Electron Microscopic Observations on the Ribonucleic Acid of Murine Leukemia Virus

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The ribonucleic acid (RNA) of murine leukemia virus (MLV) Rauscher strain was observed by the aid of electron microscopy with the use of the protein monolayer technique. RNA was observed directly after release from virus particles or after isolation by sedimentation in sucrose density gradients. Molecules were found in an extended linear form. Many of the RNA filaments released by detergent treatment contained curled regions, suggesting the linear filaments were originally coiled within the virus particle. The relationship of the curled areas to the containment of the RNA within the virus particle is discussed, and a mechanism for the inclusion of RNA in the budding virion is proposed. Treatment of the extended MLV-RNA with dimethyl sulfoxide resulted in the collapse of the molecule forming a tangled complex. Treatment with urea or heating at 50 C in 3 mM NaCl also produced this effect. Also under the conditions in which MLV-RNA was linear, RNA from Rous sarcoma virus also was linear, but Newcastle disease virus RNA and ribosomal RNA of rat liver had collapsed structures. The results indicated that the RNA of MLV, and perhaps other RNA-containing tumor viruses, has a specific unique conformation dependent upon hydrogen bonds.

The ribonucleic acid (RNA)-containing tumor viruses are unique in biological and structural characteristics and in their metabolic requirements for infection. Purified RNA from these viruses appear to be single-stranded with a high sedimentation coefficient (15), although in these studies the conformation of the RNA molecule and its structural arrangement within the virus particle were not defined.

The genomes of some viruses have been shown to consist of several RNA molecules (6, 13). It has been suggested that the large molecular weight RNA species of RNA-containing tumor viruses also are comprised of smaller molecules, since the hydrodynamic properties of these RNA molecules can be changed by treatment with agents which dissociate hydrogen bonds (3, 5, 7). Although this may be the case, other results (2) indicate that hydrogen bonds distributed over most of the molecule hold the RNA in a specific conformation.

The protein monolayer technique (12) allows the direct observation of nucleic acids with the aid of electron microscopy. The configuration of native viral RNA was determined by this technique, and treatments which dissociate hydrogen bonds were examined for their effects on viral

RNA structure. Other viral and cellular nucleic acids also were observed, and a comparison of the observed molecular structures is presented.

MATERIALS AND METHODS

Preparation of virus pellets. Splenic cells infected with and continuously producing murine leukemia virus (MLV), the JLSV_s line (18), were maintained in serial culture. Cultures were grown in monolayer at 37 C in Eagle's minimal essential medium (MEM) with 5% fetal bovine serum. For experiments involving purification of viral RNA by sucrose sedimentation, uridine 5-³H (300 µc/ml) was added to the medium of a few cultures before the next procedure. After washing the cells and adding fresh medium, cell culture fluids were collected at hourly intervals with readdition of fresh medium to the culture. The fluids were centrifuged at 2,000 × *g* to remove free-floating cells and placed in a freezer at -70 C. Hourly harvests were pooled. This procedure was necessary to insure a high proportion of active virus, unaffected by thermal inactivation at the incubator temperature. Rous sarcoma virus-Rous associated virus₁ (RSV-RAV₁) was produced by infecting chick embryo cells and collecting fluids from established cultures (1). The virus was processed similar to MLV. Newcastle disease virus (NDV) was obtained by infecting confluent chick embryo cell cultures with NDV (multiplicity of infection, 10:1) and collecting culture fluids at 2-hr intervals after 8 hr until cellular lysis was microscopically ob-

servable. Pellets of virus were prepared either (i) by sedimenting the particles directly in a centrifuge (Spinco 40 head at $100,000 \times g$) or (ii) by banding in 15 to 55% sucrose gradients (16), diluting the virus band in 10 mM tris(hydroxymethyl)aminomethane (Tris)-buffered saline (pH 7.4) containing 0.5% bovine serum albumin, and then sedimenting at $100,000 \times g$. After initial trials, only the former procedure (i) was used, since virtually all pelleted material could be identified as virus, and the rapidity of this method precluded fragmentation of intraviral RNA.

