Mode of Dimerization of HIV-1 Genomic RNA†

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Received July 1, 1993; Revised Manuscript Received August 19, 1993®

ABSTRACT: Retroviral genomes consist of two identical RNA molecules joined noncovalently near their 5′ ends, at domains called dimerization linked sequences (DLS). This physical linkage of the genomic RNAs is considered important for the control of several steps in the viral life cycle, such as recombination, translation, and encapsidation. The putative DLS of human immunodeficiency virus-1 (HIV-1), a 111-nucleotide, purine-rich stretch of RNA, has been found necessary and sufficient for a salt-induced dimerization of the genome *in vitro*. Our investigation into the mechanism of this dimerization reveals sharply varying influences of the different alkali cations on both the formation and the stabilization of the dimer, a pattern closely related to that of telomeric G-DNA complexes. To probe this phenomenon, we have carried out experiments using short antisense DNA oligomers to define the segments of the DLS that are required for dimerization and methylation protection to implicate sets of guanines in forming Hoogsteen hydrogen bonds within the dimer. Cumulatively, these data provide further evidence for the existence of guanine quartets within the dimerized HIV-1 DLS. We propose models in which guanine quartets not only allow the homodimerization of HIV-1 and other retroviral genomic RNAs but also permit the two RNA strands in a dimer to exist in an overall parallel orientation, as has been observed by electron microscopy.

Electron microscopy of RNA from retroviral virions and sedimentation studies first suggested that retroviral genomes consist of two identical RNA molecules joined noncovalently at defined sites near their 5′ ends (Bender & Davidson, 1976; Kung et al., 1976; Bender et al., 1978; Murti et al., 1981), with apparently parallel strand orientation. This physical linkage of the genomic RNAs is now considered important for the control of several steps in the viral life cycle, such as recombination [Weiss et al., 1973; Hu & Temin, 1990; reviewed by Temin (1991)], translation (Bieth et al., 1990), and encapsidation (Bieth et al., 1990; Darlix et al., 1990; Prats et al., 1990). Short (~100 nucleotides) stretches of RNA, known as dimerization linked structures (DLS), have been defined by deletion mutagenesis in a number of retroviruses [Moloney murine leukemia virus (Prats et al., 1990; Roy et al., 1990), Rous sarcoma virus (Bieth et al., 1990), and HIV-1, isolate MAL (Darlix et al., 1990; Marquet et al., 1991)]. Although these sites are probably not the only points of interstrand RNA contact within the virion, they appear to correlate well with the stable joining sites defined by electron microscopy. Darlix and colleagues (Bieth et al., 1990; Darlix et al., 1990; Prats et al., 1990; Marquet et al., 1991; Roy et al., 1990) have shown that the DLS sequences are necessary and sufficient for genomic RNA dimerization *in vitro*, regardless of whether the process is assisted by viral nucleocapsid proteins, which are thought to catalyze dimerization *in vivo*. Although DLS sequences from the different viruses show little sequence homology [the motif PuGGAPuA is conserved (Marquet et al., 1991), but the importance of this has been disputed recently (Berkhout et al., 1993)], they can form heterodimers *in vitro*. Both homo- and heterodimerization proceed at neutral pH, in the presence of salt. Non-Watson–Crick hydrogen bonding has been suspected in the dimerization process, since in each case the antisense DLS RNA fails to dimerize. The 111-nucleotide DLS of HIV-1, isolate MAL, is particularly purine-rich (Darlix et al., 1990). Figure 2a shows this sequence (DLS-112), with an extra base at the 3′ end, that we used for this work.

An early study of the *in vitro* dimerization of the HIV-1 DLS (Marquet et al., 1991) examined the influence of mono- and multivalent cations, pH, and temperature. From reductions in dimerization levels at acidic pHs, the authors concluded that $C{\cdot}C^+$ pairs and $C{\cdot}G{\cdot}C^+$ triplets were probably not involved; from the apparent efficiency of the lithium ion (compared to other alkali cations) in promoting dimerization, they concluded that guanine quartets [which are extremely stable in potassium but unstable in lithium (Pinnavaia et al., 1978; Howard & Miles, 1982; Miles & Frazier, 1978; Sen & Gilbert, 1988, 1990; Sundquist & Klug, 1989; Willamson et al., 1989; Venczel & Sen, 1993)] were also not involved. The authors proposed the involvement of novel purine quartets containing both adenine and guanine and suggested that these might be selectively stabilized by the lithium ion. Recently, however, Sundquist and Heaphy (1993) have reported a different order of stabilization of the DLS dimer by the alkali cations: they have found the order to be $K^+ > Na^+ > Li^+ > Cs^+$, the same general order found for DNA and RNA structures containing guanine quartets. On the basis of these stabilization data, they have proposed that guanine quartets are, in fact, present within the DLS dimer.

