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Secondary Structural Features in the 70S RNAs of Moloney Murine Leukemia and Rous Sarcoma Viruses as Observed by Electron Microscopy

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The secondary structural features in the 70S RNAs of the Prague strain of avian Rous sarcoma virus, subgroup A (PR-RSV-A), and Moloney murine leukemia virus (M-MuLV) were compared by electron microscopy. The PR-RSV-A genome contained two subunits joined by a linkage structure as in the genomes of M-MuLV and other mammalian retroviruses. In both viral genomes, a highly reproducible hairpin occurred at about 70 nucleotides from the 5′ end of each subunit and contained 320 ± 8 nucleotides. The stable point of linkage between the subunits in both viral genomes involved fewer than 50 nucleotides and occurred at 466 ± 9 nucleotides from the 5′ end. This places the linkage about 350 nucleotides further toward the 3′ end of the subunit than the binding site of primer tRNA. Another structural feature common to both genomes was a loop in each subunit. In M-MuLV, the loop contained 3.9 ± 0.10 kilobases (kb) and occurred at a distance of 2.2 ± 0.05 kb from the 5′ end. In PR-RSV-A, the loop was smaller (2.3 ± 0.10 kb) and further (3.3 ± 0.10 kb) from the 5′ end. When M-MuLV RNA was heated to 70, 85, or 90°C and cooled, the hairpin consistently reformed at the 5′ end. No other structures typical of the native molecules reappeared. In RNA samples heated to 70°C, a new loop reproducibly occurred near the 5′ end of each subunit, but this loop was not found in samples heated to higher temperatures. Based on all of these findings, we conclude that the genome of PR-RSV-A shares several features with M-MuLV and other mammalian retroviruses and that the primer tRNA molecules are not involved in the linkage of the two subunits in either genome. We also conclude that the dimer linkage and the loops in subunits are typical of the native molecules and that their formation requires a special environment.

The 60 to 70S genomic RNA of retroviruses has been shown by biochemical techniques to contain two or more identical subunits of about 35S and several smaller (4 to 10S) RNA species. The 5′ end of the 35S subunit contains (i) the binding site for a primer tRNA molecule, (ii) a sequence involved in the linkage with another 35S subunit, and (iii) a leader sequence that is spliced onto mRNA molecules (see reference 4 for a review). The 3′ end has a stretch of polyadenylic acid (2). The purified subunits also exhibit an extensive secondary structure (1, 6, 18).

Electron microscope analyses (8, 9, 12-15) have shown that the genomes of a number of retroviruses, e.g., endogenous feline virus (RD 114), endogenous baboon virus (BKD), sarcoma virus from woolly monkey, the Friend strain of murine leukemia virus (F-MuLV), Moloney murine leukemia-sarcoma complex [(MSV)M-MuLV], and reticuloendotheliosis virus, are each composed of two subunits joined at their 5′ ends by a morphologically recognizable linkage. In addition, these viral genomes (with the possible exception of reticuloendotheliosis virus) contain a large loop within each subunit. The loops in some genomes (BKD and sarcoma virus from woolly monkey) also contain a hairpin. The consistent occurrence of the linkage feature and the loops led to a proposal (8, 14) that all mammalian retroviral genomes share a basic structure. The functions of these features remain unknown, but suggested functions include packaging of viral genomes (14), synthesis of proviral DNA (14), and processing of viral mRNA (17). Attempts to demonstrate the same structural features in avian leukosis or sarcoma viral genomes have not been successful (7, 14, 22).

In the present ultrastructural analysis, by modification of preparative conditions, we were able to observe partially unfolded 70S RNA of the Prague strain of avian Rous sarcoma virus, subgroup A (PR-RSV-A), which revealed the linkage and other secondary structural features. We compared these features with those in the genomic RNA of M-MuLV. In addition, a clear

visualization of the linkage between the subunits in both viral genomes allowed us to conduct a more detailed structural analysis of this region than has been previously reported. The results provide new information on the structure of retroviral genomes. The data should also be useful in locating the nucleotide sequences responsible for each of the structural features when the entire nucleotide sequences of the viral genomes become available.

MATERIALS AND METHODS

Viruses. M-MuLV was obtained from A. Hackett and was passaged three times at a limiting dilution in NIH 3T3 cells. Virus was harvested at 12-h intervals from continuously producing cells. PR-RSV-A was obtained from P. K. Vogt and subsequently cloned twice. Medium from chicken embryo fibroblast cultures transformed with PR-RSV-A was collected at 4-h intervals. The virus-containing supernatants were centrifuged at 700 × *g* for 10 min to remove cell debris and frozen at −70°C until virus purification.