Fractionation of viral RNA. High molecular weight viral RNA was obtained by treatment of resuspended virus pellets with Pronase (50 $\mu\text{g}/\text{ml}$) for 5 min at room temperature before addition of sodium lauryl sulfate (SLS, 1%). The suspension was extracted twice with cold phenol. Sodium acetate (2% final concentration) and two volumes of iced ethyl alcohol were added to the aqueous layer, and, after allowing 10 min for association, the RNA precipitate was centrifuged to a pellet. The RNA pellet was resuspended in 0.2 ml of Tris-ethylenediaminetetraacetate (EDTA) and layered onto 5 to 20% sucrose gradients containing 0.1 M NaCl in Tris-EDTA (16). The gradients were centrifuged at 4 C in a swinging bucket rotor (SW 39) at $100,000 \times g$ for 2 hr. Samples of fractions were tested for radioactivity, and fractions showing peaks of radioactivity in the 60 to 70S region were pooled and diluted 1:2 with Tris-EDTA solution. The RNA was precipitated with sodium acetate-ethyl alcohol and centrifuged to a pellet. RNA from RSV-RAV₁ was prepared in an identical manner as the MLV-RNA. NDV-RNA was processed in a similar manner except that 45 to 55S material was collected after 2.5 hr of sedimentation through the sucrose gradients.

Preparations for electron microscopy. The following standard solutions were used: (i) SLS, 1% aqueous solution at room temperature; (ii) protein solutions, 0.1% cytochrome *c* in 1 M ammonium acetate (pH 7.4), 0.1% chymotrypsinogen A in 1 M ammonium acetate (pH 7.4), and 0.1% α -chymotrypsin (inactivated with diisopropyl phosphofluoridate) (Worthington Biochemical Corp., Freehold, N.J.) in distilled water; (iii) 8 M urea in distilled water; (iv) Tris-EDTA, 10 mM Tris plus 1 mM EDTA (pH 7.4); (v) SSC, 0.15 M sodium chloride and 0.015 M sodium citrate (pH 7.0); (vi) hypophase, 0.05 M ammonium acetate (pH 7.4), ice cold except when SLS was used, (vii) 2% potassium phosphotungstate (PTA, pH 6.5), (viii) 0.1% ribonuclease A (Worthington) in 0.1 M Tris buffer (pH 6.8); (ix) 0.1% pancreatic deoxyribonuclease (Worthington) in 0.1 M Tris buffer containing 0.01 M MgCl (pH 6.8); and (x) dimethyl sulfoxide (DMSO).

Direct (one step) method was used. The MLV pellet was resuspended with 100 μliter of 8 M urea and shaken for 15 min. To this was added 10 μliters of chymotrypsinogen A or chymotrypsin, and the solution was shaken for another minute. One drop of this was placed on the hypophase at 5 C. The protein film was left intact for 10 min, after which the specimen was taken by touching a carbon-collodion-coated grid to the surface of the protein film. The grid was then washed with cold distilled water and 95% ethyl alcohol for 35 sec each and air dried.

Detergent treatment of virus with protein mono-

layer technique was used. Since rupture of virions by urea alone, described above, resulted in a low yield of released RNA filaments, 20 μliters of SLS, 80 μliters of 8 M urea, and 10 μliters of chymotrypsinogen were added in this order to the virus pellet. Spreading of this suspension was greater than the protein monolayer without SLS.

Large molecular weight RNA was prepared. Viral RNA (60 to 70S) from sucrose density gradients was precipitated by the addition of sodium acetate (2%) and ethyl alcohol (2 volumes) and resuspended in various solutions. (i) The RNA was suspended in 100 μliter of SSC and was added to 10 μliter of cytochrome *c* solution. (ii) This was the same as solution i, but chymotrypsin replaced cytochrome *c*. (iii) The RNA was suspended in 100 μliters of 8 M urea, incubated 10 min at 37 C, and 10 μliters of chymotrypsin was added. (iv) After incubation with urea, the RNA was reprecipitated by absolute ethyl alcohol and resuspended in 100 μliters of SSC and 10 μliters of chymotrypsin. (v) The RNA was resuspended in 100 μliters of DMSO incubated for 10 min at 37 C, reprecipitated with sodium acetate and ethyl alcohol, and resuspended in Tris-EDTA, and 10 μliters of chymotrypsin was added. (vi) The RNA was resuspended in 100 μliters of 0.02 $\times$ SSC, heated at 50 C for 3 min, and cooled, and then 10 μliters of 10 times concentrated SSC and 10 μliters of cytochrome *c* were added.

