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Molecular Weight of RNA Subunits of Rous Sarcoma Virus Determined by Electron Microscopy

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Secondary cultures of chicken embryo fibroblasts were infected with the Schmidt Ruppin strain of Rous sarcoma virus (RSV). Five days after infection, the medium was replaced at 2-h intervals with phosphate-free Eagle medium containing 50 μCi of [^{32}P]orthophosphate per ml. Virus was collected by centrifugation, and the RNA was extracted and denatured with dimethyl sulfoxide, and the 33*S* subunit RNA was isolated by sucrose gradient centrifugation. The molecular weight of the RSV subunit RNA was determined by length measurement in the electron microscope, by using bacteriophage MS2 RNA as a length marker. Molecules of between 2.5 and 3.3 μm in length made up over 50% of the subunit RNA preparation. In this paper, we define RSV RNA subunits as that RNA released from the 70*S* RNA complex by dimethyl sulfoxide treatment, which sediments as a peak at 33*S*. Assuming the molecular weight of MS2 RNA to be 1.2×10^6, we calculate the molecular weight of RSV subunit RNA to be $3.12 \times 10^6 \pm 0.25 \times 10^6$.

RNA tumor viruses contain two major size classes of RNA; 60 to 70*S* RNA, thought to contain the viral genome, and 4 to 12*S* RNA (5). The 60 to 70*S* RNA dissociates into 30 to 40*S* subunits when the secondary structure of the RNA is destroyed (4, 7, 16, 22). The question of whether the RNA subunits are identical or different has been discussed (23), and evidence for identity (Duesberg et al., abstract, Cold Spring Harbor Symposium on Tumor Viruses, p. 43, 1974; Billeter et al., Proc. Nat. Acad. Sci. U.S.A., in press) and against (11, 21) has been reported. In both cases, the conclusions rest on the accuracy of the physical and chemical estimates of the complexity of the viral RNA. In either case, a precise value for the molecular weight of the RNA subunits is required to interpret the data. It is known that estimates of the molecular weight of large viral RNAs based on the analysis of their hydrodynamic properties can be subject to errors (see Discussion). We therefore employed length measurements of RSV (Rous sarcoma virus) RNA molecules in the electron microscope to determine molecular weight, a method independent of the hydrodynamic properties of the RNA.

Furthermore, the difficulty in isolating intact RNA from the RNA tumor viruses has led to some confusion about the physical nature of their genome. Degradation may occur during virus maturation and release (1), during purification of the virus and its RNA (20), or both. We therefore prepared RNA subunits from RSV grown under conditions which give the maximum degree of intactness (see below) and determined their molecular weight in the electron microscope by using bacteriophage MS2 RNA as a length marker. Assuming a value of 1.2×10^6 for the molecular weight of MS2 RNA we found that the RNA subunits of RSV have a molecular weight of $3.12 \pm 0.25 \times 10^6$.

MATERIALS AND METHODS

Cells and viruses. Primary chicken embryo fibroblast cultures were prepared from 11-day-old embryonated eggs (Pharmazeutisches werk Cuxhaven, Germany) as previously described (17). After 4 days of growth at 37 C in Dulbecco modified Eagle medium supplemented with 5% calf serum (Flow Laboratories, Inc., Rockville, Md.), 10% tryptose phosphate broth, and 50 U of mycostatin (complete medium) per ml, cell monolayers were subcultured into 1.5-liter glass roller bottles (Bellco Glass, Inc., Vineland, N.J.) at a concentration of 5×10^5 cells/ml and 5×10^7 cells/roller bottle. The bottles were rolled at 0.5 rpm at 37 C in the presence of 5% CO_2.

