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Role of Subunits of 60 to 70S Avian Tumor Virus Ribonucleic Acid in Its Template Activity for the Viral Deoxyribonucleic Acid Polymerase

ELI CANAANI AND PETER DUESBERG

Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720

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Heating the 60 to 70S ribonucleic acid (RNA) of Rous sarcoma virus (RSV) destroys both its subunit structure and its high template activity for RSV deoxyribonucleic acid (DNA) polymerase. In comparative analyses, it was found that the template activity of the RNA has a thermal transition of 70 C, whereas the 60 to 70S structure dissociates into 30 to 40S and several distinct small subunits with a T_m of 55 C. Analysis by velocity sedimentation and isopycnic centrifugation of the primary DNA product obtained by incubation of 60 to 70S RSV RNA with RSV DNA polymerase indicated that most, but perhaps not all, DNA was linked to small (<10S) RSV RNA primer. Sixty percent of the high template activity of 60 to 70S RSV RNA lost after heat dissociation could be recovered by incubation of the total RNA under annealing conditions. The template activity of purified 30 to 40S subunits isolated from 60 to 70S RSV RNA was not enhanced significantly by annealing. However, in the presence of small (<10S) subunits also isolated from 60 to 70S RNA, the template activity of 30 to 40S RNA subunits was increased to the same level as that of reannealed total 60 to 70S RNA. It was concluded that neither the 30 to 40S subunits nor most of the 4S subunits of 60 to 70S RSV RNA contribute much as primers to the template activity of 60 to 70S RSV RNA. The predominant primer molecule appears to be a minor component of the <10S subunit fraction of 60 to 70S RSV RNA. Its electrophoretic mobility is similar to, and its dissociation temperature from 60 to 70S RSV RNA is higher than that of the bulk of 60 to 70S RSV RNA-associated 4S RNA. The role of primers in DNA synthesis by RSV DNA polymerase is discussed.

The 60 to 70S ribonucleic acid (RNA) complexes of all known RNA tumor viruses have a subunit structure which appears to be composed of 30 to 40S RNA components representing about 80 to 90% of the mass of 60 to 70S Rous sarcoma virus (RSV) RNA (5, 13, 18). In addition, 60 to 70S avian tumor virus RNA contains heterogeneous small RNA molecules (6). Among these small RNA molecules, a 4S species has recently been shown by Erikson (12) to be a predominant component. Further, it was observed that the 60 to 70S RNA of avian tumor viruses has among all natural RNA species tested the highest template activity for the virus-associated deoxyribonucleic acid (DNA) polymerase (9, 10, 17; M. Bishop, *personal communication*). Both the subunit structure and the high template activity of the viral RNA for the viral DNA polymerase are lost after heat dissociation (9, 17). To explain the high template activity of 60 to 70S RSV RNA, it was suggested that one RNA subunit serves as template while another subunit, linked to it by limited base pairing, serves as a primer for the DNA polymerase (10). (All RNA species of the 60 to 70S RSV RNA complex are operationally referred to as subunits in this report.) We interpreted the loss of template activity of the viral RNA upon heating as due to dissociation of the primer molecules from the template subunits (10).

The present study was undertaken to determine which, if any, of the RNA subunits act as primers responsible for the high template activity of 60 to 70S RSV RNA. In addition, our preliminary evidence (10) and the evidence of others (17, 21) that primer RSV RNA is covalently linked to DNA produced by RSV DNA polymerase was further investigated. Finally, it was asked whether the thermal transition of the high template activity of 60 to 70S RNA for the viral DNA polymerase was reversible.