The following control preparations were used. Nuclear and mitochondrial deoxyribonucleic acid (DNA) species of rat liver were prepared by phenol extraction and incubated with ribonuclease. Nuclear DNA was denatured by incubation with 0.1 M sodium hydroxide in 0.2 M potassium phosphate, pH 13, then dialyzed against SSC. Rat liver ribosomal RNA was obtained by phenol extraction. The RNA was fractionated in a sucrose density gradient and the 30S region was dialyzed. These control preparations were a kind gift from C. W. Dingman.

For enzyme treatments, some of the spread RNA preparations were incubated with ribonuclease or deoxyribonuclease for 20 min at 37 C by floating the grids on the enzyme solution and then washing them.

Negative stain preparations of virus particles, purified intact virus and those treated with urea or SLS or both were mixed with PTA solution. One drop of this was placed on a carbon-coated grid, the excess was removed, and the grid was air dried.

Observations by electron microscopy. All of the spread preparations were rotary shadowed with a platinum-carbon pellet (Ladd Research Industries, Inc., Burlington, Vt.; 8 mg) at a 10° angle. For comparative study the RNA and DNA preparations were shadowed together to receive equal amounts of shadowing. The magnification was calibrated with a grating replica. Filaments were measured by tracing with a map ruler or placing a thin thread on the electron micrographs.

RESULTS

MLV-RNA directly released from virions. MLV treated with urea showed fine filaments 3 to 12 μm long. Occasionally, a small particle of 90 to 100 nm in diameter was attached to the filament as shown in Fig. 1. The association of a particle to the filament was absent in preparations treated

FIG. 1. Electron micrograph of MLV-RNA. MLV was treated with 8 M urea and spread with chymotrypsinogen A. A thin filament of 3.4 μ m long is attached to a small particle. This appears to be the virion from which RNA is partially released. $\times 80,700$. Negative stain preparation of the urea treated particles shows some unaffected virion structures. $\times 140,000$.

FIG. 2. *MLV-RNA*. *MLV* was treated with *SLS* in the presence of urea and spread with chymotrypsinogen on the surface of ammonium acetate solution. The filament is 7.1 μm long and shows a folding with a sharp edge. $\times 46,800$. Negative stain preparation of *SLS*-treated particles shows only degraded virus particles. $\times 140,000$.

FIG. 3. Curled regions in MLV-RNA. The same preparation as Fig. 2. (a) Relatively short filament with curled region at its end. $\times 176,000$. (b) A curly piece with no extended filament attached to it. $\times 176,000$. (c) A medial twisting of a filament perhaps due to the spinning force of the unwinding of the filament. $\times 62,400$. (d) Irregular winding at the end of a viral filament. $\times 70,600$.

with urea plus SLS or SLS alone followed by phenol extraction, suggesting that the particle was a viral component, perhaps collapsed envelope, from which the RNA filament was partially or completely liberated. No more than one particle per filament or more than one filament per particle was observed. Many intact virions persisted after treatment with urea alone as shown in negatively stained preparations.

Addition of SLS to the virus suspension in the presence of urea greatly increased the number of filaments visible in the shadowed preparations, and no intact particles were present in negatively stained preparations (Fig. 2). Filaments were stretched by the increased spreading action of SLS on the monolayer, allowing greater facility in length measurements. Again the filaments ranged from 3.5 to 12 μm long. Many of the filaments were folded or associated with other filaments making exact measurements difficult. Virions treated with SLS alone released extended filaments similar to SLS-urea-treated specimens.

