact, the HIV-1 leader RNA folds into a more stable alternative conformation that appears to regulate the process of RNA dimer formation (20). Specifically, we demonstrated that the poly(A) and DIS sequences can base pair, thereby masking the DIS palindrome and actively inhibiting RNA dimerization (21). This long distance interaction (LDI) extends the stem region that forms the basis of the structured PBS domain (Fig. 1, B and C), and this RNA conformation is characterized by a typical fast migration on non-denaturing gels. The LDI conformation is thermodynamically more stable than the alternative conformation that we termed the branched multiple hairpin (BMH) structure. However, the viral NC protein can induce the latter structure, concomitant with increased RNA dimerization. It thus appears that the extended PBS stem region is involved in the regulation of both the reverse transcription and dimerization reactions through masking of the PAS and DIS elements. This dynamic property of the leader RNA structure may serve to coordinate the early and late replication functions, and we proposed the idea of an RNA checkpoint that regulates different leader RNA functions in a temporal manner.

We performed a detailed in vitro analysis to further study the extended stem of the PBS domain and its influence on the structure and function of the HIV-1 leader RNA. The results indicate that the extended PBS stem can absorb minor mutations, but that progressive destabilization of the base-pairing potential does eventually trigger the LDI-to-BMH structural switch and subsequent RNA dimerization. In this sensitive assay system, we were also able to measure a modest effect of tRNA-PBS-annealing on the overall leader RNA conformation and its dimerization properties. These results provide the first evidence that the reverse transcription and dimerization reactions may be coupled through conformational changes within the leader RNA.

**EXPERIMENTAL PROCEDURES**

**HIV-1 Templates for PCR and Transcription—**Wild-type and mutant plasmids were used for PCR amplification and subsequent in vitro transcription. Phasmid Blue5-LTR (22) contains an XbaI-ClaI fragment of the HIV-1 subtype B molecular clone LAI (23). This HIV-1 fragment contains the complete 5’-LTR, PBS, leader, and the 5’-end of the gag gene (positions 454 to 376 relative to the transcriptional start site, +1), which was cloned into pBluescript KS+ (Stratagene). The construction of all mutants that target the U5 leader stem has been described previously (10). The 4-nucleotide deletion within the DIS hairpin loop of mutant GC1 was introduced as described (24).

The wild-type and mutant plasmids were used as templates for PCR amplification. The T7 promoter sequence was incorporated into the PCR product with sense primers containing the T7 promoter sequence. The T7 promoter sequence was inserted immediately upstream of positions +1 or +92 with the sense primers T7-1 and T7-92. The oligonucleotide T7 promoter sequence was inserted immediately upstream of positions 1620/1634, 1620/1640, 1620/1650, and 1620/1660. Each PCR product was gel-purified. All PCR reactions were performed with the Megashortscript T7 transcription kit, and transcripts were purified over a G50 spin column. Cy5 labeling was performed post-transcriptionally using the ULYSIS Cy5 labeling kit (Kreatech). RNA concentration and labeling density (−1 Cy5 group per 34 nucleotides) were determined by spectrophotometry.

**Array Hybridization, Data Acquisition, and Analysis—**Hybridizations were performed in a 280-μl reaction containing TEN buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 8.0), supplemented with 1% Triton X-100. Transcripts (12 fmol of RNA or 100 fmol of Cy5) were heated to 85 °C and slowly cooled to room temperature in the presence or absence of the 151/123 oligonucleotide (50 pmol) (21). Array hybridizations were performed for 16 h at room temperature under constant rotation of the hybridization chamber. Hybridization chambers were emptied and disassembled, and the array was quickly placed in a 50-ml Falcon tube containing 1× TEN buffer with 0.1% Triton, washed for 30 s by inverting the tube, placed in a second tube containing 0.1× TEN, and washed for a further 30 s with mixing. The array was slowly pulled from the final washing solution while being blown dry with a nitrogen gun at close range. The arrays were subsequently scanned in an Agilent contact array scanner at 10-micron resolution. The Agilent feature extraction software package was used to automatically detect oligonucleotide features in the hybridization image, and the resulting data were corrected for local background for each individual feature. Hybridization signals that did not pass the stringent quality tests or that were at or below background signal levels were discarded. The experimental data were exported to a tab-delimited text file and were imported in spreadsheet software for detailed analysis.

