Maturation of Dimeric Viral RNA of Moloney Murine Leukemia Virus

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We have analyzed the dimeric RNA present in Moloney murine leukemia virus (MoMuLV) particles. We found that the RNA in newly released virions is in a conformation different from that in mature virions, since it has a different electrophoretic mobility in nondenaturing agarose gels and dissociates into monomers at a lower temperature. On the basis of these results, we suggest that the RNA initially packaged into nascent virions is already dimeric but that the dimer undergoes a maturation process after the virus is released from the cell. In further experiments, we tested the possibility that this maturation event is linked to the maturation cleavage of the virion proteins, which is catalyzed by the viral protease (PR). We found that the dimeric RNA isolated from PR− mutant virions resembles that from immature virions: it has a lower electrophoretic mobility and a lower sedimentation rate, and it also dissociates at a lower temperature than does RNA from mature wild-type virions. When Kirsten sarcoma virus is rescued by a PR− mutant or by a somewhat leaky cysteine array mutant of MoMuLV, its RNA also exhibits a electrophoretic mobility lower than that in the wild-type pseudotype. These results suggest that the maturation of dimeric RNA in released virus particles requires the cleavage of the Gag precursor and the presence of an intact cysteine array in the released nucleocapsid protein.

In all retrovirus particles, the genomic RNA is in the form of a dimer. The structure of the dimer is not understood; two monomers of the same polarity are evidently linked by hydrogen bonds and/or other weak, noncovalent bonds, since the dimer is dissociated into monomers under relatively mild conditions (3, 5, 8). Electron microscopic images of partially denatured dimers suggest that the two monomers are joined in parallel orientation near, but not at, their 5′ ends (reviewed in reference 7a).

It is also not known where and when the dimer is formed. On the one hand, some early studies reported that RNA isolated from rapid-harvest avian virus is apparently monomeric; these results strongly suggested that the dimer forms only after the virus is released from the cell (6, 14). However, other investigators have analyzed the RNAs in murine leukemia (MuLV) particles after treatment of virus-producing cells with actinomycin D. Under these conditions, packaging of genomic RNA into virus particles is very limited: the majority of released virions lack genomic RNA. Remarkably, the small amount of RNA which is packaged under these conditions is still dimeric (15, 21). This observation, in contrast to the studies on rapid-harvest virus discussed above, seems to imply that the monomers of genomic RNA are not packaged independently of each other but rather are already joined into some dimeric structure when they are packaged. (Similarly, cysteine array mutants of MuLV appear to package reduced amounts of viral RNA [11, 19], but the packaged RNA is dimeric [19].) However, a recent report [2] has questioned whether these mutants are actually defective in packaging viral RNA.

One way to reconcile all of these observations is to suggest that the RNA is initially packaged as a dimer, but that this dimer is more fragile than that found in mature virions, so that it is dissociated under some extraction conditions. After the virus is released from the cell, the dimer undergoes a maturation or stabilization event. We have tested this hypothesis in the present work. We find that the dimeric RNA isolated from newly released virions of Moloney MuLV (MoMuLV) is indeed in a different, less stable physical state than that isolated from mature virus particles. We also present results for viral mutants, which indicate that the maturation of the RNA dimer is dependent on the presence of a functional viral protease (PR) and an intact cysteine array in the nucleocapsid (NC) protein. To explain these findings, we suggest that the maturation of the RNA is promoted, or catalyzed, by Gag cleavage products, including the NC protein, in the released virus particle.

MATERIALS AND METHODS

Viral mutants. Three mutants of MoMuLV were used in this study: the 32-codon deletion in the protease coding region described by Katoh et al. (12), referred to here as ΔPR; a point mutant generated in this laboratory by oligonucleotide-directed mutagenesis, changing the active-site aspartate residue in PR to leucine, referred to as D32L; and the cysteine array NC mutant Y28S (11). All of these mutants, as well as the wild-type parental clone of MoMuLV (11) designated pRR86, were full-length, nonpermutated genomes in the plasmid vector pGcCos3neo, a derivative of pSV2neo (11).