We have studied the patterns of both formation and stabilization of the DLS dimer in the presence of the different alkali cations. Our results, too, suggest the presence of guanine quartets in the DLS dimer. In this paper we describe further experiments that we have carried out to identify the broad elements of sequence within the DLS that are critical for dimerization and, at a finer level (using methylation protection), specific guanines that are likely candidates for forming G-quartets. In discussing structural models in which G-quartets provide a means for RNA *homo*dimerization, we note that certain classes of G-quartet structures, if they exist

† This work was supported by the British Columbia Health Research Foundation. D.S. is a Research Scholar of the BCHRF.

® Abstract published in *Advance ACS Abstracts,* October 1, 1993.

0006-2960/93/0432-11453$04.00/0

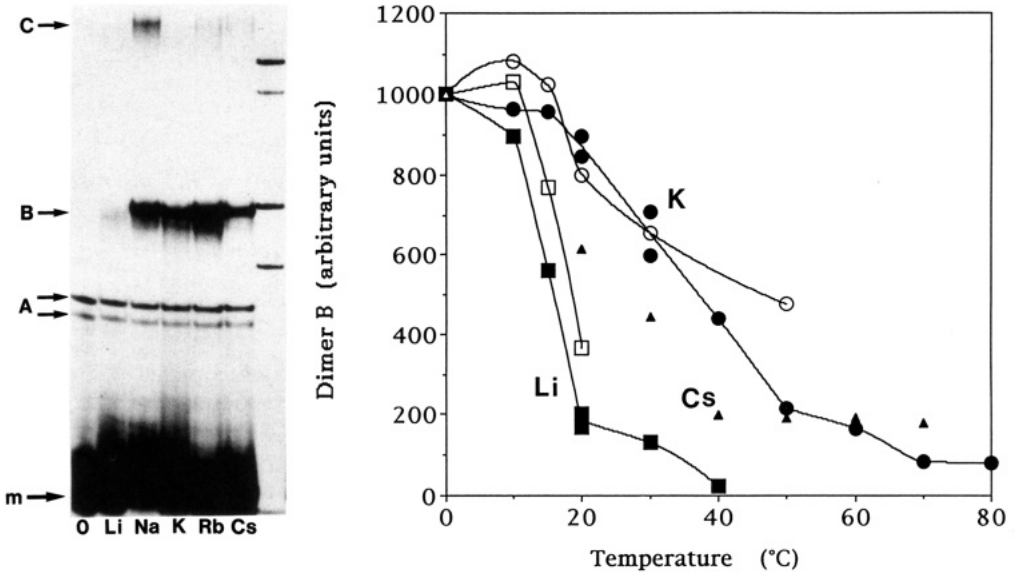

FIGURE 1: (a, left) Products of DLS-112 RNA from alkali cation incubations. Lane 0: TE buffer, pH 8.0. Lanes Li–Cs: TE + 1 M XCl (where X = Li^+, Na^+, K^+, Rb^+, or Cs^+). m: monomer, A, B, and C: salt-induced complexes. (b, right) Complex **B** melting curves. Intensities of residual dimer **B** from various Li^+, K^+, and Cs^+ melting experiments carried out on 2 μM total RNA (monomer and complexes), in TE + 70 mM XCl, plotted against temperature. The amount of **B** in each 0 °C sample has been normalized to 1000 arbitrary units. (■, ●, ▲) Melting experiments in 70 mM Li^+, K^+, and Cs^+, respectively, analyzed on a gel run in 50 mM TBE + 10 mM KCl; (□, ○) melting experiments in 70 mM Li^+ and K^+, analyzed on a gel run in 50 mM TBE + 10 mM LiCl.

within the DLS dimers, naturally permit the observed "parallel" orientation of the two RNA strands.

