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# Structure and Molecular Weight of the 60–70S RNA and the 30–40S RNA of the Rous Sarcoma Virus

**(RNA subunits/bacteriophage T4 gene-32 protein/RNA secondary structure/ RNA tumor virus infectivity/electron microscopy of RNA)**

WALTER F. MANGEL*, HAJO DELIUS†, AND PETER H. DUESBERG‡

* The Imperial Cancer Research Fund Laboratory, P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX, England; † Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York, 11724; and ‡ Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, Calif. 94720

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**ABSTRACT** **The structure and molecular weight of the 60–70S RNA complex and the 30–40S RNA species of Rous sarcoma virus were analyzed in an electron microscope after treatment of the RNAs with the bacteriophage T4 gene-32 protein to stretch out the RNA strands. Although all RNA preparations treated with gene-32 protein showed considerable heterogeneity in length, a significant fraction of the RNA retained its original sedimentation coefficient after treatment to allow the following conclusions to be made: The 30–40S RNA was confirmed to be a linear polynucleotide with a molecular weight of about $3 \times 10^6$. The 60–70S RNA exhibited a network structure with a molecular weight predominantly of about $6 \times 10^6$. Therefore, the subunit hypothesis for the 60–70S RNA is confirmed. A model for the structure and molecular weight of the 60–70S RNA postulates that the complex consists of two 30–40S RNA subunits held together at many points. This model elucidates the biological observation that the infectivity of RNA tumor viruses is proportional to the amount of 30–40S RNA in a virus preparation and not to the amount of 60–70S RNA.**

The structure and the molecular weight of tumor virus RNA have been difficult to study experimentally. The molecular weight is thought to range between a minimum of $3 \times 10^6$ and a maximum of $12 \times 10^6$ (1–10). This uncertainty is due mainly to the complex structure of the viral RNA (3) and to the difficulty in determining the exact content of RNA in purified virions (10). Extraction of the nucleic acid from virions followed by sedimentation reveals a major RNA species with a sedimentation coefficient of 60–70 S. When this RNA is placed in an environment wherein its secondary structure should be destroyed, the sedimentation coefficient decreases to 30–40 S and, at the same time, its electrophoretic mobility in sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels increases about 2-fold (11). The molecular weight of the 30–40S RNA species has been determined under denaturing conditions, and is about $3 \times 10^6$ (11). Thus, the 60–70S RNA was thought to be segmented, containing two, three, or four 30–40S RNA subunits held together by base-pairing involving short complementary sequences.

Although there is good, indirect, evidence for a subunit structure of the 60–70S RNA, nothing is known about how these subunits are linked. Linkage among 30–40S RNA subunits may be at only one site between two subunits, compatible with a relatively extended 60–70S RNA complex; or it may occur at several sites, compatible with a more compact, network-type 60–70S RNA complex. Furthermore, the available evidence does not exclude unequivocally that the conversion of tumor virus RNA from 60–70S to 30–40S may be due to an extreme conformational change rather than to a dissociation into subunits. Although there is no precedent for a conformational change that decreases the sedimentation coefficient but increases the electrophoretic mobility of nucleic acids, it was desirable to obtain independent evidence for the postulated subunit structure of the 60–70S RNA, to determine how many subunits it consists of and how these subunits are associated.

Here we report the use of the bacteriophage T4 gene-32 protein to mildly denature and to extend the 60–70S RNA and the 30–40S RNA such that, in conjunction with the protein monolayer spreading technique of Kleinschmidt (12), the structure of these RNA species can be seen in the electron microscope. Under these conditions, the length of an RNA molecule is proportional to its molecular weight (13). Although the structure of the genome of the RNA tumor virus has been studied by electron microscopy in other laboratories (2, 4, 5), the spreading techniques used either denatured the 60–70S RNA or rendered it in such a configuration as to make length measurements too difficult.