**RESULTS**

**Targeting of the Extended PBS Stem by Antisense Oligonucleotides**—To study the effect of the extended PBS stem region on the LDI-BMH equilibrium and DIS-mediated RNA dimerization, we targeted the HIV-1 leader RNA with a set of antisense DNA oligonucleotides. The transcript-oligonucleotide complexes were analyzed on non-denaturing TBE and TBM gels to distinguish the different leader RNA conformations; the blue monomer forms (the fast migrating LDI and the BMH forms) and RNA dimers. The HIV-1 transcript 1–290 that is shown in detail in Fig. 1C stably adopts the fast migrating LDI conformation on the TBE and TBM gels (lane 1 in Fig. 2A and B, respectively). Oligonucleotides targeting the TAR region (lanes 2 and 3) do not affect the LDI conformation and do not induce RNA dimer formation. Oligonucleotides that target the poly(A) domain do trigger the LDI-to-BMH rearrangement. This is clearly demonstrated on the TBE gel by the appearance of the slow migrating BMH form (Fig. 2A, lanes 4–6). Consistent with previous results (27), the LDI form refolds during electrophoresis on the TBM gel (Fig. 2B, lanes 4–6). Most
importantly, the LDI-to-BMH switch coincides with increased formation of RNA dimers (D), at least for the 104/77 and 122/99 oligonucleotides. The RNA dimers are apparent on the TBE gel (Fig. 2A, lanes 5 and 6), but dimers are more prominent on the TBM gel (Fig. 2B, lanes 5 and 6). The TBM gel yields the highest dimerization level because labile kissing-loop RNA dimers do survive electrophoresis in the presence of magnesium.

We initially tested five oligonucleotide probes that cover most of the PBS domain. All these oligonucleotides mediate the
LDI-to-BMH switch (Fig. 2, lanes 7–11), but the 181/152 and 202/182 probes show a partial transition (lanes 8 and 9). This trend translates directly into the dimerization capacity of HIV-1 RNA, which is strongly induced by oligonucleotides 151/123 and 223/200 that target the left and right side of the extended PBS stem, respectively. A very minor stimulation of RNA dimer formation is observed with probes 181/152 and 202/182, and oligonucleotide 245/216 induces an intermediate level of RNA dimers. The same samples were also analyzed on the TBM gel that stabilizes the dimer conformation (Fig. 2B). The ranking order of the dimer induction properties of the antisense oligonucleotides in the PBS domain is 151/123 > 223/200 > 245/216 > 181/152 > 202/182.

The probe 245/216 anneals very close to the DIS hairpin, apparently without preventing its dimerization function. However, we observed a severe dimerization defect when the probe is extended by three nucleotides toward the DIS element (probe 248/216, results not shown). We show two control reactions with other anti-DIS oligonucleotides (269/246 and 290/270). As expected, these probes do effectively disrupt the LDI conformation by shielding of the actual dimerization motif (Fig. 2, A and B, lanes 12 and 13). These results demonstrate that the DIS motif is solely responsible for in vitro dimerization of HIV-1 RNA. This was confirmed in studies with a mutant transcript (GCGCGC to GC) that did not form dimers under these conditions (results not shown).

