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Human Immunodeficiency Virus Type 1 RNA outside the Primary Encapsidation and Dimer Linkage Region Affects RNA Dimer Stability In Vivo

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To characterize the *cis*-acting determinants that function in RNA dimer formation and maintenance, we examined the stability of RNA dimers isolated from virus particles containing mutations in the encapsidation region of human immunodeficiency virus type 1 (HIV-1). The genomic RNAs of all mutants containing lesions in elements required for in vitro dimerization exhibited thermal stability similar to that of wild-type (WT) HIV-1. These data indicate that the eventual formation of stable dimeric RNA in vivo is not absolutely dependent on the elements that promote dimer formation in vitro. Surprisingly, mutants that lacked a large segment of the middle portion of the genome, outside the likely primary dimer linkage region, formed RNA dimers that were measurably more stable than WT. In addition, the insertion of one or multiple copies of a foreign gene, which resulted in a series of vectors that approached RNA length similar to that of WT RNA, still exhibited augmented dimer stability. These results suggest that there are regions in the HIV-1 genome outside the primary dimer initiation and dimer linkage regions that can negatively affect dimer stability.

Encapsidated full-length retrovirus RNAs are found in homodimeric form in virus particles. The association between the two RNAs is noncovalent, as evidenced by the dissociation of dimers to monomers under condition of elevated temperature or by the presence of denaturing agents (for a review, see reference 13). Based on electron microscopy, the 5′ region of both viral RNAs contains a primary contact point. This contact point is often referred to as the dimer linkage sequence (DLS) (5, 28, 33, 39).

Short synthetic retroviral RNAs can spontaneously dimerize in vitro under certain conditions (4, 6, 8, 11, 12, 14, 15, 20, 27, 29, 34, 41, 43–45), and the efficiency of dimerization can be enhanced by the viral nucleocapsid (NC) protein (14–16). Based on studies of in vitro dimer formation with deletion and substitution mutations, the presumptive primary DLS of human immunodeficiency virus type 1 (HIV-1) has been mapped to a region near the major splice donor, which is located just proximal to the beginning of the *gag* gene (4, 34, 45). Thus, the DLS overlaps with regions of the viral RNA known to be important in *cis* for encapsidation in vivo (3, 10, 22, 31).

Recent evidence suggests that there may be an additional region adjacent to the DLS important for the in vitro formation of RNA dimers. This region has been termed the dimer initiation sequence (DIS) and is located just upstream of the major splice donor (29, 38, 41, 44). Biochemical and RNA structure analyses indicate that the DIS probably forms a stem-loop structure with a hairpin loop containing a six-nucleotide (nt) palindromic sequence (11, 21). This hairpin structure has also been designated stem-loop 1 (SL1), and, based on genetic data, forms and functions in RNA encapsidation in vivo (35, 36). Ostensibly, dimer formation would occur through a kissing hairpin mechanism by which the two RNAs would form an initial loop-loop contact based on complementary antiparallel base pairing followed by the generation of a stable intermolecular structure composed of hydrogen-bonded nucleotides in the stem regions of the hairpin (29, 38, 41, 44). In support of this model, disruption of the palindromic sequence in the hairpin abrogates in vitro dimerization (12, 20, 29, 38, 41, 44).

In vivo, dimerization may include a maturation process catalyzed by the viral NC protein, which is present in virus particles, resulting in more extensive and more stable interaction between the RNA pair (14–16). Very little is known about the requirements for dimer formation in vivo, and some data suggest that there may be qualitative differences between in vitro and in vivo dimerization (14–16, 18, 19, 46). In the present study, we describe experiments that examine in vivo dimer formation and dimer stability using a series of mutants in the region that functions in in vitro dimerization and measure the stability of those dimers. Surprisingly, all of these mutants were able to form dimers that were as stable as wild-type (WT) RNA. In addition, we examined the stability of RNA dimers composed of HIV vector RNA lacking a large segment of the interior of the viral genome. These vectors formed dimers that were more stable than dimers formed from full-length RNA. These data suggest that there are internal sequences of the viral genome that negatively affect overall dimer stability.

