Analysis of the Ribonucleic Acid of Murine Leukemia Virus

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Cells producing the Rauscher strain of murine leukemia virus (MLV) were exposed to ³H-uridine, and labeled virus was collected at hourly intervals. Ribonucleic acid (RNA) extracted from virions (vRNA) had a characteristic single peak when analyzed by electrophoresis in polyacrylamide-agarose composite gels. Exposure of vRNA to dimethyl sulfoxide, urea, formaldehyde, or heat altered the mobility to a faster moving form (vRNA'). This vRNA' sedimented more slowly than native vRNA in sucrose gradients. Incubation of labeled virions at 37 C resulted in fragmentation of viral RNA which was detectable only after denaturation. Also, large differences in the temperature required for the change from vRNA to vRNA' were seen with alterations in NaCl concentration. These experiments demonstrate that the vRNA of MLV is held in a specific conformation by hydrogen bonds distributed over a large part of the molecule. The possibility that an undefined factor is associated with viral RNA is discussed.

Several ribonucleic acid (RNA)-containing tumor viruses have been shown to contain a large RNA molecule with an apparent molecular weight of approximately 10⁷ as their main RNA constituent. These RNA molecules from avian leukosarcoma, murine leukemia, or mouse mammary tumor viruses can be altered by exposure to heat or dimethyl sulfoxide (3, 7, 8, 10). The treated RNA molecules migrate faster during electrophoresis in polyacrylamide gels and sediment slower in sucrose gradients than the original viral RNA (vRNA) molecules. The conclusion was drawn that the vRNA of RNA-containing tumor viruses is composed of smaller pieces held together by hydrogen bonds, and a molecular weight of 3 × 10⁶ for the largest piece was proposed.

In studies of viral ribonucleic acid (RNA), hydrolysis of the RNA at physiological temperature is an important consideration. The infectivity of RNA-containing tumor viruses has been reported to succumb to exposure at 37 C with a half-life of less than 3 hr (6, 11, 15). This effect of temperature on infectivity may be due to an effect on virion RNA. Prolonged periods of radioactive labeling were used in earlier studies on viral RNA structure. Partial thermal degradation of the RNA polymer possibly occurred which was inapparent until the molecule was treated to dissociate structural bonds.

The incorporation of exogenous ³H-uridine into the RNA of virions of murine leukemia virus (MLV) has been found to occur within 2 hr (J. P. Bader, *in preparation*). Also, it seemed likely that the vRNA fated for virion was incorporated within 2 or 3 hr after intracellular synthesis. Therefore, it was practical after initial exposure of infected cells to ³H-uridine to collect fluids at hourly intervals to obtain MLV with radioactive RNA. Such preparations of MLV presumably contain a high proportion of active to inactive virus, and RNA of uniformly high integrity, suitable for analytical studies of the structure of MLV-RNA.

MATERIALS AND METHODS

Cells and virus. Mouse splenic cells (JLSV₈) infected with and producing MLV Rauscher strain were cultivated in a growth medium (GM) composed of Eagle's Minimal Essential Medium (MEM) and 5% fetal calf serum. Cell cultures were grown as monolayers in 10-cm plastic petri dishes, in a humidified CO₂ atmosphere incubator set at 37 C. Cells were exposed to uridine-5-³H (150 μC in 3 ml of GM) for 1 hour. The label was removed, the cultures were rinsed with warm MEM, and GM was added. At hourly intervals over the next 5 hr, the virus-laden culture fluids were collected and replaced with fresh GM. The harvested fluids were centrifuged at 3,000 × g for 10 min to remove floating cells and then placed in a -80 C freezer.

Extraction and analysis of the intravirion RNA. The pooled fluids were thawed within 48 hr of collection and centrifuged at 105,000 × g for 2 hr to pellet

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the virus. When the entire labeling pattern of vRNA was to be analyzed, a 20% sucrose solution [in 0.1 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, and 0.001 M disodium diethyl diaminetetraacetate (EDTA)] was layered under the culture fluid and virions were pelleted through this barrier. A solution containing 1% sodium lauryl sulfate (SLS), 50 μ g of polyvinyl sulfate (PVS) per ml, 1 mg of yeast RNA, 10^{-3} M EDTA, and 0.05 M Tris-hydrochloride (pH 7.4) was added to solubilize the pellets and protect the released RNA from digestion. The mixture was extracted twice with equal volumes of phenol, and the RNA was precipitated by making the solution 2% in sodium acetate and adding 2 volumes of iced ethyl alcohol.

