

Requirements for Kissing-Loop-Mediated Dimerization of Human Immunodeficiency Virus RNA

JARED L. CLEVER,¹ MEI LIE WONG,^{2,3} AND TRISTRAM G. PARSLOW^{1*}

Departments of Pathology, Microbiology, and Immunology,¹ and Department of Biochemistry² and the Howard Hughes Medical Institute,³ University of California, San Francisco, California 94143

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Sequences from the 5' end of type 1 human immunodeficiency virus RNA dimerize spontaneously in vitro in a reaction thought to mimic the initial step of genomic dimerization in vivo. Dimer initiation has been proposed to occur through a "kissing-loop" interaction involving a specific RNA stem-loop element designated SL1: the RNA strands first interact by base pairing through a six-base GC-rich palindrome in the loop of SL1, whose stems then isomerize to form a longer interstrand duplex. We now report a mutational analysis aimed at defining the features of SL1 RNA sequence and secondary structure required for in vitro dimer formation. Our results confirm that mutations which destroy complementarity in the SL1 loop abolish homodimer formation, but that certain complementary loop mutants can heterodimerize. However, complementarity was not sufficient to ensure dimerization, even between GC-rich loops, implying that specific loop sequences may be needed to maintain a conformation that is competent for initial dimer contact; the central GC pair of the loop palindrome appeared critical in this regard, as did two or three A residues which normally flank the palindrome. Neither the four-base bulge normally found in the SL1 stem nor the specific sequence of the stem itself was essential for the interaction; however, the stem structure was required, because interstrand complementarity alone did not support dimer formation. Electron microscopic analysis indicated that the RNA dimers formed in vitro morphologically resembled those isolated previously from retroviral particles. These results fully support the kissing-loop model and may provide a framework for systematically manipulating genomic dimerization in type 1 human immunodeficiency virus virions.

Retroviral virions each typically contain two copies of viral genomic RNA (6 [reviewed in reference 8]). When all other virion components are removed by extraction in vitro, these two identical RNA strands remain linked together noncovalently, so that they migrate as a heat-labile RNA dimer in sucrose gradients or on nondenaturing gels. Electron microscopic examination of such dimers from a variety of retroviruses has revealed that under conditions which denature most other elements of RNA structure, the two strands remain bound to one another at a unique short region near their 5' ends (4, 5, 18). This region of maximally stable binding between genomic strands is termed the dimer linkage structure (DLS).

The widespread (and perhaps universal) tendency of retroviral genomes to dimerize and the evolutionarily conserved 5' location of the DLS imply an important role for this phenomenon in retroviral biology. Diploidy per se makes it possible for reverse transcriptase to switch from one template strand to another during minus-strand synthesis, thereby allowing the polymerase to bypass nicks in the RNA genome to produce a complete provirus while also promoting recombination between strands (12, 20); dimer linkage would presumably facilitate such template switching by juxtaposing the two RNAs and aligning them in register. Moreover, the estimated location of the DLS (as judged by electron microscopy) coincides closely with those of the primer binding site, the major splice donor, the *gag* start codon, and the *cis*-acting 5' encapsidation signals (ψ locus) of the virus, suggesting that dimerization might somehow directly facilitate early stages of reverse transcription or

modulate splicing, translation, or encapsidation of the viral RNA. Efforts to demonstrate the biological significance of the DLS, however, have been hampered by the fact that the molecular interactions linking the two strands are unknown. As a result, it has been difficult to specify the RNA residues involved in this linkage or to design viral mutations that would selectively block dimerization without affecting other activities of the genome.

Studies of this phenomenon have gained impetus in recent years from the finding that short, synthetic RNA strands representing the 5' ends of certain retroviral genomes, including that of the type 1 human immunodeficiency virus (HIV-1), will dimerize spontaneously and efficiently in vitro (9, 15). Utilizing this assay system, several laboratories have obtained evidence that a particular stem-loop structure situated immediately upstream of the major splice donor is specifically required for in vitro dimerization of HIV-1 RNA (13, 14, 16, 17, 19, 21). The sequence of this element, termed the dimerization initiation sequence (DIS), is highly conserved and is predicted to fold into an 11-bp stem with an internal 4-base bulge, crowned by a 9-base loop (Fig. 1A). Within the loop is a six-base palindrome (GCGCGC) whose sequence is almost invariant among HIV-1 strains. The in vitro dimerization of HIV-1 5' RNA sequences can be abolished either by deletion of the entire DIS stem-loop or by introduction of a single point mutation into the loop palindrome (19). Significantly, this putative DIS stem-loop element is also found in the genomes of HIV-2 and simian immunodeficiency virus (SIV), in which many include a slightly different six-base palindrome (GUGCAC) in their loops. These observations have given rise to a hypothetical model (Fig. 1B) in which a pair of HIV-1 or HIV-2 or SIV genomic RNAs initially interact through Watson-Crick base pairing between their palindromic DIS loops; subsequently, the intrastand base pairs of the two DIS stems are postulated to melt

* Corresponding author. Mailing address: Department of Pathology, Box 0506, University of California, San Francisco, CA 94143. Phone: (415) 476-1015. Fax: (415) 476-9672. Electronic mail address: parslow@cgl.ucsf.edu.