Two hours after seeding, cells were infected by the addition of the Schmidt-Ruppin strain of RSV, subgroup D (approximately 0.1 focus-forming unit per cell). Within 48 h, the majority of cells appeared transformed as judged by their altered morphology. From this stage, medium was changed at least once per day with 50 ml of medium per bottle. Five days

after infection, the medium in each bottle was replaced with 20 ml of phosphate-free Eagle medium containing 50 μCi of carrier-free [^{32}P]orthophosphate per ml. Twelve hours later, the medium was replaced by 10 ml of phosphate-free medium, and these changes were repeated at 2-h intervals for 10 h. At the end of each 2-h period, the medium was collected, cell debris was removed by centrifugation at 12,000 $\times$ *g* for 10 min, and the medium was frozen and stored at −20 C.

Virus purification and extraction of virus RNA. Virus-containing medium (2-h collections) was thawed, and virus were recovered by centrifugation for 2 h at 45,000 rpm at 4 C, in a Spinco Ti60 rotor. The virus pellet was resuspended by using a dounce homogenizer, in 0.5 ml of NTE (50 mM NaCl, 25 mM Tris-HCl, 1 mM EDTA, pH 7.5). A 250-μg amount of proteinase K (Merck) was added together with recrystallized sodium dodecyl sulfate to a final concentration of 0.25%, and digestion was continued at 4 C for 20 min.

This solution was extracted twice with an equal volume of phenol saturated with NTE, and the combined phenol phases were extracted once with NTE. After addition of sodium acetate (pH 5.4) to 0.25 M, the RNA was precipitated from the combined aqueous phases with 2.5 volumes of ethanol. After 2 h at −20 C, the precipitate was recovered by centrifugation (12,000 $\times$ *g*, 4 C, 15 min), dried under vacuum, and dissolved in NTE at a concentration of 3 to 5 μg/ml. The RNA was stored frozen at −20 C.

Denaturation of RNA with dimethyl sulfoxide. RNA preparations were denatured in a solution of 0.2 $\times$ NTE and 80% dimethyl sulfoxide (made 1 mM in EDTA and adjusted to pH 7.2 with 1 M HCl). Samples were heated to 45 C for 5 min and were then precipitated with ethanol as described above.

Electron microscopy. RNA was prepared for electron microscopy by the following modification of the basic protein film technique (13). A 10-μliter amount of RNA (3 to 5 μg/ml) was added to 70 μliters of redistilled formamide, 10 μliter of 100 mM triethanolamine, and 10 mM EDTA (pH 8.5). The solution was incubated at 50 C for 15 min, chilled on ice for 5 min, and then mixed with 10 μliters of cytochrome *c* (1 mg/ml). A 40-μliter amount of this mixture was pipetted onto a distilled water surface (10.9 cm by 10.9 cm), and the cytochrome films were picked up on grids coated with parlodion, stained with 0.2 mM uranyl acetate (3), and rotary-shadowed with platinum. All materials which came into contact with the RNA were made nuclease-free by autoclaving or by treatment with diethyl pyrocarbonate before use.

RESULTS

^{32}P-labeled 60 to 70*S* RNA extracted from RSV as described above was centrifuged through a sucrose density gradient, and the profile obtained is shown in Fig. 1A. The RNA sedimenting as a peak at 60 to 70*S* (fractions 5 to 10) was isolated by ethanol precipitation, denatured with dimethyl sulfoxide (see above), precipitated again with ethanol, and dissolved in NTE buffer. This solution was centrifuged through a sucrose density gradient yielding the profile shown in Fig. 1B. Two main peaks were observed; a sharp peak sedimenting at 33*S*, which we consider to be the denatured viral RNA subunits and a second, rather broad peak sedimenting at 45 to 50*S*, which appears to contain only partially dissociated viral RNA complexes. In contrast, 60 to 70*S* RNA from virus grown in the presence of complete medium