We observed an almost perfect correlation between the ability of certain antisense oligonucleotides to mediate the LDI-to-BMH switch and their stimulatory effect on RNA dimerization. This result reinforces the idea that RNA dimer formation is prevented by the LDI conformation and that dimerization occurs spontaneously for BMH-folded transcripts. However, it cannot be excluded that the oligonucleotides have a more direct impact on the RNA dimerization reaction. To critically test this possibility, we used the TAR-deleted HIV-1 transcript 92–290 that exclusively folds into the BMH structure. Such transcripts indeed form dimers at a reasonable efficiency (27). Most importantly, none of the antisense oligonucleotides that stimulate dimerization of the wild-type transcript have an effect on the dimerization properties of this 5′-truncated transcript (results not shown). These combined results demonstrate that RNA dimerization is a direct consequence of the LDI-to-BMH conformational switch of the HIV-1 leader RNA.

Targeting of the PAS Stem Affects the Leader RNA Conformation—The results described thus far indicate that oligonucleotide-mediated targeting of the PBS domain can affect the LDI-BMH equilibrium but not all subdomains react similarly. Targeting of the PBS motif and the directly flanking sequences only marginally affected the level of RNA dimerization. Disruption of the PBS stem by DNA probes that bind either to the left side (151/123) or the right side (223/200) seems the most effective trigger of the LDI-to-BMH conformational switch, as measured by induction of RNA dimerization. To corroborate this result, we analyzed additional antisense oligonucleotides that target slightly shifted leader RNA domains. In brief, these oligonucleotides exhibit profound differences in their effect on the LDI-to-BMH equilibrium and concomitantly on the dimerization properties of the transcript. For instance, probe 151/123 induces RNA dimer formation as is the case when a more upstream leader sequence is targeted (152/194). However, a downstream shift (161/135) results in an almost complete loss of the ability to induce the BMH conformation and RNA dimerization (Fig. 3, A and B, lane 4). Probe 223/203 targets the right side of the LDI stem and effectively triggers RNA dimerization. Compared with this probe, a significant loss in dimerization was observed when a more upstream site is targeted (202/182, see Fig. 2). Similarly, less dimers are induced when a more downstream site is targeted (245/216 and 245/225, Fig. 3). These results indicate that maintenance of the upper part of
the PBS stem, that is the PAS-containing segment, is critical for maintenance of the extended PBS stem and stable LDI folding of the HIV-1 leader RNA.

One obvious problem with the use of oligonucleotides as antisense probes is that a length of about 20 nucleotides is required to ensure annealing onto the RNA template. Thus, the PAS stem cannot be targeted more specifically by this approach. We therefore synthesized mutant HIV-1 transcripts with more defined, precise alterations. A set of 18 transcripts with mutations in the PBS domain was analyzed, but we will focus on mutants that target the PAS-containing stem segment. The left side was altered in mutant 2L, the right side in mutant 2R, and the 2LR double mutant restores the base pairing possibility (28). As a control, we also included the 3L mutant that removes nearly the complete left side of the PBS element. All these mutations have a partial effect on the LDI-to-BMH equilibrium and RNA dimer formation.

Disruption of the PAS stem segment influences the overall folding of the HIV-1 leader RNA; the LDI-BMH equilibrium is shifted to the BMH conformation, and dimerization is induced to some extent. Interestingly, it has recently been proposed that the opening of the PAS stem occurs during initiation of reverse transcription. The tRNA\(^{Lys}\) primer first anneals onto the PBS, but an additional interaction with the PAS element is required for efficient initiation of reverse transcription (9, 10, 30). Thus, a scenario can be proposed in which reverse transcription events (the initial tRNA-PBS complex formation and subsequent RNA-PAS interaction) affect the leader RNA conformation (LDI-to-BMH switch) and possibly some of the leader RNA-associated functions (e.g. dimerization). We set out to study the effect of tRNA\(^{Lys}\)-annealing on the structure and dimerization function of the HIV-1 leader RNA. To mimic the natural reverse transcription process, one would ideally assemble the vRNA-RNA complexes with viral proteins such as the reverse transcriptase (RT) enzyme or the NC protein/Gag-p55 precursor. We tried this approach, but these protein-RNA samples turned out to be too complex to allow a straightforward identification of the different RNA structures (LDI, BMH, and D) by their electrophoretic mobility.