The stability of RNA dimers is not affected by disruption of SL1-4. In an attempt to identify specific elements that are required for association of the two HIV-1 RNAs in vivo, we introduced a variety of mutations into the *cis*-acting encapsidation region (E/Ψ) of full-length viral RNA, harboring a small deletion in the *env* gene, pMSMBA (35), which is a derivative of the infectious proviral clone pNL4-3 (1). We first tried to see whether disruption of stem-loop structures around the DIS-DLS region, which functions in in vitro dimer formation, affected the thermal stability of HIV-1 dimeric RNA. Genetic and biochemical data indicate that there are four stem-loop structures (designated SL1 to SL4) within the 5′ packaging signal region of HIV-1 (11, 35) and that SL1, SL3, and SL4 function in encapsidation (35, 36) (Fig. 1). We analyzed four mutants that individually disrupt each one of these four structures (S1, S2, S3, and S4) and one virus that had mutations in both stem 1 and stem 3 (S1S3). Construction of pMSMBAS1,

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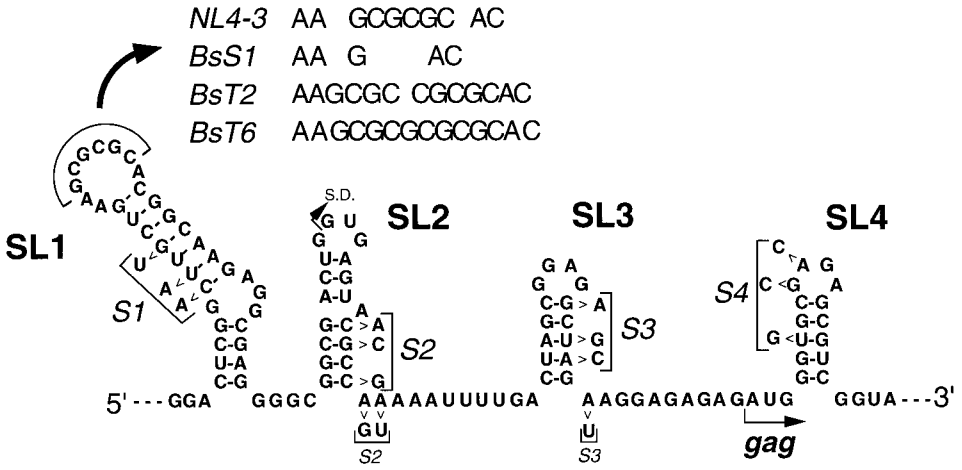

FIG. 1. Mutations introduced into the stem-loop structures SL1, -2, -3, and -4 in the E/Ψ region of HIV-1_{NL4-3} (1). BsS1, BsT2, and BsT6 contained base insertions and a deletion that would change the palindromic sequence in the hairpin loop of SL1. The natures of the substitution mutations that would destabilize the individual components of each of the stem structures are shown adjacent to the WT sequence. Evaluation of likely secondary structures for each of the mutants by Mfold (24, 25, 47) indicated that the substitutions designed to destabilize a particular stem would be unlikely to indirectly affect the formation of the other three stems. The major splice donor (S.D.) and the start codon of the *gag* gene are indicated for reference.