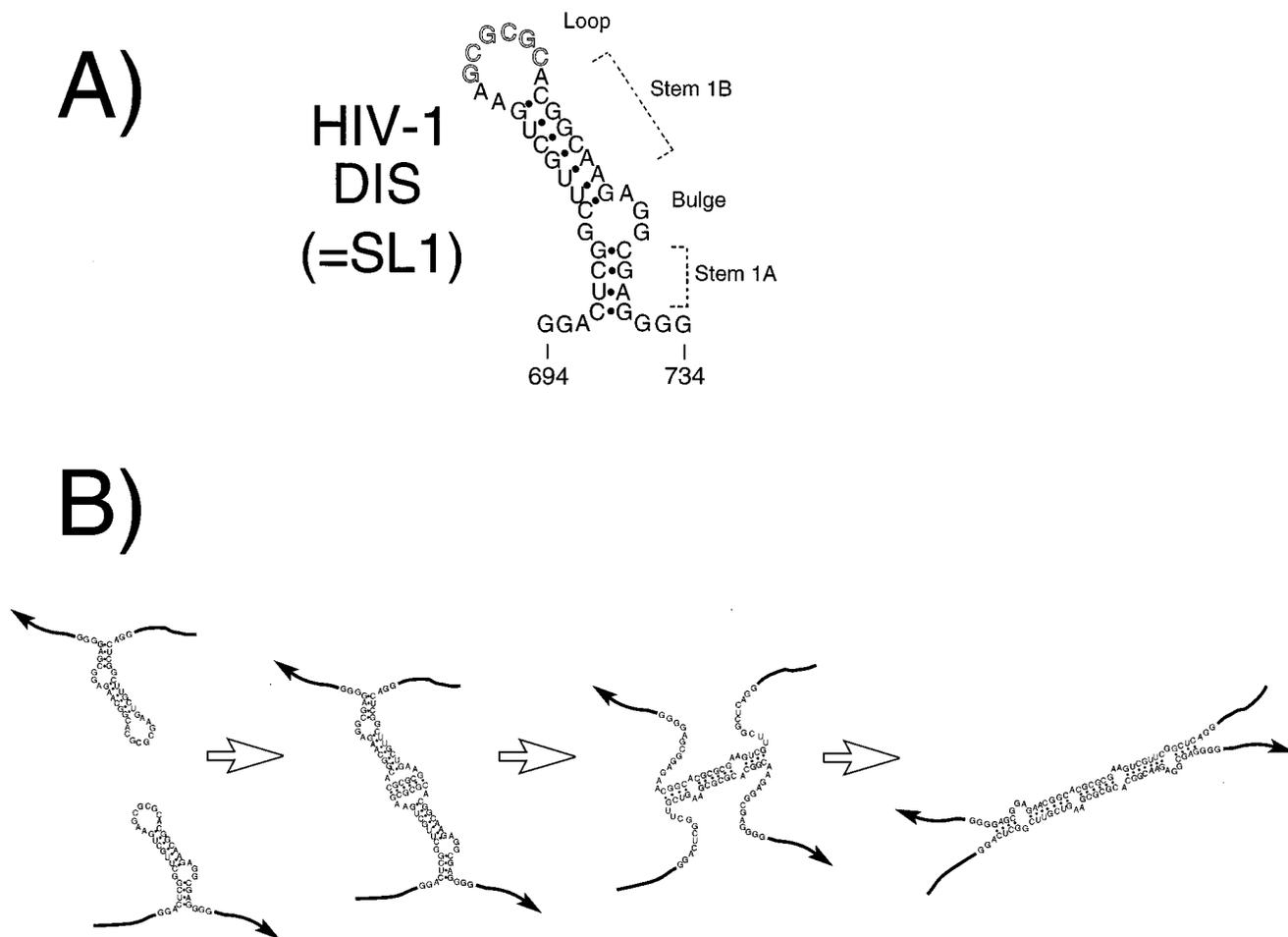


FIG. 1. Proposed mechanism of HIV-1 RNA dimerization. (A) Hypothetical structure of SL1, the putative DIS of HIV-1 LAI. The numbering of residues follows that of reference 7; the SL1 element is situated approximately 240 bases downstream from the cap site and 15 bases upstream from the major splice donor in the HIV-1 transcript. (B) The kissing-loop model of RNA dimerization, based on references 13, 19, and 21. Solid arrowheads indicate the 5'-to-3' orientation in each RNA strand.

apart and then reanneal to form a stable, interstrand duplex (13, 21). Consistent with this "kissing-loop" model, a variant HIV-1 RNA which fails to dimerize because of a point mutation (GCGCAC) in its DIS loop palindrome will nevertheless heterodimerize efficiently with a second variant that carries the complementary loop mutation (GUGCGC) (19). In the present report, we confirm and extend these earlier results by analyzing additional variant forms of the HIV-1 DIS element. Our results help to define the specific features of sequence and secondary structure required for HIV-1 RNA dimerization *in vitro* and may provide a basis for selectively manipulating this process in HIV-1 virions.

