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Multiple Regions of Harvey Sarcoma Virus RNA Can Dimerize In Vitro

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Retroviruses contain a dimeric RNA consisting of two identical molecules of plus-strand genomic RNA. The structure of the linkage between the two monomers is not known, but they are believed to be joined near their 5′ ends. Darlix and coworkers have reported that transcripts of retroviral RNA sequences can dimerize spontaneously in vitro (see, for example, E. Bieth, C. Gabus, and J. L. Darlix, Nucleic Acids Res. 18:119–127, 1990). As one approach to identification of sequences which might participate in the linkage, we have mapped sequences derived from the 5′ 378 bases of Harvey sarcoma virus (HaSV) RNA which can dimerize in vitro. We found that at least three distinct regions, consisting of nucleotides 37 to 229, 205 to 272, and 271 to 378, can form these dimers. Two of these regions contain nucleotides 205 to 226; computer analysis suggests that this region can form a stem-loop with an inverted repeat in the loop. We propose that this hypothetical structure is involved in dimer formation by these two transcripts. We also compared the thermal stabilities of each of these dimers with that of HaSV viral RNA. Dimers of nucleotides 37 to 229 and 205 to 272 both exhibited melting temperatures near that of viral RNA, while dimers of nucleotides 271 to 378 are quite unstable. We also found that dimers of nucleotides 37 to 378 formed at 37°C are less thermostable than dimers of the same RNA formed at 55°C. It seems possible that bases from all of these regions participate in the dimer linkage present in viral RNA.

The genomic RNA in a retrovirus particle is dimeric. Two molecules of plus-strand genomic RNA are joined together in a noncovalent linkage; the bonds in the dimeric structure are presumably hydrogen bonds and/or other weak bonds, since the dimer is dissociated into monomers under relatively mild conditions (for a review, see reference 4).

It has been proposed (5, 8, 9) that the dimeric structure is a prerequisite for encapsidation of genomic RNA. Obviously, precise knowledge of the nature of this structure would be of great importance. However, this is an extremely challenging problem, given the large size of retroviral genomic RNAs and the low amounts of virus released by virus-producing cells.

Darlix and coworkers have recently made the important finding that relatively short RNA molecules containing retroviral sequences can dimerize in vitro (e.g., see references 3, 5, and 16). This discovery appears to offer the prospect of major new insights into the dimeric structure, since (i) analysis of structures present in shorter molecules is far simpler than analysis of full-length viral RNA; (ii) synthetic transcripts can easily be produced in much larger amounts than are available from standard virus preparations; and (iii) the sequences in the transcripts can readily be altered by deletion and mutation, so that sequence requirements for dimer formation can be determined easily.

Taking advantage of these possibilities, Darlix and others have shown that the retroviral sequences capable of dimerization in vitro are, in general, near the 5′ end of the viral genome (3, 5, 16); this is the region believed to be involved in the dimer structure present in virion RNA, and it is also known to be required for RNA encapsidation (4, 13, 17). Marquet et al. have also reported that dimerization is a specific property of plus-strand transcripts of retroviral sequences, since the complementary RNAs generally do not dimerize in vitro (14). Finally, the thermal stability of these dimers formed in vitro (IVD) is, at least in some cases, similar to that of the dimers found in virions (e.g., see reference 18).

The present report describes experiments mapping retroviral sequences required for dimerization in vitro. These experiments used Harvey sarcoma virus (HaSV); the genomic RNA of this virus does not encode viral proteins but is efficiently encapsidated by proteins of the mammalian type C viruses. Its sequence is nearly identical to that of Moloney murine leukemia virus (Mo-MuLV) for the 5′ 206 nucleotides but is quite different 3′ of this position (20). While this work was in progress, a report identifying an HaSV sequence capable of dimerization in vitro appeared (23). Our results are consistent with these previously published results but show that the situation is unexpectedly complex: at least two distinct, nonoverlapping regions within the 5′ 378 nucleotides are independently capable of dimerization in vitro. At present, we are unable to say which, if any, of the dimeric structures formed by the transcripts in vitro corresponds to the structure actually present in HaSV virion RNA. However, the results raise the possibility that several regions of the genome are involved in the structure of viral RNA.