pMSMBAS2, pMSMBAS3, pMSMBAS4, and pMSMBAS1S3 has been described elsewhere (35). Mutants S1, S3, S4 and S1S3 are significantly impaired in encapsidation compared to WT RNA (35, 36). Nonetheless, since some detectable genomic RNA is packaged in these mutants, we thought it would be feasible to compare the stability of those mutant dimeric RNAs with that of WT. To obtain virus particles containing these mutant RNAs, we transfected 293tat cells (40) with equivalent amounts of WT (pMSMBA) or mutant DNAs using the calcium phosphate precipitation method (2). Two or three days later, virus was harvested and purified from the media by centrifugation through a 20% sucrose cushion and viral RNA was then extracted from purified virus. To isolate RNA from particles, virions were disrupted by the addition of sodium dodecyl sulfate to 1% and treatment with proteinase K (300 μg/ml) at room temperature for 60 min followed by phenol (pH 8.0)-chloroform extraction, chloroform extraction, and ethanol precipitation. Pelleted RNA was then resuspended in T buffer (10 mM Tris-Cl [pH 7.5], 1 mM EDTA, 1% sodium dodecyl sulfate, 100 mM NaCl, 10% formamide). The thermostability of dimeric viral RNA was determined by incubating RNA aliquots for 10 min over a range of temperatures. The proportions of dimer and monomer were measured by electrophoresis in a nondenaturing 0.75% agarose gel containing 0.5× Tris-borate-EDTA (TBE) buffer (30). Northern (RNA) hybridization analysis was performed as described previously (30). To generate an RNA probe specific for HIV-1 RNA, we subcloned a *Kpn*I fragment, corresponding to a segment of the *pol* gene (nt 3831 to 4159 of NL4-3), into pGEM3Z to create pT7pol. Labeled RNA was then generated by using T7 polymerase. To assess the conversion of dimeric to monomeric RNA, the relative amounts of both RNA species were quantitated with a Molecular Dynamics PhosphorImager.

The mobility of both the dimeric and monomeric RNAs was indicative of heterogeneous RNA structure within both RNA populations. This is not surprising, since the RNAs were necessarily analyzed on a nondenaturing gel. Similar electrophoretic profiles have been previously observed for retroviral RNAs (9, 20, 30). Nonetheless, we could generally distinguish between these two RNA populations, generate dissociation curves for each RNA, and determine whether the mutations affected the thermal stability of the dimer. The results of this experiment indicated that the thermal stability of the RNA dimers from each of the mutants was similar to WT (Fig. 2). Thus, these data are consistent with the surprising possibility that the eventual stability of the dimer does not rely on any of these hairpin structures.

SL1 has been shown to play a central role in the formation of RNA dimers in vitro (29, 38, 41, 44). In particular, the palindromic sequence that comprises the hairpin loop of SL1 appears to function in initial interaction between the two RNAs in a "kissing loop" association (12, 20, 29, 38, 41, 44). Consequently, we then tried to examine the role of the loop of SL1 in virion RNA dimerization in vivo. The loop of SL1 includes 6-nt palindromic sequence (GCGCGC), which is also a *Bss*HII site. We introduced nucleotide deletions or insertions at this site (Fig. 1) and examined the stability of dimeric RNA from particles. pMSMBABsS1 was created by digestion of pMSMBA with *Bss*HII, treatment with S1 nuclease, and ligation with T4 DNA ligase. pMSMBABsT2 and pMSMBABsT6 were created in essentially the same way as pMSMBABsS1, except that T4 DNA polymerase instead of S1 nuclease was used. The mutant BsS1 would lack palindromic sequence in the hairpin loop of SL1 as a result of deletion such that the initial interaction between two SL1 sequences would be ablated. In contrast, BsT2 and BsT6 contain 3 and 4 additional nt, respectively. As a consequence of these insertions, BsT6 would have a longer palindromic sequence, which would potentially lead to more stable interaction between two SL1 hairpins, while BsT2 contains an interrupted palindrome that might interfere with stable association between the hairpin in the two RNAs. Based on previous analysis of mutants containing lesions in the hairpin loop (12, 20, 29, 38, 41, 44), the mutations in BsS1 and BsT2 would be expected to abrogate dimer formation in vitro. However, examination of RNAs from virus particles indicated that regardless of whether the palindromic sequence was disrupted (BsS1 and BsT2) or elongated to 10 nt (BsT6), the thermal stability of RNA dimers from particles was still very similar to that of WT (Fig. 2C).