MATERIALS AND METHODS

Preparation of HIV-1 RNA fragments. DNA fragments encoding HIV-1 sequences (from strain LAI) were subcloned into pBluescript II KS+ (Stratagene) and then transcribed *in vitro*. In general, sense RNAs were transcribed from the T7 promoter and antisense RNAs were transcribed from the T3 promoter, after linearization of the plasmids with either *Xho*I or *Eco*RI, respectively. Production of fragments A, B, and I has been described previously (7).

The Δ SL1 construct was created by a multistep procedure whose final effect was to replace SL1 (nucleotides [nt] 697 to 731) with an *Sfi*I restriction site within an HIV-1 DNA fragment extending from the unique *Kas*I site (nt 637) to the unique *Cla*I site (nt 829) subcloned into pBluescript II KS+ as described before (7). Mutant forms of SL1 were then subcloned into the *Sfi*I site of Δ SL1 with overlapping synthetic oligonucleotides with *Sfi*I ends and a *Pvu*I site immediately prior to the 3' end to facilitate screening for the orientation of the

inserts. The sequences of all of the clones were confirmed by double-stranded plasmid sequencing. The orientation of these fragments within the vector was such that sense RNAs were transcribed with T3 RNA polymerase. Radiolabelled RNAs were prepared from *Eco*RI-cleaved plasmids, and longer, unlabelled transcripts (≈ 1.0 or 2.8 kb) were prepared from *Ssp*I- or *Afl*III-cleaved plasmids, respectively.

Radiolabelled RNAs were prepared exactly as described previously (7). Unlabelled RNAs were prepared in 100- μ l reaction mixtures containing 40 mM Tris-HCl (pH 8.0), 6 mM MgCl₂, 10 mM dithiothreitol, 2 mM spermidine, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.5 mM UTP, 100 U of RNase inhibitor (Boehringer Mannheim), 2 μ g of linearized plasmid DNA, and 40 U of T3 RNA polymerase and incubated for 1 h at 37°C. Template DNA was removed by treatment of the reaction mixtures with 5 U of RQ1 DNase (Promega) for 15 min at 37°C, after which the reaction mixtures were phenol-chloroform extracted, ethanol precipitated, resuspended in 40 μ l of RNase-free H₂O, and then stored at -70°C.

RNA dimerization assay. Each sample contained either 1 to 3 μ g of unlabelled RNA or 250,000 cpm (≈ 0.05 μ g) of ³²P-labelled RNA in 4 μ l of RNase-free water. These samples were heated to 85°C for 2 min, placed on ice for 2 min, and then either mixed with 4 μ l of 2 \times monomer buffer (1 \times monomer buffer is 50 mM sodium cacodylate [pH 7.5], 0.1 mM MgCl₂, and 40 mM KCl) and incubated for 30 min on ice or mixed with 4 μ l of 2 \times dimer buffer (1 \times dimer buffer is 50 mM sodium cacodylate [pH 7.5], 5 mM MgCl₂, and 300 mM KCl) and incubated for 30 min at 25°C. After addition of 0.04% bromophenol blue, 0.04% xylene cyanol, and 5% glycerol, the reaction mixtures were electrophoresed through nondenaturing 3.5% or 5% polyacrylamide gels in TBM buffer (89 mM Tris, 89 mM borate, 0.1 mM MgCl₂) at 4°C (13, 15, 21).

RNA electron microscopy. Unlabelled ≈ 1.0 -kb synthetic RNAs were spread by the method of Wellauer and Dawid, bound to grids, shadowed, and examined by electron microscopy exactly as described previously (23), except that denaturation was performed in 75% formamide without added urea.