Virus preparation and RNA isolation. All cell culture media were supplemented with 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. In studies on virion maturation, virus was collected from NIH 3T3 mouse cells productively infected with wild-type MoMuLV and grown in Dulbecco modified Eagle’s medium supplemented with 5% fetal calf serum. In all other cases, mass cultures of stable transfectants were obtained by G418 selection after transfection of the molecular clones described above. In these experiments, studies of MoMuLV were performed in CHO hamster cells, while studies involving Kirsten sarcoma virus (KiSV) were carried out in KiSV-

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transformed rat cells (11). Culture fluids were collected from confluent cultures every 24 h for a period of 3 days and filtered through a 0.45-μm-pore-size filter. Virions were pelleted by centrifugation at 25,000 rpm for 30 min at 4°C in a Beckman SW27 rotor. Virion pellets were resuspended in lysis buffer (50 mM Tris [pH 7.4], 10 mM EDTA, 1% sodium dodecyl sulfate [SDS], 100 mM NaCl, 50 μg of yeast tRNA [Bethesda Research Laboratories] per ml, 100 μg of proteinase K [Bethesda Research Laboratories] per ml) and incubated for 30 min at 37°C. RNA was then extracted from the viral lysate with an equal volume of phenol, followed by an equal volume of chloroform. This extraction was repeated, and RNA was precipitated with 95% ethanol.

Immature virions were collected from confluent cultures in 100-mm-diameter culture dishes. The culture medium in the dish was replaced with 4 ml of fresh medium at 37°C in a water bath. The medium was collected every 5 min, kept below 0°C, and centrifuged at 25,000 rpm for 10 min in a Beckman SW27 rotor at 0°C. Virions were lysed and RNA was extracted as described above.

Electrophoretic analysis of viral RNAs. Viral RNA was dissolved in R buffer (10 mM Tris [pH 7.5], 1 mM EDTA, 1% SDS, 50 mM NaCl) and analyzed by nondenaturing Northern (RNA) blot analysis (13). Briefly, RNA was electrophoresed in nondenaturing 1% agarose gels in 39 mM Tris (pH 8.3)–89 mM boric acid–2.5 mM EDTA. After electrophoresis, the gels were incubated in 6% formaldehyde at 65°C for 30 min, and the RNA was then blotted onto nylon membranes. The damp membranes were baked at 80°C for 2 h under vacuum before hybridization. In some experiments, MoMuLV RNA was detected with a 32P-labeled probe made by random-primer labeling of pRR88 DNA, using the conditions specified by the manufacturer (Boehringer Mannheim). These blots were washed twice at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.5% SDS, then for 30 min at 65°C in the same buffer, and finally for 30 min at room temperature in 0.1× SSC. In other experiments, 32P-labeled MoMuLV ribo-probes were made by linearizing the pMXH clone (10) with XbaI and transcribing this template with T7 polymerase as instructed by the manufacturer (Promega). These filters were washed twice in 0.1× SSC–0.1% SDS at room temperature for 5 min, twice at 70°C for 15 min in the same buffer, and at room temperature in diethyl pyrocarbonate-treated H2O. KiSV RNA was hybridized with 32P-labeled random primer probes of the c-Ki-ras clone pSW11.1 (18) after digestion of the plasmid with PstI. In some experiments, melting temperatures of dimeric RNAs were determined by incubating aliquots of the RNA for 10 min in R buffer at temperatures ranging from 42.5 to 60°C before analysis.

Sedimentation analysis of viral RNAs. Flasks (150 cm2) of CHO cells producing wild-type MoMuLV were labeled overnight with 60 μCi of [5-3H]uridine (27 Ci/mmol; Amer sham) per ml in α-modified Eagle’s medium supplemented with 10% fetal calf serum. In other experiments, CHO cells producing the D32L mutant of MoMuLV were labeled with 100 μCi of [32P]phosphate (carrier free; Amer sham) per ml overnight in minimal Eagle medium containing 5% of the normal level of phosphate, supplemented with 5% dialyzed fetal calf serum and (as in α-modified Eagle’s medium) 40 μg of proline per ml in 150-cm2 flasks.