Purification of viruses and viral RNA. The viruses were concentrated by precipitation with 8% (wt/vol) polyethylene glycol (6,000 to 7,000 M_r) after the addition of NaCl (final concentration, 0.4 M) to virus-containing culture supernatants (3). The pellets (10,000 × *g*, 30 min) were suspended in 0.01 volume of 0.1 M NaCl–0.01 M Tris-hydrochloride (pH 7.5)–0.001 M EDTA (NET buffer). The PR-RSV-A suspension was sedimented through 25% sucrose onto a 50% sucrose cushion containing NET buffer. This partially purified PR-RSV-A and the resuspended polyethylene glycol pellet of M-MuLV were layered on top of 15 to 55% (wt/wt) sucrose gradients in NET buffer and centrifuged at 96,000 × *g* for 15 h at 4°C. The viral bands were collected from the top, diluted at least three-fold with NET buffer, and pelleted by centrifugation at 4°C for 1.5 h at 96,000 × *g*. The virus pellets were resuspended in NET buffer (0.5 to 1 mg of virus protein per ml); sodium dodecyl sulfate (0.5%, wt/vol) and proteinase K (100 μg/ml) were added. After incubation at 22°C for 30 min, RNA was extracted three times at 22°C with an equal volume of phenol-chloroform (1:1, vol/vol). The final aqueous phase was made 0.2 M with potassium acetate and precipitated at −20°C with 2.5 volumes of ethanol. The RNA precipitate was dissolved in 0.01 M Tris-hydrochloride (pH 7.5)–0.001 M EDTA (TE buffer) and sedimented in 10 to 30% sucrose gradients in NET buffer at 35,000 rpm in an SW41 rotor for 3.6 h at 4°C. Fractions (0.5 ml each) were collected, and those containing 60 to 70S RNA were identified by measurement of absorbance at 260 nm. These gradient fractions were pooled, subjected to two successive ethanol precipitations as above, dissolved in TE buffer at a concentration of 100 μg/ml, and stored in aliquots at −70°C.

Electron microscopy. The purified RNAs from M-MuLV, PR-RSV-A, and Qβ (external size standard; Miles Laboratories, Inc.) were prepared for electron microscopy by the formamide-urea method (19, 23) modified as follows. A stock solution of 5.0 M urea (Ultrapure/Schwarz/Mann) was made in purified formamide (Matheson, Coleman and Bell), and the concentration of this solution in the spreading medium was adjusted with 2× distilled water. The spreading solution (100 μl) contained 0.1 to 0.2 μg of RNA, 50% formamide, 2.5 M urea (or 40% formamide and 2.0 M urea), 30 mM Tris-hydrochloride, 1 mM EDTA (pH 8.5), and 30 μg of cytochrome *c* (2× crystallized; Calbiochem) per ml. A total of 10 to 20 μl of the solution was allowed to flow onto the surface of double-distilled water in a Teflon trough. The protein monolayer was adsorbed to Parlodion-coated, 400-mesh copper grids, stained with ethanolic uranyl acetate, and shadowed with platinum-palladium (80:20; Ted Pella Co.) alloy. The grids were viewed in a Philips 301 electron microscope operated at 80 kV. Photographs were taken at magnifications of between ×25,000 and ×57,000. Magnifications in the electron microscope were calibrated, using a carbon grating replica (2,160 lines per mm; E. F. Fullam Co.). Contour lengths of molecules were measured in prints enlarged 5- to 10-fold with a Numonics 1224 graphic digitizer. Measured lengths were converted into the number of nucleotides, using the relationship between the length and the number of nucleotides of Qβ RNA. Measurements were given as mean values ± the standard error of the mean (standard deviation/$\sqrt{n}$).

Denaturation and renaturation of M-MuLV RNA. Viral 70S RNA (1 μg in 10 μl of 20 mM Tris-hydrochloride [pH 7.4]–1 mM EDTA) was heated for 3 min at 70, 85, or 90°C. The samples were cooled to 22°C over a period of 15 min and prepared for electron microscopy as described above. In some experiments, the reannealing was continued for 48 to 72 h at 22°C in the presence of 0.1 M NaCl.

RESULTS

General structure and sizes of RNAs. The purified 70S RNAs from M-MuLV and PR-RSV-A, spread from a solvent containing 50% formamide and 2.5 M urea, were examined in the electron microscope. A majority of M-MuLV RNA molecules contained a clearly recognizable dimer linkage structure, loops in the subunits, and hairpins within the loops (Fig. 1). A new feature resolved in this study was a hairpin between the point of linkage of the subunits and their 5′ ends. In PR-RSV-A molecules, spread under similar conditions, all of the above structural features were observed but at a lower frequency. The dimer linkage structure was clearly seen in some molecules (Fig. 2C and D) but not detectable in others (Fig. 2E). Molecules containing two loops (i.e., one in each subunit) were rarely observed. A slight lowering of the concentration of the denaturing solvent to 40% formamide and 2 M urea greatly enhanced the frequency of molecules with loops and linkage structures (Fig. 2A and B), but most of these molecules were too condensed for accurate length measurements. Therefore, the size, structure, and location of the secondary structural features in both viral genomes were analyzed, using RNAs spread in 50% formamide–2.5 M urea.