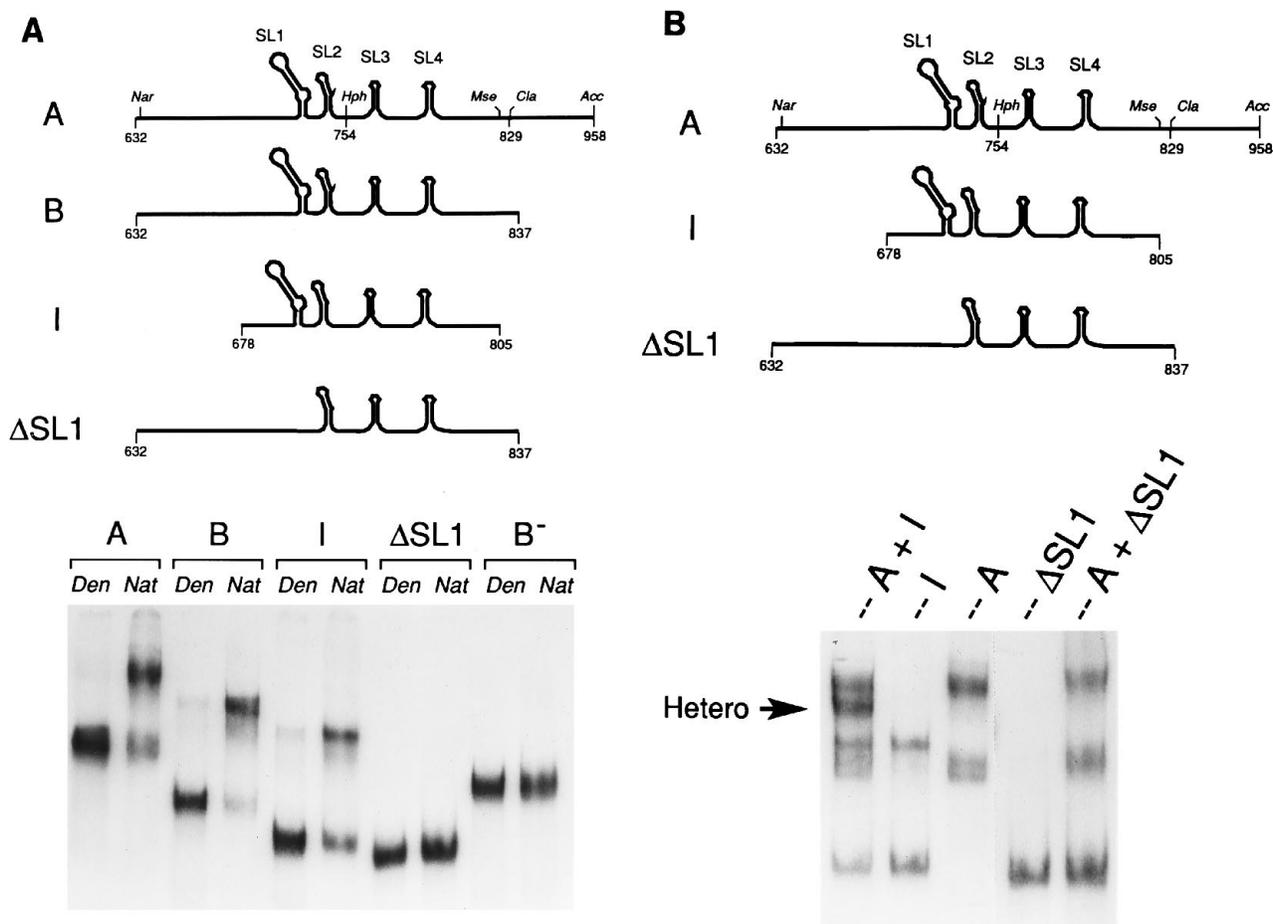


FIG. 2. SL1 dependence of RNA dimer formation. (A) Homodimerization. Synthetic RNAs spanning the indicated HIV-1 residues (above) were tested for dimer formation in the electrophoretic assay (below). The locations of SL1 and of three other potential stem-loops (7) are indicated. Each was assayed after denaturation in low-salt buffer (Den) and under native, high-salt conditions (Nat). B⁻, antisense form of construct B. (B) Heterodimerization. The three constructs shown (above) were incubated under native conditions individually and in pairs, and then they were assayed for dimerization (below). Hetero, apparent A/I heterodimer.

RESULTS

We have previously (7) formulated a working model for the secondary structure of the 5' packaging signal of HIV-1 RNA, focusing on a region that spans nt 686 to 823 and so extends from 36 bases downstream of the primer binding site through the first 34 bases of the *gag* coding sequence (Fig. 1A). We propose that this region is organized in four independent stem-loops, designated SL1 to SL4. The largest of these stem-loops (SL1) corresponds to the element identified independently by other groups as the DIS of HIV-1 (13, 19, 21).

In agreement with earlier reports, we found that various short (127 to 326 bases) synthetic HIV-1 RNA sequences that included SL1 each migrated as two discrete bands on non-denaturing gel electrophoresis after a brief incubation at high ionic strength (Fig. 2A, constructs A, B, and I). Typically, 50 to 90% of each RNA sample was found in the slower-migrating, presumptive dimer form under these native conditions (Nat), but shifted entirely to the fast-migrating (monomer) form at low ionic strength (Den). When two such RNA constructs of different lengths (constructs A and I) were combined under native conditions (Fig. 2B, lane A + I), the mixture yielded an additional band of intermediate mobility that was not observed with either construct alone (lanes A and I) and that presumably represented a heterodimer (Hetero). This confirmed that

the slower-migrating species are indeed dimers and provided an assay for both homodimerization and heterodimerization. The antisense forms of these sequences did not dimerize (e.g., Fig. 2A, construct B⁻). We have previously shown that some sequences within this region can be deleted without affecting dimerization in this assay (7). For the present study, we created a mutant form of construct B from which SL1 was precisely deleted and replaced by a unique *SfuI* restriction site. This mutant, designated ΔSL1, not only failed to homodimerize (Fig. 2) but also yielded no detectable heterodimer when combined with construct A (Fig. 2B, lane A + ΔSL1). Thus, an RNA lacking SL1 neither homodimerized nor formed heterodimers with wild-type HIV-1 RNA.