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MATERIALS AND METHODS

Molecular clones. A molecular clone containing HaSV proviral sequences was originally obtained from Douglas Lowy (National Cancer Institute, Bethesda,

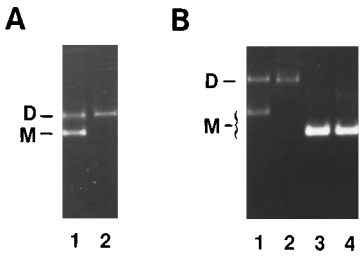

FIG. 1. Dimer formation by RNAs consisting of nucleotides 37 to 378 and 271 to 378. RNAs consisting of nucleotides 37 to 378 (A) and of nucleotides 271 to 378 and 205 to 262 (B) were tested for their ability to form dimers as described in the text. (A) Lanes 1 and 2, 37-to-378 RNA. (B) Lanes 1 and 2, 271-to-378 RNA; lanes 3 and 4, 205-to-262 RNA. Lanes 2 in panel A and 2 and 4 in panel B were incubated in buffer D at 37°C to allow dimer formation as described in Materials and Methods. Note that some dimer is formed even without this incubation (lanes 1 in both panels). D, dimer; M, monomer.

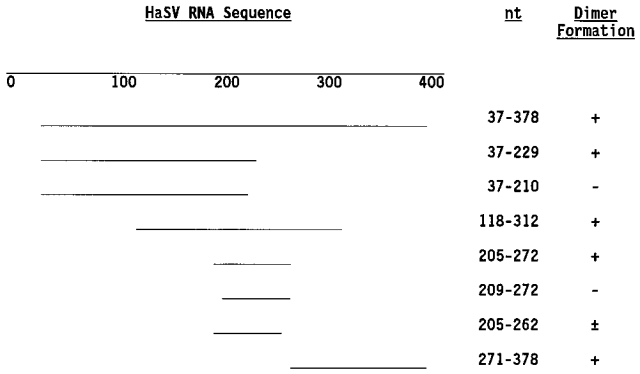

FIG. 2. Transcripts from different regions of the HaSV genome were tested for their ability to dimerize as described in Materials and Methods. +, ≥50% dimerization; ±, ~20% dimerization; −, ≤10% dimerization; nt, nucleotide(s).

Md.) (27). Sequences between the *Kpn*I site at nucleotide 37 and the *Bam*HI site at nucleotide 378 (20) were cloned into a synthetic polylinker downstream of an SP6 promoter sequence, in a plasmid vector originally derived from pCMVneo (12); details of this construction are available on request.

Subclones were made in the same plasmid vector by digestion with restriction enzymes, by PCR, or by direct insertion of synthetic oligonucleotides. Each clone made by PCR or by direct insertion was sequenced; in each case, the sequence agreed with the known HaSV sequence (20). The clones analyzed in the present study contained the following stretches of HaSV sequence: nucleotides 37 to 378, 37 to 229, 37 to 210, 118 to 312, 205 to 272, 209 to 272, 205 to 262, and 271 to 378.

RNA synthesis. Plasmid DNA was purified by buoyant density centrifugation in CsCl-ethidium bromide gradients. It was linearized by restriction enzyme digestion and transcribed by use of SP6 RNA polymerase (Promega Corp., Madison, Wis.) in the presence of RNAsin (Promega). After transcription, the DNA template was digested with DNase (Promega) and the RNA was extracted with phenol-chloroform and precipitated with ethanol. The precipitated RNA was redissolved in RNase-free water (Promega) and purified through a Sephadex G-25 spun column (Boehringer Mannheim, Indianapolis, Ind.). The purity of the RNA was examined by electrophoresis in 2.5% Metaphor (FME, Rockland, Maine) in 50 mM Tris-borate (pH 8.0)–1 mM EDTA followed by staining with ethidium bromide.