MATERIALS AND METHODS

RNA Synthesis. A synthetic DNA oligomer (Pharmacia Gene Assembler) incorporating the DLS-112 template and the T7 RNA polymerase promoter was made double-stranded by PCR, restricted at introduced sites, and cloned into pUC18. Selected clones were sequenced, linearized, and then used in transcription reactions on a 0.5-mL scale, following the protocol of Puglisi (1989). RNA was either internally labeled with [α-^{32}P]GTP or end-labeled with [γ-^{32}P]ATP following dephosphorylation with calf intestinal alkaline phosphatase (CIP).

Formation of Higher Order Products. ^{32}P-Labeled DLS-112, denatured at 95 °C for 3 min in TE (10 mM Tris, pH 8.0, and 1 mM EDTA) buffer and chilled, was aliquoted into equal volumes of TE + 2 M XCl (where X = Li^+, Na^+, K^+, Rb^+, or Cs^+), to give 5-μL final samples of RNA (8 μM) in TE + 1 M XCl. Samples were incubated at room temperature for 1 h, mixed with 5 μL of loading dye (10% glycerol in TE + loading dyes), and run on 6% non-denaturing polyacrylamide gels in 50 mM TBE + 50 mM KCl at 4 °C. Other gels, run in 50 mM TBE + 10 mM Na/KCl or 100 mM TBE, gave comparable results.

Melting Experiments. Labeled DLS-112 was heat-denatured, chilled, and incubated at an RNA concentration of 26 μM in 10-μL samples of TE + 1 M XCl, as above. One hundred thirty microliters of ice-cold TE was added to each sample, to give 140 μL of 2 μM total RNA (monomer and **B**). Fifteen-microliter aliquots of these solutions, kept on ice, were subjected for 8 min to a particular temperature between 0 and 80 °C and then returned to the ice. Samples were analyzed on non-denaturing gels run at 4 °C in both 50 mM TBE + 10 mM KCl and 50 mM TBE + 10 mM LiCl, to control for any distorting effect of either cation on the results. All gels were dried onto DEAE paper backed by Whatman 3MM filter paper; very light exposures (within the linear range of the film) were then analyzed by densitometry.

Antisense Strand Binding. Five-microliter incubations in 1 M NaCl (as above) had 5 molar equiv of one of the oligomers **R1**–**R5** or **RA**–**RC** (see Figure 2a) added per sample prior to the denaturation step. Following incubation, products were analyzed on a 6% non-denaturing gel run at 4 °C in 50 mM TBE + 50 mM NaCl. For determination of the strand stoichiometry of complex **B**, 0–1.2 molar equiv of **R5** was used.

Methylation Protection and Interference Experiments. End-labeled RNA, at 52 μM concentration, was denatured, chilled, and incubated in TE + 1 M XCl (where X = Li^+, Na^+, or K^+) for 1 h at room temperature. Ten-microliter samples, thawed on ice, were each diluted with 30 μL of ice-cold 100 mM X-cacodylate, stored on ice for 30 min, and made to 2% DMS (from a fresh ethanolic stock). Samples were incubated on ice for 45 min; loading dyes were added, and the samples were loaded on 6% non-denaturing gels in 50 mM TBE + 10 mM KCl at 4 °C (an extra lane was loaded with unmodified RNA). Gels were exposed wet to film; the appropriate monomer and dimer bands were cut out, and their RNA was eluted and treated with sodium borohydride followed by acidic aniline, according to the protocol of Peattie (1979). Aniline was removed by vacuum drying, and the samples were dissolved in 95% formamide, 5 mM Tris, pH 7.7, and 1 mM EDTA before precisely equal counts of each sample were loaded on 6% sequencing gels. After being run, the gels were dried on DEAE paper and autoradiographed with an intensifying screen at –70 °C.

For the interference experiment, RNA was denatured and pretreated with 1% DMS in 50 mM lithium cacodylate, pH 7.7, and 1 mM EDTA at room temperature for 15 min. Dimerization was allowed to proceed at 1 M KCl and at 200 mM KCl.