MATERIALS AND METHODS

Solutions and chemicals. Standard buffer contained 0.1 M NaCl, 1 mM ethylenediaminetetraacetate (EDTA), and 0.01 M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.4; low-salt buffer contained 0.01 M NaCl, 1 mM EDTA, and 0.01 M Tris, *p*H 7.4. Deoxyribonuclease buffer contained 6 mM $MgCl_2$ and 0.06 M Tris, *p*H 8.0. ^{3}H-deoxythymidine triphosphate (^{3}H-dTTP) and ^{3}H-deoxycytidine triphosphate (^{3}H-dCTP), 15 to 20 Ci/mmole, and ^{3}H-uridine, 40 Ci/mmole, were purchased from New England Nuclear Corp. $H_3{}^{32}PO_4$ was purchased from Schwartz BioResearch, Inc.

Virus. Prague RSV of subgroup C (PR RSV-C) was used in all experiments. Virus growth and purification followed published procedures (6). Medium was collected from virus-producing cultures every 3 to 5 hr for preparation of RNA, because it has been shown previously that the integrity of subunits of 60 to 70*S* RSV RNA decreases with increasing incubation periods of the virus in the growth medium at 38 to 40 C (1, 8, 13). To grow ^{3}H-RNA virus, 50 to 100 μCi of ^{3}H-uridine was added to virus-producing cultures in 6 ml of medium 199 supplemented with 2% tryptose phosphate, 1% calf serum, 1% chicken serum, 1% dimethylsulfoxide (DMSO), and 0.5 μg of amphotericin B per ml. The medium was collected three times every 3 to 5 hr. This labeling procedure was repeated twice. $H_3{}^{32}PO_4$ labeling was performed in a similar way with 1 mCi of ^{32}P in 6 ml of phosphate-free medium supplemented with 1% dialyzed calf serum, 1% dialyzed chicken serum, 1% DMSO, 0.5 μg of amphotericin B per ml, and 0.2% $NaHCO_3$.

RNA. RNA was prepared by the phenol-sodium dodecyl sulfate (SDS) method (5). To free viral RNA from residual ribonuclease, detectable during hybridizations, it was dissolved in 3 ml of standard buffer containing 0.2% SDS and incubated with 30 μg of predigested Pronase per ml for 45 min at room temperature. The RNA was then phenol-extracted three times, precipitated twice with ethanol, and dissolved in 0.2 ml of standard buffer. It was then diluted to 4 ml with deoxyribonuclease buffer and digested with 20 μg of deoxyribonuclease I free from ribonuclease (Worthington Biochemical Corp.) per ml for 30 min at room temperature. Ribonuclease encountered in some preparations of deoxyribonuclease I was inactivated by treatment with iodoacetate (22). After three more phenol extractions, the 60 to 70*S* RNA was prepared by sucrose gradient sedimentation. Preparations of 4*S* RNA from chick embryo fibroblasts were twice treated with deoxyribonuclease and fractionated by sucrose gradient sedimentation.

In vitro synthesis of DNA. Conditions of the reaction and partial purification of the PR RSV-C DNA polymerase by zone sedimentation have been described (9). The standard DNA polymerase assay described previously (10) was used unless stated otherwise, except for the concentration of ^{3}H-dNTP which was 2.5 μM.

RESULTS

Thermal transition of the 60 to 70S structure and of the high template activity of RSV RNA for