A characteristic feature of SLS or urea-treated MLV particles was a curly region at one or both ends of the filaments (Fig. 3). The curly areas were uniform in thickness (16 nm) and varied in length from a small piece to one 300 nm long. Some of the filaments showed an irregular winding (Fig. 3d). This suggested an intermediate form of unwinding from a tightly coiled structure to the extended filament, as described in detail in a later section. Long curled regions were usually attached to short filaments, whereas short curls were attached to long filaments. The relationship of the lengths between the two components is shown in Fig. 4.

Purified MLV-RNA. Viral RNA was extracted from viruses by using SLS and phenol and separated from possible contaminating RNA by sedimentation through sucrose gradients. Electron micrographs of the RNA showed filaments, 6 to 14 μm long, and short filaments of less than 2 μm (Figs. 5 and 6). Molecules often were found folded back and twisted upon themselves, and overlapping strands were difficult to resolve in many cases. This prevented an accurate definitive determination of length of the native viral RNA, and an estimate of the molecular weight of the RNA was not possible. The distribution of measured lengths is presented in Fig. 7.

Physicochemical treatments of MLV-RNA. Other studies have shown that treatments which dissociate hydrogen bonds altered the hydrodynamic properties of MLV-RNA (2, 3). Exposure of native viral RNA to DMSO (preparation v) and resuspension in aqueous buffer resulted in collapse of the filaments forming complex tangles as shown in Fig. 8a and b. Measure-

FIG. 4. Relation of curled to extended regions of MLV-RNA. Twenty-two filaments were measured after treatment of MLV with SLS-urea. Thick lines on the left represent curly regions and thin lines on the right the extended linear portions of filaments observed in electron micrographs. There is an inverse relation in the lengths of the two components in individual filaments, suggesting that the filament is compressed in the curly area, presumably by coiling.

ment of the lengths of the tangled filaments was impossible, although the overall size of the collapsed filaments was fairly uniform.

Addition of urea to the viral RNA (preparation iii) did not bring about any visible difference in the dimension and profile of the filaments as shown in Fig. 11b. However, incubation at 37 C of the RNA with urea with subsequent reprecipitation by ethyl alcohol and resuspension in salt solution (preparation iv) resulted in collapse of the filaments similar to that seen after DMSO treatment (Fig. 8c).

To examine the effect of heat on viral RNA structure, precipitated RNA was dissolved in 3 mM NaCl, incubated for 3 min at 50 C, and the NaCl concentration then was increased to 0.15 M. About half of the observed molecules were found in the collapsed form, whereas the other half remained extended. Many of the RNA filaments after heating were extremely thin (Fig. 9).

All of the filaments and aggregates disappeared completely with ribonuclease treatment.

Other nucleic acid molecules. Mitochondrial and nuclear DNA molecules were examined to insure that the above observed structures were in fact viral RNA and to compare the presumptive single-stranded viral RNA with known double- and single-stranded nucleic acids.

The native DNA preparations were considerably thicker than single-stranded molecules, smoother in profile, and clearly distinguishable from MLV-RNA (Fig. 10). As in the case of

FIG. 5. MLV-RNA from 60 to 70S region of sucrose density gradient. This preparation was spread with cytochrome *c* in the absence of urea. The filament is folded forming a side-by-side association, which differs from strand separation of a denatured double-stranded nucleic acid molecule. The arrows indicate the same region of a molecule photographed in separate plates. $\times 52,000$.

FIG. 6. The same specimen as Fig. 5, but chymotrypsin was used for spreading. Irregular winding with small curves or sharp edges are characteristic of the RNA filaments observed. This filament is 11 μm long. Spreading with chymotrypsin followed by washing with water and alcohol provided a high contrast image in electron micrographs. $\times 64,000$.

FIG. 7. Histogram of length distribution of MLV-RNA isolated from the 60 to 70S region of a sucrose density gradient.

heated MLV-RNA, denatured DNA was found as thin filaments (Fig. 10d and e). Most of the denatured DNA was found in collapsed tangled forms, whereas native MLV-RNA was always in the extended form.

Rat liver ribosomal RNA (30S) appeared in a pine-leaf shape, and extended forms were not observed. The addition of urea did not alter the noted configuration of ribosomal RNA.