Dimerization conditions. Conditions for dimerization of RNA were essentially those described by Roy et al. (18). RNA was diluted to 0.5 to 1.0 μg/10 μl, denatured at 95°C for 2 min, and placed on ice. A portion (1/10 volume) of 10× buffer D was then added (1× buffer D is 0.25 M NaCl, 1 mM $MgCl_2$, 10 mM Tris [pH 7.0]), and the RNA was incubated at 37°C for 2 h (or, where indicated below, at 55°C for 30 min). The extent of dimerization was determined by electrophoresis in Metaphor as described above. In many cases, dimerization of a transcript was partially completed at the end of the transcription reaction (before incubation in buffer D), but no dimers were seen in the case of negative transcripts.

Analysis of thermal stability of dimers. RNA which had been fully dimerized (as determined by agarose gel electrophoresis under native conditions, as described above) was diluted to 10^{-7} M and microdialyzed, with a type V6 filter (pore size, 0.025 μm; Millipore Corp., Bedford, Mass.), for 45 min against buffer A (50 mM NaCl, 10 mM Tris-Cl [pH 7.0], 1 mM EDTA). Aliquots were then removed and incubated for 10 min at temperatures ranging from 37 to 70°C. Another aliquot was incubated for 2 min at 90°C. The RNAs were then analyzed by electrophoresis as described above, to determine the extent of dissociation of the dimer resulting from the incubation at each temperature.

Analysis of viral RNAs. Plasmids containing either wild-type or the D32L protease mutant (8) of Mo-MuLV (in a vector derived from pSV2neo [21]) were transfected (10) into NRK rat cells transformed by HaSV (also a gift of Douglas Lowy), and stable transfectants were selected with G418 (Life Technologies Inc., Gaithersburg, Md.). RNA was extracted from viral pellets as described elsewhere (8), and HaSV RNA was analyzed by nondenaturing Northern (RNA) gel analysis (11) with ^{32}P-labeled pBS9 DNA as a *ras* probe (6). Thermal stability measurements were in buffer A with 1% sodium dodecyl sulfate (SDS), as described elsewhere (8).

RESULTS

RNAs representing portions of the genomic RNA of HaSV were tested for their ability to form IVD as follows. Segments of cloned HaSV DNA were transcribed with SP6 RNA polymerase, and the resulting RNAs were incubated in buffer D to allow dimer formation, as described in Materials and Methods.

The capability of a relatively large 5′ RNA molecule, encoded by the HaSV genome between the *Kpn*I site at nucleotide 37 and the *Bam*HI site at nucleotide 378, to dimerize in vitro was tested initially. As shown in Fig. 1A, it was found that this RNA dimerizes readily under the standard incubation conditions.