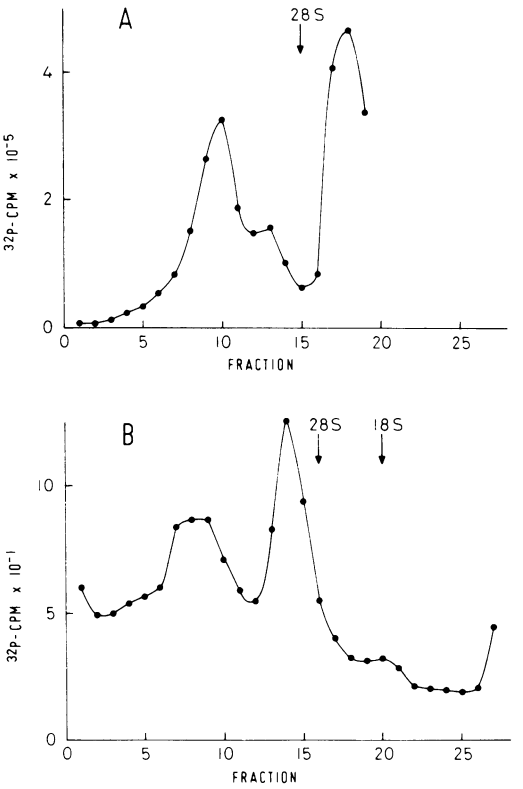

FIG. 1. (*A*) *Sucrose density gradient analysis of total ^{32}P-labeled RSV RNA. A 4-μg amount of RSV RNA (prepared as described earlier) was dissolved in 0.1 ml of NTE and centrifuged through linear, 4-ml, 5 to 20% sucrose gradients in NTE, at 50,000 rpm for 70 min at 4 C in an International SB405 rotor. Fractions were collected by puncturing the bottom of the tube and were assayed for radioactivity by liquid scintillation counting and for RNA concentration by measuring absorbancy at 260 nm. The position of the 28S rRNA marker was taken from a separate gradient run in parallel. The direction of sedimentation is from right to left. (B) Sucrose density gradient analysis of dimethyl sulfoxide treated 60 to 70S RSV RNA. The RNA was taken from the 60 to 70S peak of the gradient shown in (A) (fractions 5 to 10) and after denaturation with dimethyl sulfoxide and subsequent ethanol precipitation, the sample (in 0.1 ml of NTE) was centrifuged as described for (A), but for 2 h in this instance.*

(see above) is completely denatured to subunits and fragments under the same conditions. All of the fractions (13 to 17) of the 33S peak of the gradient shown in Fig. 1B were pooled, and the RNA was precipitated with ethanol. The RNA was prepared for analysis in the electron microscope (see above).

An electron micrograph of part of the RSV RNA preparation used in these studies shows that the RNA molecules are well distended, and little or no secondary structure can be seen, allowing for accurate length measurements (Fig. 2).

In Fig. 3, the results obtained from measurements of 93 molecules of RSV RNA subunits and 84 molecules of MS2 RNA are presented. Figure 3A shows a plot of the percentage of total RNA (RSV subunits) present in each length class from 0.2 to 3.2 μm in length. It can be calculated that over 50% of the total RNA examined is between 2.5 and 3.3 μm in length, with a mean length of 2.86 $\pm$ 0.23 μm.

These data are plotted in Fig. 3B to show the actual number of molecules which fall into each size class. Although there are many fragmented molecules found from 0.6 to 2.2 μm in length, there is a considerable number of molecules ranging from 2.5 to 3.3 μm in length. Since these larger molecules constitute over half of the total RNA in the preparation (see Fig. 3A), we assume that the large molecules are representative of the RSV RNA subunits and that the smaller molecules are degradation products.

Eighty-four molecules of bacteriophage MS2 RNA were measured, and the results are plotted in a histogram to show the number of molecules occurring in each length class (Fig. 3C). It is clear that the MS2 RNA preparation contains a homogeneous population of molecules, whose mean length is calculated to be 1.12 $\pm$ 0.09 μm. The molecular weight of the RNA of the related bacteriophages MS2 and R17 have been reported to range from 1.05×10^6 to 1.3×10^6 (2, 9); if we assume that the molecular weight of MS2 RNA is 1.2×10^6, one can calculate a value of $3.12 \times 10^6 \pm 0.25 \times 10^6$ for the molecular weight of the RNA subunits of RSV.

