A Short Autocomplementary Sequence Plays an Essential Role in Avian Sarcoma−Leukosis Virus RNA Dimerization†

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ABSTRACT: Retroviral genomes consist of two identical RNA molecules joined noncovalently near their 5′-ends. Recently, two models have been proposed for RNA dimer formation on the basis of results obtained *in vitro* with human immunodeficiency virus type 1 RNA and Moloney murine leukemia virus RNA. It was first proposed that viral RNA dimerizes by forming an interstrand quadruple helix with purine tetrads. The second model postulates that RNA dimerization is initiated by a loop−loop interaction between the two RNA molecules. In order to better characterize the dimerization process of retroviral genomic RNA, we analyzed the *in vitro* dimerization of avian sarcoma−leukosis virus (ASLV) RNA using different transcripts. We determined the requirements for heterodimer formation, the thermal dissociation of RNA dimers, and the influence of antisense DNA oligonucleotides on dimer formation. Our results strongly suggest that purine tetrads are not involved in dimer formation. Data show that an autocomplementary sequence located upstream from the splice donor site and within a major packaging signal plays a crucial role in ASLV RNA dimer formation *in vitro*. This sequence is able to form a stem−loop structure, and phylogenetic analysis reveals that it is conserved in 28 different avian sarcoma and leukosis viruses. These results suggest that dimerization of ASLV RNA is initiated by a loop−loop interaction between two RNA molecules and provide an additional argument for the ubiquity of the dimerization process via loop−loop interaction.

A ubiquitous property of retroviruses is that the virion genomic RNA is a dimer formed of two identical single-stranded RNA molecules (Coffin, 1984). The encapsidated RNA dimer is in the form of a 60−70S complex with the two molecules held together by several RNA−RNA interactions (Mangel *et al.*, 1974). The nature of the linkage between the monomers is probably via noncovalent bonds since the RNA dimer is dissociated into monomers under relatively mild conditions (Mangel *et al.*, 1974; Stoltzfus & Snyder, 1975; Darlix *et al.*, 1978; Fu *et al.*, 1994). Electron microscopy under partially denaturing conditions suggests that the two monomers are joined at their 5′-ends by a major interaction called the dimer linkage structure (DLS)[1] (Bender & Davidson, 1976; Murti *et al.*, 1981). Interestingly, location of the dimerization sequences in several retroviruses coincides with that of a major encapsidation signal (Bieth *et al.*, 1990; Prats *et al.*, 1990; Torrent *et al.*, 1994); it has been postulated that the dimeric structure is necessary for genomic RNA packaging (Bieth *et al.*, 1990; Housset *et al.*, 1993; Rein, 1994). Furthermore, the dimeric structure plays probably a role in other steps of the retroviral cycle, such as (i) translation (Bieth *et al.*, 1990), (ii) recombination during reverse transcription (Hu & Temin, 1990; Stuhlmann & Berg, 1992; Mikkelsen *et al.*, 1996), and (iii) stabilization of the genomic RNA against nuclease degradation (Darlix *et al.*, 1978).

Recent studies have provided evidence that RNA transcripts containing the 5′-end of the viral genome can dimerize spontaneously *in vitro* (Bieth *et al.*, 1990; Prats *et al.*, 1990; Roy *et al.*, 1990; Marquet *et al.*, 1991) and that this process is activated by nucleocapsid protein (Bieth *et al.*, 1990; Prats *et al.*, 1990; Torrent *et al.*, 1994). Several results obtained with RNA transcripts generated *in vitro* have favored the notion that HIV-1 RNA dimerization occurs through formation of purine tetrads (Marquet *et al.*, 1991; Awang & Sen, 1993; Sundquist & Heaphy, 1993). However, the thermal stability of the dimeric RNA isolated from HIV-1 virions was not significantly affected by the identity of the monovalent cation, indicating that purine quartets are probably not implicated in the structure of HIV-1 RNA dimer (Fu *et al.*, 1994). Furthermore, additional studies suggest that purine tetrads are dispensable for RNA dimerization *in vitro* of HIV-2 (Berkhout *et al.*, 1993), HIV-1 (Marquet *et al.*, 1994; Paillart *et al.*, 1994; Muriaux *et al.*, 1995; Laughrea & Jetté, 1996) and MoMuLV (Girard *et al.*, 1995). Indeed,

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[1] Abbreviations: ALV, avian leukosis virus; ASV, avian sarcoma virus; ASLV, avian sarcoma−leukosis virus; BLV, bovine leukemia virus; DLS, dimer linkage structure; HaMSV, Harvey mouse sarcoma virus; HIV-1 and HIV-2, human immunodeficiency virus types 1 and 2; MoMuLV, Moloney murine leukemia virus; nt, nucleotide(s); Pr-B, Rous sarcoma virus Prague-B; Pr-C, Rous sarcoma virus Prague-C; R, purine; RSV, Rous sarcoma virus; SD, splice donor site; SNV, spleen necrosis virus.

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an autocomplementary sequence able to adopt a stem−loop structure appears to be responsible for dimerization of short HIV-1 (Laughrea & Jetté, 1994, 1996; Paillart *et al.*, 1994; Skripkin *et al.*, 1994; Muriaux *et al.*, 1995, 1996) or MoMuLV (Girard *et al.*, 1995) RNA transcripts *in vitro*. On the basis of these data, it has been proposed that dimer formation is initiated by the annealing of these autocomplementary sequences via a loop−loop interaction between the two monomers (Laughrea & Jetté, 1994, 1996; Paillart *et al.*, 1994; Skripkin *et al.*, 1994; Girard *et al.*, 1995; Muriaux *et al.*, 1995, 1996).

Surprisingly, dimerization of avian sarcoma−leukosis virus (ASLV) RNAs has been studied less extensively. Electron micrographs of partially denatured dimer molecules suggest that Rous sarcoma virus (RSV) RNA DLS is a small region of less than 60 nucleotides centered on nucleotide 511 (Murti *et al.*, 1981). Rapid and extensive dimerization of synthetic RSV RNA transcripts containing the 5′ leader sequence was shown to be promoted by nucleocapsid protein NCp12 *in vitro* (Bieth *et al.*, 1990). Under these conditions, sequences between positions 208−270 and 400−600 were found to be necessary; however, the first 63 nucleotides of the RNA transcripts were nonviral and might therefore have interfered with the authentic dimerization process. Recently, Lear *et al.* (1995) showed that RNA transcripts representing portions of the genomic RNA of Rous sarcoma virus Prague C (Pr-C) can dimerize *in vitro* in the presence of a very high $MgCl_2$ concentration (50 mM). This study suggests that Pr-C RNA contains at least two dimerization signals. One, located between nt 531 and 634, might involve Watson−Crick pairing of an imperfect inverted repeat, while the second signal, located between nucleotides 496 and 530, requires a tetraguanine sequence which is not involved in an identifiable Watson−Crick interaction or in guanine tetrad formation.