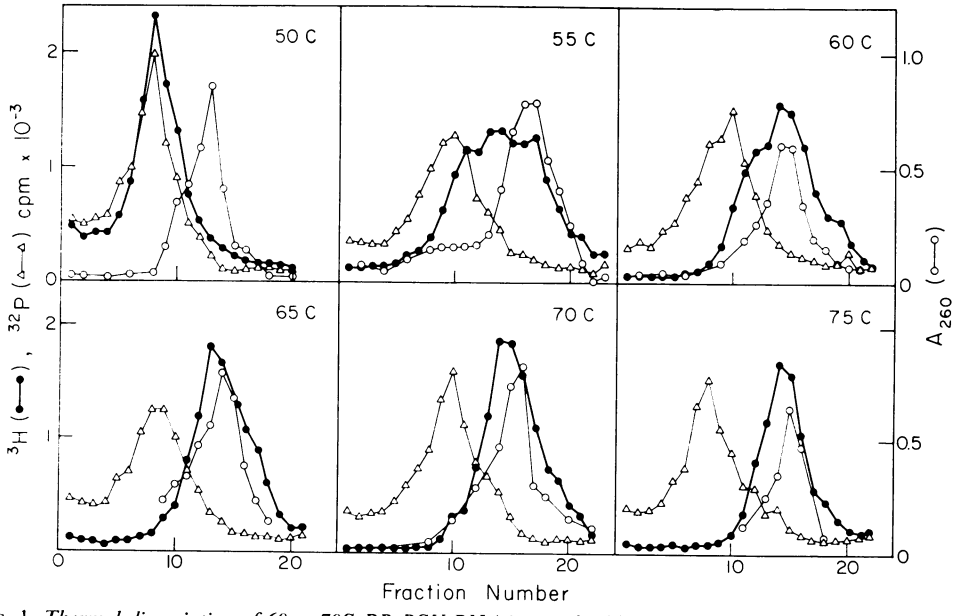

FIG. 1. *Thermal dissociation of 60 to 70S PR RSV RNA in standard buffer (Materials and Methods). Thermal transition of the 60 to 70S structure was determined by incubating equal portions of 60 to 70S ^{3}H-RSV RNA in standard buffer containing 0.1% sodium dodecyl sulfate for 3 min at a given temperature in a sealed ampoule. The RNA was then cooled in melting ice and mixed with unheated 60 to 70S ^{32}P-PR RSV RNA and tobacco mosaic virus RNA. The mixture was analyzed by sedimentation in a sucrose gradient (5 to 20%, w/v) in standard buffer containing 0.1% sodium dodecyl sulfate for 40 min at 20 C in a Spinco SW 65 rotor. Analysis of the gradient was as described previously (9).*

the viral DNA polymerase. The thermal transition of the 60 to 70*S* RNA structure is shown in Fig. 1. It was determined by exposing samples of 60 to 70*S* ^{3}H-RSV RNA in 0.1 M NaCl to various temperatures for 3 min. These samples were subsequently analyzed by sucrose gradient sedimentation. The percentage of ^{3}H-RSV RNA heated to a given temperature that coincided with a 60 to 70*S* ^{32}P-RSV RNA marker is plotted in Fig. 2. The midpoint of the thermal transition (T_m) of the 60 to 70*S* form of RSV RNA to 30 to 40*S* subunits and RNA molecules of smaller size was at about 55 C.

The transition of the template activity of 60 to 70*S* RSV RNA was determined by taking samples of viral RNA exposed to various temperatures and incubating this RNA with RSV DNA polymerase as described previously (10). Figure 2 shows that the transition profile of the template activity of 60 to 70*S* RSV RNA with heat was broader and about 15 C higher than the T_m of its 60 to 70*S* structure. At a temperature of 70 C, at which all of the 60 to 70*S* RNA was already converted to RNA forms with lower sedimentation coefficients, about 50% of the template activity was retained. We conclude, therefore, that linkage among the major 30 to 40*S* subunits of 60 to 70*S* RSV RNA contributes little to the high template activity of the viral RNA for RSV DNA polymerase. Since heating beyond the T_m of 60 to 70*S* structure further reduced the template activity of the viral RNA without detectably further reducing its sedimentation coefficient, it appears likely that this loss of template activity reflects the dissociation of intermolecular bonds between individual 30 to 40*S* subunits and small RNA subunits. This hypothesis is based on the assumption that most or all intramolecular secondary structure of single-stranded RNA lost after heating is recovered instantly at room temperature (19) and would not be measured in the assay.