Partial identification of dimerization sequences. We then

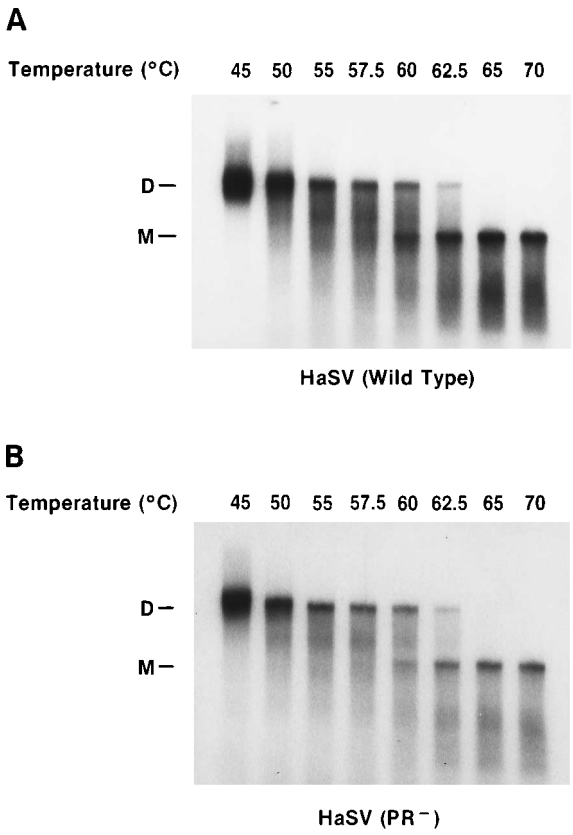

FIG. 3. Melting profiles of HaSV RNA isolated from virions after rescue of HaSV with either wild-type (A) or PR^- (B) Mo-MuLV. RNAs extracted from the respective virions were dissolved in buffer A supplemented with 1% SDS at room temperature. Aliquots of the RNAs were heated for 10 min at the indicated temperatures. The RNA samples were analyzed by nondenaturing Northern blotting, with a pBS9 probe to detect the HaSV genome, as described in Materials and Methods. D, dimer; M, monomer.

subdivided this 342-nucleotide region, in an attempt to localize the sequences required for dimerization. Results of all these tests are summarized in Fig. 2. At the 5′ end of this region, we found that RNA composed of HaSV nucleotides 37 to 229 can dimerize (see below). In addition, a transcript of the central portion of the region, consisting of nucleotides 118 to 312, could also dimerize. Finally, sequences from the 3′ end of the region, i.e., nucleotides 271 to 378, were also capable of dimerization (Fig. 1B), although the dimerization of this transcript was frequently incomplete and, as noted below, these dimers were quite unstable.

The subclones described above were also subdivided further (Fig. 2). Within the 5′ region (nucleotides 37 to 229), an RNA consisting of nucleotides 37 to 210 was unable to dimerize in vitro.

Within the central region, nucleotides 205 to 272 were capable of dimerization, in agreement with results of Torrent et al. (24). Nucleotides at both ends of this transcript are evidently involved in dimerization, since RNAs composed of nucleotides 209 to 272 (Fig. 2) did not form IVD, while transcripts of nucleotides 205 to 262 formed IVD with very low efficiency (Fig. 1B and 2). The fact that many transcripts fail to dimerize (Fig. 2) demonstrates the specificity of the assay.

Thermal stability of dimers. The studies described above showed that the 5′ region of HaSV RNA contains at least three stretches capable of dimerization under the incubation conditions used: nucleotides 37 to 229, 205 to 272, and 271 to 378 (Fig. 2). It would be important to known which, if any, of these stretches is involved in the linkage between full-length HaSV genomic RNAs in virions.

One property which can be used to characterize dimeric RNAs is thermal stability. We therefore compared the dissociation temperatures of the dimers formed in vitro with that of genomic HaSV RNA, in hopes of identifying an IVD which resembled the dimers present in virions.

We initially determined the dissociation temperatures of HaSV viral RNAs. The helper viruses used were wild-type Mo-MuLV and protease-negative (PR^-) Mo-MuLV; it was important to test both of these preparations, since the melting temperatures of Mo-MuLV and human immunodeficiency virus type 1 (HIV-1) viral RNAs are affected by PR function (7, 8) and it seemed likely that the same would be true of HaSV RNA. The results of these tests are shown in Fig. 3; it can be seen that the HaSV dimers in both samples dissociated at approximately 60°C.

Melting profiles of selected IVD are shown in Fig. 4. These tests were performed in the same buffer as that used in analysis of viral RNAs except that SDS was not included in the tests on IVD; this buffer (buffer A) is of significantly lower ionic strength than the solutions used for dimer formation in vitro and contains EDTA rather than Mg^{2+}. Briefly, the results showed that IVD of nucleotides 37 to 229 dissociate between 55 and 65°C (Fig. 4A); dimers of nucleotides 205 to 272 dissociate at 65 to 70°C (Fig. 4B); and dimers of nucleotides 271 to 378 are quite unstable under the conditions of the melting experiments (i.e., in buffer A rather than the dimerization buffer), since they are partially dissociated at 37°C and completely dissociated at 55°C (Fig. 4C).