In an attempt to get a more comprehensive view of the mechanism of ASLV RNA dimerization, we have analyzed *in vitro* the spontaneous dimerization of ASLV RNA under physiological salt conditions. We examined dimerization of ASLV RNA transcripts with various 5′- and 3′-ends, dimerization in the presence of antisense DNA oligonucleotides, formation of heterodimeric RNAs, and stability of the RNA dimers in the presence of various cations. In addition, we performed a phylogenetic comparison to examine the functional relevance of a stem−loop structure involved in ASLV RNA dimerization *in vitro*.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Digestion. *Escherichia coli* DH5α was used for plasmid amplification. Standard procedures were used for restriction enzyme digestion and plasmid construction (Sambrook *et al.*, 1989). Restriction endonucleases were purchased from New England Biolabs. Plasmid pLADI contains the proviral DNA of a highly infectious avian leukosis virus of subgroup A (ALV-RSA; termed ALV in the text) (Bieth & Darlix, 1992). Polymerase chain reaction was performed on pLADI with oligonucleotide O1, carrying an *Eco*RI site, T7 RNA polymerase promoter, and 15 nucleotides corresponding to the complementary part of positions 1−15 of the ALV genome, and oligonucleotide O2, carrying an *Xba*I site and 13 nucleotides corresponding to the complementary part of positions 617−629 of the ALV genome. The amplified product was inserted in the pSP64 plasmid (Promega) after *Eco*RI and *Xba*I digestions and gave rise to plasmid pCG44. The pCG44 plasmid was digested with restriction endonucleases *Xho*I, *Ava*I, *Mse*I, and *Dde*I and transcribed with T7 RNA polymerase to generate transcripts starting at position 1 of ALV RNA and ending at positions 626, 516, 387, and 226, respectively, corresponding to RNAs 1−626, 1−516, 1−387, and 1−226, respectively (Figure 1).

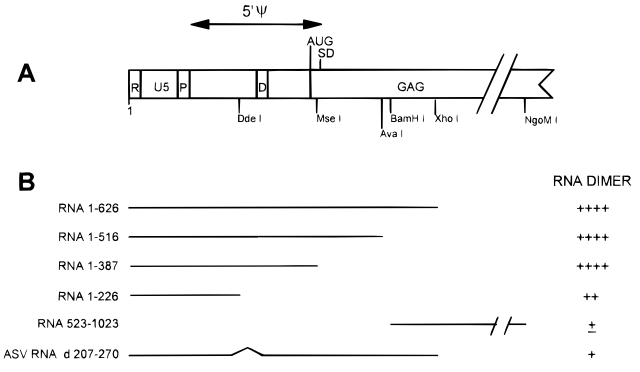

FIGURE 1: Mapping of sequences required for ALV RNA dimerization *in vitro*. (A) Representation of the 5′-end of ALV RNA: R, terminal repeat; U5, sequence unique and untranslated at the 5′-end; P, the replication primer $tRNA^{Trp}$ binding site (PBS); 5′Ψ, nt 120−387 required for encapsidation of genomic RNA (Koyama *et al.*, 1984; Katz *et al.*, 1986; Anderson *et al.*, 1992) and sufficient to direct encapsidation of heterologous RNA into virions (Aronoff *et al.*, 1993); D, autocomplementary sequence (258−274) involved in ALV RNA dimerization *in vitro*; AUG, initiation codon for Pr76gag synthesis; SD, splice donor site at position 387. (B) RNA transcripts from different sizes are precisely described in Experimental Procedures. Numbering is relative to the genomic RNA cap site (+1). The column labeled RNA dimer indicates the level of dimeric RNA: (±) 5−10%, (+) 25−35%, (++) 40−50%, and (++++) 75−95% (mean values based on at least three experiments).

Plasmid pLAD9 contains a fragment of the ALV genome from positions 523 to 1913 (Bieth *et al.*, 1990). After digestion with *Ngo*MI, pLAD9 was transcribed with T7 RNA polymerase to generate RNA 523−1023 (Figure 1). The non-ALV sequence of 24 nt and of plasmidic origin is present at the 5′-end of this transcript. Plasmid pLAD8 contains a viral fragment from position −52 to +2312 lacking nucleotides 207−270 of the avian sarcoma virus genome (Bieth *et al.*, 1990; Katz *et al.*, 1986). This plasmid digested by *Xho*I was transcribed with SP6 RNA polymerase to generate ASV RNA d207−270 (Figure 1). ASV RNA d207−270 carries 61 additional nt (9 of the plasmid and 52 of the 3′-end of U3) upstream from the viral cap site (+1).

Purification and End-Labeling of DNA Oligonucleotides. DNA oligonucleotides were synthesized using an Applied Biosystems Synthesizer. The oligonucleotides were loaded onto a 15% denaturing polyacrylamide gel in 89 mM Tris-borate and 2 mM EDTA, electrophoresed, visualized by UV shadowing, excised from the gel, and purified by elution and ethanol precipitation. The concentration was determined by UV spectroscopic measurement at 260 nm. DNA oligonucleotides were labeled at the 5′-end using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (Amersham). The 5′-end-labeled oligonucleotides were purified by electrophoresis on a 15% denaturing polyacrylamide gel and isolated by elution and ethanol precipitation. The specific radioactivity was about 4×10^5 cpm/pmol of DNA oligonucleotide. DNA oligonucleotides at 3×10^4 cpm/pmol used in the dimerization assays were obtained by mixing

unlabeled oligonucleotides with end-labeled oligonucleotides. The DNA oligonucleotides 25, 189, 223, 273, 270, 430, 462, 528, 531, 540, 556, 582, and 610 were complementary to positions 6−25, 170−189, 204−223, 254−273, 259−270, 411−430, 443−462, 509−528, 512−531, 517−540, 536−556, 563−582, and 591−610 of the ALV RNA sequence, respectively. Oligonucleotide in273 has the reverse sequence (5′-CCCTCGAGGTCCCGGGCCTC-3′) of oligonucleotide 273 (5′-CTCCGGGCCCTGGAGCTCCC-3′).