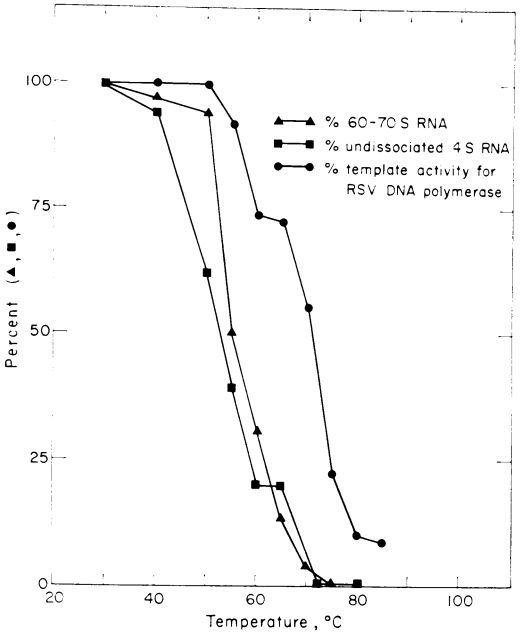

FIG. 2. *Comparisons of the thermal transitions of the subunit structure and the template activity for RSV DNA polymerase of 60 to 70S RSV RNA. Thermal transition of the 60 to 70S structure of RSV RNA was performed as described for Fig. 1. The amount of ^{3}H-RSV RNA of a given heated sample that coincided with the 60 to 70S ^{32}P-RSV RNA marker was compared with that of an unheated sample to determine the percentage of surviving 60 to 70S structure. Thermal transition of the template activity of 60 to 70S RSV RNA was determined by incubating 20-μliter samples which had been heated to various temperatures in standard buffer, in a standard RSV DNA polymerase assay (10). Thermal dissociation of the 4S subunit species of 60 to 70S RSV DNA was determined by gel electrophoresis of heated 10-μliter samples as described for Fig. 3.*

To test the possibility that small RNA subunits of 60 to 70*S* RSV RNA, perhaps the 4*S* species described by Erikson (12), are responsible for the high template activity of 60 to 70*S* RSV RNA, we have analyzed the melting profile of the 4*S* RNA species (Fig. 2). Dissociation of the 4*S* RNA species from the 60 to 70*S* RSV RNA complex was monitored by polyacrylamide gel electrophoresis of RNA samples heated to various temperatures. Examples of this analysis can be seen in Fig. 3, which shows the electrophoretic distribution of 60 to 70*S* RNA after incubation at 40, 65, and 73 C. Three very small RNA species, termed 1, 2, and 3 (Fig. 3), were resolved. In addition, larger heterogeneous species were observed which penetrated the 6% polyacrylamide gel (Fig. 3). The 4*S* species (12) is presumably the RNA termed 1 in Fig. 3. Dissociation of RNA species 2 and 3 was complete, and dissociation of RNA species 1 was about 80%, at a temperature of 65 C. By contrast, 75% of the template activity survived heating to 65 C. We conclude that most of the high template activity of 60 to 70*S* RSV RNA is due either to 20% of the 4*S* subunit species or to unidentified primer molecules.

Size range of the primer RNA of 60 to 70S RSV RNA linked to the DNA produced by the viral DNA polymerase. It has been shown that the DNA polymerase of RSV can synthesize DNA by adding deoxynucleotides to the 3′ OH end of DNA or RNA primers complementary to the template (2, 15, 17, 21). DNA produced by the viral DNA polymerase with endogenous viral RNA as template is small, about 6*S* (7), after KOH or ribonuclease treatment. If 30 to 40*S*