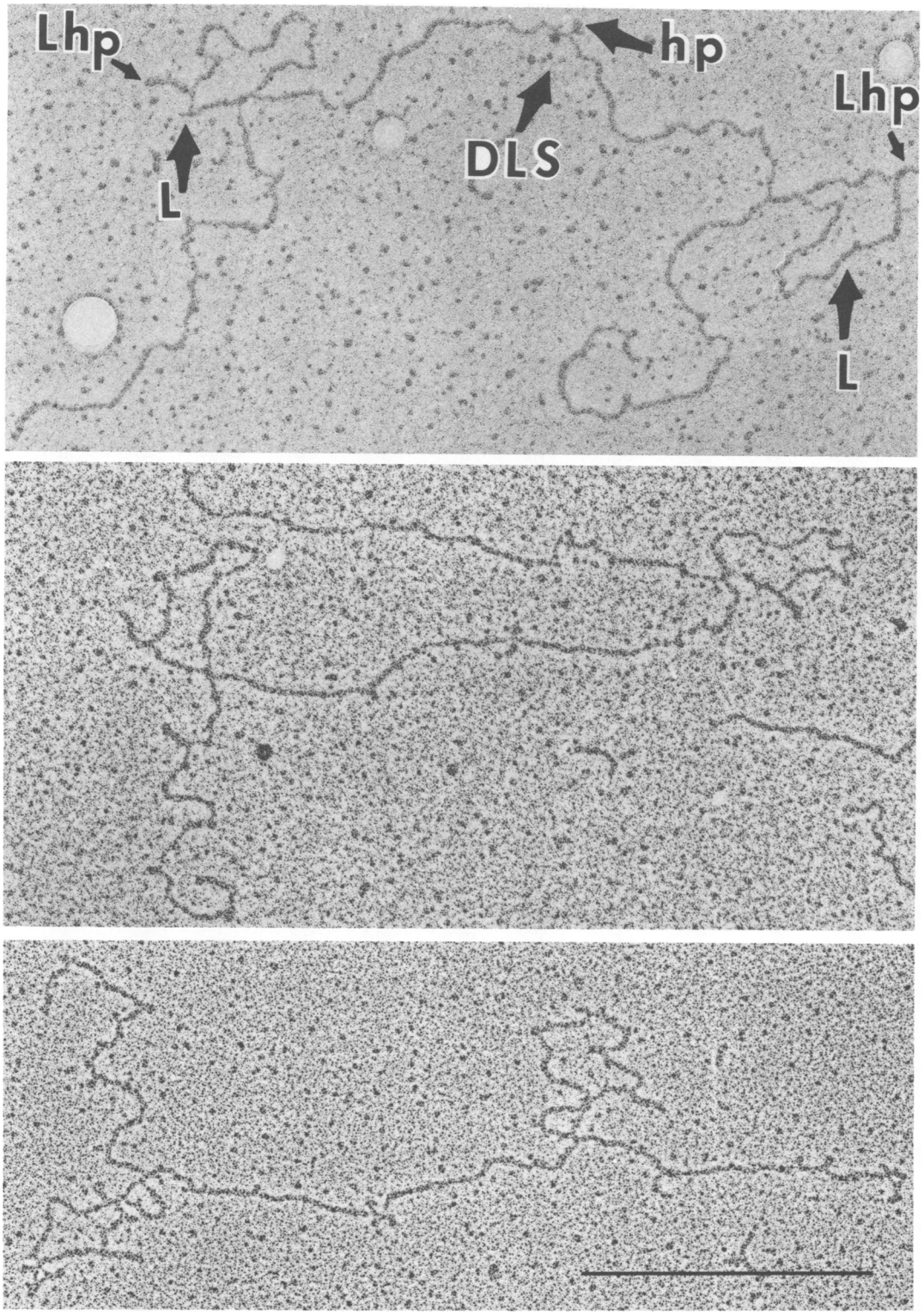

FIG. 1. *Electron micrographs of 70S M-MuLV RNA molecules spread in 50% formamide–2.5 M urea. Note the dimeric structure, linkage between the subunits (DLS), small hairpin at the 5′ end (hp), loop (L) in each subunit, and hairpin (Lhp) within each loop. Bar = 0.5 μm.*