## MATERIALS AND METHODS

Tobacco mosaic virus (TMV) was a gift of C. A. Knight, University of California, Berkeley, and PM2 DNA was a gift from Loren Day, Public Health Research Institute, New York. Prague Rous sarcoma virus of subgroup B (PR RSV-B) and a recombinant between PR RSV of subgroup A and Rous associated virus (RAV-2) were obtained from P. K. Vogt, University of Southern California. Rous sarcoma virus (RSV) was propagated as described (14). Media harvested from infected cultures at 3- to 5-hr intervals were used for virus purification (3). After extraction of the nucleic acid from purified virions by phenol–$NaDodSO_4$ (8), the RNA was incubated in standard buffer [0.1 M NaCl, 0.01 M Tris·HCl (pH 7.2), 0.001 M EDTA] containing 0.1% $NaDodSO_4$ and 100 μg/ml of Pronase (predigested in standard buffer for 2 hr at 37°) for 30–60 min at room temperature. Subsequently the solution was extracted three times with phenol. It was then dissolved in standard buffer containing 0.1% $NaDodSO_4$, incubated at 40° for 5 min, and fractionated by

Abbreviations: TMV, tobacco mosaic virus; RSV, Rous sarcoma virus; $NaDodSO_4$, sodium dodecyl sulfate; PR RSV, Prague Rous sarcoma virus; RAV, Rous associated virus.

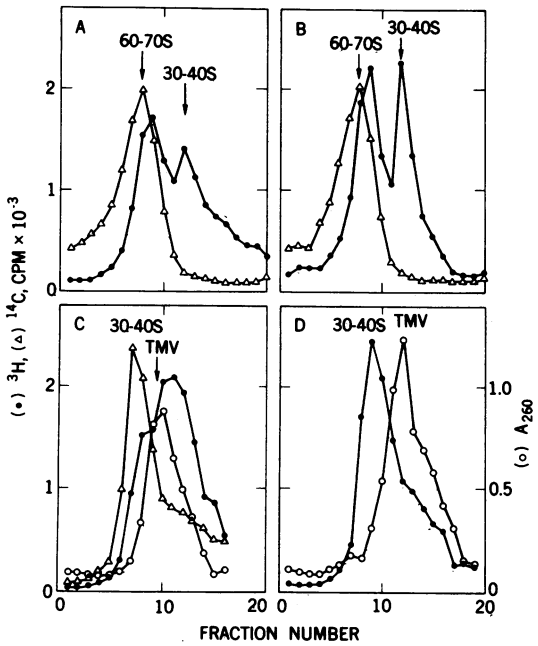


FIG. 1. Effects of an incubation with gene-32 protein on the subsequent sedimentation behavior of 60–70S RSV RNA and on 30–40S RSV RNA. (A) About 12,000 cpm of 60–70S RSV [$^3$H]-RNA in 10 μl of 0.01 M Tris·HCl (pH 7.4), 0.001 M EDTA were mixed with about 1 μg of gene-32 protein in 2 μl of phosphate buffer (*Materials and Methods*). The mixture was then incubated at 38° for 20 min. Afterward, the following were sequentially added: 200 μl of standard buffer (*Materials and Methods*) containing 0.1% NaDodSO$_4$, 30 μg of Pronase in 3 μl of standard buffer (predigested in standard buffer for 2 hr at 37°), and some 60–70S RSV [$^{14}$C]RNA for a sedimentation marker. The mixture was again incubated for 20 min at 38° and then layered on a linear, 15–30% glycerol gradient containing standard buffer and 0.1% NaDodSO$_4$. Sedimentation was in a Spinco SW50.1 rotor at 49,000 rpm for 60 min at 20°. (B) The 60–70S RSV RNA was treated as in (A) but without gene-32 protein. (C) To obtain 30–40S RSV RNA, 60–70S RSV [$^3$H]RNA was heated at 100° for 30 sec in low-salt buffer containing NaDodSO$_4$ (8). The RNA was then precipitated with ethanol and redissolved in low-salt buffer as described in (A). About 10,000 cpm of [$^3$H]RNA in 10 μl were then incubated with about 1 μg of gene-32 protein, as described in (A). After the addition of 200 μl of standard buffer and 30 μg of Pronase as described above, 40 μg of TMV RNA and 6000 cpm of heat-dissociated, 60–70S RSV [$^{14}$C]RNA were added as sedimentation markers. The RNAs were sedimented for 105 min, as described in (A). (D) The 30–40S RNA was treated as in (C) but without gene-32 protein. No heat-dissociated, 60–70S RSV [$^{14}$C]RNA was added as marker.