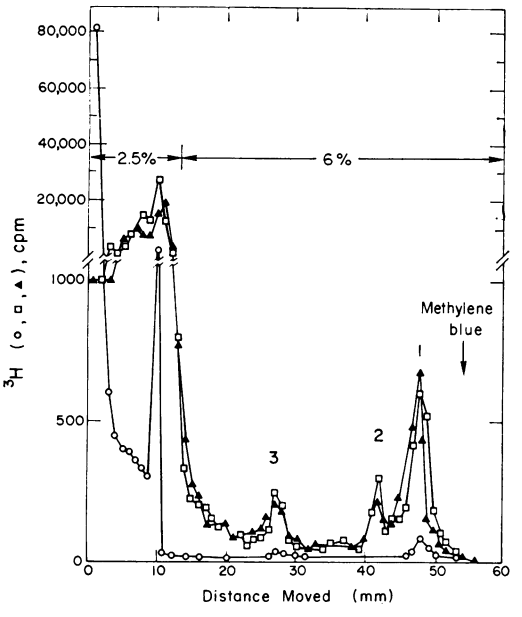

FIG. 3. *Polyacrylamide gel electrophoresis of ^{3}H-labeled 60 to 70S RSV RNA heated to 40 C (○), 65 C (□), and 73 C (▲) in standard buffer containing 0.2% SDS. Samples (10 μliters) were incubated in sealed ampoules for 3 min at the temperatures indicated. Subsequently, they were cooled in melting ice and mixed with 20 μliters of electrophoresis sample buffer (5), and were analyzed by gel electrophoresis as described by Erikson (12) with the use of the buffer systems described previously (11). Electrophoresis was for 2 hr at 15 v/cm until a methylene blue marker reached the bottom of the gel. After electrophoresis, gels were sliced with stacked razor blades. Radioactivity of slices was determined after incubating each slice with 0.9 ml of NCS (Nuclear-Chicago Corp.) and 0.1 ml of water for 2 hr at 60 C and subsequent addition of 10 ml of toluene-based scintillation fluid in a Tri-Carb liquid scintillation counter.*

RNA subunits serve as a primer, the primary DNA product, still linked to such RNA, should have a high sedimentation coefficient. Alternatively, if a small RNA serves as primer, the DNA product should have a low sedimentation coefficient if the DNA remains linked to the RNA primer.

The experiment depicted in Fig. 4 shows that, after a 15-min reaction of the partially purified viral DNA polymerase with 60 to 70S ^{32}P-RSV RNA as template, most of the DNA product sediments at 3 to $5S_{w,\ 1.1MCH_2O}$ after denaturation in 1.1 M formaldehyde. [We would like to recall that the sedimentation coefficient of nucleic acid is reduced after reaction with formaldehyde (3).] The sedimentation coefficient of a small fraction of the DNA, however, extended to the 7 to $10S_{w,\ 1.1MCH_2O}$ range based on a $17S_{w,\ 1.1MCH_2O}$ (3) tobacco mosaic virus (TMV) RNA marker (Fig. 4A). Most of the 60 to 70S ^{32}P-RSV RNA template sedimented as a sharp peak slightly faster than TMV RNA after reaction with formaldehyde (not shown). After incubation with the enzyme, however, the ^{32}P-RSV RNA sedimented more slowly than TMV RNA but farther than the DNA product (Fig. 4A). This indicates the presence of ribonuclease activity in our enzyme preparation. After incubation with KOH, all DNA produced by the enzyme had a sedimentation coefficient of 2 to $4S_{w,\ 1.1MCH_2O}$, which was slightly lower than the sedimentation coefficient of untreated DNA product. Essentially all ^{32}P-RSV RNA template was solubilized by KOH (Fig. 4B). The average buoyant density in Cs_2SO_4 containing 1.1 M CH_2O of the untreated primary DNA product was about 1.51 g/ml. This is significantly higher than that of KOH-treated DNA product or marker DNA, which banded at about 1.46 g/ml (7). However, a fraction of the primary DNA product had a density which was only little higher than that of a salmon DNA marker.