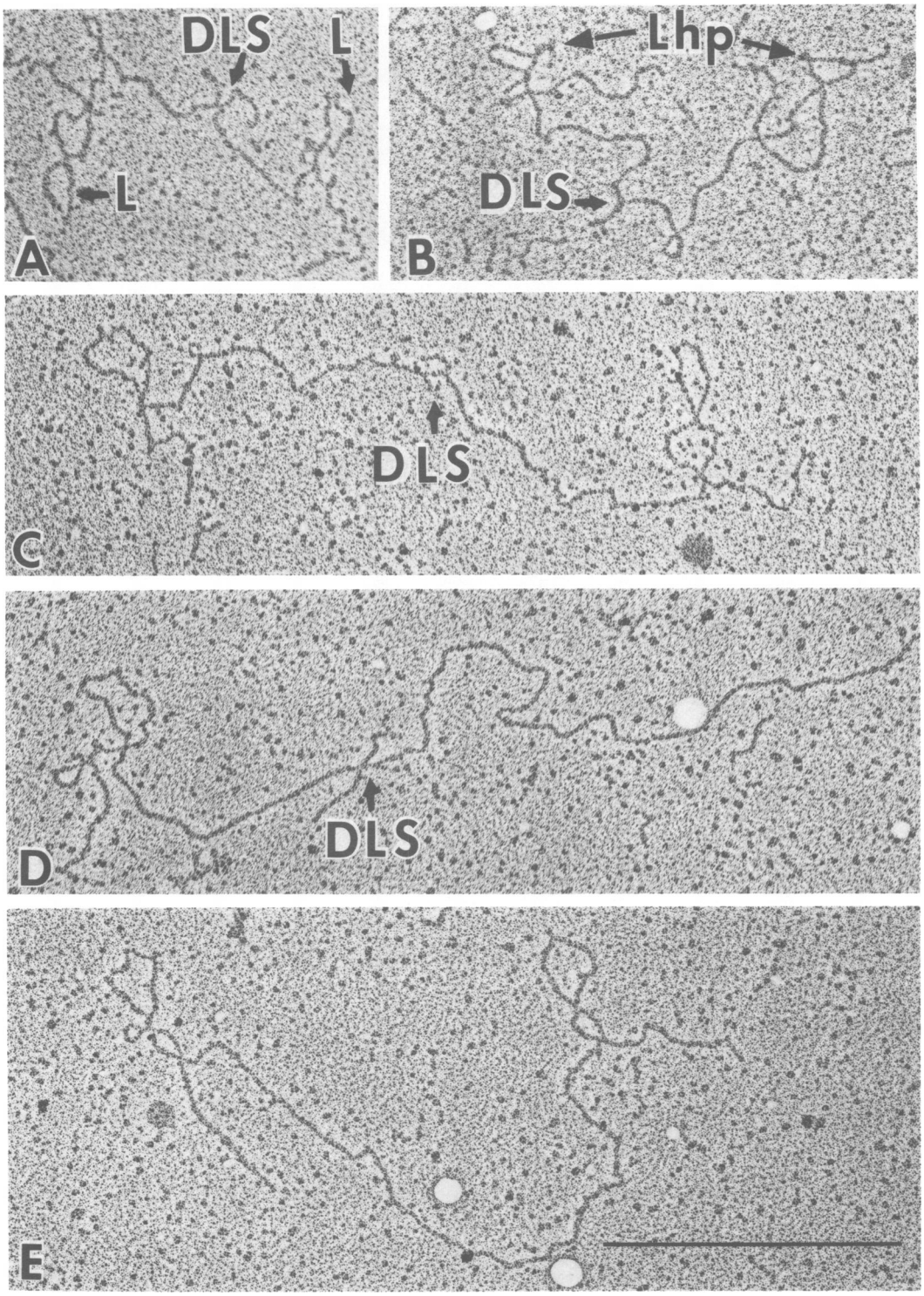

FIG. 2. *Electron micrographs of 70S PR-RSV-A RNA spread in (A and B) 40% formamide–2.5 M urea and (C, D, and E) 50% formamide–2.5 M urea. Abbreviations are as in Fig. 1. Bar = 0.5 μm.*

The length distributions of the RNAs of M-MuLV, PR-RSV-A, and Qβ (external size standard) are shown in Fig. 3. The 70S RNA molecules of M-MuLV ranged in length from 3.00 to 5.60 μm (Fig. 3A), with a mean of 3.96 μm and a standard error of the mean of ±0.11 μm, and those of PR-RSV-A ranged from 3.20 to 5.80 μm (Fig. 3B), with a mean of 3.94 ± 0.08 μm. Qβ RNA molecules had lengths that ranged from 0.60 to 1.20 μm (Fig. 3C), with an average of 1.03 ± 0.01 μm. When Qβ RNA is used as a standard (4,790 nucleotides [21]), the calculated numbers of nucleotides in M-MuLV and PR-RSV-A 70S RNAs are 18.4 ± 0.51 and 18.3 ± 0.37 kilobases (kb), respectively. These values agree with the molecular weights of both RNAs determined by other methods (11). In M-MuLV, the length of the subunits, as determined by measurements from the free end to the end involved in dimer linkage, was 1.97 ± 0.03 μm or 9.2 ± 0.14 kb (Fig. 3A). In PR-RSV-A molecules with identifiable linkage structures, the length of the subunits was 1.93 ± 0.03 μm or 9.0 ± 0.14 kb (Fig. 3B).