Viral RNAs were isolated as described above from the supernatants of the labeled cultures. They were washed once with 70% ethanol and resuspended in 0.3 ml of R buffer. The labeled RNA samples were mixed, layered on a 15 to 30% (wt/wt) sucrose gradient in R buffer, and centrifuged for 3 1/3 h at 37,500 rpm in a Beckman SW41 rotor at 23°C. Fractions of 0.5 ml were collected from the bottom of the tube; 0.5 ml of 10% trichloroacetic acid at 0°C was added to each fraction, and the fractions were filtered through 0.45-μm-pore-size membrane filters (Millipore). The filters were rinsed with 5% ice-cold trichloroacetic acid and with 70% ethanol and were dried and counted in a scintillation counter.

Estimation of protein content of viral RNA preparations. The degree to which our viral RNA preparations were contaminated with protein was estimated as follows. Virus-producing CHO cells were labeled overnight with [35S]cysteine. Viral RNA was isolated as described above and divided into two portions. Each portion was subjected to nondenaturing agarose gel electrophoresis and transfer to a GeneScreen Plus nylon membrane as in the nondenaturing Northern blot analysis procedure (13). One of the resulting membranes was analyzed directly for the presence of RNA-associated protein by autoradiography, while the other was probed for the presence of viral RNA by hybridization (13). No bands of 35S label were detectable in the membrane, although there was a faint haze of radioactivity distributed throughout each lane in the autoradiographic analysis (data not shown). The absence of detectable protein bands suggested that very little, if any, protein remained associated with the viral RNA. We attempted to quantitate the relative amounts of RNA and protein in the RNA preparations by calibrating the Northern blot with a known quantity of synthetic viral RNA (transcribed from pMXH [10]) and by counting the radioactivity in the region of the membrane containing the viral RNA and determining the specific activity of 35S labeling in extracts of the virus-producing cells. The results of these measurements indicated that we had removed >99.7% of the viral protein from the RNA; it seems unlikely that any protein is bound to the RNA, although because of the haze of radioactivity, we cannot exclude the presence of a few molecules of NC or other protein in association with each RNA molecule. Similar results were obtained for RNA preparations from wild-type and D32L virus.

In other experiments (data not shown), we also found that incubation of dimeric RNAs from wild-type and PR MoMuLV with 0.2 U of RQI DNase (Promega) per ml for 30 min at 30°C had no effect on their electrophoretic mobilities in nondenaturing gels.

RESULTS

Maturation of the dimeric RNA of MoMuLV. As discussed in the introduction, one plausible interpretation of prior studies on RNA from rapid-harvest virions (6, 7, 14) is that newly released virions contain dimeric RNA, but that this RNA is physically different from that present in mature virions. To test this possibility, we extracted RNA from MoMuLV virions isolated every 5 min. Electrophoretic analysis of the RNA from these rapid-harvest virions is shown in Fig. 1. It can be seen that a single species of RNA was obtained, but this RNA migrated more slowly than that isolated from control (24-h) virus preparations. These RNAs are the dimeric viral RNAs, as shown by the fact that they can be dissociated into a much more rapidly migrating, monomeric band by mild heating (see below). The difference in mobility between bulk rapid-harvest and control dimeric RNAs (Fig. 1) supports the idea that the dimeric RNA undergoes some change in state after the virus is released from the cell.

We also tested the idea, discussed in the introduction, that
the dimeric structure of the RNA is newly released virus is less stable than that in mature virus. RNA from rapid-harvest or mature virus preparations was incubated at different temperatures as described in Materials and Methods before electrophoretic analysis. In each case, the proportion of RNA which was still in the dimeric form was determined. As shown in Fig. 2A, dimeric RNA from rapid-harvest virus dissociated into monomers at a slightly lower temperature than that from mature virus. The autoradiographs in Fig. 2A were analyzed by densitometry; as shown in Fig. 2B, the \( T_m \) (the temperature at which only half of the RNA remains in the dimeric form) of rapid-harvest viral RNA was about 51.3°C, while that of the mature viral RNA was about 53.8°C.

Conformation of dimeric RNA in mutants of MoMuLV. The results presented above show that the dimeric RNA present in a MoMuLV virion undergoes a maturation event after the particle is released from the cell. It was of interest to determine whether there is any connection between this event and the well-known maturation cleavage of the Gag polyprotein, catalyzed by the viral protease, which also occurs after virus release. We therefore investigated the conformation of the dimeric RNA in PR-deficient mutant particles of MoMuLV.