These results are compatible with the view that at least some DNA produced by the enzyme is covalently linked to RNA primer. However, since most of the RNA-linked DNA product appeared to have a relatively low sedimentation coefficient (3 to $5S_{w,\ 1.1MCH_2O}$) and a buoyant density which was significantly lower than that of RNA (1.64 g/ml), it seems plausible that mainly small RNA molecules had served as primers for DNA synthesis. The argument that the small RNA primers derived from randomly acting ribonuclease degradation of large RNA is considered unlikely, because the bulk of the viral RNA was only slightly degraded by the enzyme (Fig. 4).

Recovery of high template activity of heat-dissociated RSV RNA for RSV DNA polymerase by incubation of RSV RNA subunits under annealing conditions. To determine whether the 10-fold thermal reduction of the high template activity of 60 to 70S RSV RNA for viral DNA polymerase (Table 1, experiments 1 and 2) can be reversed, the annealing experiments listed in Table 1 were carried out. About 60% (experiment 5) of the template activity of native (experiment 1) 60 to 70S RNA was recovered by incubation under annealing conditions.

Purified 30 to 40S subunits, obtained by sucrose gradient sedimentation of heat-dissociated 60 to 70S RNA (Fig. 5), had only 10% of the template activity (experiment 3) and after annealing not more than 15 to 20% (experiment 6) of the template activity of native 60 to 70S RSV RNA. The slowly sedimenting <10S subunits obtained by sucrose gradient fractionation of heat-dissoci-