To define the sequence requirements for dimerization, we reinserted various mutant forms of SL1 into the *SfuI* site of ΔSL1 and tested their ability to support homodimerization with the electrophoretic assay. The initial series of mutants involved the two double-stranded regions of SL1 (which we designated stems 1A and 1B) and the bulge that separates them (Fig. 1A). The results are summarized in Fig. 3, and electrophoretic patterns for some representative mutants are shown in Fig. 4. We found that mutations or deletions in the bulge were well tolerated (mutants mB.1 and mB.2); indeed, the entire bulge could be deleted without appreciably interfer-

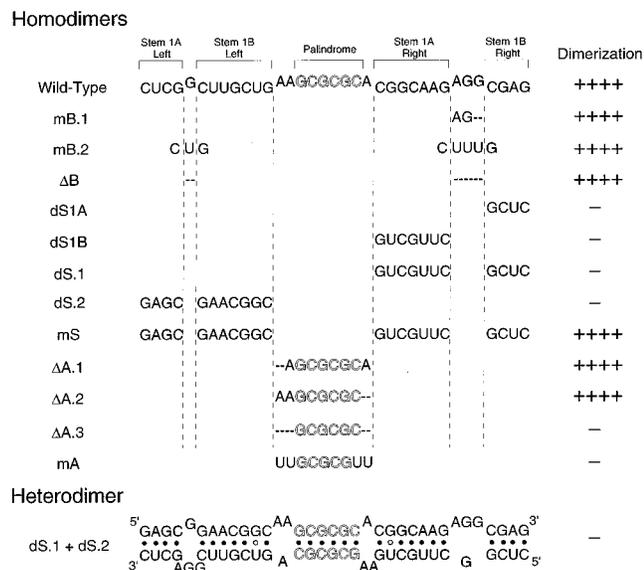


FIG. 3. In vitro dimerization of HIV-1 RNAs harboring mutations in SL1. The linear sequence of wild-type SL1 is shown at top, with major conformational domains demarcated. Mutants contained the wild-type SL1 sequence at all positions, except those indicated; dashes denote deleted bases. Each was inserted into the ΔSL1 construct and tested for homodimerization; two mutants (dS.1 and dS.2) were also tested for heterodimerization with each other. The proportion of labelled RNA migrating in the slower (dimer) electrophoretic band is indicated to the right for each: -, less than 5% dimer; +, 5 to 10% dimer; ++, 10 to 50% dimer; +++, 50 to 75% dimer; +++++, more than 75% dimer.

ing with dimerization (mutant ΔB). In contrast, mutations that disrupted base pairing in the stems, either individually (mutants dS1A and dS1B) or together (mutants dS.1 and dS.2), completely eliminated dimerization. When both stems were replaced by heterologous sequences that preserved base pair-

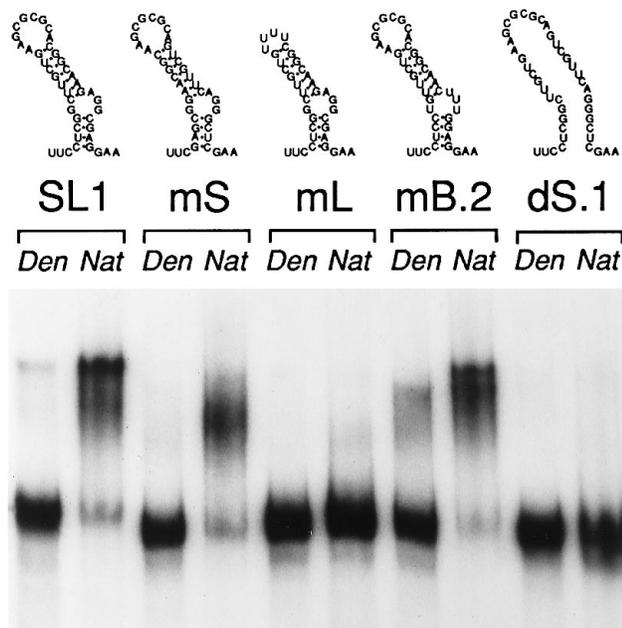


FIG. 4. Dimerization of HIV-1 RNAs containing mutant forms of SL1. Synthetic RNAs representing HIV-1 residues 637 to 829 and containing either the wild-type SL1 sequence or each of the four mutants shown above were tested for dimerization under denaturing (Den) and native (Nat) conditions.