Dimeric RNAs with internal deletions exhibit increased stability. The length of the HIV RNA genome is about 9,200 nt. Although it is widely accepted that the primary DLS overlaps

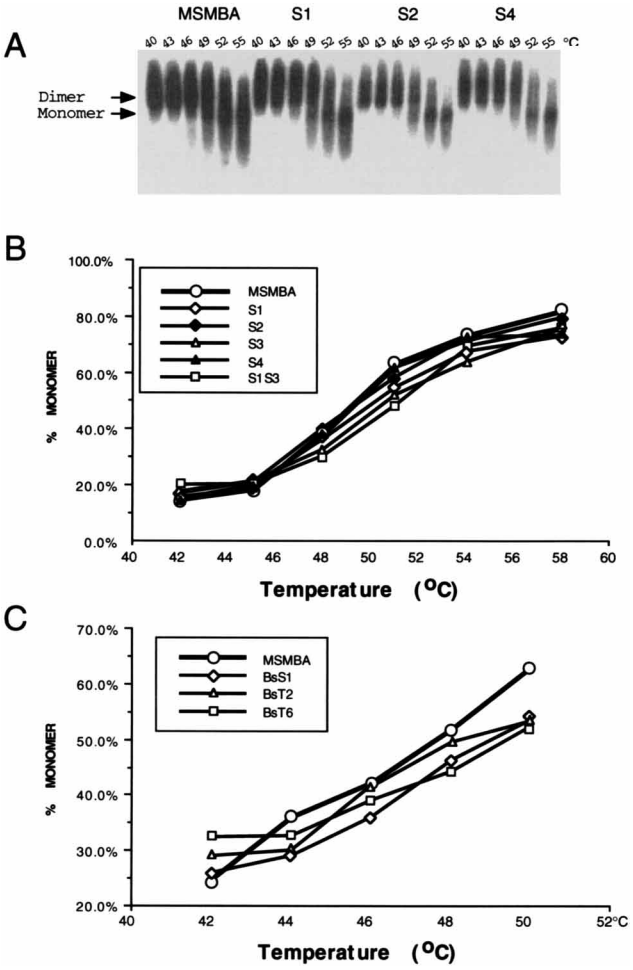

FIG. 2. Thermal stability of WT and mutant RNA dimers. Aliquots of RNA extracted from virions were resuspended in T buffer and incubated for 10 min in parallel reactions at various temperatures and then analyzed on a native agarose gel. (A) PhosphorImager analysis of RNA detected by Northern blotting. Positions of dimer and monomer RNAs are indicated. Various temperatures in which aliquots were incubated are indicated for each lane. (B and C) Thermal dissociation kinetics of RNA dimers. The relative amounts of monomeric and total RNAs in each lane were quantitated with a PhosphorImager, and the percentages of monomeric RNA were calculated for each RNA sample. Similar results were obtained in three separate experiments.

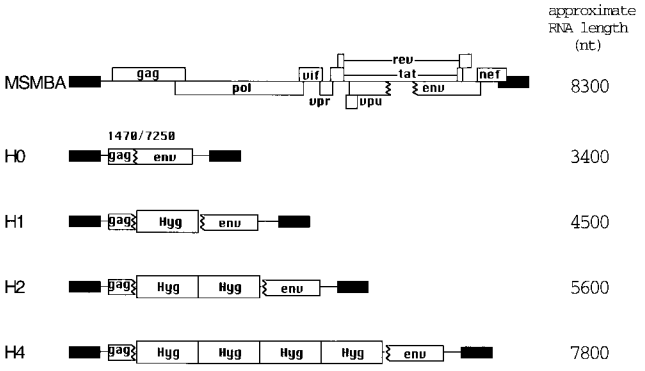

FIG. 3. H series of HIV-1 packaging vectors. The origin and construction of these plasmids are described in Materials and Methods. Hyg, hygromycin B phosphotransferase gene. The lengths of transcripts that are expected from constructs are indicated to the right.