Dimer linkage structure. In this study, the region of linkage between the subunits was much more clearly resolved than in previous studies on these or other viruses (8, 9, 12–15). Examples of this region in M-MuLV are shown in Fig. 4. In a majority of the subunits, the region between the 5′ end and the linkage contained a hairpin. In a few dimers, the hairpin was present in one of the subunits but absent in the other, and on rare occasions hairpins in both subunits were totally extended. The point of linkage between the subunits occurred at an average distance of 0.10 ± 0.002 μm (466 ± 9 nucleotides) from the 5′ end of each subunit (Fig. 5). Measurements of the size and location of the hairpin gave the following values. The hairpin occurred at an average distance of 70 nucleotides from the 5′ end and contained 320 ± 8 nucleotides. The region between the hairpin and the linkage again contained about 70 nucleotides. The length of sequence involved in the linkage was too small to measure but must be less than 50 nucleotides.

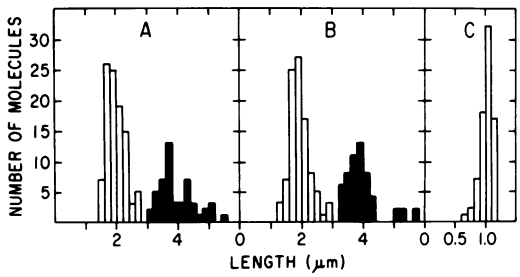

FIG. 3. *Histograms of contour lengths of RNAs of M-MuLV (A), PR-RSV-A (B), and Qβ (C) spread in 50% formamide–2.5 M urea. In (A) and (B), solid blocks represent measurements of dimer molecules, and open blocks illustrate the lengths of subunits in the dimers.*

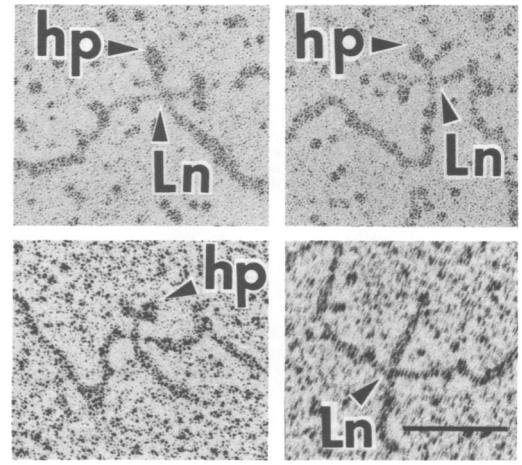

FIG. 4. *Examples of dimer linkage region of M-MuLV RNA shown at a high magnification. Note the point (Ln) of actual linkage and a hairpin (hp) near the 5′ end. Bar = 0.1 μm.*

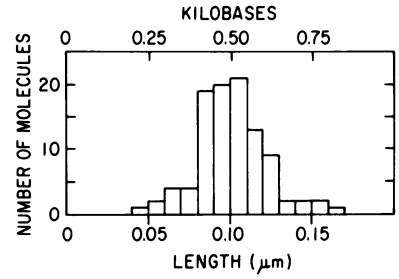

FIG. 5. *Histograms showing the length measurements from the 5′ end to the actual point of linkage of M-MuLV subunits. Since the molecules with fully extended 5′ ends were rare, the measurements included many molecules which contained the hairpin at the 5′ end.*

In PR-RSV-A genomes, the region between the 5′ end and the linkage (Fig. 6) contained structural features similar to those in M-MuLV. A hairpin or, more frequently, a partially opened loop of about the same size and location as in M-MuLV was observed within this region. The regions were measurable in 22 of the 54 dimers examined. The linkage between subunits occurred at an average distance of 0.11 ± 0.006 μm (511 ± 28 nucleotides) from the 5′ end.

Loop within subunits. A large loop was found in most M-MuLV subunits. Measurements of the distance from the loop junction to the 5′ end, loop size, and the distance from the loop junction to the 3′ end are shown in Fig. 7A, B and C, respectively. The loop occurred at an

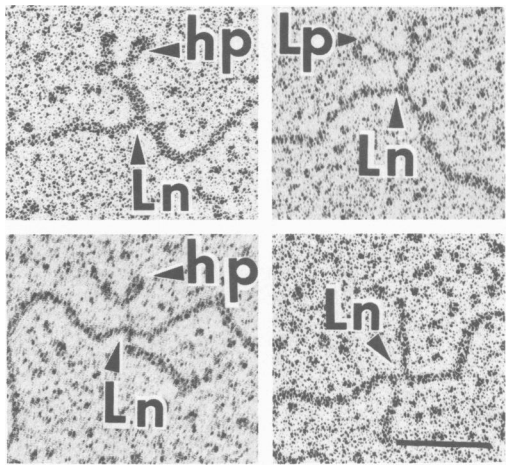

FIG. 6. *Examples of dimer linkage region of PR-RSV-A. Abbreviations are as in Fig. 4. Note the partially opened hairpins (Lp) in the top right section. hp, Hairpin. Bar = 0.1 μm.*