Two PR- mutants were used in these experiments: ΔPR, a 32-codon deletion described previously (12), and D32L, in which the only change is replacement of aspartate at the active site with leucine. Figure 3 shows a comparison between the dimeric RNAs isolated from these two mutants (lanes 3 and 4, respectively) and that of wild type (lanes 1 and 5) with respect to electrophoretic mobility. It can be seen that the RNA of the mutants migrated more slowly than the wild-type RNA. Indeed, the RNA of the mutants migrated even more slowly than that isolated from rapid-harvest, wild-type virions (lane 2).

We also compared the sedimentation rates of wild-type and D32L dimeric RNA in a sucrose gradient. Cells producing wild-type or D32L virions were metabolically labeled with [\(^3\)H]uridine or \[^{32}\)P\]PO\(_4\), respectively. RNAs were extracted from the virions produced by these cultures, mixed, and layered on a sucrose gradient. Fractions were collected from the gradient after centrifugation, and each fraction was analyzed for \(^3\)H and \(^{32}\)P in a scintillation counter. As is shown in Fig. 4, the dimeric RNA isolated from D32L particles, with a peak in fractions 6 and 7, sedimented slightly more slowly than that from the wild-type particles, with a peak in fraction 5.

The stability of RNA dimers from the mutant particles was also compared with that from wild-type virions. Figure 5A shows the electrophoretic analysis of mutant and wild-type
RNAs after heating to various temperatures; these results were analyzed densitometrically to generate the graph shown in Fig. 5B. It can be seen that in the mutants, less than half of the RNA was in the dimeric form after exposure to 50°C, and no dimeric RNA could be detected after incubation at 55°C. In contrast, about half of the wild-type RNA was still dimeric after exposure to 52.5°C, and dissociation was not complete until exposure to 57.5°C. The melting temperatures estimated from this experiment are approximately 48.5°C for both mutant RNAs and 53.5°C for wild-type RNA.

It is interesting to note that the RNA isolated from rapid-harvest virions was intermediate in its properties between PR⁻ and wild-type RNAs with respect to both electrophoretic mobility (Fig. 3) and melting temperature (compare Fig. 2 and 5; data from side-by-side comparisons are not shown). One interpretation of these results is that PR⁻ viral RNA is completely immature, while that in our rapid-harvest virus preparations is already partially matured. In other experiments, immunoblotting with anti-p30\textsuperscript{CA} antiserum showed that the Gag polyprotein precursor in our rapid-

harvest virus preparations was almost completely cleaved (data not shown). In summary, therefore, virions present in 5-min harvests have almost completely processed Gag proteins but appear to contain only partially matured dimeric RNA.

**Dimeric RNA of KiSV: an effect of NC on RNA conformation.** We have shown above that the dimeric RNAs isolated from PR⁻ mutants of MoMuLV have a conformation different from that of wild-type MoMuLV. In principle, this difference could be due either to the effect of PR function on the genomic RNA in the virion or to differences in the sequences of the RNAs themselves. To test the latter possibility, we determined the effect of PR function on the

FIG. 3. Electrophoresis of RNA dimers from rapid-harvest, wild-type, and PR⁻ virions. RNAs were extracted from rapid-harvest (lane 2), wild-type (lanes 1 and 5), ΔPR (lane 3), and D32L (lane 4) virions and analyzed with a MoMuLV riboprobe as described in Materials and Methods.

FIG. 4. Sedimentation of dimeric RNAs from wild-type and D32L virions. Virus-producing CHO cells were labeled with [\textsuperscript{3}H]luridine (wild-type) or [\textsuperscript{32}P] (D32L). The viral RNAs were extracted, mixed, and analyzed by velocity sedimentation through a 15 to 30% sucrose gradient as described in Materials and Methods. Only the bottom 14 of 25 gradient fractions are shown.