In Vitro RNA Synthesis and Purification. Five micrograms of the linearized plasmid was transcribed with T7 RNA polymerase or SP6 RNA polymerase for pLAD8 under the conditions stipulated by the RiboMAX large-scale RNA production system (Promega). The DNase treatment was stopped by adding SDS and proteinase K to final concentrations of 0.5% and 0.2 mg/mL, respectively, and the mixture was incubated for 30 min at 37 °C. The sample was subjected to three phenol−chloroform extractions after proteinase K treatment, and the RNA transcripts were precipitated three times in order to remove nucleotides (Sambrook *et al.*, 1989). The three precipitations were performed in the presence of (i) ethanol and 0.8 M lithium chloride, (ii) 1.8 M lithium chloride, and (iii) ethanol and 0.3 M sodium acetate, pH 5.2. The RNA precipitate was washed twice with 80% ethanol, dissolved in double-distilled water, and dialyzed (Millipore filters, Type V6, 0.025 μm) for 2 h against sterile double-distilled water. The purity and integrity of the RNA transcripts were checked on denaturing polyacrylamide gels and the concentration was determined by UV spectroscopic measurement at 260 nm.

Monomer and Dimer Formation. In a standard experiment, RNA (3.8 pmol), alone or with antisense oligonucleotide(s), in 6 μL of double-distilled water, was heated at 90 °C for 2 min and chilled for 2 min on ice. Four microliters of 2.5-fold concentrated standard buffer was added (final concentrations: 50 mM Tris-HCl, pH 7.5, 125 mM KCl, and 5 mM $MgCl_2$) and the sample was incubated for 15 min at 20 °C. Then, the sample was incubated for 60 min at 20 or 50 °C to obtain a majority of RNA monomers or dimers, respectively. At the end of incubation, all samples were put on ice, mixed with 2 μL of loading buffer [50% (w/v) glycerol, 0.05% (w/v) bromophenol blue, and 0.05% (w/v) xylene cyanol], loaded on a 1.2% agarose gel, and electrophoresed at 5 V/cm and 4 °C in 0.5X TBM buffer (Laughrea & Moore, 1977; Marquet *et al.*, 1994) containing 45 mM Tris-borate, pH 8.3, 0.1 mM $MgCl_2$, and 0.12 μg/mL ethidium bromide. Gels were scanned for fluorescence by the Bioprofil apparatus (Vilbert-Lourmat, France). Peak areas, corresponding to the monomeric and dimeric forms, were quantified, and the percentage of dimer was defined as the area of the dimer peak divided by the sum of the areas of the monomer and dimer peaks. Experiments with labeled oligonucleotides were also analyzed on a 1.2% agarose gel as described above. After being stained with ethidium bromide, gels were photographed under UV illumination using Polaroid films and then fixed for 10 min in 10% trichloroacetic acid and dried for 40 min under vacuum at room temperature. The hybridization of [^{32}P]DNA oligonucleotides to monomer and dimer RNAs was detected by autoradiography.

Cation-Dependent Thermal Dissociation of Dimeric RNA. RNA 1−626 (30.4 pmol) in 48 μL of double-distilled water was heated at 90 °C for 2 min and chilled on ice for 2 min. Thirty-two microliters of 2.5-fold concentrated buffer was added (final concentrations 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, and 125 mM XCl, where X = Li^+, Na^+, or K^+) and the samples were incubated at 20 °C for 15 min, followed by incubation at 50 °C for 60 min. The resulting dimers were then dialyzed (Millipore filters, Type V6, 0.025 μm) for 2 h at 4 °C against 10 mM Tris-HCl, pH 7.5, and 10 mM XCl (where X = Li^+, Na^+, or K^+). Aliquots (10 μL) of each sample were incubated for 5 min at temperatures ranging from 4 to 65 °C and electrophoresed as described above. After ethidium bromide staining of the gels, the percentage of dimer was measured (see above).

RNA Secondary Structure Analysis. GenBank identification codes for all the viral sequences referred to in the text are in parentheses: AEV (AEVPDNA), AEVES4 (REAEV5TR), ALV (ALVCG), AMV (ACMHBIPB), CT10 (REASVXX), EV-1 (AC2EV1LTR), EV-2 (AC2EV2LTR), FSV (ACF), H-19 (RERSVH19), HPRS-103 (ALV103J), IC4 (GGRVIC4L), IC10 (REIC10), IC11 (ALERMILAA), MC29 (REMC29Z), MH2 28-Z (AC2MILA), MH2E21 (AC2E21CG), PR2257 (ALRSRCAC), PR2257T (ASVPR-225T), Pr-B(HM) (RERSVHM1), Pr-B(LA23) (ALRLR, ALRASEG01, and ALRBSEG01), Pr-C (ALRCG), da Pr-C (RSVSEQ), RAV-0 (CHKER5LD), RAV-2 (ALEENV), SR-A (ALRDA1), SR-D (DO10652), UR2 (ACSUR2CG), and Y73 (ACSY73). RNA secondary structures were predicted by using the MFOLD program (kindly provided by M. Zuker) of Zuker and co-workers (Jaeger *et al.*, 1989). As the folding rules and energy parameters are not accurately known, the MFOLD program allows the determination of suboptimal foldings, within 5−10 % of the calculated minimun free energy, which might be of biological significance.

RESULTS

To investigate ASLV RNA dimerization, RNA transcripts representing portions of the genomic RNA were generated by *in vitro* transcription and examined for their ability to dimerize. RNAs 1−626, 1−516, 1−387, and 1−226 generated *in vitro* started at the cap site (+1 position) and ended with an authentic ALV sequence. Therefore, most of the RNA transcripts used in our study do not contain additional sequences (resulting from DNA plasmid construction) which might interfere with the dimerization process.

Spontaneous Dimerization of ALV RNA 1−626. RNA 1−626, corresponding to the 5′ first 626 nucleotides of ALV RNA, encompasses sequences that are involved in ASLV RNA dimerization (Murti *et al.*, 1981; Lear *et al.*, 1995). After heat denaturation and renaturation by incubating at 20 °C for 15 min (see Experimental Procedures) in a buffer formed of 50 mM Tris-HCl, pH 7.5, 125 mM KCl, and 5 mM $MgCl_2$, RNA 1−626 was mostly monomeric (Figure 2, lane 2) and this did not change after an additional incubation at 20 °C for 60 min (Figure 2, lane 3). In contrast, RNA 1−626 dimerized spontaneously and extensively *in vitro* when an additional incubation was carried out at 50 °C for 60 min (Figure 2, lane 4). The dimerization yield of RNA 1−626 was about 50% when incubation was performed at 37 °C (Figure 4C, lane 2).