DISCUSSION

There have been many reports of the determination of the molecular weight of tumor virus RNA and the methods used may be grouped as follows: (i) those making use of the hydrodynamic properties of the RNA; (ii) those measuring the nucleotide complexity of viral RNA; and (iii) those measuring the length of the molecules in the electron microscope.

(i) The RNA of RSV was first isolated and analyzed by sucrose density gradient centrifugation, and the molecular weight of the 60 to 70S RNA was calculated to be 9.6×10^6 (18). Subsequently, values of 2.3×10^6 to 3.4×10^6 have been reported for the molecular weight of the RNA subunits by using a variety of sedimentation and electrophoretic methods under native and denaturing conditions (4, 6, 16). Although the variations between these results may be partly due to the different strains of virus analyzed, they may also reflect the unequal degree of denaturation of the viral RNA relative to the various RNA markers used. For instance, it has been shown that paramyxovirus RNAs behave anomalously when analyzed under standard denaturing conditions, with molecular weight values reported to range from 2.3×10^6 to 10^7 (6, 14; Kolakofsky, personal communication).

(ii) The determination of the nucleotide complexity of tumor virus RNA has been attempted by using two main experimental approaches, polynucleotide association kinetics, and chemical analyses of the viral RNA. Association kinetics analyses have yielded values of 2.5×10^6 to 3.4×10^6 (Baluda et al., abstract, Cold Spring Harbor Symposium on Tumor Viruses, p. 47, 1974) and of 10^7 (11, 21) for the molecular weight of tumor virus RNA, depending on the procedures used. It is unclear why experimentally related methods should give such different results. Recently, molecular weight values of 2.5×10^6 (Duesberg et al. Cold Spring Harbor Symposium on Tumor Viruses, p. 43, 1974) and $3.4 \times 10^6 \pm 0.9 \times 10^6$ (Billiter et al., in press) have been obtained by chemical analysis of tumor virus RNA. Although this approach appears to be the method of choice, its main limitation is the accuracy with which the yield of the isolated oligonucleotides can be ascertained.

(iii) Length measurements of molecules in the electron microscope have been performed on RNA from avian myeloblastosis virus (10) and from murine (12) and feline (25) leukemia viruses; a value of about 10^7 was reported for the molecular weight of these RNAs. These high values are surprising, since to display single-stranded RNA in the electron microscope, the molecules must first be denatured, releasing the RNA subunits from the 60 to 70S complex. It has been recently suggested that contaminating DNA molecules may have been mistaken for viral RNA in these studies (24), but it is also conceivable that RNA aggregates were produced. Analysis of RNA preparations from mouse mammary tumor viruses revealed only fairly short molecules, the largest having a molecular weight of 3.6×10^6 (19). Two studies