average distance of 0.48 ± 0.01 μm (2.2 ± 0.05 kb) from the 5′ end (Fig. 7A) and contained 0.84 ± 0.02 μm (3.9 ± 0.10 kb) of RNA (Fig. 7B). Both measurements closely correspond to those previously noted for M-MuLV (15) and to those of other mammalian retroviruses, RD 114 and BKD (13, 14). The distribution of lengths from the loop to the 3′ end was rather broad (Fig. 7C), probably due to degradation at the ends. In PR-RSV-A, the loops were seen in about half of the subunits, but they showed a wide variation in size and location. The loops contained one to three crossover points, and these interfered with an accurate determination of loop size and location. Nevertheless, measurements in 108 subunits gave the following values. The loop occurred at an average distance of 0.70 ± 0.02 μm (3.3 ± 0.10 kb) from the 5′ end (Fig. 7D) and had a mean contour length (Fig. 7E) of 0.47 ± 0.02 μm (2.3 ± 0.10 kb). When compared with M-MuLV or other mammalian retroviruses (14), the loop in PR-RSV-A was smaller and occurred further from the 5′ end. The average length from the loop to the 3′ end in PR-RSV-A (Fig. 7F) was 0.49 ± 0.02 μm (2.4 ± 0.10 kb).

A hairpin containing 500 to 600 nucleotides of single-stranded RNA (Fig. 2) was seen within the loop in a majority of M-MuLV subunits. A similar hairpin was also described in BKD and woolly monkey sarcoma virus subunits but not in RD 114 (14). In PR-RSV-A, a smaller hairpin containing 170 to 250 nucleotides was frequently observed within the loop when the RNA was spread from 40% formamide–2 M urea (Fig. 3B). Based on its location in the approximate center of the subunit, we suggest that this hairpin corresponds to the double-stranded fragment of about 300 nucleotide pairs obtained by Perdue et al. (17) after RNase digestion of PR-RSV-C subunits. The hairpin observed here is smaller, possibly due to unfolding of some of the base-paired regions under the denaturing conditions used to spread the molecules.

Reannealed M-MuLV RNA. To determine whether the observed structural features are present in native genomes or are formed during RNA preparation, 70S RNA was heated and cooled as described above and examined in the electron microscope. When the RNA was heated to 70, 85, or 90°C and cooled, the sample contained molecules ranging in length from 0.50 to 2.30 μm; no molecules corresponding in size to the dimers were detected. About 50% of the molecules had lengths of between 1.57 and 2.30 μm, with an average length of 1.96 ± 0.08 μm. These presumably represent the individual subunits. None of these molecules contained the loop typical of the native subunits. In about 75% of the subunits in all three samples (70, 85, or 90°C) a hairpin was seen at one end (Fig. 8A and

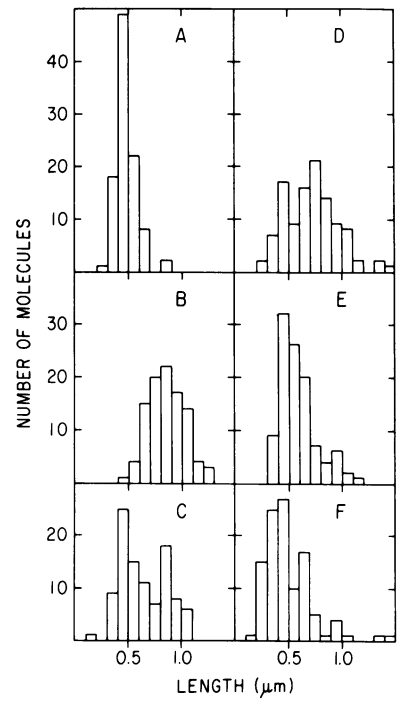

FIG. 7. *Histograms illustrating the size and location of the loop in M-MuLV (A, B, and C) and PR-RSV-A (D, E, F) subunits. The data illustrate the length from the 5′ end to the loop junction (A and D); the contour length of the loop (B and E); and the distance from the loop to the 3′ end (C and F). See text for details.*

B). The hairpin was similar in size and structure to that seen at the 5′ end of the subunits in native dimers (Fig. 4). In samples heated to 70°C, about half of the subunits contained a new loop of highly reproducible size and location (Fig. 8B and C). The loop measured 0.32 ± 0.004 μm (1.5 ± 0.02 kb) in circumference and occurred at a distance of 0.26 ± 0.005 μm (1.2 ± 0.02 kb) from the end that contained the hairpin. The loop was less frequent (~5% of the subunits) is samples heated to 85°C and was not present in samples heated to 90°C.