glycerol gradient sedimentation as described in the legend of Fig. 1. Preparation of 30–40S RNA is also described in the legend of Fig. 1. The electron microscopy, preparation of bacteriophage T4 gene-32 protein, and the techniques for length measurements have been described (13). Preparation of the RNA for analysis in the electron microscope is described in the legend of Fig. 2.

## RESULTS

*Effect of the Bacteriophage T4 Gene-32 Protein on the Sedimentation Properties of the 60–70S RNA and the 30–40S RNA of RSV.* The gene-32 protein binds cooperatively to single-stranded nucleic acids and is capable of partially unwinding double-helical nucleic acid structures (13). Thus, the possibility existed that the presence of the gene-32 protein would result in the dissociation of those base-paired regions in the 60–70S RNA complex that presumably hold the putative 30–40S RNA subunits together. Therefore, the effects of the gene-32 protein on the sedimentation properties of the two species of RNA were assessed.

Incubation of sedimentation-purified 60–70S RNA with gene-32 protein at low ionic strength and 38° did not significantly alter the sedimentation distribution of the RNA after removal of the gene-32 protein by Pronase digestion (Fig. 1a) when compared to a control treated identically but without gene-32 protein (Fig. 1b). The only difference was more trailing in the RNA that had been incubated with gene-32 protein (fractions 14–20 of Fig. 1a). Both 60–70S RNA samples were partially melted upon incubation at low ionic strength and 38°. This is evident from the slight shift of the 60–70S RNA peak, compared to a 60–70S RNA marker added after the incubations, and from the appearance of some 30–40S RNA. Because the melting temperature for the conversion of 60–70S RNA to 30–40S RNA is about 46°, the partial melting of the 60–70S RNA under the conditions used for analysis in the electron microscope was not unexpected (11, 15).

When purified 60–70S RNA is heated to obtain 30–40S RNA and then incubated with gene-32 protein, the resultant sedimentation profile is different compared to a control treated identically but in the absence of gene-32 protein. The heated 60–70S RNA incubated with gene-32 protein sedimented more slowly, after removal of the protein, than a 31S TMV RNA marker (16–18) (Fig. 1c), whereas the peak of the 30–40S RNA control, which was not exposed to gene-32 protein, sedimented faster (Fig. 1d). About 20% of the heated 60–70S RNA that was incubated with gene-32 protein sedimented with a 30–40S RNA marker, which was added after the incubation (fractions 6–8 of Fig. 10), while about 50% of the control sedimented at 30–40S (fractions 7–10 of Fig. 1d). Thus, compared to a control treated identically but without gene-32 protein, more than half of the resultant 30–40S RNA from heated 60 to 70S RNA seemed to be degraded.

We conclude that the presence of the bacteriophage T4 gene-32 protein under the conditions used for analysis in an electron microscope does affect the sedimentation properties of the 60–70S RNA and of the 30–40S RNA, but does so differentially. Besides being partially melted, some 60–70S RNA is slightly degraded. Conversely, the sedimentation coefficient of most of the 30–40S RNA is reduced significantly after incubation with the gene-32 protein. In each case, however, a portion of the original 60–70S RNA and the 30–40S RNA retained its sedimentation coefficient after treatment; consequently the upper limit in the length distribution, as measured in an electron microscope, can be correlated to a hydrodynamic property.