FIG. 5. Melting curves of dimeric RNAs from wild-type and PR⁻ virions. (A) RNAs extracted from the respective virions were heated for 10 min to the temperatures shown and analyzed in a nondenaturing Northern blot using a MoMuLV DNA probe. (B) The autoradiograms shown in A were analyzed by densitometry.
mobility of dimeric RNA of KiSV. (KiSV is a replication-defective derivative of MuLV that carries the Ki-ras oncoprotein but encodes no viral proteins. KiSV genomic RNA is packaged efficiently by MuLV proteins.) As shown in Fig. 6, KiSV dimeric RNA migrated more slowly in nondenaturing gel electrophoresis when it was packaged by PR− viral proteins (ΔPR in lane 1; D32L in lane 5) than when it was packaged by wild-type proteins (lanes 2 and 4). The fact that the difference between wild-type and PR− RNAs is seen with KiSV RNA, as well as with MoMuLV RNA itself, shows that PR acts in trans to affect the structure of dimeric viral RNA.

In general, mutants in the cysteine array motif of the NC region of Gag package very little intact viral RNA (11, 19). However, at least some cysteine array mutants of MoMuLV are only partially, rather than completely, defective with respect to packaging of KiSV RNA (11). This leakiness in packaging of KiSV RNA made it possible to investigate the properties of viral RNA isolated from a cysteine array mutant in which the Gag polyprotein is cleaved normally by the PR, but the structure of the cysteine-array motif in the NC protein has been disrupted by mutation. We therefore isolated RNA from a virus preparation in which KiSV RNA had been packaged by the Y28S mutant of MoMuLV (11). As shown in Fig. 6, lane 3, this dimeric RNA, like that packaged by PR− MoMuLV, migrated more slowly in nondenaturing gel electrophoresis than did KiSV RNA packaged by wild-type MoMuLV.

**DISCUSSION**

As outlined in the introduction, a review of earlier studies on viral RNA led to the suggestion that retroviral RNAs may be initially packaged in a dimeric form and that the RNA dimers may undergo a maturation event after the virus is released from the cell. This would be contrary to the original interpretations of some of these experiments on avian retroviruses (6, 14), although it is consistent with the report that rapid-harvest avian virus contains a dimeric RNA which melts at a lower temperature than does that from mature virions (25). Another study (7) reported that rapid-harvest avian virus preparations contain a 55S RNA, i.e., an RNA sedimenting more rapidly than monomers but more slowly and more heterogeneously than dimers from mature viruses. It seems likely that this RNA, like that described by Stoltzfus and Snyder (25), represents the immature dimer of avian virus.

This report has dealt with murine rather than avian retroviruses. We found that rapid-harvest MoMuLV particles contain dimeric RNA, but this RNA differs from that in mature virions in several respects. First, the dimer from rapid-harvest virus migrates more slowly in nondenaturing gel electrophoresis than does that from mature virus (Fig. 1). (In contrast, monomers obtained after incubation of these RNAs at 65°C have the same mobility in these gels, regardless of the age of the virus [data not shown].) Second, the dimer from rapid-harvest virus is dissociated into monomers at a slightly lower temperature (about 2.5°C under our experimental conditions) than is that from mature virus (Fig. 2). Thus, our results for MuLV are completely consistent with our interpretation, given above, of the earlier studies on avian retroviruses.

Since it is well known that the proteins of a retrovirus particle undergo an extracellular maturation step, i.e., cleavage of the Gag and Gag-Pol polyprotein precursors by the viral PR, we tested the effects of PR mutations on the properties of the RNA dimers isolated from virions. We found that RNAs isolated from PR− particles differ from wild-type RNAs in that they migrate more slowly in nondenaturing gel electrophoresis (Fig. 3) and also in sedimentation (Fig. 4). The melting temperature of PR− dimeric RNA is also approximately 5°C lower than that from mature wild-type particles (Fig. 5). These differences are due to the lack of PR function in the mutants rather than to the sequence differences between the mutant and wild-type RNAs, since (i) identical results were obtained for two PR− mutants, a 2-base change (D32L, in which a GAU aspartate codon has been replaced with a CUU leucine codon) and a 96-base deletion (12), and (ii) a similar difference in electrophoretic mobility was observed between dimeric KiSV RNA molecules packaged by PR− MuLV and those packaged by wild-type MuLV (Fig. 6). These results all support the hypothesis that PR function is required for the RNA maturation event.