The tRNA\(^{Lys}\) primer or the anti-PBS DNA probe 202/182 were heat-annealed onto the 1–290 transcript, and the complexes were analyzed on TBE and TBM gels (Fig. 5, A and B). We included oligonucleotide 151/123 that triggers the LDI-to-BMH switch and RNA dimerization (lane 7) and anti-DIS oligonucleotide 269/246 that also mediates the switch.

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**Fig. 4.** HIV-1 RNA mutants and the effect on the LDI-BMH equilibrium and RNA dimer formation. The PAS-containing stem segment is mutated in mutant 2L (118UGUGU122 to ACGCA) and mutant 2R (118ACCAGAG123 to UGGUCUC), and 2LR is the double mutant. The segment upstream of the PAS is mutated in 3L (118UGUGU122 to ACGCA). The deletion mutant D1 lacks nucleotides 112–148. The wild-type and mutant 1–290 transcripts were incubated as described, and the samples were analyzed on TBE and TBM gels (panels A and B). The positions of the fast migrating LDI form, the alternative BMH fold, and RNA dimers (D) are marked with arrowheads.

**Fig. 5.** The effect of tRNA\(^{Lys}\) primer annealing on the LDI-BMH equilibrium and HIV-1 RNA dimerization. The 1–290 (lanes 1–7) and 92–290 (lanes 8–14) transcripts were incubated with the reagents indicated on top of the gel. We tested three tRNA concentrations (1, 4, and 8 µg). The samples were analyzed on TBE and TBM gels (panels A and B). For transcript 1–290, we marked the position of the fast migrating LDI form, the LDI\(^{rRNA}\) complex and the RNA dimer (D) on the left. For transcript 92–290, we marked the positions of the BMH conformation, the BMH\(^{rRNA}\) complex, and the dimer (D) on the right.
but effectively blocks dimerization (lane 6). We tested three tRNA concentrations (1, 4, and 8 µg) none of which triggered RNA dimerization of the 1/290 transcript by tRNA-annealing (lanes 2–4). A similar result was obtained with the anti-PBS probe, although a low level of RNA dimer is formed (lane 5).

The HIV-1 transcript clearly runs slower during electrophoresis upon incubation with 4 and 8 µg of tRNA (lanes 2 and 3), which may suggest LDI-to-BMH refolding. Even though this complex migrates at the position that is expected for a wild-type BMH transcript, the reduced gel migration could also be the direct result of tRNA-annealing and shifting of the transcript band on the gel (the 76-nucleotide tRNA is significantly larger than the DNA probes). To discriminate between these two possibilities, we used the 5′-truncated transcript 92–290 that exclusively folds the BMH conformation, such that the magnitude of the tRNA bandshift can be compared. The transcript-tRNA complexes were analyzed on TBE and TBM gels (Fig. 5, A and B, lanes 8–14). Whereas the full-length 1–290 transcript adopts the ground-state LDI conformation, the 92–290 transcript favors the BMH conformation, and this transcript is dimerization-prone (lane 8). We observed the same bandshift upon tRNA-annealing to either the mutant BMH transcript (lanes 9 and 10) or the wild-type LDI transcript (lanes 2 and 3). This suggests that tRNA-annealing on the wild-type transcript only triggers a tRNA bandshift, and no further shift is observed that is caused by the LDI-to-BMH conformational switch.