*Length Measurements of 30–40S RSV RNA.* The 30–40S RNA species, in the presence of the gene-32 protein, is a linear molecule (Fig. 2a). Each molecule has two ends and appears to be fully coated with gene-32 protein. In the absence of gene-32 protein (Fig. 2b), the 30–40S RNA molecules exhibit the high degree of secondary structure observed with most single-stranded RNA molecules under these conditions (13). This pattern of loops and branches probably arises

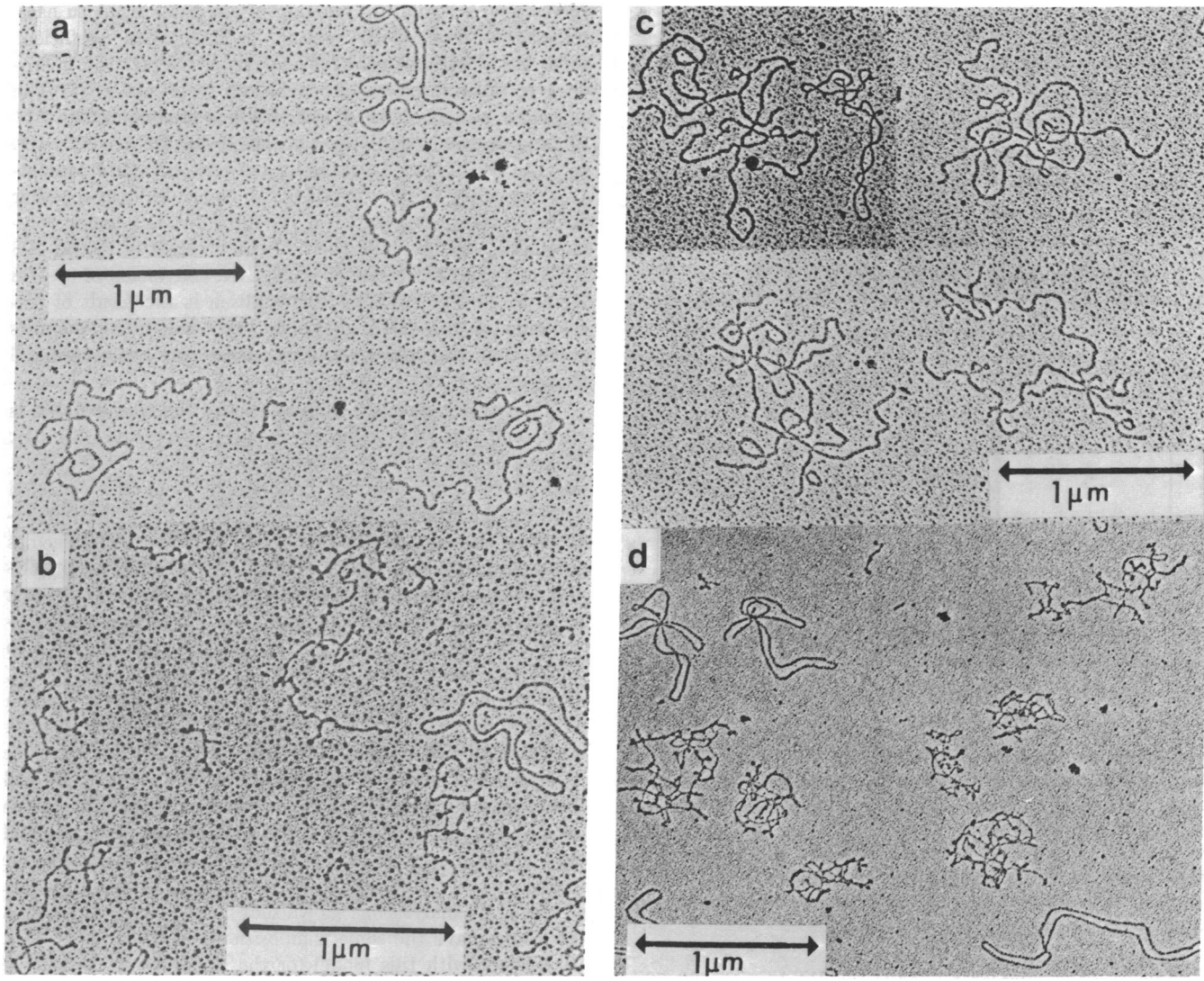


FIG. 2. Electron micrographs of RSV RNA. (a) 30–40S RNA–bacteriophage T4 gene-32 protein complexes; (b) 30–40S RNA without gene-32 protein; (c) 60–70S RNA–bacteriophage T4 gene-32 protein complexes; and (d) 60–70S RNA without gene-32 protein. For preparation of RNA–bacteriophage T4 gene-32 protein complexes, RNA (500 μg/ml) was diluted 10-fold into 0.1 M NaCl, 0.01 M Tris·HCl (pH 8), and 0.001 M EDTA and incubated for 5 min at 40°. One microliter of this RNA solution was then mixed with a 9-μl solution of 0.01 M potassium phosphate, 0.001 M EDTA (pH 7), containing 120 μg/ml of bacteriophage T4 gene-32 protein. After an incubation of 10 min at 37°, 1 μl of 1% glutaraldehyde was added. Incubation was continued for another 15 min at 37°, in order to fix the protein to the RNA. Preparation of RNA–bacteriophage T4 gene-32 protein complexes or RNA for electron microscopy, using the cytochrome spreading technique (12), was done as described (13). PM2 DNA was mixed with samples after glutaraldehyde fixation to provide an internal length standard. Pictures were taken with a Siemens Elmiskop 101 at magnifications of 4000–8000 at 40 kV.