Influence of Monovalent Cations on the Thermal Stability of ALV RNA Dimer. The presence of potassium results in significantly greater thermal stability of quadruple helical

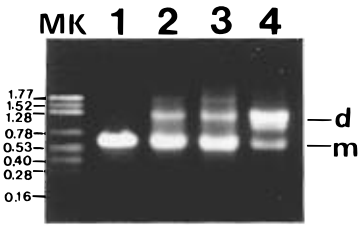

FIGURE 2: Spontaneous dimerization of ALV RNA 1−626. Heat-denatured RNA (0.38 μM) (lane 1) was incubated in 50 mM Tris-HCl, pH 7.5, 125 mM KCl, and 5 mM $MgCl_2$ for 15 min at 20 °C (lane 2). Subsequently, samples were incubated for 60 min at 20 °C (monomer conditions, lane 3) or at 50 °C (dimer conditions, lane 4). Samples were analyzed by 1.2% agarose gel electrophoresis as described in Experimental Procedures. Lane MK, 0.16−1.77-kb RNA ladder (Life Technologies, Inc.). m and d indicate the monomer and dimer forms of RNA 1−626, respectively.

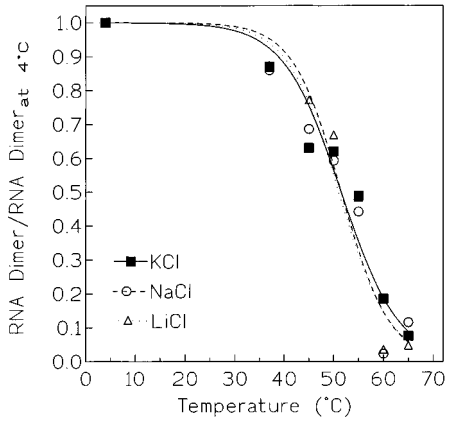

FIGURE 3: Influence of monovalent cations on the thermal stability of dimeric RNA 1−626. Melting curves were determined as described in Experimental Procedures. Data are normalized to the dimer yield at 4 °C.

structures formed by guanine base tetrads (G-tetrads) than do other larger or smaller monovalent cations (Awang & Sen, 1993; Sundquist & Heaphy, 1993). If G-tetrads are involved in RNA 1−626 dimeric structure, then the thermal dissociation of the dimer should be cation dependent. The influence of monovalent cations on the thermal stability of ALV dimeric RNA was investigated by monitoring dimer stability at various temperatures in the presence of 10 mM Tris-HCl, pH 7.5 and 10 mM LiCl, NaCl or KCl, respectively. As shown in Figure 3, the thermal stability of dimeric RNA 1−626 is cation independent. Therefore, specific cation-RNA interactions are probably not essential for the stability of the ALV RNA dimer structure. Thermal transition from dimer to monomer occurred at 51 °C.

Mapping of the Sequences Involved in ALV RNA Dimer Formation Using Complementary DNA Oligonucleotides. To map the dimerization domain of ALV RNA, 5′-end-labeled DNA oligonucleotides 223, 430, 462, 528, 582, and 610, which are complementary to sequences containing purine tracks, were tested for their ability to interfere with dimer formation (see Experimental Procedures). Among them, oligonucleotides 223, 462, 528, and 610 target stretches of at least four guanines (G212−G216, G450−G453, G515−G518, and G592−G595 and G605−G609). It should be noted that the four guanines 515−518 of ALV correspond to the tetraguanine sequence located at nt 523−526 of Pr-C (Schwartz et *al.*, 1983; Bieth & Darlix, 1992). This tetraguanine sequence is required for dimerization of short Pr-C RNA transcripts (Lear et *al.*, 1995). Oligonucleotides 430, 462, and 582 are complementary to the RGGARA sequences proposed by Marquet *et al.* (1991) as candidates for the formation of purine quartets involving both adenine(s) and guanine(s). As shown in Figure 4A, none of these oligonucleotides inhibited RNA 1−626 dimer formation. In all cases, the different [^{32}P]DNA oligonucleotides hybridized to both the monomer and dimer forms of RNA 1-626 (see autoradiogram in Figure 4A). RNA 1−626 contains five stretches of four guanines or more, and any one of these sequences could interact with any G-rich stretch present in the second RNA molecule. Therefore, it is conceivable that RNA 1−626 uses alternatively different guanine motifs to dimerize. To test this possibility we incubated all four oligonucleotides 223, 462, 528, and 610 together with RNA 1−626, but dimerization still occurred (Figure 4A, lanes 4OL). Similarly, oligonucleotides 430, 462, and 582, targeting the RGGARA sequences were unable to inhibit the dimerization process when they were all incubated together with RNA 1−626 (Figure 4A, lanes 3OL). Schwartz *et al.* (1983) identified an autocomplementary sequence in Pr-C, corresponding to the 513−540 region of ALV, that may contribute to the DLS. It is interesting that oligonucleotide 528 did not inhibit RNA 1−626 dimerization (Figure 4A), although it interfered partially with the autocomplementary sequence of Schwartz *et al.* (1983). In order to completely exclude a putative effect of this autocomplementary sequence, we used oligonucleotides 531 and 540, which are complementary to nt 512−531 and 517−540 of ALV RNA sequence, respectively. Oligonucleotides 531 and 540 did not inhibit RNA 1−626 dimerization, even at an oligonucleotide/RNA ratio of 5/1, and were found to hybridize to both monomeric and dimeric RNA (Figure 5). Bieth *et al.* (1990), using antisense oligonucleotides, had implicated the 544−564 region in the dimerization process of RSV RNA. Oligonucleotide 556 is directed against the 536−556 region of ALV which corresponds to the 544−564 region described by Bieth *et al.* (1990). As shown in Figure 5, oligonucleotide 556 was unable to interfere with the dimerization process of RNA 1−626. Recent reports show that autocomplementary sequences, which can form a stem−loop structure, are involved in the RNA dimerization of HIV-1 (Paillart *et al.*, 1994; Laughrea & Jetté, 1996; Muriaux *et al.*, 1996) and MoMuLV (Girard *et al.*, 1995) *in vitro*. Interestingly, the 5′ first 626 nucleotides of ALV and RSV SR-A differ only by two nucleotides (Bieth & Darlix, 1992; Swanstrom *et al.*, 1982). On the basis of a phylogenetic analysis, Hackett *et al.* (1991) proposed two secondary structures for the first 499 nucleotides of the 5′-end of RSV SR-A. On the basis of these possible secondary structures, we selected DNA oligonucleotides 25, 189, 223, 273, and 462 to target the autocomplementary sequences capable of forming a stem−loop structure. RNA secondary structures in the region encompassing nucleotides 500−626 were predicted using the Zuker algorithm (Jaeger *et al.*, 1989) and allowed to select oligonucleotide 528 directed against a putative autocomplementary stem−loop structure. Oligonucleotides 25, 189, 223, 462, and 528 did not inhibit RNA 1−626 dimerization, even at an oligonucleotide/RNA ratio of 5/1 (Figure 4A, B) and were found to hybridize to both monomeric and dimeric RNA. In contrast, oligonucleotide 273, complementary to the stem−loop termed L3 (Hackett *et al.*, 1991), strongly inhibited dimerization at an oligonucleotide/RNA ratio of 2/1 and hybridized solely to