These results argue against the hypothesis that PBS-tRNA-annealing and the subsequent PAS interaction affect the overall leader RNA structure. Nevertheless, we observed some other interesting effects in the context of the mutant BMH transcript 92–290 (Fig. 5, A and B, lanes 8–14). First, tRNA-annealing seems slightly less efficient for the BMH transcript compared with the wild-type LDI transcript, and this effect is most obvious in the experiment with the intermediate amount of tRNA (4 µg, lanes 3 and 10). This result may suggest that the PBS is more accessible within the LDI conformation. Second, we observed a small, but significant effect of tRNA-annealing on the dimerization capacity of the 92–290 transcript. This tRNA effect is most apparent on the TBM gel that yields extremely high dimerization efficiencies. A residual amount of monomeric HIV-1 RNA is present in the absence of tRNA (Fig. 5B, lanes 8 and 11) but disappears at a certain tRNA concentration (lanes 9 and 10). The intermediate tRNA sample that was analyzed on the TBE gel is also very informative as it shows a mixture of monomeric and dimeric RNA with and without an annealed tRNA primer (Fig. 5A, lane 10). In this sample, 38% of the tRNA-free transcript is in the dimeric form, whereas at least 61% of the tRNA-bound transcript has dimerized. The results obtained in this sensitive dimerization assay with the 92–290 transcript provides the first in vitro evidence that reverse transcription events (tRNA-PBS-annealing and/or PAS interaction) may influence the structure and function of the viral RNA template. Because similar effects are seen with the anti-PBS probe (lane 12), these tRNA effects do not provide independent evidence for a PAS-mediated effect.

To elaborate on this result, we tested primer annealing on the set of mutant HIV-1 transcripts. The wild-type and mutant transcripts were either mock-incubated or incubated with the anti-PBS probe 202/182 or the tRNA\textsuperscript{Lys} primer, and samples were analyzed on TBE and TBM gels (Fig. 6, A and B). Occupation of the PBS did not trigger dimerization in most cases, but a minor stimulatory effect of the anti-PBS probe is apparent. Oligonucleotide-induced RNA dimerization is most pronounced in combination with the 2R mutant transcript that is more dimerization-prone because of the partial LDI-to-BMH shift (lanes 7 and 8). The mutant 3L transcript also shows a prominent oligonucleotide-induced effect and, more importantly, a minor tRNA effect (lanes 15). Most informative is the analysis of the D1 mutant that stably adopts the BMH conformation, which coincides with a relatively high intrinsic dimerization capacity. Approximately 19% dimeric RNA is scored on the TBE gel for the D1 mutant (Fig. 6A, lane 16) that increases to 53% on the TBM gel (Fig. 6B, lane 16). Annealing of the anti-PBS DNA oligonucleotide or the natural tRNA primer has a significant stimulatory effect on the level of RNA dimerization, which is more pronounced on the TBM gel (lanes 17 and 18). These combined results argue that PBS-annealing can induce a conformational switch in the leader RNA that modulates dimerization, but it is also obvious that tRNA-annealing is not sufficient to induce the switch.

The effects of tRNA-annealing on the conformation of the monomeric RNA are complex, but interesting. The mutations in the 2L and 2R transcripts open up the PAS stem segment, and both changes cause a partial shift of the LDI-to-BMH equilibrium (Fig. 4). It is therefore remarkable that the corresponding vRNA-tRNA complexes differ in their migration (Fig. 6A, lanes 6 and 9). It is possible that the slow migrating vRNA-tRNA complex that is selectively formed by the 2R mutant has established the PAS-antiPAS interaction (lane 9). This interaction is not possible for the 2L and 2R mutants because the PAS motif is mutated, and these transcripts do not form the slow migrating vRNA-tRNA complex (lanes 6 and 12). The wild-type template exhibits an intermediate phenotype (lane 6), probably because the PAS is not directly available for base pairing with the tRNA. Formation of the PAS-antiPAS interaction on the 2R template will destabilize the extended PBS stem and may therefore favor the BMH folding. The slow mi-
migration of this 2R complex is indeed reminiscent of the BMH fold, but we did not observe induced RNA dimerization, except for the deletion mutant D1 (lane 13). Interestingly, formation of the slow migrating vRNA-trNA complex for the 2R mutant coincides with an increased reverse transcription activity of this template (9). This correlation holds up for the other templates, with the ranking order 2R > wt > 2L, 2L.R. Mutants 3L and 3R also destabilize the extended PBS stem and thereby indirectly expose the PAS element. Indeed, these transcripts form a slowly migrating vRNA-trNA complex (Fig. 6A, lane 15 for mutant 3L and results not shown for mutant 3R). Thus, it seems that the additional PAS-antiPAS interaction induces a conformational switch of the vRNA-trNA complex that makes it an efficient template for reverse transcription. The slow migration of this complex suggests that formation of the PAS-antiPAS interaction facilitates the LDI-to-BMH switch.