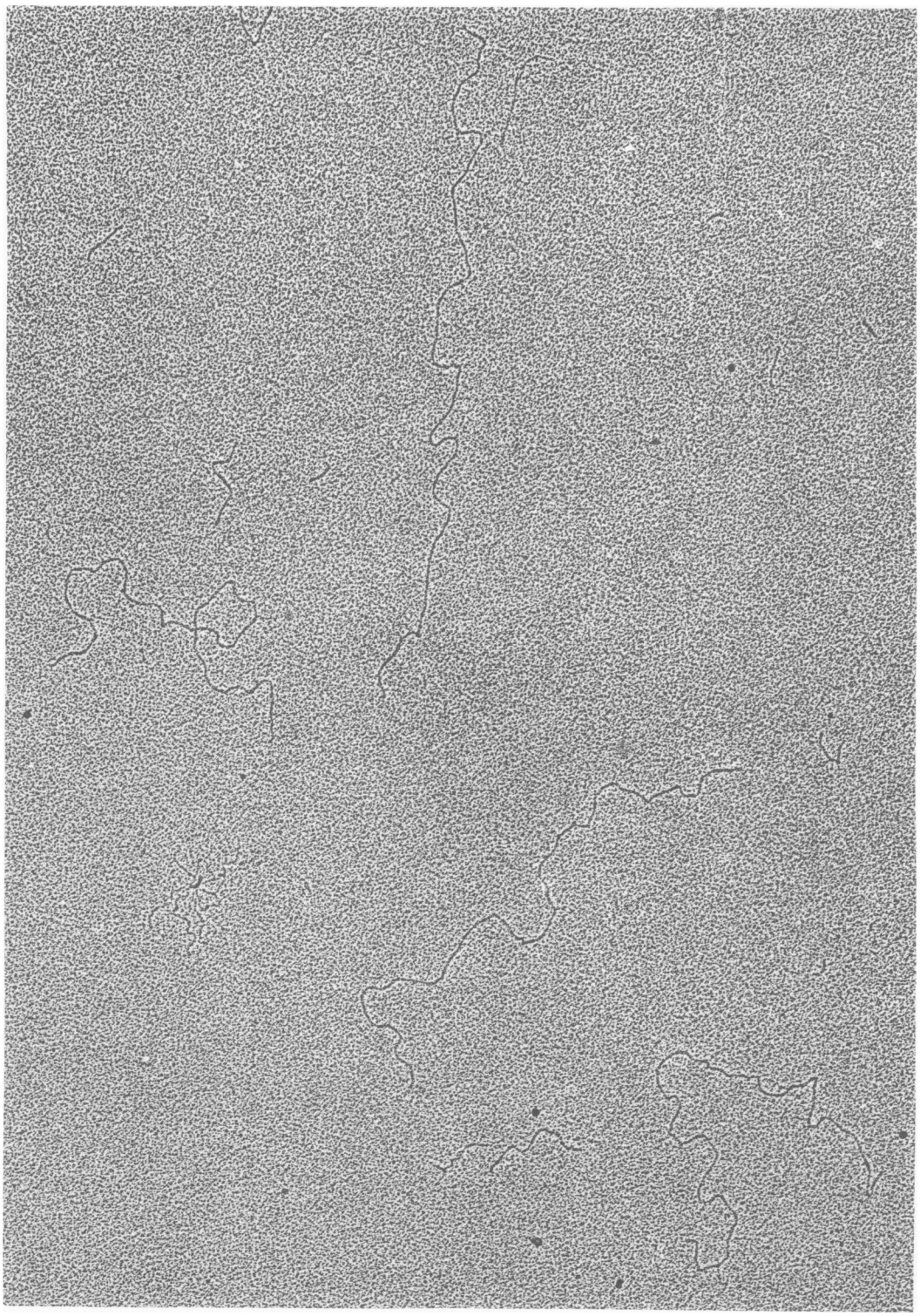

FIG. 2. *An electromicrograph of RSV RNA taken from fractions 13 to 17 of the sucrose gradient shown in Fig. 1B. The magnification used was 40,000×.*