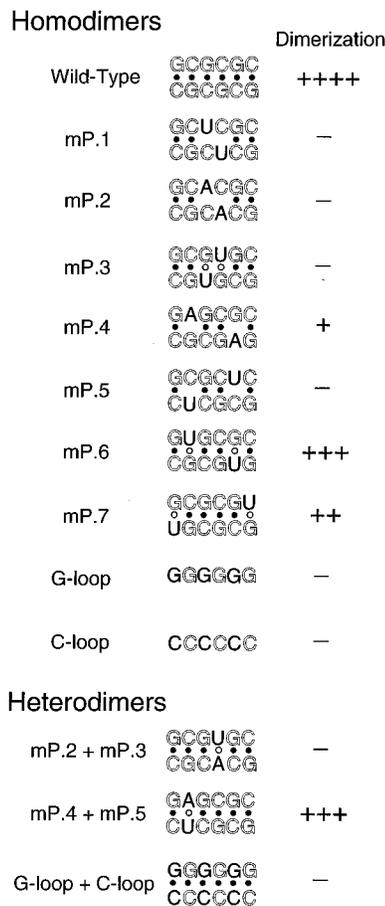


FIG. 5. Effect of mutations in the SL1 palindrome on in vitro dimer formation. SL1 sequences containing either the wild-type palindrome or the indicated mutations were inserted into the ΔSL1 construct and tested either individually (for homodimerization) or in pairs (for heterodimerization). Sequences shown are for the hypothetical duplexes that might be formed, with closed circles denoting GC base pairs and open circles denoting AU or GU base pairs. The proportion of labelled RNA migrating in the slower (dimer) electrophoretic band is indicated at right for each: -, less than 5% dimer; +, 5 to 10% dimer; ++, 10 to 50% dimer; +++, 50 to 75% dimer; +++++, more than 75% dimer.

ing (mutant mS), homodimerization was also preserved, indicating that the stem structure is critical for this activity but its sequence is not. This conclusion was reinforced by the finding that the disrupted-stem mutants dS.1 and dS.2, neither of which homodimerizes, also failed to heterodimerize when combined, even though their sequences are complementary and the loop palindrome is preserved (Fig. 3).

We then tested mutations that altered the SL1 loop sequence in the context of the wild-type stems and bulge. One such mutant (mL), which replaces the loop of SL1 with four U residues, abolished dimer formation (Fig. 4). In the wild-type loop, the six-base palindrome is flanked by three unpaired A residues. We found that deletion of the single downstream A (mutant ΔA.2) or one of the two upstream A's (mutant ΔA.1) individually had no effect, but that deleting all three A's abolished homodimerization (mutant ΔA.3). Interestingly, replacement of all three A's with U's also eliminated dimer formation (mutant mA), suggesting a sequence-specific role for these nonpalindromic bases; however, this mutant also changes the last C in the palindrome to a U residue, which by itself affects the efficiency of dimerization (Fig. 5, mP.7).

The effects of single point mutations within the loop palindrome also revealed strong sequence- and position-specific effects. In principal, any single-base substitution within the palindrome would be expected to affect 2 of the 6 GC bp involved in the initial kissing-loop interaction. As illustrated in Fig. 5, we found that mutations involving the two central bases of the palindrome were very poorly tolerated: homodimerization was completely inhibited not only by mutations that prevented base pairing (mutations mP.1 and mP.2) but also by the introduction of potential GU pairs at these positions (mutation mP.3). In contrast, although mutations that eliminated base pairing at positions 2 and 5 also abolished homodimerization (mP.4 and mP.5), GU base pairing at these flanking positions (mP.6) or at the outer residues of the palindrome (mP.7) was well tolerated. This suggested that, although the central GC residues were specifically required, the flanking and outer residues could tolerate some mutations, provided that base pairing was preserved. Further support for this interpretation came from experiments in which homodimerization-defective mutants from this series were tested for their ability to heterodimerize with complementary mutants (Fig. 5): whereas mutants mP.4 and mP.5 dimerized with nearly wild-type efficiency, constructs mP.2 and mP.3 failed to associate, even though the predicted thermodynamic stabilities of the two heteroduplexes are equivalent. We also tested two additional mutants (G-loop and C-loop) in which the palindrome was replaced by six G's or six C's, respectively. As expected, neither homodimerized, but, surprisingly, they also failed to heterodimerize appreciably with each other in this assay, despite their potential to form a highly stable interstrand duplex.

Having confirmed that these short, HIV-1-derived RNAs exhibited SL1-dependent dimerization, we then asked whether they retained this capability when linked to heterologous sequences. For these studies, we synthesized unlabelled T3 transcripts that not only encompassed the wild-type or mutant forms of HIV-1 residues 637 to 829 but also extended downstream into the adjacent plasmid sequences; this yielded transcripts approximately 1.0 or 2.8 kb in length, each with 193 bases of HIV sequence at its 5' end. As illustrated in Fig. 6A, we found that transcripts containing the wild-type HIV-1 sequence dimerized efficiently, whereas those containing Δ SL1 or a subtler mutation (mA) in the SL1 loop did not. Constructs containing these HIV-1 sequences linked to 2.6 kb of plasmid-derived RNA gave similar results; plasmid transcripts containing no HIV-1 RNA did not dimerize detectably (data not shown). These sequences thus behaved in the heterologous context as they had in isolation. To evaluate the morphology of these *in vitro* dimers, a preparation of 1.0-kb synthetic plasmid transcripts containing the wild-type HIV-1 sequence at their 5' ends was exposed to partially denaturing conditions (75% formamide) and then examined by electron microscopy. As depicted in Fig. 6B, many of the transcripts (18 of 42 strands examined) appeared as simple linear, presumably monomeric, forms (left), but a substantial proportion (24 of 42) appeared to form dimers (center and right). In each of these dimers, the two strands appeared to be linked in parallel near one end, but they remained separate along the remainder of their lengths—a morphology closely recapitulating that previously observed in authentic RNA genomic dimers isolated from retroviral particles (4, 5, 18).