Electrophoresis in polyacrylamide gels. Resolution of RNA was made by electrophoresis in slab gels (13) containing a mixture of 1.9% acrylamide, 0.1% N'-methylenebisacrylamide, 0.5% agarose, and 0.1% sodium lauryl sulfate. Ammonium persulfate (0.05%) and dimethylaminopropionitrile (0.4%) were added for polymerization. The slab gels were slotted to accommodate 8 to 12 samples per gel. "Electrophoresis buffer," pH 7.2, contained Tris-hydrochloride (0.09 M), boric acid (0.09 M) and disodium EDTA (0.0025 M). Gels were run at 12 C, 11 v/cm, 50 ma for 90 min.

Strips from the slab gels were sliced into 1-mm sections. These were exposed overnight to 0.1 ml of NaOH (0.3 M); 1 ml of NCS reagent (Nuclear Chicago Inc.) was added, and samples were counted in 25:1 toluene-Liquifluor (New England Nuclear Corp., Boston, Mass.) in a Packard scintillation counter.

Rat embryo cytoplasmic RNA extracted from cells in culture served as markers. After electrophoresis, strips containing marker were rinsed in water, then stained with methylene blue (0.002% in 0.01 M acetic acid) overnight. Stained bands regularly were found at regions expected for mobilities of 28S, 18S, and 5S ribosomal RNA fractions and 4S transfer RNA fractions. Occasionally cellular deoxyribonucleic acid (DNA) also was included as marker.

Sucrose gradient sedimentations. RNA to be examined was layered in 0.15 ml over 5 to 20% linear gradients of sucrose containing 0.1 M NaCl, 0.01 M Tris-hydrochloride, and 0.001 M disodium EDTA at pH 7.2. The gradients were centrifuged at 5 C for 2.5 hr at an average $105,000 \times g$. Collected fractions were mixed with NCS reagent and radioactivity counted after addition of toluene-Liquifluor.

RESULTS

Analysis of virion RNA. The labeled RNA found in semipurified MLV virions was analyzed in polyacrylamide-agarose gels after extraction by the SLS-PVS-phenol method. Radioactivity was found in the region expected for the presumptive high molecular weight vRNA (Fig. 1). No smaller molecular species of RNA were found radioactive, suggesting that the slowly migrating RNA is the only RNA in the virion. Peacock and Dingman (13) have proposed a method for the calculation of molecular weights of single-stranded RNA molecules on the relation of migration rate to

FIG. 1. Polyacrylamide gel electrophoresis of MLV-RNA. Radioactive viral RNA was prepared by the rapid labeling procedure with ^3H -uridine. Half of the ethyl alcohol-precipitated sample was exposed to 95% DMSO for 5 min at 37 C. Both samples were then reprecipitated with sodium acetate (2%) and 2 volumes ethyl alcohol, resuspended in "electrophoresis buffer," and applied to polyacrylamide (2%)-agarose (0.5%) gels. Migrations were analyzed after 1.5 hr at 11 v/cm, 50 ma, at 12 C.

molecular size. By this method, the molecular weight of the vRNA was estimated to be 1.2×10^7 , which agrees with values proposed by sedimentation in sucrose gradients (9). However, such estimates of molecular weight require a knowledge of molecular structure and therefore cannot be applied to the native RNA of MLV.

Virus collected after prolonged labeling periods often contained RNA with a broad distribution of radioactivity following the 5S RNA markers. Little significance is attached to these presumably degraded RNA molecules.

Modification of vRNA. Exposure of the vRNA to 95% dimethyl sulfoxide (DMSO) resulted in an increased migration of the RNA (Fig. 1). This altered form will be referred to as vRNA'. The vRNA' migrates at a position identical to 45S intranuclear RNA under these gel electrophoresis conditions, suggesting an approximate molecular weight of 4×10^6 for vRNA'. The sharp symmetrical peak, representing 90% of the radioactive counts found in the original vRNA, suggests that herein is the only important constituent of the vRNA. The remainder of the radioactivity migrated heterogeneously as a slight leading edge to the main peak and as small fragments of less than 10^5 molecular weight. These fragments undoubtedly represent degraded RNA.