with E/Ψ, near the 5′ end of the RNA, it is possible that there are secondary DLS sites in the viral genome that stabilize the dimer or that the presence of RNA outside the DLS somehow contributes to RNA stability nonspecifically. To test these possibilities, we constructed a series of vectors of HIV-1, designated H0, H1, H2, and H4 (Fig. 3). To create H1, H2, and H4, a bacterial hygromycin resistance (*hyg*^{r}) gene fragment was obtained from pGB108 (17) by cleavage with *Xba*I and *Nhe*I. This 1.1-kb *hyg*^{r} gene was inserted between nt 1470 and 7250 of pMSMBA. The resulting construct expresses mRNA from the MSMBA long terminal repeat. H1 has one *hyg*^{r} gene, whereas H2 and H4 have two and four tandem repeated *hyg*^{r} genes, respectively. H0 was created by deletion between nt 1470 and 7250 of pMSMBA. H0 was expected to express an RNA of about 3,400 nt, and the transcripts from H1, H2, and H4 should be 4,500, 5,600, and 7,800 nt of RNA, respectively. Since the transcribed RNA length of pMSMBA was expected to be 8,300 nt, H4 RNA approaches the same length as of that of pMSMBA. 293tat cells were cotransfected with H0, H1, H2, or H4 along with an expression construct designated pCMV$_{259}$MSMBAΔ21 (35). pCMV$_{259}$MSMBAΔ21 expresses amounts of the viral gene products and produces amounts of virus particles following transfection similar to those for WT. However, pCMV$_{259}$MSMBAΔ21 is profoundly impaired in encapsidation due to the absence of multiple elements that function in *cis* during encapsidation (35). In these experiments, each of the vector RNAs were packaged at a relative efficiency that was about 10 times greater than that of pCMV$_{259}$MSMBAΔ21, as evidenced by an RNase protection assaying to measure encapsidation (35–37) (data not shown). Since CMV$_{259}$MSMBAΔ21 RNA was encapsidated very inefficiently compared to the vectors, heterodimeric RNA composed of CMV$_{259}$MSMBAΔ21 and vector RNA could represent only a small proportion of the packaged RNA population.

To generate probes that could detect vector RNA, an *Nhe*I fragment containing the *hyg*^{r} gene from pGB108 was subcloned into the *Xho*I site of pGEM3Z to create pT7hygro. In addition, the 5′ leader region (nt 410 to 910) was amplified by using PCR with primers containing terminal *Sal*I and *Xba*I sites, and the resulting DNA product was cleaved with *Sal*I and *Xba*I and subcloned into pGEM11Zf(−) to generate pT7HIV-1$_{410–910}$. pT7HIV-1$_{410–910}$ was transcribed to generate an RNA probe that would anneal to each of the vector RNAs. In some experiments we also used an RNA probe from transcription of pT7hygro. The virion RNAs were purified from transfected media and the thermal stability of dimers was measured. As shown in Fig. 4, the stability of dimeric RNA from each of these vectors was similar. However, the stability of each of these dimeric vector RNAs was significantly higher than that of WT RNA (pMSMBA). In particular, the temperature at which 50% of the total RNA was converted to monomeric form was consistently about 5°C higher for the vector RNAs than for WT. These surprising data suggest that deletion of a large internal portion of the viral RNA or substitution with a foreign gene does not abrogate the formation of RNA dimers in virions. Moreover, these data are consistent with the idea that the interior of the viral genome decreases the stability of the RNA dimer. The observation that H4 has stability similar to that of H0 indicates that stabilization is due to the absence of the internal viral sequences rather than to augmented stabilization due to the presence of the *hyg*^{r} sequence.

It is possible that the character of dimeric RNA formed in vivo is different from that formed in vitro. Our results are consistent with some recent reports (7, 20) which suggest that the DLS-DIS region is dispensable for both the formation and