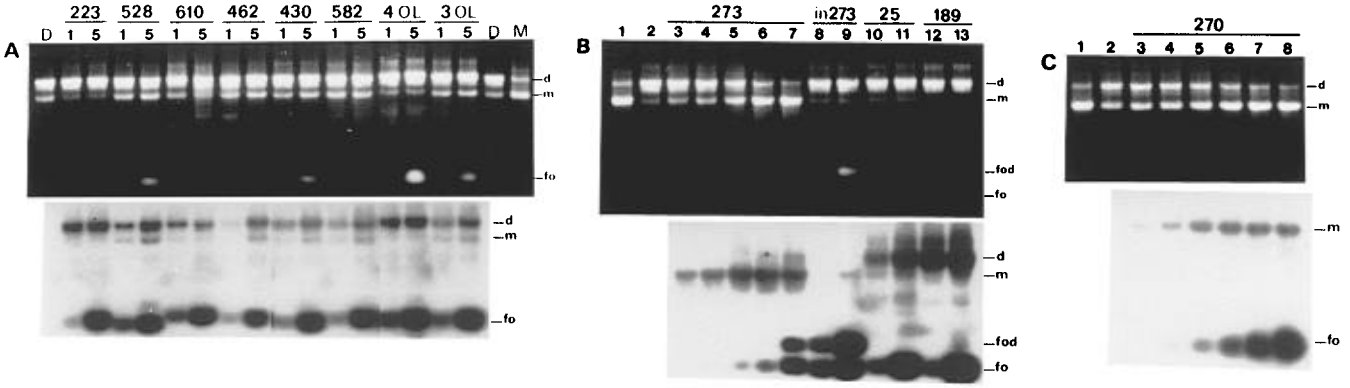

FIGURE 4: Analysis of ALV RNA 1−626 dimer formation in the presence of oligonucleotides targeting either purine tracts or autocomplementary sequences. (A) RNA 1−626 (0.38 μM) was incubated at 50 °C under dimerization conditions with [^{32}P]DNA oligonucleotide 223, 528, 610, 462, 430, or 582 at an oligonucleotide/RNA ratio of 1/1 or 5/1 (lanes 1 and 5). Dimerization was also carried out in the presence of several oligonucleotides together (223, 528, 610, and 462 in lanes 4OL; 462, 430, and 582 in lanes 3OL), each of them at 1 and 5 molar equiv of RNA 1−626 (lanes 1 and 5). Lanes D and M, RNA 1−626 without oligonucleotide was incubated under dimer and monomer conditions, respectively. (B) Dimerization of RNA 1−626 without oligonucleotide (lane 2), with oligonucleotide 273 at oligonucleotide/RNA ratios of 1/6, 1/4, 1/2, 1/1, and 2/1 (lanes 3−7), or with oligonucleotides in273, 25, and 189 at an oligonucleotide/RNA ratio of 1/1 (lanes 8, 10, and 12) or 5/1 (lanes 9, 11, and 13). RNA 1−626 was also incubated under monomer conditions (lane 1 in panels B and C). (C) RNA 1−626 was incubated at 37 °C without oligonucleotide (lane 2) or with oligonucleotide 270 at oligonucleotide/RNA ratios of 1/4, 1/2, 1/1, 2/1, 4/1, and 8/1 (lanes 3−8). m, d, fo, and fod indicate RNA 1−626 monomer, RNA 1−626 dimer, free oligonucleotide, and duplex of the free oligonucleotides 273 and in273, respectively. The nucleic acids were analyzed by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining (top) and autoradiography (bottom). Exposure time of the film was increased in the experiments performed with oligonucleotides 430 and 582 (A).

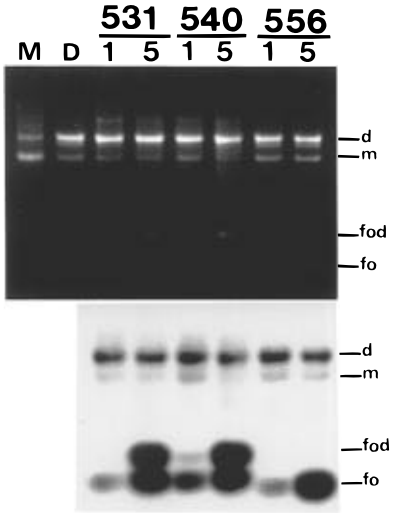

FIGURE 5: Analysis of ALV RNA 1−626 dimer formation in the presence of oligonucleotides targeting the autocomplementary sequence of Schwartz et al. (1983). RNA 1−626 (0.38 μM) was incubated at 50 °C under dimerization conditions with [^{32}P]DNA oligonucleotide 531, 540, or 556 at an oligonucleotide/RNA ratio of 1/1 or 5/1 (lanes 1 and 5). Lanes D and M, RNA 1−626 without oligonucleotide was incubated under dimer and monomer conditions, respectively. m, d, fo, and fod indicate RNA 1−626 monomer, RNA 1−626 dimer, free oligonucleotide, and duplex of the free oligonucleotides 531 and 540, respectively. The nucleic acids were analyzed by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining (top) and autoradiography (bottom).

monomeric RNA 1−626 (Figure 4B). In the same conditions, oligonucleotide in273, with the reverse sequence of oligonucleotide 273, did not inhibit dimerization. To map more precisely the minimal sequence required for dimer formation, we used oligonucleotide 270 (12 nucleotides long), which is shorter than oligonucleotide 273 (20 nucleotides long). Oligonucleotide 270 is complementary to nt 259−270, which are a part of the autocomplementary sequence spanning nt 258−274 (Figure 9A). Dimerization was performed at 37 °C to allow hybridization of oligonucleotide 270 to RNA 1−626 and this oligonucleotide was found to bind only to monomeric RNA and to inhibit dimerization strongly at an oligonucleotide/RNA ratio of 8/1 (Figure 4C).