The Extended PBS Stem Can Absorb Destabilizing Effects—We measured a minor effect of RNA-annealing on the structure and dimerization properties of the viral RNA template in a sensitive experimental setting. In particular, we used mutant templates in which the LDI-BMH equilibrium is shifted toward the BMH conformation and that therefore have a relatively high intrinsic dimerization capacity. We were not able to provide more direct evidence that RNA-annealing onto the PBS and PAS elements can trigger the LDI-to-BMH switch on a wild-type template. Either gross mutations (e.g. the D1 deletion) or multiple assays on the PBS domain (e.g. mutant template plus tRNA-annealing) are required to induce RNA dimerization. In other words, it appears that the extended PBS stem of the LDI conformation is relatively resistant to mutational attack.

To directly test this idea, we used the method of oligonucleotide-scanning arrays (31, 32). The arrays consist of tethered antisense oligonucleotides that target all possible sequences within the 1–290 HIV-1 leader RNA. This novel methodology can be successfully used to probe the accessibility of different regions of the HIV-1 leader RNA. We used this method to score the structural effects of annealing of the antisense probe 151/123. This probe targets the left side of the extended PBS stem and is one of the most effective inducers of the LDI-to-BMH switch and RNA dimerization. The array technology allows us to discriminate between local melting and complete melting of the extended PBS stem. If base pairs are melted locally, one would expect exposure of template nucleotides that flank the annealed probe and nucleotides on the opposite side of the PBS stem region (around leader position 220). However, more dramatic changes are expected in case the extended PBS stem is completely melted, in particular in the poly(A) and DIS regions that actively participate in this interaction.

We hybridized the array with the Cy5-labeled HIV-1 transcript 1–290 that was either mock-incubated or incubated with the 151/123 probe. The differential annealing activity is plotted in Fig. 7 as a function of the position on the leader RNA. The score of 1 indicates that no changes in template accessibility are apparent upon annealing of the 151/123 probe. Peaks indicate increased accessibility for oligonucleotide binding upon annealing of the 151/123 probe. Obviously, no information is obtained for the region that is targeted by the 151/123 probe itself. Increased template accessibility is apparent in the region directly upstream of the annealed oligonucleotide, and such an effect is even more pronounced in the sequences that are immediately downstream. The accessibility of the latter region (152–192) increases up to 5.5-fold upon annealing of the DNA probe, which is consistent with opening of the U5-top hairpin

Fig. 7. Oligonucleotide array probing of PBS stem destabilization. The 1–290 HIV-1 transcript was incubated with and without the 151/123 antisense probe that is an effective inducer of the LDI-to-BMH switch and RNA dimerization (see e.g. Fig. 2, lane 7). An array with all possible 25-mer antisense oligonucleotides (25/1, 26/2–30/306, 31/307) was used to probe the conformational differences between these two HIV-1 RNA samples. In fact, the array also contained all possible 24-, 23-, 22-, 21-, 20-, 19-, 18-, 17-, 16-mers, but we have focused on the results with the 25-mer oligonucleotides. The differential annealing activity is plotted versus the annealing position of the 3′-end of the antisense probes. On top we indicated the functional domains of the HIV-1 leader RNA. Differential annealing data could not be calculated for oligonucleotides that completely or partially overlap with the 151/123 probe (areas indicated by the black and white bar) because of competition for the same target-binding site. The peak of differential annealing activity near the PBS of the RNA template is represented by probe 196–220.