Dimethyl sulfoxide can separate double-stranded RNA molecules held together by hydrogen bonding (19). The effects of urea and formaldehyde, also known to dissociate hydrogen bonds, were examined. Both substances modified the vRNA to the rapidly migrating form, vRNA' (Fig. 2). Formaldehyde was found to be variable

in its action, however, occasionally slowing the migration of the larger vRNA. Other chemical interactions of the formaldehyde with RNA may be responsible for this result.

The slowly migrating vRNA was treated with other agents which might remove complexed molecules and alter the hydrodynamic behavior of the RNA. Exposure to Pronase (50 $\mu\text{g}/\text{ml}$, 30 min, 37 C), deoxyribonuclease (10 $\mu\text{g}/\text{ml}$, 30 min, 37 C), 1 M NaCl, EDTA (10^{-2} M), or water alone had no effect on the mobility of the slowly migrating vRNA.

Sedimentation in sucrose. The migration of the vRNA and vRNA' in acrylamide-agarose gels could be interpreted in two ways: (i) two or more molecular species, held together by hydrogen bonds, comprised the native vRNA, or (ii) the vRNA is a single molecule whose conformation, held together by hydrogen bonds, is changed to a more rapidly migrating form. Sedimentation in sucrose gradients was performed in an attempt to discriminate between these possibilities. The greater degree of compactness expected in the latter case (ii) should cause the vRNA' to sediment as fast as, or faster than, vRNA, whereas slower sedimentation might be expected in the former case (i).

Viral RNA exposed to DMSO or urea sedimented slower than the 65 to 70S native vRNA. Sedimentation values of 35 to 44S were calculated in several experiments, indicating a reduction in size or a decrease in compactness of the vRNA molecule. The molecular weight of vRNA' estimated from sedimentation values is 3×10^6 to 4×10^6 .

FIG. 2. Effects of urea and formaldehyde on vRNA. ^3H -Uridine labeled vRNA was exposed to urea (8 M) or formaldehyde (4%) for 10 min at 37 C. RNA was precipitated and run in polyacrylamide gels as in Fig. 1.

Effect of virion incubation on vRNA and vRNA'. Exposure of various stocks of labeled virus to DMSO gave variable results with respect to electrophoretic migration in gels. As in other published reports (3, 7, 8), a long hetero-disperse region of radioactivity occasionally preceded the major vRNA' peak. This heterogeneity was rarely observed in virus stocks collected hourly within a few hours after initial labeling and was always found when prolonged labeling periods were used. It seemed likely that this effect was a time-dependent result of exposure of released virions to 37 C. To test this, culture medium containing freshly labeled virions was incubated at 37 C for 4 hr, and the effect on the integrity of vRNA was determined. The vRNA isolated from these incubated preparations showed the same radioactive peak as unincubated control samples. However, upon treatment with DMSO, the incubated RNA disintegrated, giving a heterogeneous distribution of radioactivity containing only a remnant of the expected vRNA' peak (Fig. 3).

A more precise analysis of the effect of prolonged incubation at 37 C on vRNA and vRNA' was performed by incubating fresh virion-containing medium for various times. The fraction of radioactivity remaining in undenatured vRNA and in DMSO-treated vRNA' was measured as a function of duration of incubation of virions. No loss of radioactivity was detected in the undenatured vRNA peaks during a 3-hr incubation

FIG. 3. Effect of 37 C incubation on vRNA and vRNA'. ^3H -Uridine-labeled virions in cell culture medium were incubated at 37 C for 4 hr. After virus concentration, RNA was extracted, one-half was treated with DMSO, and then both samples were analyzed by gel electrophoresis.

(Fig. 4). A time-related loss in radioactivity was found in the $vRNA'$ peak after DMSO treatment. The loss corresponded to a half-life of 2 hr. These results suggest that the $vRNA$ contains extensive internal, noncovalent bonding which maintains a stable secondary structure. Otherwise, strand scissions which result in disappearance of radioactivity from the denatured $vRNA'$ would also be manifested by a loss of radioactivity from the native $vRNA$.