An Autocomplementary Sequence Is Required for Efficient Dimerization of ASLV RNA. Results of oligonucleotide mapping prompted us to synthesize five ASLV RNA transcripts: ALV RNAs 1−516 and 1−387, containing the L3 stem−loop, and ALV RNAs 1−226 and 523−1023 and ASV RNA d207−270, which are devoid of the autocomplementary sequence (Figure 1A). The 207−270 sequence of ASV corresponds to the 208−270 sequence of ALV (Katz et *al*., 1986; Bieth & Darlix, 1992). Results show that RNAs 1−516 and 1−387 dimerized very efficiently (Figure 1B; Figure 6A, B, lanes 3). Furthermore, oligonucleotide 273, targeting the L3 stem−loop, abolished dimerization at an oligonucleotide/RNA ratio of 2/1 (Figure 6A, B, lanes 6). RNA 523−1023 was unable to form more than 10% dimer (Figure 1B; Figure 6C, lane 4) while ASV RNA d207−270 dimerized poorly (Figure 1B; Figure 7C, lane 6). Unexpectedly, up to 50% of RNA 1−226 was in a dimeric form (Figure 7B, lane 6). Thus, region 1−226 contains sequences which can interact to convert 40−50% of monomers into dimers under the present conditions. If ALV RNA dimer formation proceeds by the same mechanism for RNA 1−626 and 1−226, then these two transcripts should form heterodimers of the form RNA 1−626/1−226. However, no clear band migrating between the positions of the monomeric and dimeric forms of RNA 1−626 was observed (Figure 7B, lane 5), suggesting that the primary site for RNA 1−626 dimerization is not located within the region spanning nucleotides 1−226. Finally, to determine whether dimerization of RNA 1−626 and RNA 1−387 occurred via the same mechanism, we tried to form heterodimers with such RNAs. As shown in Figure 7A (lane 5), the new band between dimeric RNA 1−626 and RNA 1−387 corresponds to heterodimer 1−626/1−387. These data indicate that a major domain necessary for dimerization

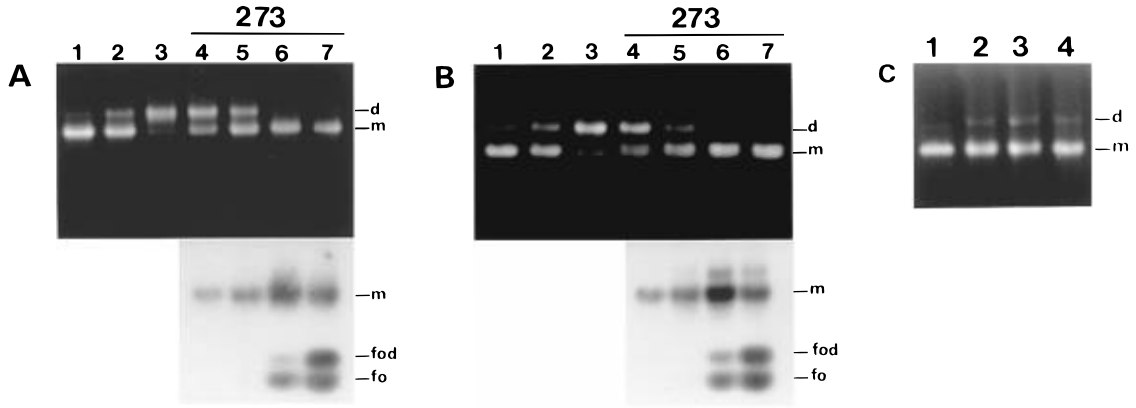

FIGURE 6: Gel analysis of dimer formation with RNAs 1−516 (A), 1−387 (B), and 523−1023 (C). RNA transcripts (0.38 μM) in 10 μL of standard buffer were incubated at 20 °C for 15 min (panels A and B, lane 1; panel C, lane 2) before incubation at 20 °C for 60 min (monomer conditions; lane 2 in panels A and B, lane 3 in panel C) or at 50 °C (dimer conditions; lane 3 in panels A and B, lane 4 in panel C). Under dimerization conditions, [^{32}P]DNA oligonucleotide 273 was present at oligonucleotide/RNA ratios of 1/2, 1/1, 2/1, and 4/1 in lanes 4−7 (panels A and B). Heat-denatured RNAs 523−1023 is shown in lane 1 of panel C. RNAs were analyzed by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining. Autoradiograms, below the agarose gels stained with ethidium bromide (panels A and B), show hybridization of [^{32}P]DNA oligonucleotide 273 to RNAs 1−516 and 1−387, respectively.

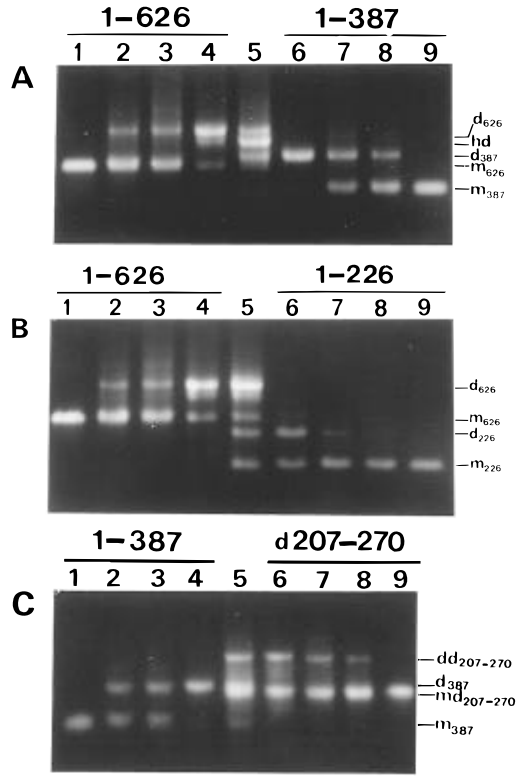

FIGURE 7: Gel analysis of heterodimer formation with different ASLV RNA transcripts. Lanes 1 and 9 show heat-denatured RNAs, and lanes 2 and 8 show RNAs after 15 min at 20 °C in standard buffer. RNAs were incubated in monomer (lanes 3 and 7) or dimer (lanes 4 and 6) conditions. RNA 1−626 (0.38 μM) was incubated in dimer conditions with either RNA 1−387 (0.38 μM) (panel A, lane 5) or RNA 1−226 (0.38 μM) (panel B, lane 5). (C) Lane 5, ASV RNA d207−270 (0.38 μM) was incubated in dimer conditions with RNA 1−387 (0.38 μM). Monomer (m) and dimer (d) positions of the different RNA transcripts are indicated. hd indicates heterodimer formed of RNA 1−626 and RNA 1−387.