We also examined the thermal stability of dimers of the entire region under study, i.e., nucleotides 37 to 378. Preliminary experiments suggested that the melting profile of these IVD might depend upon the conditions used for dimerization. To test this possibility directly, we performed the following experiment. RNA consisting of nucleotides 37 to 378 was prepared and divided in two aliquots. One was incubated in dimerization buffer (buffer D) at 37°C, while the other was incubated in the same buffer at 55°C. Dimerization was complete under both conditions. The thermal stabilities of the dimers were then compared. As can be seen (Fig. 5), the dimer which had been formed at 37°C had a very broad (probably biphasic) melting profile: some dimers were dissociated by exposure to 45°C, but dissociation was only complete at a temperature between 70 and 90°C. In contrast, the dimers which had been formed at 55°C exhibited a much sharper melting profile, in which no dissociation was seen until the IVD were incubated at 65°C.

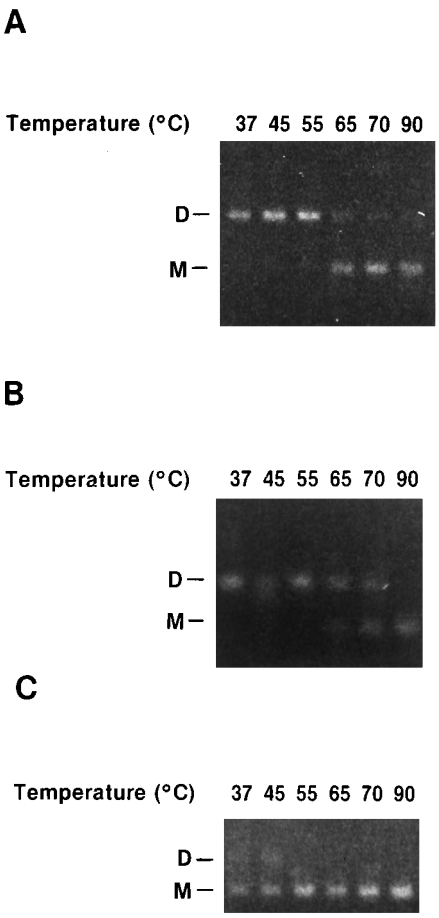

FIG. 4. Melting profile of HaSV IVD. After dimerization, RNA was microdialyzed into buffer A and aliquots were incubated for 10 min at the indicated temperatures. The RNAs were then analyzed by agarose gel electrophoresis. (A) 37-to-229 RNA; B, 205-to-272 RNA; C, 271-to-378 RNA. D, dimer; M, monomer.

We also tested the possibility that the melting temperatures of other IVD would be dependent on conditions used in dimer formation. However, dimers of nucleotides 37 to 229, 205 to

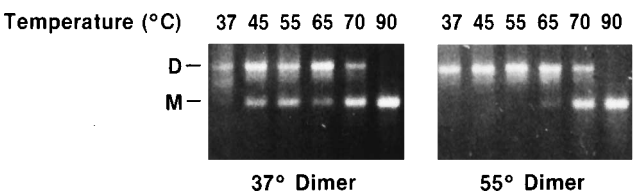

FIG. 5. Melting profiles of dimeric RNAs of nucleotides 37 to 378. The effects of dimerization temperature upon melting profiles are shown. RNA composed of nucleotides 37 to 378 was incubated in buffer D at either 37 or 55°C. Dimerization was complete at both temperatures. Melting profiles of the dimers were then determined as for Fig. 4. D, dimer; M, monomer.

272, 118 to 312, and 271 to 378 formed at 55°C showed melting profiles indistinguishable from those of the corresponding dimers formed at 37°C (data not shown).

DISCUSSION

As noted above, the sequences and structures involved in the dimeric linkage of the genomic RNAs in retrovirus particles are not yet known with precision. One approach to the analysis of these structures is the identification of viral sequences which can form dimers in vitro. In the present work, we have attempted to identify such sequences in RNAs transcribed from the 5′ region of the HaSV genome.

The results of these experiments can be briefly summarized as follows. Transcripts from several regions can dimerize: these include nucleotides 37 to 229, 205 to 272, and 271 to 378 (Fig. 2). Thus, the 5′ 378 nucleotides of HaSV RNA do not contain a unique dimerization sequence.