from the base-pairing of more or less complementary regions in the RNA which, in the presence of the gene-32 protein, become stretched out into a linear configuration.

All of the 30–40S RNA preparations, whether treated with gene-32 protein or not, showed a rather large content of short RNA fragments. A histogram of a representative preparation is shown in Fig. 3a. The RNA found in the position presumably corresponding to intact 30–40S RNA, i.e., the upper length limit, lay between 2.8 μm and 3.5 or 4.0 μm (Fig. 3a and Table 1). Based upon the length of the circular, double-stranded DNA, PM2, which has a molecular weight of $6.4 \times 10^6$ (19) and is present as an internal length standard, the maximal molecular weight of the 30–40S RNA in two different RNA preparations is 2.76 and $2.73 \times 10^6$ (Table 1). These data for the limit molecular weight are in excellent agreement with the molecular weight of 30–40S RNA that was determined under denaturing conditions (8, 11) and by complexity analysis (20).

*Length Measurements of the 60–70S RSV RNA.* Representative electron micrographs of the 60–70 RNA appear in Fig. 2c. In most cases, the 60–70S RNA–gene-32 protein complexes appear to consist of several linear molecules held together at many different points. There are between four and eight free ends per molecule (6.4 ± 2 ends as averaged from 30 molecules). Most of the RNA is coated with gene-32 protein, consequently extensive double-stranded regions are not found. Since double-stranded nucleic acids do not bind gene-32 protein, those regions would appear thinner than the single-stranded, protein-coated regions. For example, the double-stranded, circular DNA marker, PM2, is not as thick