RESULTS AND DISCUSSION

Cation Dependence of Dimer Formation and Stability. We investigated in detail the *in vitro* dimerization of DLS-112. Figure 1a shows the distribution of products from 1-h incubation of the RNA, at room temperature and in 10 mM

Tris, pH 8.0, and 1 mM EDTA (TE buffer) containing 1 M XCl (where X = Li^+, Na^+, K^+, Rb^+, or Cs^+). Salt concentrations lower than 1 M also gave the same pattern of products, although at slower rates of formation. Three complexes, **A**, **B**, and **C**, were seen: **A** formed independently of RNA concentration, while both **B** and **C** formed in proportion to the starting RNA concentration, suggesting that they were multimers of DLS-112 RNA. Figure 1a shows that neither lithium nor cesium is an efficient producer of the major complex, **B**. Here the yields followed the order $Rb^+ > Na^+ > K^+ \gg Cs^+ > Li^+$. The general order, derived from several experiments carried out at different ionic strengths and temperatures, was Na^+, $Rb^+ \gg Cs^+$, Li^+, with K^+ producing complexes at anomalous levels between the two extremes. This pattern of yield was strikingly similar to that found for the formation of G4-DNA (containing guanine quartets) by telomeric DNA oligomers under comparable conditions of salt incubation (Sen & Gilbert, 1990). The poor kinetics of G4-DNA formation in potassium reflected the high degree of stabilization by potassium of alternative G-quartet structures and pathway intermediates (Sen & Gilbert, 1990). A similar explanation may also apply for the formation of DLS complex **B**.

We tested the relative stabilization of putative dimer **B** by the five alkali cations. Complex **B** was formed first by incubation in 1 M XCl. The solutions were diluted with TE buffer at 0 °C to a final XCl concentration of 70 mM; aliquots of each solution were then treated individually at temperatures from 10 to 80 °C and analyzed in gels. Figure 1b plots dimer **B** band intensities for Li^+ and K^+ experiments (showing also data points for Cs^+, for comparison). The difference in the T_m of **B** in 70 mM KCl versus 70 mM LiCl is ~20 °C, a remarkably large difference in the dimer-stabilizing ability of the two cations. The overall order measured was $K^+ > Rb^+ > Na^+ \sim Cs^+ > Li^+$, an order that is consistently found for the stabilization of DNA or RNA complexes containing guanine quartets (G-DNA or RNA). With G-DNA complexes, the large differences in stabilization by individual alkali cations reflect the crown-ether-like property of the G-quartet, whose inner cavity coordinates potassium optimally but binds the other alkali cations, both smaller and larger, with progressively less affinity (Pinnavaia et al., 1978; Howard & Miles, 1982; Miles & Frazier, 1978).

Sequences Important for Dimerization. To determine which segments of DLS-112 were required for the formation of the major product, **B**, we synthesized nine DNA oligomers, **R1**–**R6** and **RA**–**RC**, each16–20 nucleotides long, that were complementary to different stretches of DLS-112 (Figure 2a). Inclusion of a 5-fold molar excess of each oligomer in separate dimerization incubations defined the segments of DLS-112 that were critical for dimerization. Oligomers complementary to important sequences inhibited the formation of **B** and **C** (Figure 2b; shown in uppercase letters in Figure 3b). It was significant (a) that the "required" sequences were even more purine-rich than DLS-112 itself and (b) that the single largest contiguous stretch of pyrimidines in DLS-112, the five uracils at positions 319–323, was not required for dimerization. These data further suggested the importance of non-Watson–Crick bonding in product **B**.

The analysis above implicated two distinct stretches of sequence (complementary to **R4** and to **R1**–**R2**) as being important for the formation of **B**. It should be noted, however, that a positive result does not directly implicate a particular stretch of sequence in bonding, for constrained structures such as hairpin loops connecting domains that *are* involved in bonding would also show a "positive" result. This particular set of data was derived from permissive conditions for hybridization, i.e., 1 M NaCl at room temperature. Future analysis will focus on more stringent hybridization conditions to provide more detailed information on the importance of the different sequence motifs.