that is located just upstream of the PBS (Fig. 1C). The PBS sequence does not show an altered accessibility upon probe annealing. A distinct peak of increased template accessibility is apparent on the opposite side of the PBS stem, with a more than 3-fold increase for template position 196–220. This second-site effect of annealing of the probe 151/123 is restricted to the 190–226 region, and the differential accessibility returns to the value of 1 for nucleotides that are positioned further upstream and downstream. This finding provides independent evidence for the existence of the base-paired PAS stem as shown in the secondary RNA structure model of Fig. 1C. There is a small but significant increase in the accessibility of the DIS region, which is consistent with the observed ability of probe 151/123 to induce RNA dimerization. On the other hand, it is clear that the DIS is not fully exposed, and we measured no increased accessibility in the poly(A) domain. These results confirm that the extended LDI stem has the capacity to resist a certain level of destabilization. Similar results were obtained with shorter antisense oligonucleotides such as 24-mer, 23-mer, etc. (data not shown).

DISCUSSION

We previously reported that the HIV-1 leader RNA can adopt two mutually exclusive RNA secondary structures, and an RNA switch mechanism was proposed that regulates some of the leader-encoded functions such as RNA dimerization (20, 21). The DIS hairpin motif is involved in RNA dimerization and is exposed in the BMH leader conformation. However, the DIS motif is masked by base-pairing with the upstream poly(A) domain in the alternative LDI conformation, which is thermodynamically more stable. This LDI interaction extends a base-paired stem that encloses the central PBS domain (Fig. 1C).

Using different experimental approaches, we attacked the PBS stem to measure how much this extended structure should be destabilized to trigger the LDI-to-BMH switch and concomitant RNA dimerization. The biological rationale for this study is to

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Conformational changes within the leader RNA orchestrate in this study we specifically addressed the possibility that an absolute requirement for the packaging of apparently reg...

We did not include the process of RNA packaging in this mechanistic becomes accessible after the switch to the BMH structure, thus facilitating interaction may occur in the LDI-context, but the PAS motif may only dimerization (this study); bottom, NC protein facilitates the LDI-to-BMH switch and RNA dimer formation (21). The initial tRNA-PBS interaction may occur in the LDI-context, but the PAS motif may only become accessible after the switch to the BMH structure, thus facilitating initiation of reverse transcription. See the text for further details. We did not include the process of RNA packaging in this mechanistic model, but packaging may be coupled to RNA dimerization.

analyze whether structural changes in the upper PBS domain (during reverse transcription) can influence the overall RNA folding and some of the leader-encoded functions. Specifically, the tRNA<sup>Lys<sub>3</sub></sup> primer anneals to the PBS motif and subsequently to the PAS motif that forms the upper segment of the extended PBS stem (9, 10, 30). Indeed, we measured a small but significant effect of tRNA-annaeling on the LDI-BMH equilibrium and the dimerization properties of the viral RNA. This is the first demonstration that the processes of reverse transcription and RNA dimerization may be functionally coupled. This result was obtained in a simplified in vitro assay system with naked RNA transcripts, which is likely to differ substantially from the in vivo situation in virion particles. We tried to mimic the in vivo situation by inclusion of the viral RT enzyme and the RNA chaperone protein NC, but these conditions are incompatible with our experimental approach as no discrete RNA conformations could be distinguished that were due to protein-mediated bandshifts (results not shown).