Effect of salt and temperature on dissociation. Base-paired, double-stranded nucleic acids typically are converted readily to single strands at characteristic temperatures, and the dissociation temperature rises with increasing salt concentration. The complete conversion of $vRNA$ to $vRNA'$ required heating at 80 C in 0.3 M NaCl (Fig. 5). Similar conversion in 0.003 M NaCl was effected at 50 C. Conversion was more gradual at the higher salt concentration, and at 70 and 75 C intermediate forms migrating between the $vRNA$ and $vRNA'$ were found (Fig. 6). This occurrence of intermediate forms suggests a partial denaturation of native $vRNA$.

DISCUSSION

The RNA content of murine leukemia virions has been analyzed by electrophoresis in composite polyacrylamide-agarose gels and found to migrate as a single entity. The rapid labeling procedures used in these analyses precluded contamination of virions by labeled cellular constituents and protected intravirion RNA from degradation. It seems unlikely that the minor small-molecular weight species of RNA, or transfer RNA species,

FIG. 4. Effect of incubation at 37 C of virions on $vRNA$ and $vRNA'$. Culture medium containing labeled virions was incubated for the designated times at 37 C. RNA was extracted from pelleted virus, and one-half was treated with DMSO. Radioactivity in the denatured $vRNA'$ peaks and in untreated $vRNA$ peaks was determined and compared to RNA from unincubated virions.

FIG. 5. Effect of salt and temperature on transition of $vRNA$. RNA was extracted from uridine- 3H -labeled virus and suspended in 0.3 M NaCl + 0.03 M sodium citrate (pH 7.0) or a 1:100 dilution of this solution. These RNA solutions were then heated at the indicated temperatures for 3 min, the salt concentration of the diluted sample was increased to equal the higher level, and samples were directly analyzed by gel electrophoresis for the relative proportion of radioactivity in the $vRNA$ and $vRNA'$ bands. At 70 and 75 C in 0.3 M NaCl conversion was estimated by the relative change in migration distance, since forms intermediate between $vRNA$ and $vRNA'$ occurred (see Fig. 6).

FIG. 6. Partial conversion of $vRNA$ or $vRNA'$. Samples of $vRNA$ in 0.3 M NaCl were heated to 60, 70, or 80 C and analyzed by gel electrophoresis. The observed pattern at 70 C indicates an incomplete denaturation of $vRNA$, resulting in forms intermediate between $vRNA$ and $vRNA'$.

noticed by others (2, 9, 10) are integral components of RNA-containing tumor viruses. The results presented here suggest rather that the slowly sedimenting RNA often found associated with virions are derived from ruptured cells or are products of $vRNA$ degradation.

Radioactivity in the regions characteristic of ribosomal RNA migration also was rarely found in rapidly labeled virion preparations. Cellular cytoplasmic RNA fractions (28S, 18S, 5S, and 4S) are heavily labeled within 2 hr after addition of 3H -uridine, the time at which radioactive $vRNA$ is first detected in virions (J. P. Bader, *in preparation*). One would not expect that only older, possibly degraded cellular RNA species are contained in virions, and such RNA species when found (2, 5) probably should be regarded as

extraviral contaminants. It is possible that occasional cells lyse and membrane vesicles (similar to rough microsomes) form containing cellular RNA species. These vesicles would have a size and density similar to virions, and the RNA would be resistant to added ribonuclease.

Exposure of the vRNA to various denaturing treatments changes the RNA to a new form, more rapidly migrating in gels, but less rapidly sedimenting in sucrose gradients. These results are consistent with dissociation of the native vRNA into smaller units, as previously suggested (3, 7, 8, 10). If molecular dissociation takes place upon denaturation, smaller molecules of essentially identical size result, since a single symmetrical peak is found by gel electrophoresis analysis. However, configurational changes in a single RNA molecule could give similar results. If this be the case, the molecular weight of vRNA (10^7 to 1.2×10^7) determined from hydrodynamic data (9) may be an overestimate. The estimated molecular weight of vRNA', 3×10^6 to 4×10^6 , is closer to the size of other viral RNA molecules, and may be the true molecular weight of RNA tumor virus RNA.