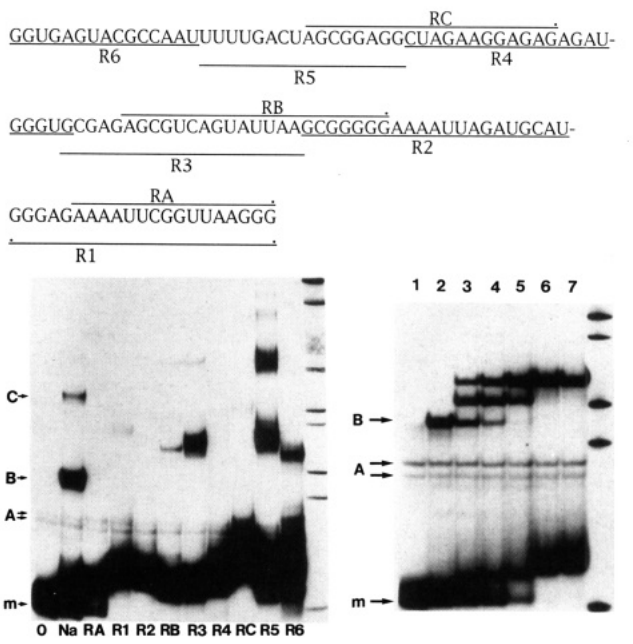

FIGURE 2: (a, top) Sequence of DLS112, showing the regions of complementarity of various anti-sense DNA oligomers. (b, bottom left) Determination of the segments of DLS-112 crucial for dimerization. DLS-112 was incubated in TE + 1 M NaCl in the presence of a 5× molar excess of each of the complementary DNA strands shown in panel a. (c, bottom right) Stoichiometry of complex **B**. Fixed concentrations of DLS-112 RNA were incubated in TE + 1 M NaCl in the presence of (lanes 2–7) 0, 0.33, 0.53, 0.73, 1.0, and 1.2 molar equiv of the DNA oligomer **R5**, complementary to nucleotides 320–335 of DLS-112. Lane 1 shows DLS-112 incubated in TE alone.

Product B Is a Dimer. The DNA oligomer **R5**, which bound to DLS-112 but did not inhibit dimerization (it gave rise to a "dimer" band of reduced gel mobility), was used to determine the stoichiometry of complex **B**. Fixed concentrations of DLS-112 were incubated for dimerization in the presence of molar ratios of **R5** between 0 and 1.2. Figure 2c shows that complex **B** is a strand-dimer (the three **B** bands containing, respectively, zero, one, and two **R5** molecules bound to each dimer). Complex **C** was harder to define since it gave fuzzy bands on binding **R5**; however, preliminary indications are that it, too, is a dimer.

Hoogsteen Hydrogen Bonding in the Dimer. The formation of guanine quartets necessitates Hoogsteen hydrogen bonding, which renders the relatively nucleophilic N7-positions of participating guanines inaccessible to methylation by dimethyl sulfate (Maxam & Gilbert, 1980). Such protected guanines can then be identified by borohydride reduction followed by aniline cleavage of the modified RNA (Peattie, 1979). Figure 3a shows the reactivity patterns of the monomer (m) and of **B**, formed in LiCl, NaCl, and KCl, to dimethyl sulfate (DMS). In all three incubations, a number of guanines, mainly in the 3′ half of DLS-112, were reproducibly resistant to DMS in **B** relative to the monomer. Also reproducible were the characteristic differences in protection intensities for each cation, although overall, the three patterns were remarkably similar (summarized in Figure 3b, as derived from a number of independent experiments).