We also attempted to identify structures that might be present in viral RNA by comparing the thermal stabilities of short IVD (Fig. 4) with that of authentic dimeric RNA from virions (Fig. 3). Indeed, IVD of nucleotides 37 to 229 and 205 to 272 had dissociation temperatures in the same range as that of viral RNA, while IVD of nucleotides 271 to 378 did not. However, we also found that one IVD, viz., that of the entire region, nucleotides 37 to 378, does not have an intrinsic dissociation temperature (Fig. 5). Rather, the melting profile depends upon the conditions at which the dimer was formed. In other words, this region is capable of forming more than one distinct dimeric structure, with different thermal stabilities. This may also be true of other transcripts; in any case, the phenomenon raises the possibility that observed melting temperatures cannot be used alone to positively identify sequences involved in the dimer linkage in virion RNAs.

Mechanisms of dimerization. Torrent et al. (23, 24) have studied dimer formation by a single region in HaSV, i.e., nucleotides 205 to 272. Our results on this region are completely consistent with theirs: we found that nucleotides 205 to 272 can dimerize, while 209 to 272 cannot and 205 to 262 dimerizes only to a very limited extent (Fig. 2). Since the dimerization apparently requires both the extreme 5′ and extreme 3′ ends of this RNA (as also shown by Torrent et al. [23, 24]), it seems possible that the dimerization involves an interaction between these ends. Indeed, Torrent et al. suggested (on the basis of results with a mutant with multiple changes in the region 205 to 218) that the purine-rich sequence at the 5′ end of this sequence may play a crucial role in dimerization of this transcript (23, 24). The failure of the nucleotide 209 to 272 RNA (lacking nucleotides GGCA at its 5′ end) to dimerize (Fig. 2) lends additional support to this proposal.

We also found (Fig. 2) that a transcript consisting of nucleotides 37 to 229 can dimerize while one consisting of nucleotides 37 to 210 cannot. In this case, the results suggest that sequences between nucleotides 210 and 229 interact with sequences in the 5′ portion of the 37-to-229 transcript during dimerization.

In addition to the empirical studies described here, we have analyzed the HaSV RNA sequence for possible secondary structures, using the MFOLD program of Zuker and coworkers (10a). As shown in Fig. 6, nucleotides 205 to 271 can be folded into a pair of stem-loops. It is intriguing to note that one of these stem-loops, i.e., nucleotides 205 to 226, contains an inverted repeat (GGCC) in the loop. A structure of this type has been implicated in the dimerization by HIV-1 transcripts, and a "kissing-loop" mechanism of dimerization, based on the inverted-repeat character of the loop, has been proposed (11b, 19). Since HaSV nucleotides 205 to 226 are present in the overlap between nucleotides 37 to 229 and 205 to 272, it seems possible that this stem-loop is involved in formation of IVD by both of these HaSV transcripts (Fig. 2). In general, the results of Torrent et al. on mutants of nucleotides 205 to 272 (23, 24) are all consistent with this proposed mechanism of dimerization by this transcript.

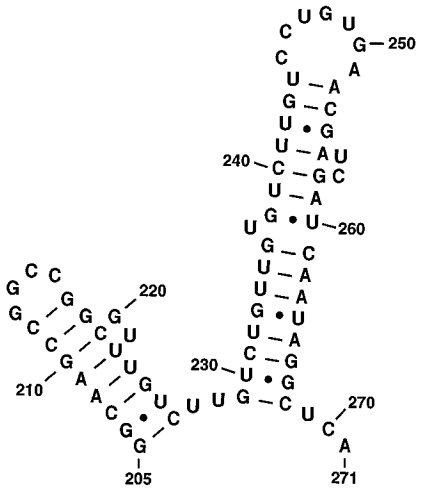

FIG. 6. Proposed secondary structure of RNA composed of HaSV nucleotides 205 to 272.

Torrent et al. (24) pointed out that the sequence UGUCU UGUC is found twice in the 205-to-272 transcript, i.e., at nucleotides 223 to 231 and 237 to 245. They considered the possibility that this sequence may also be important for formation or stabilization of dimers. However, the 37-to-229 transcript does not contain a complete copy of this repeat; thus, this repeat is not required for dimerization of RNAs extending in the 5′ direction to nucleotide 37.