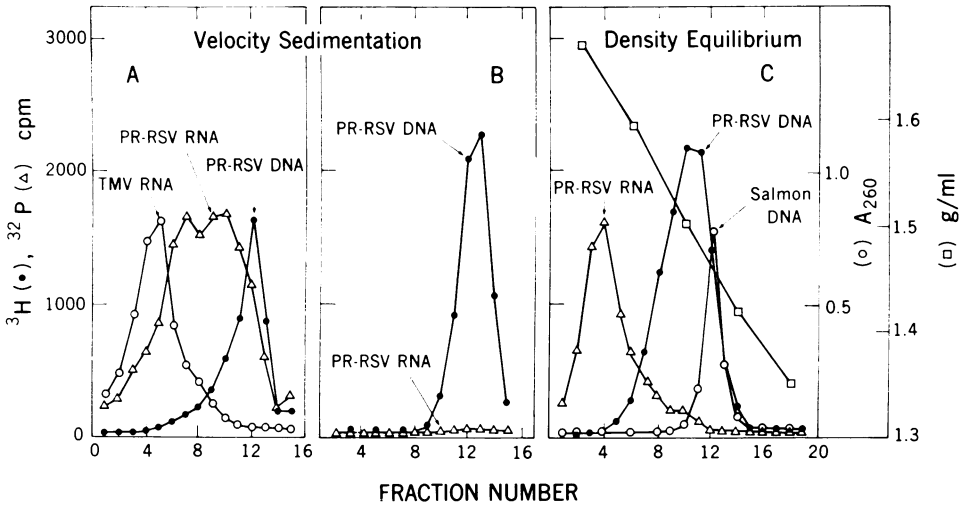

FIG. 4. *Analysis of the 60 to 70S ^{32}P-RSV RNA template and the ^{3}H-DNA produced after a 15-min reaction with the RSV DNA polymerase. The 60 to 70S ^{32}P-RSV RNA was prepared (Materials and Methods) from virus which had been released in the growth medium of an infected culture during 3-hr intervals (Materials and Methods). The RNA was placed in 400 μliters of a standard polymerase assay solution (10) in which the concentration of ^{3}H-dCTP was 10 μM. After 15 min at 40 C, the nucleic acids were isolated by the phenol-SDS method (Materials and Methods) and redissolved in 600 μliters of buffer containing 0.1 M NaCl, 0.02 M EDTA (pH 7.0), and 0.1% SDS. Three equal portions were prepared. The first (Fig. 4A) was mixed with about 60 μg of TMV RNA and one-tenth volume of 37% CH_2O, and was heated in a sealed ampoule for 15 min at 63 C. Thereafter, the RNA was layered on a 5 to 20% (w/v) sucrose gradient containing the same buffer and concentration of CH_2O as the sample. Centrifugation was for 7 hr in a Spinco SW 65 rotor at 46,000 rev/min at 20 C. After fractionation, the absorbance at 260 nm was determined. The radioactivity was measured by placing an appropriate sample in a solution containing 20% NCS (Nuclear-Chicago Corp.) and 80% toluene-based scintillation fluid and by counting in a Tri-Carb liquid scintillation counter. The second sample (Fig. 4B) was incubated in 0.3 N KOH at 60 C for 30 min, and after neutralization with 1 N acetic acid it was analyzed as described for Fig. 4A. The third sample was diluted with 1.5 ml of buffer (described above) containing 3.7% CH_2O and mixed with 60 μg of salmon DNA. It was then treated as described for Fig. 4A and mixed with about 1.4 ml of a saturated solution of Cs_2SO_4 containing 3.7% CH_2O to attain a final density of about 1.5 g/ml. The solution was centrifuged in a Spinco SW 50.1 rotor at 33,000 rev/min for 62 hr at 20 C. After fractionation, solution density was determined by weighing 100-μliter portions. Absorbance at 260 nm was measured as described for Fig. 1. Trichloroacetic acid-precipitable radioactivity was measured after the addition of 100 μg of yeast RNA and an equal volume of 10% trichloroacetic acid. The precipitate was washed on membrane filters (Millipore Corp.), and after drying the filters were placed in toluene-based scintillation fluid and counted in a Tri-Carb liquid scintillation counter.*

ated 60 to 70S RSV RNA (Fig. 5) had only <10% of the template activity of 60 to 70S RSV RNA with or without prior incubation under annealing conditions (experiment 4). However, if 30 to 40S RSV RNA was reannealed in the presence of <10S subunits of 60 to 70S RSV RNA, about the same percentage of template activity for viral DNA polymerase was recovered as if unfractionated 60 to 70S RNA was reannealed (experiment 7). In experiment 7, the 30 to 40S and <10S RNA subunits were reannealed at the same relative concentration at which they were obtained by melting of 60 to 70S RSV RNA (see Fig. 5). The 60% recovery of template activity of RNA subunits could not be enhanced significantly by annealing in the presence of concentrations of small RNA subunits higher than those used in experiment 7 (experiment 9). Reducing the ratio of <10S RNA to the 30 to 40S RNA during annealing reduced the recovery of template activity of reannealed RNA (experiment 8). The <10S RNA subunits of 60 to 70S RSV RNA did not enhance the template activity of TMV RNA or 28S ribosomal RNA after incubation under annealing conditions (experiments 10–13). The 4S species of 60 to 70S tumor virus RNA was shown to resemble cellular transfer RNA. However, the template activity of 30 to 40S RSV RNA was not affected after incubation under annealing conditions with 4S RNA prepared for chick embryo fibroblasts (experiment 14).

The thermal reduction of the template activity of 30 to 40S subunits reannealed with <10S subunits of 60 to 70S RSV RNA (Fig. 6) had a T_m

TABLE 1. *Effects of heating and reannealing on the template activity of RNA for DNA polymerase of RSV*

Expt	RNA	Treatment[a]	[^{3}H]dTMP incorporated[b]
1	60–70*S* PR RSV	–	104,372
2	60–70*S* PR RSV	Heated	11,599
3	30–40*S* PR RSV	Heated + fractionated	10,334
4	<10*S* PR RSV	Heated + fractionated	5,221
5	60–70*S* PR RSV	Heated + reannealed	63,442
6	30–40*S* PR RSV	Heated + reannealed	16,129
7	30–40*S* PR RSV + <10*S* PR RSV[c]	Heated + reannealed	60,124
8	30–40*S* PR RSV + <10*S* PR RSV[c]	Heated + reannealed	35,601
9	30–40*S* PR RSV + <10*S* PR RSV[c]	Heated + reannealed	62,790
10	TMV	Heated + reannealed	9,181
11	TMV + <10*S* PR RSV[d]	Heated + reannealed	14,683
12	28*S* ribosomal	Heated + reannealed	21,690
13	28*S* ribosomal + <10*S* PR RSV[e]	Heated + reannealed	22,014
14	30–40*S* PR RSV + cellular 4*S*[f]	Heated + reannealed	10,821