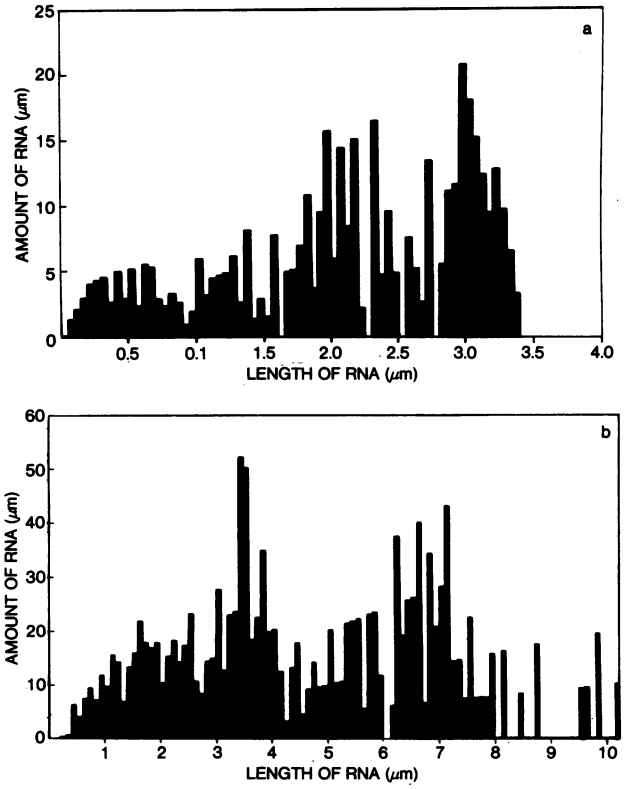


FIG. 3. Histograms of (a) 30–40S RNA–bacteriophage T4 gene-32 protein complexes and (b) 60–70S RNA–bacteriophage T4 gene-32 protein complexes. The amount of RNA (μm) is the sum of the lengths of all the RNA molecules measured that have the lengths indicated on the abscissa. Length measurements were made on prints after a 5- to 10-fold photographic enlargement, using an X-Y measuring stage connected to a Wang calculator. The length increments in (a) are 0.05 μm, and in (b) 0.1 μm.

in these electron micrographs as is the RNA in the 60–70S RNA–protein complexes. A representative electron micrograph of 60–70S RNA that had not been treated with gene-32 protein is shown in Fig. 2d. Here also the RNA appears to be an aggregate of several molecules, but now exhibiting extensive secondary structure, which prevents the recognition of ends and makes reliable length measurements impossible.

To determine the mass of the 60–70S RNA complexes, we measured the total length of the RNA chains in each RNA–gene-32 protein complex. A representative histogram of 60–70S RNA appears in Fig. 3b. The upper length limit was between 6 and 8 μm, comprising 28% by weight of the total RNA (Table 1). The average length was 6.8 μm, which corresponds to a molecular weight for the 60–70S RNA of $6.1 \times 10^6$. A few of the complexes (5 out of 400) were 9 μm in length. There is a second peak in the length distribution, between 2.8 and 4.2 μm (Fig. 2b). This corresponds to the upper length limit distribution of 30–40S RNA (Table 1). Approximately 66% of these molecules are 30–40S RNA molecules, i.e., a linear molecule with two free ends or a 30–40S RNA molecule with a small molecule attached to it.

## DISCUSSION

Although none of our 60–70S RNA and 30–40S RNA preparations appeared to be homogeneous when seen in the electron microscope, a significant fraction of the total RNA in each preparation retained its original sedimentation coefficient under the conditions used for analysis in the electron microscope to allow three major conclusions to be made: (*i*) The molecular weight of the 60–70S RNA is predominately $6 \times 10^6$, and the molecular weight of the 30–40S RNA is $3 \times 10^6$. (*ii*) The 60–70S RNA exhibits a network structure presumably consisting of two 30–40S RNA molecules held together at many different points. The 30–40S RNA is a linear polynucleotide. (*iii*) Because the molecular weight of the 60–70S RNA is twice that of the 30–40S RNA, conversion of 60–70S RNA to 30–40S RNA by denaturation is the result of dissociation of the RNA molecules into individual subunits rather than the consequence of an extreme conformational change.

The network structure we observe for the 60–70S RNA is consistent with the following earlier observations (21–24): Virus collected at 12- to 24-hr intervals yields a 60–70S RNA peak that is very similar to that obtained from virus collected at 3- to 5-hr intervals. Heating the 60–70S RNA of 12- to 24-hr virus, however, results in a wide distribution of molecules sedimenting between zero and 40 S; whereas heating the 60–70S RNA of virus collected at 3- to 5-hr intervals yields a relatively sharp peak of 30–40S RNA. This may be interpreted by assuming a network structure for 60–70S RNA in which the two subunits are linked together at many points. One unique property of such a structure is that a random nick will not, in most cases, change the mass of the network although two free ends will be created. When that network is dissociated, however, the mass of the individual units will be decreased in proportion to the number of nicks. Thus, the origin of the RNA molecules that sediment between zero and 40 S upon the heating of 12- to 24-hr virus 60–70S RNA is the nicking of the RNA within the virion prior to its extraction. Fingerprint analysis has shown that most of these small RNA molecules have the same composition as 30–40S RNA (20). Consistent with this model for the structure of the 60–70S RNA is the biological observation that the infectivity of RNA tumor viruses is proportional to the amount of 30–40S RNA obtainable from a virus preparation and not necessarily proportional to the amount of 60–70S RNA (22).