RNA species from other viruses. The electron microscopic appearance of the purified RNA from NDV was strikingly different from MLV-RNA. The molecules were found in the collapsed form; no extended filaments were observed (Fig. 11c). Addition of urea or treatment with DMSO did not change this conformation (Fig. 11d). The RNA molecules of RSV were, in contrast, all in the extended linear form (8 to 11 μ m long) in the absence or presence of urea similar to MLV-RNA (Fig. 10a). The effects on RSV-RNA of DMSO, urea, or heating were not examined.

DISCUSSION

The RNA of MLV was examined in the electron microscope by using the protein monolayer technique of Kleinschmidt et al. (12). Only extended linear filaments were found when RNA was released directly from virions by urea or SLS, or after fractionation by zonal centrifugation. Measurements of molecular length revealed a wide distribution of sizes. Several factors could be responsible for this size inconsistency, including fine irregular wrinkles and folding which impeded accurate measurements, shearing or other degrading influences during the preparation for electron microscopy, and the difficulty in resolving aggregates. Granboulan et al. (9) in their observations on the RNA of avian myeloblastosis virus likewise found a variety of sizes. Since it is unlikely that the viral genomes are composed of several RNA molecules of different sizes (2), other methods must be developed to insure the structural integrity of the native molecule.

Alteration in sedimentation rates in sucrose gradients and migration rates in polyacrylamide gel electrophoresis have been observed after DMSO treatment of RNA species from oncogenic viruses (2, 3, 5, 7). These results suggested that the viral genome may be composed of two or more molecules of RNA of equal size. The electron micrographs revealed no obvious connecting structures in the 60 to 70S native MLV-RNA. Treatments which dissociate hydrogen bonds resulted in molecular collapse and no estimate of molecular length could be made; the determination of the total RNA per virion remains unresolved. However, conformational changes in the MLV-RNA were observed after exposure to DMSO, urea, or heat, and these changes may be sufficient to explain the alteration in the hydrodynamic properties of MLV-RNA after such treatments.

The intact MLV-RNA and RSV-RNA maintained extended linear forms after isolation, whereas ribosomal RNA, NDV-RNA, and denatured DNA formed tangled complexes. Possibly the MLV-RNA has intramolecular base pairing which determines an extended linear conformation but differs from that found in double-stranded nucleic acids. An alternative explanation is the association of a non-RNA factor which prevents the collapse of MLV-RNA.

It is possible also that chemical changes in MLV-RNA are imposed by the manipulations for electron microscopy, and that the configuration of native MLV-RNA differs from that described. It is unlikely that simple denaturation of hydrogen bonds could produce this effect, however, since other RNA molecules and denatured DNA and MLV-RNA were found in collapsed forms.

Whether the observed folding and side-by-side associations of the filaments (Fig. 5) are natural forms of the viral RNA or are a consequence of preparation is unknown. The lengths of each side of the loops which were formed by intermittent association of a folded filament were found to be unequal, indicating that the structures were not formed by strand separations from hydrogen bound, double-stranded molecules. It is clear that MLV-RNA is not a classical base-paired double-stranded molecule. The base composition of MLV-RNA is inconsistent with complementary base-pairing, and the RNA is digestible by pancreatic ribonuclease (15).

Interestingly, however, the change in sedimentation rate of tumor virus RNA species with varying salt concentration is relatively minor compared to the changes observed with other viral RNA species (4, 7, 15). Generally, the sedimentation rate of RNA, particularly single-stranded RNA, is markedly dependent on the ionic strength. The observed extended conforma-

FIG. 8. MLV-RNA treated with DMSO or urea. (a) and (b) MLV-RNA exposed to DMSO, reprecipitated, and resuspended in SSC, showing collapse of the filaments. No extended filaments were observed after DMSO treatment. $\times 55,000$. (c) MLV-RNA incubated with urea at 37 C for 5 min. The viral RNA was reprecipitated with ethyl alcohol and resuspended in SSC. Note the loss of the extended linear structure. $\times 49,000$.