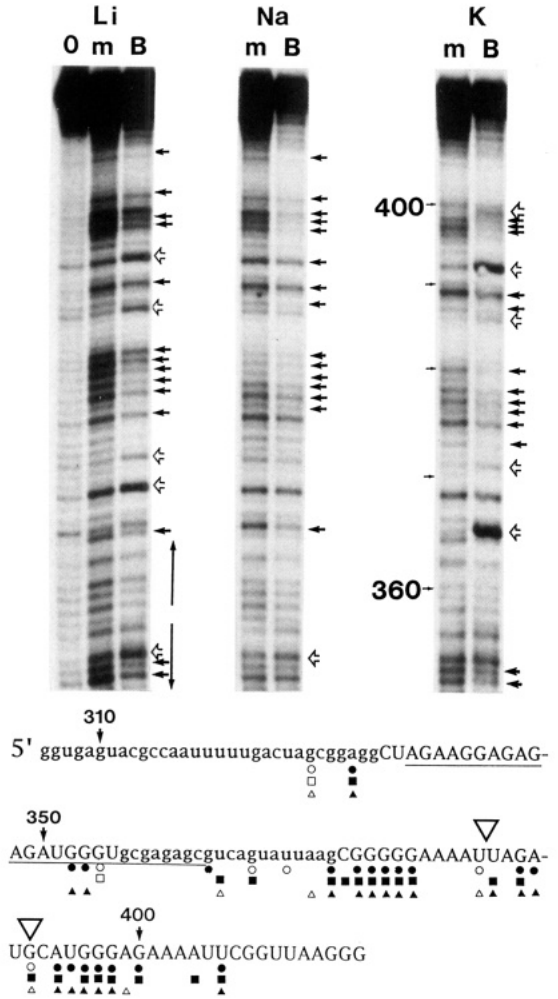

FIGURE 3: (a, top) Dimethyl sulfate (DMS) probing of the dimer (B) and monomer (m) of DLS-112. Lane 0: RNA control, not treated with DMS but worked up alongside treated samples. Equal counts of each sample were loaded. Cleavage protections are indicated by black arrows; enhancements, by open arrows. (b, bottom) Sequence of DLS-112. Uppercase sequences indicate regions required (as determined by complementary strand binding) for dimerization. DMS protection and enhancement sites (derived from a number of independent experiments) are indicated by filled and open symbols, respectively: Li (●, ○), Na (■, □), and K (▲, △). The underlined sequence shows a region of slight but unstable methylation protection/enhancement. The large, inverted triangles indicate possible bends in the RNA backbone in the dimer. Modification patterns of the 3′-most nine nucleotides are not shown, since these could not be reliably gauged.

Two major regions of protection were found: around the five contiguous G's at positions 377–381 and around the three G's at 396–398. A second block of three contiguous guanines, at 352–354, showed an unreliable pattern of methylation. Two spots (indicated by large inverted triangles in Figure 3b) between the two blocks of protected guanines showed a high variability in the extent of methylation in the presence of different cations. Possibly, these are bending or turning points in the RNA backbone, where small differences in structure in the three cationic environments are picked up by methylation. We believe that the methylation protection results shown in Figure 3a indicate Hoogsteen hydrogen bonding rather than coordination to the N7-positions of guanines of divalent or higher valent cations, since such cations were rigorously absent from the solutions used.

An exception to this pattern of reproducibility of methylation was shown by the stretch of primarily alternating G's and A's between positions 338 and 365 (underlined in Figure 3b); the purines within this stretch showed either slight protection or slight enhancement in the different incubations, often with the same cation.