DISCUSSION

The results obtained in this study strongly verify the critical role of the SL1 locus in initiating HIV-1 RNA dimerization *in vitro* and are fully compatible with the “kissing-loop” model

for dimer interaction (13, 19, 21). Our data also help delineate specific molecular characteristics of SL1 that are necessary for this interaction:

First, contact between viral RNA strands is proposed to occur initially at a conserved, six-base, GC-rich palindrome in the SL1 loop (13, 19, 21). We find that AU or GU base pairs can be tolerated at some locations within the six-base duplex, but that GC base pairs appear to be specifically required at the two central positions. Significantly, the DIS loop palindrome found in many HIV-2 and SIV genomes (GUGCAC) also conforms to this requirement. The palindromic nature of the wild-type HIV-1 and HIV-2 or SIV sequences may simply reflect a requirement that two identical RNA strands be complementary to each other at a common site in order to anneal, because we find that some complementary sequences can heterodimerize even in the absence of palindromic symmetry (Fig. 5, constructs mP.4 + mP.5). Interestingly, however, complementarity alone is not sufficient for dimer formation, even between GC-rich sequences, as revealed by the failure of the G-loop and C-loop mutants to heterodimerize. This indicates that the specific sequence of the contact region determines its capacity for kissing-loop interactions, presumably by influencing its conformation or accessibility. In this regard, it may be significant that many loop palindromes of both HIV-1 and HIV-2 or SIV consist of alternating purines and pyrimidines.

Second, the palindrome in the native SL1 loop is flanked on each side by one or two A residues. We find that a single A can be removed from either side without effect, but that deletion of all three abolishes dimer formation. This may indicate that a certain minimum loop length (perhaps eight bases) is required. Replacement of these three residues by U's, however, also prevents dimer formation, implying that they serve a sequence-specific function. Although this hypothetical function remains to be determined, one possibility is that the flanking A's might form homopurine contacts—a type of non-Watson-Crick base pairing interaction that has been observed between A and G residues in RNA loops from a variety of sources (2, 10, 24)—with one or more G's in the loop palindrome, and thereby might stabilize its conformation in a way that promotes dimerization.

Third, the palindrome and flanking A's normally reside within the loop atop an 11-bp stem structure interrupted by a four-base bulge. Our studies (Fig. 3) show that the bulge is dispensable, at least *in vitro*, but that truncation of the stem to only 7 bp prevents dimerization. It is not clear whether it is the length *per se* or the stability of the longer stem that confers activity. We found that dimer formation was eliminated by mutations which disrupt base pairing in the stem (dS1A, dS1B, dS.1, and dS.2), but it could be fully restored by complementary mutations that could potentially restore this structure (mS); this implies that complementarity of this region, rather than its sequence, is critical for dimerization. Under the kissing-loop model, such complementarity is required initially for folding of the intrastrand SL1 stem and later for formation of the interstrand duplex. Our finding that mutants dS.1 and dS.2, which each contain the wild-type palindrome with flanking A's and which could together form an essentially wild-type duplex, nevertheless failed to heterodimerize (Fig. 3) confirms that the stem itself is critical. Its importance may lie in imposing a proper conformation onto the loop sequence or in ensuring the accessibility of the loop within the highly structured context of the viral leader region (3, 7, 11).

We have also examined the morphology of the *in vitro* RNA dimers by electron microscopy and find that they closely resemble the authentic genomic dimers isolated from retroviral virions in earlier studies (4, 5, 18). This finding lends additional