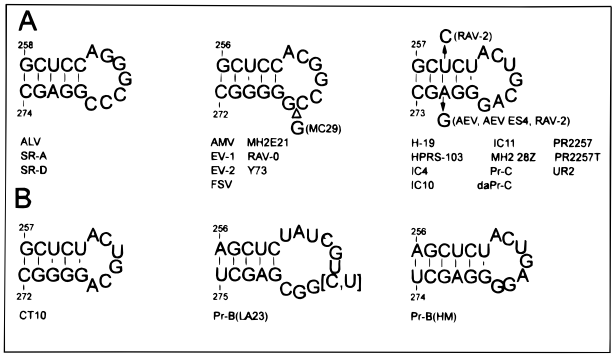

FIGURE 8: Comparison of predicted stem−loop structures in the L3 domain of 28 ASLV. Arrows indicate the nucleotides present in the viruses listed in parentheses. The G insertion in MC29 is indicated by a triangle. Bases in square brackets vary in the Pr-B(LA23) isolates (Darlix & Spahr, 1983). Nucleotides are numbered according to Pr-C (Schwartz *et al.*, 1983).

in vitro is located between nt 227 and 387. When oligonucleotide 273 was hybridized to the 258−274 autocomplementary sequence, RNAs 1−626 and 1−387 were unable to form heterodimers (data not shown). In addition, ASV RNA d207−270 lacking the L3 stem−loop was unable to form heterodimers with RNA 1−387 (Figure 7C, lane 5). Taken together, these results show that the 258−274 autocomplementary sequence is critical for *in vitro* dimerization of an ASLV RNA transcript encompassing the 5′ leader region.

Phylogenetic Comparison. To examine the functional relevance of the L3 stem−loop structure located between positions 258 and 274 of ALV RNA, we searched for its conservation among 28 ASLV sequences. The analysis of 13 ASLV sequences has revealed that a conserved secondary structure is present in the 237−300 region encompassing the L3 structural element (Hackett *et al.*, 1991). We used the MFOLD program (Jaeger *et al.*, 1989) to predict the secondary structures of 28 ASLV sequences containing the 237−300 region. The free energy minimization predictions favored the L3 stem−loop structures presented in Figure 8. An autocomplementary sequence within the loop is present in 27 RNA secondary structures. Among them, 25 exhibit a six-nucleotide-long autocomplementary sequence (Figure 8A). Pr-B(HM) does not display a loop with an autocomplementary sequence (Figure 8B), but the G and A residues in the middle of the loop should not prevent pairing because the noncanonical A-G pair is not rare in RNAs (Woese & Pace, 1993). Naturally occurring compensatory sequence (covariant) changes were found between ALV and other ASLV at positions G264 and G265 facing nucleotides C269 and C268, respectively (Figure 8A). It is highly

unlikely that it is a coincidence that two pairs of compensatory substitutions are adjacent.

DISCUSSION

Our results show that T7-generated RNA transcripts containing the complete 5′ leader of ALV genomic RNA can dimerize extensively *in vitro* under physiological salt concentrations (Figures 1 and 2). Thermal stability of dimeric RNA 1−626 was not affected by the monovalent cation present, either K^+, Na^+, or Li^+ (Figure 3), and half of the RNA dimers were found to be denatured at 51 °C, a temperature close to that found for the dimeric RNA genome (Mangel *et al.*, 1974; Stoltzfus & Snyder, 1975). DNA oligonucleotides complementary to purine-rich sequences hybridized to dimeric RNA and were unable to inhibit the dimerization process (Figure 4A). In addition, ALV RNA 1−387 lacking the consensus RGGARA purine sequences dimerized to a high level (Figure 6B). Therefore, these findings indicate that purine tetrads are most probably not directly involved in the dimeric structure of ALV RNA, which is in agreement with recent results on Pr-C (Lear *et al.*, 1995).

On the basis of sequence (Schwartz *et al.*, 1983) and electron microscopy data (Murti *et al.*, 1981), Schwartz *et al.* (1983) originally proposed that the DLS of Pr-C genomic RNA corresponds to canonical base-pair interactions involving residues 521−548. The 521−548 sequence of Pr-C corresponds to the 513−540 sequence of the ALV strain used in our study. DNA oligonucleotides 528, 531, and 540 complementary to the 513−540 region did not inhibit dimerization of ALV RNA 1−626 and hybridized to the dimeric RNA (Figures 4A and 5). Bieth *et al.* (1990), using antisense oligonucleotides, found that the 544−564 sequence of RSV, which is equivalent to the 536−556 sequence in our study, could correspond, at least in part, to the DLS. However, a DNA oligonucleotide complementary to nucleotides 536−556 was able to hybridize to dimeric RNA 1−626 without altering the dimerization process *in vitro* (Figure 5). The simplest explanation for the discrepancy between our results and those of Bieth *et al.* (1990) is the use of different RNA transcripts. We used RNA transcripts starting at the cap site (+1 position) and ending with an authentic ALV sequence in order to mimic *in vitro* the genomic RNA dimerization. In contrast to our study, all RNA transcripts tested by Bieth *et al.* (1990) contained 63 additional nucleotides (resulting from DNA plasmid construction) upstream from the cap site. RNA transcripts with additional nucleotides could form structures which are not adopted by the genomic RNA and by RNA transcripts starting at the cap site. It should be emphasized that Bieth *et al.* (1990) only stated that an antisense oligonucleotide targeting the 544−564 region of RSV hybridized poorly to dimeric RNA. We think that dimeric RNA, generated by Bieth *et al.* (1990), adopted a peculiar structure which prevented the binding of oligonucleotide to the 544−564 sequence. However, the linkage between the monomers was probably not within the region spanning nt 544−564. In accordance with our hypothesis, Bieth *et al.* (1990) showed (see Figure 4, RNAs 3, 4, and 5) that RNA transcripts containing the 544−564 region and devoid of sequences forming the L3 stem−loop were not efficient for dimerization. Lear *et al.* (1995) also reported that nt 544−564 are not necessary for Pr-C RNA dimerization *in vitro*. They found that Pr-C RNA dimerization *in vitro* was dependent upon a region spanning nucleotides 496−634, which encompasses the putative DLS. However, these authors used very high salt concentrations, 250 mM NaCl and 50 mM $MgCl_2$, that do not mimic physiological conditions and which could explain that a low level of dimeric Pr-C RNA 1−634 was observed (Lear *et al.*, 1995). Under these high salt conditions only short Pr-C RNA transcripts 496−634 were able to dimerize extensively, in contrast to previous findings (Bieth *et al.*, 1990) and the present ones showing that sequences upstream from the SD are essential for ASLV RNA dimerization (Figure 1). In agreement with these data, it should be emphasized that sequences upstream from the SD of HIV-1 (Laughrea & Jetté, 1994; Marquet *et al.*, 1994; Paillart *et al.*, 1994; Skripkin *et al.*, 1994) and BLV (Katoh *et al.*, 1993) are critical for viral RNA dimerization *in vitro*. Furthermore, it should be noted that only one team has been able to detect and locate the DLS (position 511 ± 28 nt) of ASLV by electron microscopy of partially denatured RNAs (Murti *et al.*, 1981). Another electron microscopy study, using a different spreading technique, suggested that the dimer consists of two RNA subunits held together at many points (Mangel *et al.*, 1974). Thus, the postulated DLS might not be the only or even the main linkage site between the two subunits. Consistent with this suggestion is a recent report which indicates that the sequences present in a heterologous RNA, which lacks both the PBS and the DLS but bears the region located between the PBS and the DLS, are sufficient to allow detection of dimers and higher-order complexes in nondenaturing gel systems (Aronoff *et al.*, 1993).