FIG. 9. *MLV-RNA heated at 50 C for 3 min in 3mM NaCl. Various aggregated and extended forms were noted. Some filaments appeared thinner than those in unheated samples and these showed extensive inter- and intrastrand aggregation. $\times 49,000$.*

FIG. 10. Comparison of MLV-RNA with other single- and double-stranded polynucleotide chains. All were spread with cytochrome c and ammonium acetate and equally shadowed with platinum carbon. (a) Rat liver mitochondrial DNA. (b) Rat liver nuclear DNA. (c) 30S ribosomal RNA of rat liver. (d) Alkaline-denatured nuclear DNA. (e) MLV-RNA. Note that double-stranded polynucleotide chains (a and b) are thicker than single-stranded molecules. Ribosomal RNA molecules are all in a collapsed form; no extended molecules were found. Alkaline denatured DNA molecules were found in both extended and collapsed forms. Intact MLV-RNA was always in the extended form and was slightly thicker than denatured DNA. $\times 70,600$.

FIG. 11. Comparison of MLV-RNA with RNA molecules from other viruses. (a) Purified Rous sarcoma virus RNA from sucrose density gradient (60 to 70S) spread with chymotrypsin without adding urea. The extended linear molecule is 11.7 μm long. $\times 44,000$. (b) MLV-RNA; same preparation as Fig. 6 but with urea added. In this 14- μm long filament, no noticeable change in appearance was found. $\times 37,000$. (c) Newcastle disease virus RNA; (d) after treatment with DMSO, no conformational change was observed. $\times 40,000$.

tion of MLV-RNA may be reflected in the hydrodynamic behavior of the molecule.

The small curly regions frequently observed at one or both ends of the filaments were another feature of the MLV-RNA. Such structures were not observed in the DNA and ribosomal RNA preparations studied here or in previous reports dealing with different species of DNA and RNA (8, 9, 11, 12, 17; T. Kakefuda, T. Tanaka, and R. Kinoshita, *Abstr. 5th Int. Congr. Electron Microscopy*, vol. 2, p. 10-11, 1962). There was an inverse relationship between the length of the extended areas and the length of the curly areas of individual filaments. Transitional forms were also noted, suggesting an unwinding process (Fig. 4d) and the compression of the MLV-RNA into a coil in the curled region. The compression of the extended filament by coiling was estimated to be 1:32, although a definitive number must await absolute resolution of the extended length of native MLV-RNA.

The completion of MLV by a process of envelopment and budding from the cell membrane has been adequately established in other studies. Disposition of RNA within the developing virion must be highly oriented, maintaining juxtaposition with the intermediate membrane and the inner surface of the budding membrane. This inner electron-dense RNA layer bounding the nucleoid measures 16 nm and cannot consist of a single linear strand of RNA. Since only extended filaments of RNA were seen in the present studies, it is unlikely also that the electron-dense area is composed of multi-stranded RNA. The dimension of the curled regions (16 nm) suggests that the electron dense area is in fact linear viral RNA in a coiled configuration.

On the basis of these considerations a mechanism for the incorporation of RNA of oncogenic viruses into virions is proposed (Fig. 12). Viral RNA, perhaps in association with another substance, attaches to an appropriate site on the developing intermediate membrane at the cellular periphery. This site presumably has a critical concentration of viral structural protein assembled. The RNA is attached either at the middle of the molecule or, we prefer, at one end and begins twisting into a tight coil (16 nm diameter). This twisting process occurs only at the site of incorporation, since electron-dense material of these dimensions has never been observed except in the immediate budding area. The tight coil forms a larger ropelike super-coil within the budding particle, developing into a hollow sphere of coiled and super-coiled RNA, the electron-dense nucleoid with its electron lucent center. The tight coiling imposes a rigidity to the linear molecule which forces the RNA against the

FIG. 12. Diagrammatic representation of the proposed incorporation of MLV-RNA into the developing virion. The RNA is coiled into a springlike configuration, as it is included in the budding particle. These coils and filaments form larger, ropelike coils which eventually constitute an empty sphere, the nucleoid of the virions. Disruption of the coil would result in collapse of the nucleoid.

inner surface of the intermediate membrane. So-called maturation, or the transition of nucleoids to electron-dense centers, we suggest occurs by disruption of the RNA super-coil within the completed virion, perhaps by accumulation of temperature-dependent scissions in the RNA (2).

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