Finally, we found (Fig. 1 and 2) that transcripts of nucleotides 271 to 378 can also form IVD. However, these dimers are notably less stable in buffer A than those of authentic viral RNA (Fig. 4C). Analysis of nucleotides 271 to 378 for possible secondary structures revealed the two conserved stem-loops previously pointed out by Konings et al. (11a), containing GACG in each loop (data not shown). Examination of these hypothetical structures does not reveal an obvious mechanism for dimerization. The instability of IVD of this transcript (Fig. 4C) suggests that the linkage formed by these nucleotides in vitro is not alone responsible for joining genomic RNAs in virions; however, it is still possible that interactions involving these nucleotides contribute to the stability of dimers of larger RNA molecules, such as those found in virions.

Two observations made during this work point to the complexity underlying the thermal stability of dimeric RNA structures. First, it is striking that the melting profiles of IVD of nucleotides 37 to 378 (Fig. 5) are different from those of nucleotides 37 to 229, 205 to 272, and 271 to 378 (Fig. 4), despite the fact that all of these sequences are contained within the 37-to-378 transcript. It seems clear that small sequences can adopt structures when they are expressed alone which they do not adopt when they are part of a larger molecule. A similar finding with HIV-1 RNA was made by Marquet et al. (15): IVD of nucleotides 311 to 415 are considerably more stable than IVD of nucleotides 1 to 615 and are apparently stabilized by very different types of structures (2, 22). In the case of HIV-1, it seems likely that the dimeric structure formed by

nucleotides 311 to 415 is not present in full-length viral RNA (7, 15, 19).

The second observation concerning thermal stability of IVD is the existence of two dimeric forms of nucleotides 37 to 378 with different melting profiles. We have recently reported (7, 8) that full-length genomic RNAs of Mo-MuLV, Kirsten sarcoma virus, and HIV-1 can each exist in two alternative forms; however, this has not previously been observed in IVD. Indeed, we could not have predicted that this would be true of such short transcripts. We found (Fig. 5) that annealing at a high temperature (55°C) resulted in a more stable dimer than annealing at a lower temperature (37°C). Presumably, some intramolecular bonds present in monomers incubated at 37°C are broken at 55°C, so that more bases are available for intermolecular interactions at the latter temperature and a more stable dimer is generated.

In authentic viral RNAs, the transition from one form to the other is dependent upon the activity of the viral PR (7, 8). We have suggested (8) that PR cleaves the Gag polyprotein and that one of the cleavage products, the nucleocapsid (NC) protein, binds to the viral RNA and alters the conformation of the dimer. With this hypothesis in mind, we are now investigating the effect of purified NC protein upon IVD of nucleotides 37 to 378 formed at 37°C.

In both Mo-MuLV and HIV-1, the dimeric form found in virus particles containing functional PR is more thermostable than that in PR^- particles (7, 8). It seems reasonable to suppose that NC in the PR^+ particles catalyzes a transition of the dimer to a more stable, more thermodynamically favored form (26). However, in the present work we were unable to distinguish the dissociation temperatures of HaSV genomic RNAs packaged into virions by wild-type Mo-MuLV proteins from those packaged by PR^- ones (Fig. 3). Other experiments (not shown) demonstrated that these HaSV RNAs were nevertheless in different conformations, since they exhibited different electrophoretic mobilities in nondenaturing agarose gels. It is, of course, possible that HaSV dimers from wild-type particles are slightly more stable than those from PR^- particles.

In summary, at least two different regions of HaSV RNA can form IVD. This finding raises the possibility that more than one region contributes to the dimeric structure in the virus particle. Alternatively, some of the structures formed in vitro may not exist in vivo, as is evidently the case with HIV-1 RNA (7, 15, 19). Ultimately, determining the structure of the RNA in the virus will require direct biochemical analysis. It is likely that IVD will provide useful models for this analysis, since the dimeric structure described for Moloney MuLV IVD (25) is quite similar to that present in the virus (1).

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