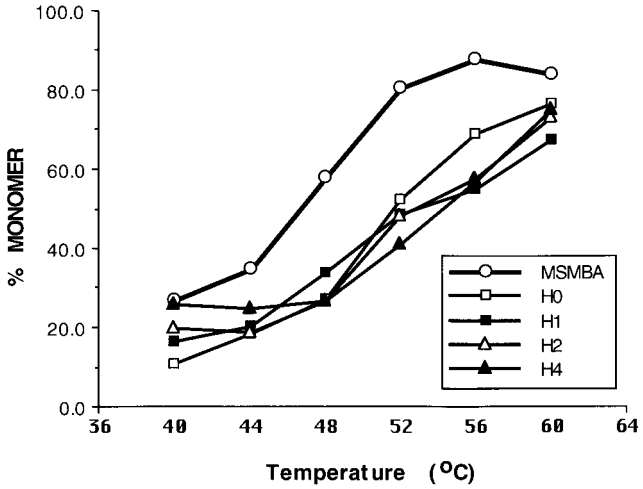

FIG. 4. Comparison of the thermal stabilities of WT and vector RNA dimers. The thermal stabilities of RNA dimers and monomers were measured as described for Fig. 2. The vectors are described in the text and are shown in Fig. 3. Similar results were obtained in three separate experiments.

stability of dimers in vivo. However, we think it likely that the elements that function in in vitro dimer formation may still be relevant for understanding in vivo RNA-RNA association. Isolation of RNA from particles necessarily involves the collection of particles over a relatively protracted period of time. Thus, it is possible that the rate of dimer formation is faster in the presence of a functional SL1, which contains the DIS for in vitro dimer formation, but that over extended time the two RNAs become stably associated even when the DIS or the DLS is absent. A corollary of this scenario is that overall RNA stability is a result of specific or nonspecific multiple contacts outside of the primary dimer linkage site. Recent electron microscopic analysis of WT virion RNAs is consistent with two primary interactions near the 5′ end of the RNA (23). It should be possible to assess the role of individual elements within E/Ψ through similar qualitative analysis of RNAs harboring mutations in that region.

The temporal order of RNA dimer formation relative to encapsidation remains unclear. Several of the mutants in the E/Ψ region that we used in this study of dimer stability are defective in encapsidation. For example, S1, S3, and S1S3 are markedly reduced in encapsidation efficiency (35, 36). If encapsidation efficiency is reduced for these mutants and RNA monomers can be encapsidated, this should decrease the efficiency at which two RNAs are packaged into each particle. However, for these mutants the relative amounts of monomeric and dimeric RNAs isolated from particles were similar to those of WT. On the surface, these data support the idea that dimer formation precedes encapsidation; formation of dimers before encapsidation would result in an equivalent monomer-to-dimer ratio even when overall packaging efficiency is reduced. On the other hand, there is evidence that encapsidation occurs prior to stable dimer formation. Genetic analysis of SL1 and SL3 indicates that these elements are true intramolecular hairpin structures that function in encapsidation; there is *cis* but not *trans* complementation between primary mutations, which ablate packaging, and second-site reversion mutations, which restore encapsidation (35, 36). In addition, the RNAs from rapid harvest virus particles are not as stably associated with each other as the RNAs from mature particles. Similarly, when particle maturation is blocked by mutation of the viral protease, the RNA dimers are less stable (18, 19).

Our data indicate that retroviral vectors can form stable dimers and that RNA length per se does not markedly influence dimer stability. In fact, deletion of the interior of the genome or substitution with a foreign nucleic acid sequence led to dimeric RNA that was more stable than WT viral RNA. These observations are consistent with two interesting conclusions. First, the viral RNA located in the interior of the genome is not required for stable dimer formation. Although it is probable that there is stable interaction between regions of the viral genome outside of E/Ψ in full-length WT viral RNA, those interactions are likely to be nonspecific. Second, the centrally located sequences in HIV-1 RNA may partially interfere with the formation of stable dimers. If regions at the termini are involved in specific interactions leading to stable dimer formation, these interior sequences may simply compete with the establishment of those interactions leading to alternative, less-stable dimers. Reduced dimer stability would not necessarily be a liability for retrovirus replication. Dimer formation is required for recombination during reverse transcription (26, 32, 42). It is possible that there may be an optimal stability for the dimer. When association between the two RNAs is too robust, dissociation of the RNAs may become inefficient.

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