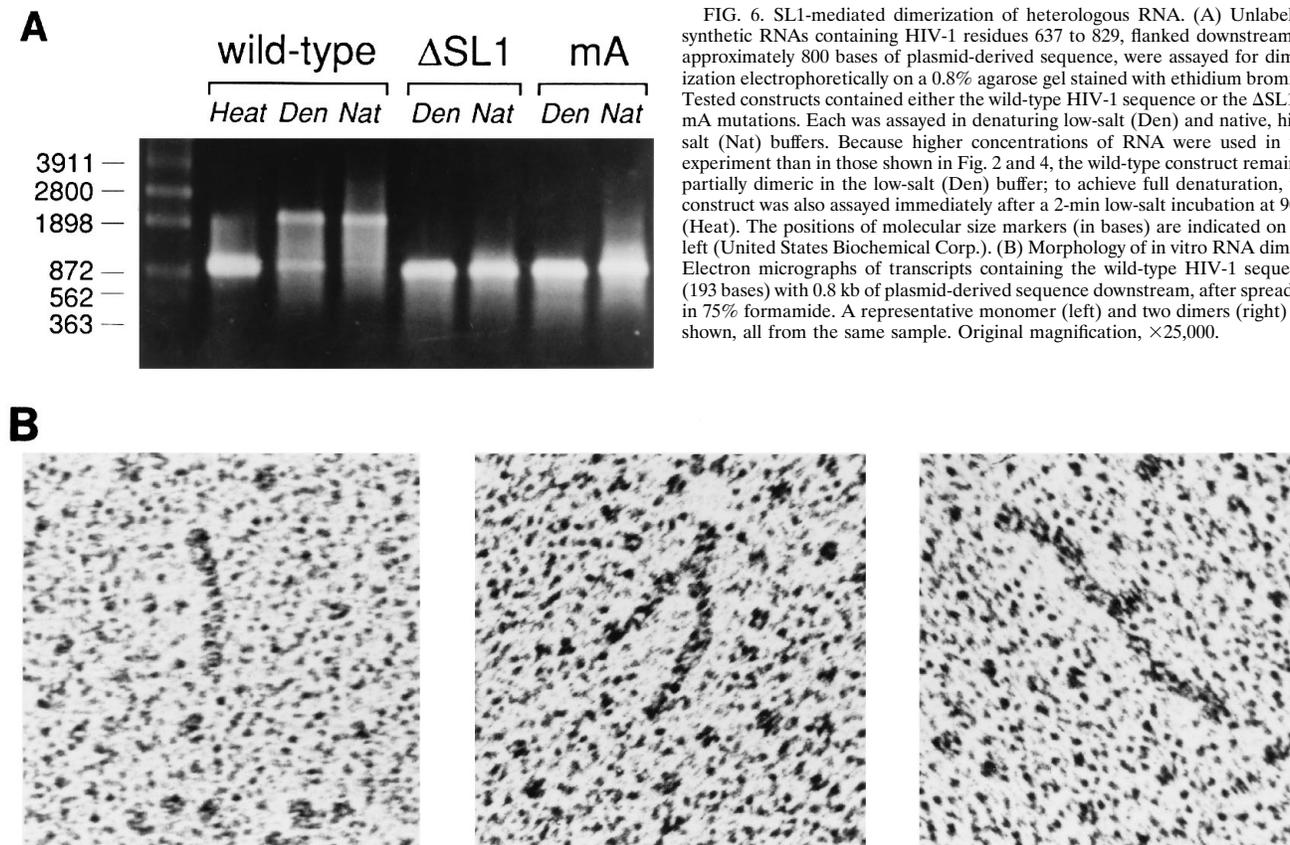


FIG. 6. SL1-mediated dimerization of heterologous RNA. (A) Unlabelled synthetic RNAs containing HIV-1 residues 637 to 829, flanked downstream by approximately 800 bases of plasmid-derived sequence, were assayed for dimerization electrophoretically on a 0.8% agarose gel stained with ethidium bromide. Tested constructs contained either the wild-type HIV-1 sequence or the Δ SL1 or mA mutations. Each was assayed in denaturing low-salt (Den) and native, high-salt (Nat) buffers. Because higher concentrations of RNA were used in this experiment than in those shown in Fig. 2 and 4, the wild-type construct remained partially dimeric in the low-salt (Den) buffer; to achieve full denaturation, this construct was also assayed immediately after a 2-min low-salt incubation at 90°C (Heat). The positions of molecular size markers (in bases) are indicated on the left (United States Biochemical Corp.). (B) Morphology of in vitro RNA dimers. Electron micrographs of transcripts containing the wild-type HIV-1 sequence (193 bases) with 0.8 kb of plasmid-derived sequence downstream, after spreading in 75% formamide. A representative monomer (left) and two dimers (right) are shown, all from the same sample. Original magnification, $\times 25,000$.

credence to the view that the in vitro assay mimics in vivo dimer formation. It should be noted that, whereas the kissing-loop model predicts a locally antiparallel linkage between DIS elements (Fig. 1B), the RNA pairs in most of the dimers we examined microscopically appeared to be aligned in parallel (Fig. 6B), as is the case with most virion-derived dimers (4, 5, 18). This may indicate that the initial, essential duplex formation mediated by SL1 induces secondary annealing events at adjacent sites, perhaps through isomerization of other nearby stem-loops (7, 11) or by formation of AG quartets (1, 22). Two or more such linkages occurring within a short region of the genome could give rise to a globally parallel alignment of RNA strands, even if the linkages themselves involve locally antiparallel interactions.

The results of this study extend earlier evidence that SL1 provides signals required for initiating HIV-1 RNA dimerization in vitro (13, 14, 16, 17, 19, 21). Further research will be needed to evaluate whether SL1 is uniquely important in this regard and how sequences elsewhere in the genome might contribute to the stabilization of such dimers. It will also be of interest to investigate whether the RNA conformational changes that accompany dimerization affect the biological activities of other *cis*-acting RNA elements, such as the viral major splice donor, *gag* start codon, or primer binding site, all of which are located in the vicinity of SL1. Most importantly, it remains to be established whether SL1 is a predominant signal for HIV-1 RNA dimerization in the intact virus, as it appears to be in vitro. If so, then the mutations characterized here may prove useful for probing the biological significance of dimerization in the retroviral life cycle.

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