Results presented here show that a short autocomplementary sequence, located between positions 258 and 274 in the 5′ noncoding region and able to form a stem−loop structure, plays an essential role in ALV RNA dimerization *in vitro*. In fact, DNA oligonucleotides complementary to nt 258−274 were the only ones capable of preventing RNA 1−626 dimerization when the dimer was formed at 37 °C or at 50 °C (Figure 4B, C). Therefore, the same autocomplementary sequence is involved in dimerization mechanism of both dimers formed at 37 or 50 °C. Deletions of nt 1−522 (RNA 523−1023) or nt 207−270 (ASV RNA d207−270), thus removing the putative L3 stem−loop, resulted in a strong inhibition of ASLV RNA transcripts dimerization, as previously shown for dimerization activated by the nucleocapsid protein NCp12 (Bieth *et al.*, 1990). Nevertheless, a low level of dimeric RNAs 523−1023 and d207−270 was observed (Figures 1B, 6C, and 7C), suggesting that nt 523−626 could weakly contribute to ASLV RNA dimerization. However, the formation of heterodimers between two ASLV RNA transcripts was only possible if sequence 258−274 was present in both transcripts (Figure 7). Surprisingly, RNA 1−226 was found to dimerize moderately and the nature of that dimer is not yet understood. However, RNA 1−226 did not form heterodimers with RNA 1−626, while RNA 1−387 containing the L3 stem−loop was able to direct heterodimerization (Figure 7). The absence of heterodimer formation between RNA 1−226 and RNA 1−626 is clear evidence that ALV RNA transcripts are unable to link within region 1−226 when this region is a part of a large molecule like RNA 1−626. These observations suggest that nt 1−226, once expressed alone, can form structures which are not adopted when they are part of a larger molecule. It seems obvious that the dimerization of short transcripts does not

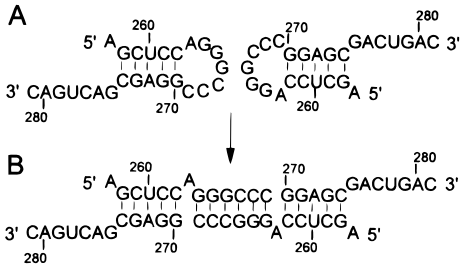

FIGURE 9: Model for ALV RNA dimerization initiation. (A) Postulated stem−loop structure of the monomers involved in dimer formation. (B) Proposed structure upon dimer formation. Numbering is in relation to the cap site (+1).

always reflect the dimerization of genomic RNA. In the case of ASLV as described for HIV-1 (Marquet *et al.*, 1994; Paillart *et al.*, 1994; Laughrea & Jetté, 1996), the most appropriate models for mimicking the dimerization of full-length genomic RNA *in vitro* are probably large transcripts including the entire 5′ noncoding region (for example, RNAs 1−626, 1−516, and 1−387). Taken together, results presented here strongly suggest that the autocomplementary sequence 258−274 forming a putative stem−loop structure, named L3, is the initial site of ALV RNA dimerization *in vitro*. This finding is also supported by the fact that 28 ASLV sequences conserve this stem−loop structure with a short autocomplementary sequence in the loop. Therefore, the phylogenetic analysis favors the notion that the L3 stem−loop is functionally relevant. Thus, the model proposed for HIV-1 (Skripkin *et al.*, 1994; Muriaux *et al.*, 1996; Laughrea & Jetté, 1996) and MoMuLV (Girard *et al.*, 1995) in which dimer formation is initiated via loop−loop interactions may well hold true for ASLV RNA (Figure 9). A "kissing complex" may be formed between complementary nucleotides in loops. The opening of both stem−loops during the interaction could lead to a double-stranded region through Watson−Crick base pairing. Recent biochemical and genetic analyses on HaMSV RNA (Torrent *et al.*, 1994; Feng *et al.*, 1995) suggest that dimerization of this recombinant MoMuLV-VL30 RNA probably proceeds via a similar mechanism.

The ASLV L3 stem−loop structure is within the 5′ Ψ region, located between the PBS and the SD, which is required for encapsidation of genomic RNA (Koyama *et al.*, 1984; Katz *et al.*, 1986; Anderson *et al.*, 1992) and sufficient to direct encapsidation of heterologous RNA into virions (Aronoff *et al.*, 1993). Thus, the L3 element is probably necessary for genomic RNA dimer packaging into virions. However, it is most probably not sufficient for genomic RNA dimer packaging as indicated by genetic data on ASLV (Darlix, 1986; Knight *et al.*, 1994). Indeed, it has been found that the 5′ stem−loop termed O3, located within the 5′ Ψ region, is required for efficient ASLV RNA packaging (Knight *et al.*, 1994). Recently, Yang and Temin (1994) showed that two stem−loop structures are required for SNV RNA encapsidation. The next challenge will be to examine the structure−function relationships of the L3 stem−loop structure in ALV genomic RNA dimerization and packaging.

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