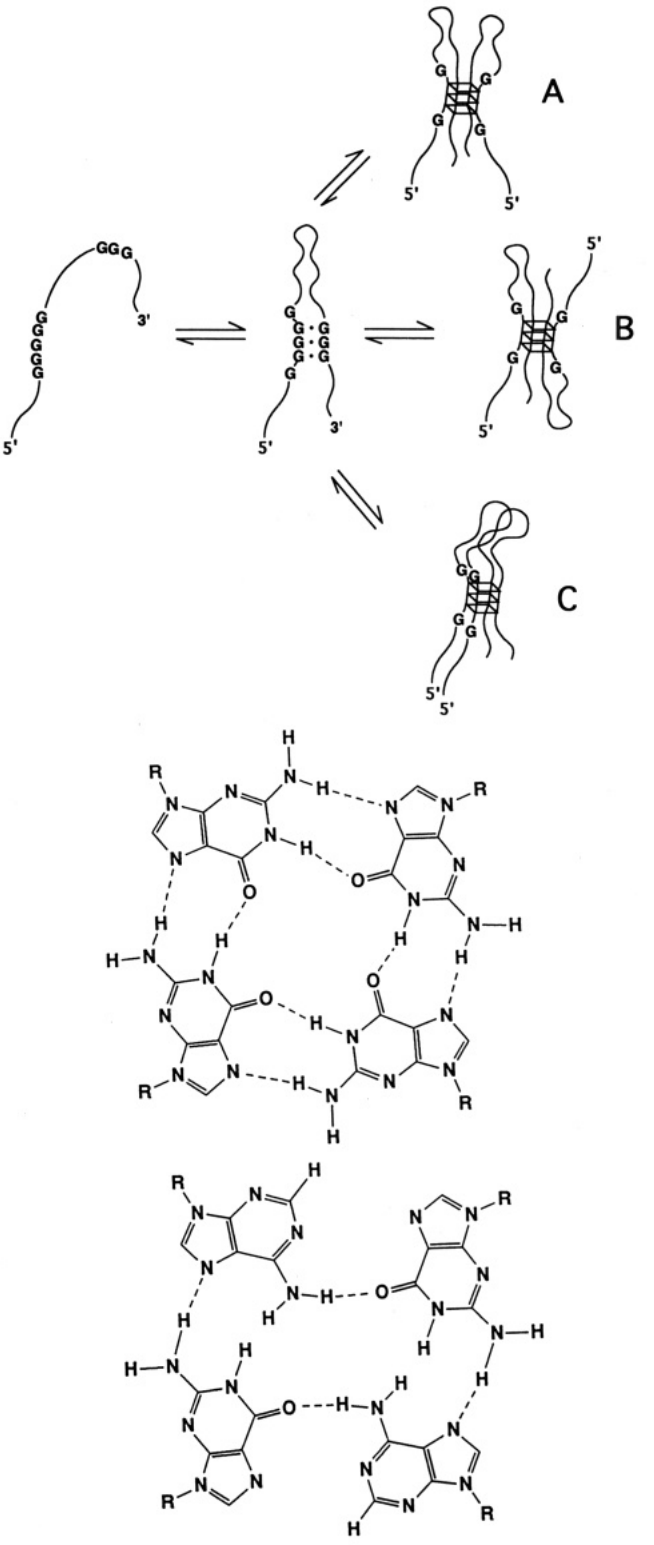

FIGURE 4: (a, top) General models (A, B, and C) for homodimerization of retroviral DLS sequences. (b, bottom) A standard guanine quartet (upper) and a putative two-guanine/two-adenine quartet (lower).

Preliminary carboxyethylation protection patterns with DEPC indicate that several stretches of adenines (such as A382–A385, contiguous to the protected G's G377–G381) are indistinguishably reactive in the monomer and the dimer and are hence unlikely to be involved in the dimerization.

Structural Models for Complex B. Our methylation protection data suggest that the two most promising guanine motifs for G-quartet formation are located at positions 375–381 and 396–400 [conversely, the G_3 motif at 352–354 and the GGAGG motif at 331–335 proposed by Sundquist and Heaphy (1993) are variably methylated in our experiments]. A model for the dimer would have DLS RNA from positions 375 to 400 form an approximate stem–loop structure, held together primarily by non-Watson–Crick interactions. The putatively antiparallel G·G base-paired stem would then be able to dimerize in a number of possible orientations by forming G-quartets (Figure 4a).

Guanine-mediated tetraplexes as a class are distinctively polymorphic. Thus, a given telomeric DNA oligomer is able to form, under different conditions, a parallel strand-tetramer as well as more than one kind of antiparallel strand-dimer, the latter distinguishable by their overall strand orientations as well as by their distribution of glycosidic conformations (Kang et al., 1992; Smith & Feigon, 1992; Mohanty & Bansal, 1993). Certain classes of antiparallel tetraplexes within the dimerized DLS would allow a net parallel orientation for the two exiting 5′ strands of the genomic RNA (models A and C in Figure 4a), in keeping with electron-microscopic observations made on various retroviral RNA dimers purified from virions and spread under partially denaturing conditions.

We carried out two methylation-interference experiments (data not shown) for dimerization in the presence of 1 M and 200 mM KCl, respectively. By sharp contrast with the protection data, however, both interference experiments showed that no single guanine N7 was absolutely required for the dimerization of DLS-112. These data, in conjunction with the deletion analysis carried out by Sundquist and Heaphy (1993), suggest a flexibility in the way the DLS itself or its fragments are able to dimerize *in vitro*. The latter study clearly established the importance of the 375–381 guanine motif for dimerization, in agreement with our results. However, elimination of the 396–400 motif in that study did still allow dimerization by the truncated DLS. It is of course conceivable that different fragments of the DLS may dimerize differently *in vitro*, forming G-quartets using alternative guanine motifs. Therefore, to successfully model the *in vivo* dimerization with *in vitro* experiments, it will be necessary to probe larger fragments of HIV-1 genomic RNA, possibly even in the context of the intact virion.

Finally, the stabilization and chemical protection data presented in this paper, though strongly suggestive of the presence of guanine quartets in **B**, cannot rule out the mixed A–G quartets proposed by Marquet et al. (1991). As commented upon by a number of investigators (Marquet et al., 1991; Sundquist & Heaphy, 1993; Williamson, 1993), such quartets formed by purines (including noncontiguous bases on a given strand) would provide a general but flexible mechanism for RNA homodimerization, one that could be exploited alike by distantly related retroviruses with dissimilar DLS sequences. It remains to be seen, however, whether putatively stable quartets composed of two guanines and two adenines (such as that visualized in Figure 4b) or, indeed, other purine–purine or purine–pyrimidine combinations (Johnson & Morgan, 1978; Lee et al., 1980) are able to coordinate cations with the selectivity shown by G-quartets.

ACKNOWLEDGMENT

We thank Sachdev Sidhu, Craig Martin, Loren Williams, Thor Borgford, and colleagues at Simon Fraser University.

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