The forces that hold the network structure of the 60–70S RNA together are unknown. Certainly, these forces are resistant to $NaDodSO_4$ and Pronase, yet sensitive to the application of heat or the presence of dimethyl sulfoxide. For these reasons, they were postulated to be hydrogen bonds (11). Because reassociation to 60–70S RNA from 30 to 40S RNA has not been observed experimentally, even under optimal reannealing conditions, the extent of double-strandedness in 60–70S RNA at any point must be small. We could have observed, in these electron micrographs, contiguous regions of double-strandedness of approximately 100 base pairs. Such lack of extensive double-strandedness is consistent with the low melting temperature and high salt dependence for the transition of 60–70S RNA to 30–40S RNA (11, 22).

The 60–70S RNA of RNA tumor viruses appears to be polyploid, i.e., the primary sequence of bases in each subunit is the same. Fingerprint analysis of the unique oligonucleotides produced after ribonuclease T1 digestion of 60–70S RNA yields a unique sequence molecular weight of $2.7 \times 10^6$ (20). Our result that 60–70S tumor virus RNA has a mass

TABLE 1. *Length measurements of bacteriophage T4 gene-32 protein–RNA complexes*

| | Total length measured (μm) | Limits of RNA (μm) | Fraction of RNA in limits | Mean length* (μm) | Molecular weight* ($10^{-6}$) |
|---|---|---|---|---|---|
| Rous 30–40S RNA | | | | | |
| 1st determination | 450 | 2.80–3.45 | 0.25 | 3.13 ± 0.15 | 2.76 ± 0.13 |
| 2nd determination | 441 | 2.80–4.00 | 0.32 | 3.12 ± 0.15 | 2.73 ± 0.13 |
| Rous 60–70S RNA | | | | | |
| 60–70S RNA | 1374 | 6.00–8.00 | 0.28 | 6.89 ± 0.48 | 6.18 ± 0.43 |
| 30–40S RNA† | 1374 | 2.80–4.20 | 0.24 | 3.48 ± 0.36 | 3.12 ± 0.12 |

The total length measured is the sum of the lengths of all the RNA molecules measured in each determination. The mean length and molecular weight were determined on those molecules whose lengths were measured within specific limits (limits of RNA), relative to the length measured of PM2 DNA, which was corrected to 3.45 μm. The fraction of RNA in limits is defined as the sum of the lengths of RNA within the limits divided by the total length of RNA measured. A molecular weight-to-length ratio of 870,000/μm was calculated for the RNA as seen in the electron microscope after the coating and extension of the RNA by gene-32 protein and preparation by the Kleinschmidt technique (13). The double-stranded DNA of phage PM2 was used as an internal length standard. Since gene 32 does not bind to double-stranded nucleic acids, the length of the DNA standard compared with the length of the RNA–gene-32 protein complex might vary in a range of up to 10% (13).

* Values are ± SEM.

† The 30–40S RNA region from the histogram of 60–70S RNA (see Fig. 3).

of predominately $6 \times 10^6$ daltons is compatible with the view that the complexity of the RNA is about $3 \times 10^6$ and that most 60–70S RNA molecules contain two identical 30–40S RNA subunits. If distinct patterns of secondary structure (25) can be detected in our 30–40S RNA, it should be possible to determine independently, by analysis in the electron microscope, the ploidy of the RNA tumor virus genome.

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