MuLV particles formed by proteins with mutations in the cysteine array region of the NC protein contain very little intact MuLV RNA (11, 19). However, they can package detectable levels of KiSV RNA (11). We found that KiSV RNA dimers packaged by such a mutant, like those packaged by PR− mutants, have a lower electrophoretic mobility than do those encapsidated by wild-type MuLV (Fig. 6). This observation suggests that RNA maturation requires an intact, unsubstituted cysteine array as well as PR function.

On the basis of these results, we propose the following maturation pathway for MuLV particles: (i) Gag polyprotein molecules assemble into a nascent virion containing an immature dimer; (ii) the particle is released from the cell, and the Gag polyprotein is cleaved by PR, generating cleavage products including NC; and (iii) NC protein, in a step requiring the cysteine array, acts on the dimer, converting it from immature to mature form. (Our data cannot, of course,
exclude the alternate possibility that monomers are initially packaged but are converted to immature dimers more rapidly than we can isolate viral RNA.)

Since the structure of the dimer is so poorly understood, it is difficult to be precise about the difference between immature and mature dimeric forms. However, the dimer isolated from PR- particles appears to have a more extended, less compact conformation than does the wild-type dimer, since it migrates more slowly in both electrophoresis and sedimentation experiments. We do not know whether the greater stability and more compact conformation of the mature form reflect increases in base-pairing, base-stacking, or tertiary interactions. Another feature of viral RNA which might affect our results, but which we have not investigated here, is 70S-associated 4S RNA (5).

It is interesting that RNA isolated from rapid-harvest virus is intermediate in its properties, i.e., electrophoretic mobility and melting temperature, between PR- viral RNA and wild-type RNA (Fig. 2, 3, and 5). This observation is consistent with the idea that rapid-harvest RNA is partially matured by the time it is isolated for analysis. We have been unable to resolve the dimeric RNA from rapid-harvest virus into two species, one with the electrophoretic mobility of wild-type RNA and the other with that of PR- RNA (data not shown); these results would tend to suggest that the maturation process is gradual, rather than concerted, in any given molecule.

In the original description of cysteine array mutants of MuLV (11), it was noted that the relative level of KiSV RNA packaged by these mutants was far higher than the level of infectious KiSV in these virus preparations. The present results (Fig. 6) suggest that this RNA fails to mature; it is conceivable that maturation is essential for infectivity and that this failure is the explanation for the lack of infectivity of the mutant particles containing KiSV RNA.

In light of the data presented here, it seems possible that MuLV particles never package monomeric RNAs; the idea that only dimeric RNAs are encapsidated should be considered for retroviruses in general. A number of reports have described situations in which viral RNA appears to be a mixture of monomers and dimers. However, these may all be situations in which the dimeric RNA is less stable than that in mature dimeric virus. Perhaps in each of these experiments, some step in the extraction or analysis of the RNAs was harsh enough to partially dissociate these relatively fragile dimers. They include rapid-harvest avian virus (6, 7, 14), which, like rapid-harvest MoMuLV (this report), contains dimers which dissociate at a lower temperature than do those from wild-type virus (25); PR- avian virus (22, 24), which may resemble PR- MoMuLV in having a reduced melting temperature (this report); and avian cysteine array mutants (4, 9, 20), which may contain RNA like that in PR- particles, as in the case of KiSV (this report). A further similarity between the present results and those obtained with avian viruses is that dimers isolated from PR and NC mutants of avian virus also migrate more slowly in non-denaturing gel electrophoresis than do those from wild-type virions (20, 24).

The idea that only dimeric RNAs are packaged in turn raises the possibility that the dimeric structure is an element of the packaging signal recognized by the Gag polyprotein during virus assembly. Thus, perhaps experiments designed to determine which viral sequences are necessary (e.g., reference 17; reviewed in reference 16) or sufficient (1, 2) for encapsidation of the RNA are really determining sequence requirements for dimerization. In this connection, it is intriguing that plus-strand retroviral RNAs containing sequences required for encapsidation can dimerize spontaneously in vitro, as reported by Darlix and colleagues, e.g., reference 23. This observation lends strong support to the idea that viral RNAs can dimerize before encapsidation in vivo and that there is an intimate connection between dimerization and encapsidation. It will be interesting to learn the degree of correlation between the ability to dimerize in vitro and the ability to be packaged in vivo.

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REFERENCES

MATURATION OF VIRAL RNA OF MuLV