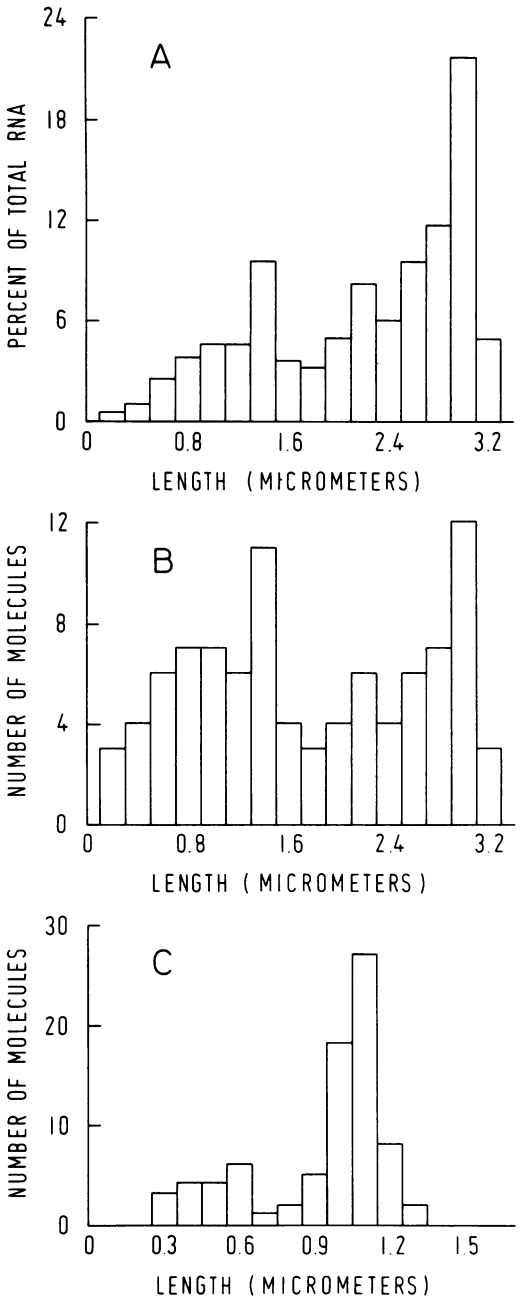

FIG. 3. *(A) Histogram of length measurements of RSV RNA subunits. RNA from the 33S peak shown in the gradient of Fig. 1B (fractions 12 to 15) was recovered by ethanol precipitation, and spread for measurement in the electron microscope. The percentage of total RNA is plotted as a function of molecular length in micrometers. The total additive value of the length of all molecules measured is 100%. (B) Histogram of length measurements on RSV RNA subunits. The data of (A) are plotted here to show the number of molecules occurring in each length class. (C) Histogram of length measurements on bacteriophage MS2 RNA. Bacteriophage MS2 RNA prepared as described by Fiers et al. (8) was purified by sucrose gradient centrifugation, and the RNA peak sedimenting at 30S was isolated. This RNA was spread for examination in the electron microscope, and the data obtained are expressed as for (B).*

with different spreading techniques performed at the time same time as ours report values of 2.8×10^6 (Delius et al., abstract, Cold Spring Harbor Symposium on Tumor Viruses, p. 42, 1974) and 3.3×10^6 (Kung et al., abstract, Cold Spring Harbor Symposium on Tumor Viruses, 1974) for the molecular weight of RSV RNA; these are reasonably close to the value we obtain ($3.12 \times 10^6 \pm 0.25 \times 10^6$).

Several comments must be made when discussing the results presented here and in comparing them with those from other laboratories. First, our value for the molecular weight of the 33S subunits of RSV RNA is based on that of 1.2×10^6 for MS2 RNA; since published figures for the latter range from 1.05×10^6 (9) to 1.3×10^6 (2), these differences must be noted in assessing our results and comparing them with those obtained with other RNA standards. Second, it is important to point out that the differential stretching of viral RNA molecules between different spreadings may be a further source of error; mixing the length marker RNA with the RNA to be measured would obviate this error, but this seems unwise, considering how little is known at present about the length distribution of tumor virus RNA. Third, our value for the molecular weight of RSV RNA subunits is an average of molecules ranging from 2.5 to 3.3 μm in length; such a range is that usually found in a homogeneous population of molecules (see Fig. 3C and ref. 15). In this respect, we should point out that our measurements were made on the molecules isolated from the whole subunit peak (Fig. 1B); measuring molecules taken from the leading edge of such a peak would be misleading. In addition, an essentially similar length distribution was obtained for RNA from the 45 to 50S peak of the gradient shown in Fig. 1B.

In all these studies of the molecular weight of tumor virus RNA, a major difficulty has been the preparation of intact RNA. The histogram of MS2 RNA (Fig. 3C) shows that the denaturing conditions used in spreading RNA for measurements in the electron microscope are unlikely to be a cause of the length heterogeneity in the RSV RNA preparations. Rather, we find that the presence of serum and other additives in the culture medium during virus release leads to significant degradation of the RNA;

for RSV prepared in the presence of complete medium, less than 1% of the total RNA is found in molecules of length greater than 2.5 μm as compared to 50% when the RNA is prepared from virus grown under the conditions detailed earlier. Clearly, more work is required to define conditions under which the integrity of tumor virus RNA is fully conserved.

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