Although speculative, one could propose that the cross-talk between reverse transcription and other leader-mediated processes plays an important biological role in the viral replication cycle. One possibility is that the leader RNA conformation is designed to coordinate the accurate timing of the different leader-encoded functions. In this scenario, it seems possible that early reverse transcription events induce other leader functions such as RNA dimerization and packaging (Fig 8, top). This may sound counterintuitive as the order of events is thought to be reversed in vivo, with initial dimerization and packaging of the RNA and subsequent reverse transcription. However, we only probed the effect of tRNA primer annealing, which is the first step in reverse transcription that may occur relatively early in the virus-producing cell. This mechanism may be regarded as a safety feature to ensure that only viral genomes with a properly associated tRNA primer are allowed to dimerize and to be packaged into virion particles. Studies with virus mutants are required to critically test this hypothesis, although we realize that such an analysis will be complicated due to the presence of multiple overlapping signals in the HIV-1 leader RNA (2). Furthermore, the presence of a functional PBS sequence with an annealed tRNA<sup>Lys<sub>3</sub></sup> primer is not an absolute requirement for the packaging of apparently regular RNA dimers into HIV-1 particles (33). The presence of a dimeric RNA genome in retrovirus particles is clearly instrumental in the later steps of reverse transcription (33–35), but in this study we specifically addressed the possibility that conformational changes within the leader RNA orchestrate early functions during virion assembly. It remains possible that the virus replication mechanisms are coordinated differently in vivo than suggested by our in vitro studies. For instance, it seems possible that RNA dimerization and/or packaging is mediated by the viral NC protein through induction of the LDI-BMH switch (21). Due to destabilization of the extended PBS stem, the PAS may become accessible for annealing to and activation of the tRNA primer for reverse transcription (Fig. 8, bottom). Whatever the precise mechanism, the presence of an RNA switch mechanism allows the coordination of different RNA-mediated functions. It is also possible that the same leader RNA switch is involved in the decision of a full-length viral RNA to act as mRNA for translation by ribosomes or as genomic RNA that is encapsulated in virions. This possibility becomes more realistic because we have recently found interactions between the upstream and downstream leader domains, which include the translational initiation region and the AUG start codon. Although viral proteins may modulate this RNA switch, we propose that it is the RNA itself that forms the intrinsic checkpoint for the production of infectious virus particles with a dimeric- and reverse transcription-competent RNA genome.

Another intriguing finding is that a major destabilization of the lengthy PBS-LDI stem is required to induce the LDI-to-BMH conformational switch. The extended nature of the PBS-LDI stem makes it relatively resistant to the impact of minor destabilizing mutations or antisense oligonucleotides. In a biological setting, this may indicate that HIV-1 needs tight control over the process of RNA dimerization. Premature RNA dimerization may for instance interfere with the process of mRNA translation (36) and should therefore be restricted to viral RNA genomes that participate in the assembly of virus particles. Interestingly, we previously demonstrated that the viral NC protein can mediate the LDI-to-BMH switch (21). Because this RNA chaperone protein is released form the Gag precursor protein during the late stage of virus assembly, this mechanism will ensure the proper timing of RNA dimerization. Even though the LDI stem can adsorb mutations because of its extended nature, this RNA structure is not excessively stable in thermodynamic terms (21). This is due to the presence of multiple destabilizing bulge or internal loop elements (Fig. 1C). In fact, we previously demonstrated that further stabilization of this duplex leads to a severe replication defect, and such a mutant template cannot easily be reverse-transcribed (29). These mutant RNA templates also demonstrate a severe in vitro RNA dimerization defect, thereby confirming the inverse correlation between LDI folding and RNA dimer formation.

The apparent damage tolerance of the extended PBS stem in the LDI conformation is similar to what has been described as error and attack tolerance of complex biological networks (37). The ability of the base-paired stem to absorb mutations may explain a paradox that could not be resolved previously. The individual hairpin motifs of the BMH conformation are readily supported by phylogenetic analysis. Different virus isolates show many base-pair co-variations that change the nucleotide sequence but not the base pairing within the poly(A) and DIS hairpins (24, 38). However, there is no such support for the base pairs of the LDI conformation. We proposed the LDI base-pairing scheme based on multiple experimental lines of evidence (21), but we found little phylogenetic support for this interaction in terms of base-pair co-variations. We now realize that base pairs in the extended LDI stem are under limited evolutionary pressure because single point mutations will not.

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4 H. Huthoff and B. Berkhout, unpublished results.

3 T. E. M. Abbink and B. Berkhout, unpublished results.

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have a major impact on LDI folding and function. In contrast, the same point mutation is much more likely to have a significant destabilizing effect on the hairpins of the BMH conformation, and second-side mutations will primarily be selected for their property to repair the hairpins.

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