Direct viewing of MLV-RNA by electron microscopy (12) reveals long extended linear molecules, an unusual configuration for isolated RNA in 0.1 M NaCl. Exposure of this RNA to DMSO or urea results in the collapsing of the molecule to an entangled form. These observations support the hypothesis of a structural form of vRNA which yields to treatments which dissociate hydrogen bonds.

Between low (0.003 M) and high (0.3 M) NaCl concentrations a large difference was noted in the temperature required for transition from vRNA to vRNA'. Reovirus RNA, composed of double-stranded molecules, is denatured into single strands at temperatures changing from 80 C in 0.003 M NaCl to 87 C in 0.3 M NaCl (18). It seems unlikely, therefore, that MLV-RNA contains long regions of base-pairing as found in reovirus RNA. This is substantiated by the extensive degradation of vRNA by pancreatic ribonuclease (9).

The intravirion degradation of MLV-RNA at 37 C is manifested by the dissolution of denatured vRNA' but not untreated vRNA. This suggests that the presumptive hydrogen bonding holding vRNA together is distributed over a large portion of the molecule. It appears, therefore, that the series of short hydrogen-bonded regions as detected by heating experiments is distributed over the length of the molecule. It is perhaps these regions which are responsible for maintaining the vRNA in an extended linear conformation under conditions in which other single-stranded polynucleotides are found in collapsed forms. The

electron microscopic observations demonstrating such extended vRNA molecules are reported in the companion paper (12).

The possibility that molecules other than RNA are involved in maintaining the configuration of vRNA has not been excluded. Treatments which usually dissociate ionically bound ribonucleoproteins, i.e. 1% SLS, 1 M NaCl, and Pronase, had no effect on the mobility of vRNA. Likewise, cross-linking by Mg^{2+} was excluded by the failure of EDTA to affect vRNA mobility. Deoxyribonuclease also had no effect, as anticipated by the failure to find DNA within virions of RNA-containing tumor viruses (4, 15). Thus, a series of treatments with various modes of action failed to alter the mobility of vRNA, whereas the several treatments (heat, DMSO, urea, and formaldehyde) which dissociate hydrogen bonds were uniformly and identically effective in changing the vRNA to vRNA'.

The decrease in intact vRNA' of MLV after incubation of virions at 37 C occurs at about the same rate as the loss in infectivity of RNA tumor viruses (6, 11, 15). This suggests that the thermal inactivation of these viruses at 37 C is due to the scission of the viral RNA molecule, and that the intact conformation of the vRNA is insufficient to allow replication. Whether vRNA' disintegration is due to an intravirion enzymatic process or some other hydrolytic process has not been determined.

The present studies have defined qualitatively the RNA content of MLV and offer possibilities for a structural definition of the native molecule. It is unlikely that the native vRNA is composed of a single RNA molecule of molecular weight 1.2×10^7 . Configurational changes in such a large molecule would be insufficient to explain the alteration in sedimentation in sucrose gradients or mobility in polyacrylamide-agarose gels after denaturation. A base-paired double-stranded structure for vRNA is excluded on the basis of sedimentation data, the large difference in "conversion" temperature with changing salt concentration, and sensitivity to pancreatic ribonuclease. The occurrence of intermediate forms upon heating of vRNA and the high sedimentation rate of untreated vRNA argue against a double-stranded structure with intermittent base pairing. The retention of vRNA structure after virion incubation at 37 C suggests a wide distribution of bonds over the molecule and indicates that a base-paired joining of ends of smaller molecules is not responsible for the slowly migrating vRNA.

While difficult to envision, the possibility that hydrogen bond dissociations change the vRNA from a specific compact form to a random, less compact form has not been excluded. Also, the

possibility of a non-RNA hydrogen-bonded factor which holds the vRNA in its specific configuration is suggested.

These data, however, do not exclude the possibility suggested by others (3, 7, 8, 10) that two or more molecules comprise the native vRNA, although certain considerations argue against it. Multiple identical strands are unlikely since inactivation kinetics by a variety of physical agents are uniformly "single-hit" (1, 6, 11, 17). Multiple RNA strands having different genetic functions might be expected to result in a high frequency of recombinants when coinfection with a related virus strain occurs. Despite the high incidence of phenotypic mixtures resulting from dually infected cells, genetic recombinants among RNA tumor viruses have yet to be reported.

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