DISCUSSION

In this study, we have demonstrated that the 70S RNA of PR-RSV-A is very similar to that of the mammalian retroviruses. It is a dimer with a dimer linkage virtually identical to that of M-MuLV. It also possesses a loop within each subunit, although the size and location of the loop differed from that of M-MuLV and other mammalian retroviruses.

The analysis of the linkage region in the dimers of both M-MuLV and PR-RSV-A provided two new findings. These concern the location of the point of linkage between the two subunits and a secondary structural feature associated with the 5′ end of each subunit. Based on a sequence analysis of the 5′ end of PR-RSV-C RNA, Haseltine et al. (10) have postulated that the primer tRNA molecules and the sequences flanking their binding sites are involved in the linkage of the subunits. Our data for both M-MuLV and PR-RSV-A suggest that the point of linkage between the subunits occurs at 466 ± 9 nucleotides from the 5′ end of each subunit. This places the linkage point at about 350 nucleotides from the primer tRNA binding site of both viral genomes (5, 10). Thus, it appears unlikely that the primer tRNA's are involved in the physical linkage of the subunits. From the location of the initiator codon for the *gag* precursor protein of RSV (396 nucleotides from the 5′ end [D. Schwartz, personal communication]), we suggest that the dimer linkage occurs in the 5′ portion of the coding region for the *gag* gene. Additional work, however, is required to determine whether the structures or sequences involved in the dimer linkage have a role in the processing of mRNA's or in the regulation of translation.

The second feature in both M-MuLV and PR-RSV-A subunits is a hairpin containing 320 ± 8 nucleotides which begins at about 70 nucleotides from the 5′ end. Based on its location, we believe that it contains the primer tRNA binding site (5, 10). In addition, the 3′ end of this hairpin is near the 3′ end of the leader sequence (350 nucleotides from the 5′ end of RSV [G. Gasic, personal communication]) which is spliced onto subgenomic mRNA's (16).

Our findings from the denaturation and renaturation studies of M-MuLV confirm and extend the results obtained by others with RD 114 (12), BKD (14), and large subunits in (MSV)M-MuLV (15). The reannealed M-MuLV subunits contained a hairpin at one end resembling that at the 5′ end of native molecules. They also contained a new loop near the end with the hairpin. The new loop is identical to that reported by Maisel et al. (15) in the large subunits of (MSV)M-MuLV heated to 40°C in 58% formamide and rapidly cooled. These authors observed new loops in rare, undenatured dimer molecules and established their location near the 5′ ends of the subunits. Based on this observation, we suggest that the terminal hairpin and the new loop in reannealed subunits observed here are at the 5′ end. The reformation of the hairpin after denaturation indicates that the native structure is formed by pairing of complementary sequences in the vicinity of the primer binding site.

It has been suggested that the new loops and analogous structures in the reannealed subunits of (MSV)M-MuLV (15), BKD (14), and RD 114 (12) contain some sequences of the native loop paired at a different location. This is consistent with our measurements. However, in our studies, the formation of the new loop depended on the temperature of initial denaturation. The loop was seen in half of the subunits after the RNA was heated to 70°C and cooled but absent in subunits reannealed after heating to 90°C. The reason for the dependence of the formation of the new loop on temperature is unclear.

Our denaturation studies, like those of others (12–15), raise questions concerning the nature of interactions responsible for the formation of the dimers and the loops in native subunits. If the dimer linkage and the loops are due to base pairing, then they must form in a special environment either inside the cell or within the virion. Their inability to reform after denaturation may be due to more rapid formation of new structures which block the original interactions. Alternatively, the interactions in the linkage and the loop may involve small linker RNAs which are removed during denaturation (14). Finally, the interactions may involve proteins, although extensive digestion of M-MuLV 70S RNA with proteinase K before spreading neither dissociated the dimers nor eliminated the loops (unpublished data).

The occurrence of common structural features (dimer linkage, loops, and hairpins) in all retrovirus genomes so far examined suggests specific

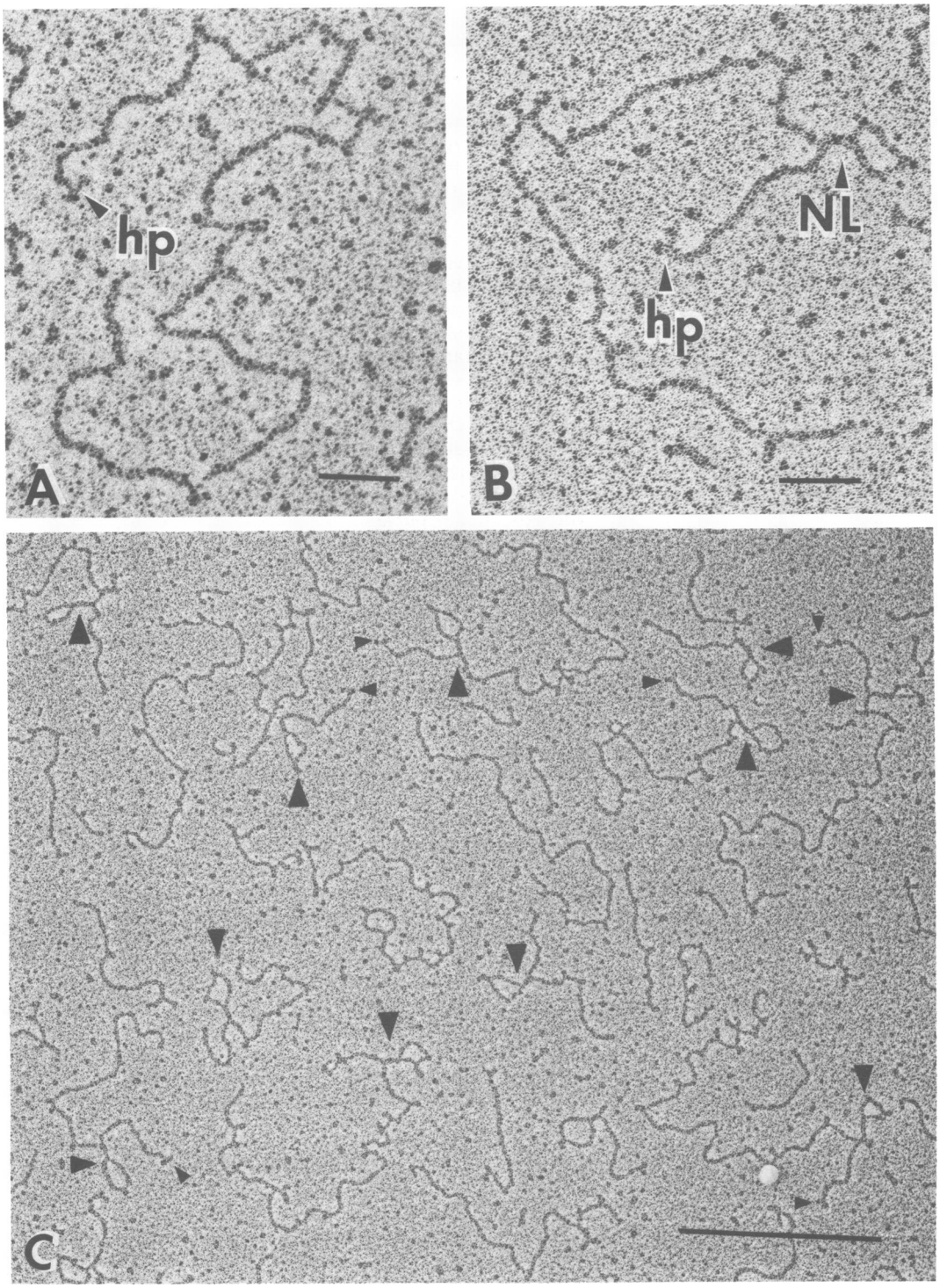

FIG. 8. *Electron micrographs of M-MuLV RNA heated to 70°C and cooled. The sample had linear subunits with a reproducible hairpin (hp) at one end (A) and subunits with a new loop (NL) and a hairpin (hp) at the end (B). (C) Field illustrating the frequency of molecules with new loops. Large arrowheads point to the subunits and fragments with the new loop, and small arrowheads point to the hairpin at the end. Bar = 0.1 μm in (A) and (B) and 0.5 μm in (C).*

and important functional roles for each of these features. The hairpin at the 5′ end occurs in an area important in (i) initiation of proviral DNA synthesis, (ii) splicing of the leader sequence onto subgenomic messengers, and (iii) initiation of *gag* protein synthesis. If secondary structure is important in any of these functions, then a correlation of the hairpin with mRNA and complementary DNA sequence data may provide insight into the enzymatic recognition mechanisms involved in such a function. The dimer linkage structure, while maintaining the diploid state of the retrovirus genome, may also have additional functions. Its location in the 5′ portion of the *gag* gene could block translation and insure that the incoming dimers are directed into reverse transcription. In addition, if dimerization of new transcripts occurs inside the cell nucleus (20), the linkage structure may prevent splicing as well as translation and preserve the dimers for packaging into virions.

Our measurements indicate that the intrasubunit loop in the M-MuLV genome encompasses the *pol* gene, with the *gag-pol* junction falling within the hairpin of the loop. In PR-RSV-A, on the other hand, the loop encloses portions of both *pol* and *env* genes. Perhaps the loops simply fold the genomes for packaging into the virions. Such folding may also bring the 5′ and 3′ ends of the subunits together to facilitate the synthesis of proviral DNA (4). In any case, all of the structural features described here should provide a basis for locating the nucleotide sequences responsible for each structure when the complete sequences of the retrovirus genomes become available. This, in turn, should lead to a better understanding of the functional significance of each structure.

ACKNOWLEDGMENTS

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