[a] RNA samples were heated in 0.01 M NaCl, 2 mM EDTA, 0.01 M Tris (*p*H 7.4), and 0.1% SDS at 100 C for 45 sec in sealed ampoules and were then cooled in melting ice. A 6- to 10-μg amount of RNA was annealed in 50 μliters of 6 × SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate, *p*H 7.0) containing 0.1% SDS. Annealing was for 30 min at 80 C followed by slow cooling over an 8- to 10-hr period to room temperature. RNA was recovered after 20-fold dilution with standard buffer by addition of 2 volumes of ethanol. RNA was once more reprecipitated from standard buffer and then redissolved in 0.05 M NaCl, 0.01 M Tris, *p*H 7.4. Some RNA samples were annealed in 0.5 M NaCl, 0.2 M Na_2HPO_4-NaH_2PO_4, *p*H 7.0, containing 0.1% SDS. After annealing, the RNA samples were freed from phosphate by sedimentation on a sucrose gradient and recovered by ethanol precipitation.

[b] Template activity of RNA samples in 20 μliters of 0.05 M NaCl, 0.01 M Tris, *p*H 7.4, was determined by incubation for 2 hr in a standard polymerase assay (13) which contained [^{3}H]dTTP at 2.5 μM. All assays contained 0.5 μg of RNA. Results show the counts per minute of deoxythymidine monophosphate incorporated.

[c] Amounts of 6 μg of 30–40*S* PR RSV RNA were annealed in the presence of 1 μg (experiment 7), 0.5 μg (experiment 8), or 3 μg (experiment 9) of fractionated <10*S* PR RSV RNA.

[d] A 6-μg amount of TMV RNA was annealed in the presence of 1 μg of <10*S* PR RSV RNA.

[e] A 6-μg amount of ribosomal 28*S* RNA was annealed in the presence of 1 μg of <10*S* PR RSV RNA.

[f] A 6-μg amount of 30–40*S* PR RSV RNA was annealed in the presence of 1 μg of 4*S* RNA isolated from chicken embryo fibroblasts (Materials and Methods).

of 70 to 75 C, which is very similar to that of native 60 to 70*S* RSV RNA (Fig. 2).

Because not more than 60% of the template activity of RSV RNA could be recovered by reannealing of 30 to 40*S* and small RNA subunits, we asked whether the reannealed product was homogeneous with regard to template activity or whether it consisted of a mixture of material with very high and very low template activity. To answer the question, reannealed RSV RNA was fractionated by sucrose gradient sedimentation, and its template activity for viral DNA polymerase was examined. The size range of reannealed RSV RNA with high template activity for the viral DNA polymerase was rather broad, extending from 10 to about 70*S*, with a peak at 50*S* (Fig. 6). The specific template activities of all reannealed RSV RNA fractions sedimenting faster than 10*S* were very similar (not shown). Since fast-sedimenting complexes of radioactive RSV RNA were also obtained after annealing under various conditions that did not yield RNA with high template activity for the enzyme (30 to 40*S* RSV RNA only, low concentrations of 30 to 40*S* RSV RNA and TMV RNA; experiments not shown), the specificity of fast-sedimenting RSV RNA aggregates obtained by annealing and their similarity to native 60 to 70*S* RSV RNA could not be determined.

We deduce from these experiments that at least 60% of the template activity of 60 to 70*S* RSV RNA for the viral DNA polymerase lost after heat dissociation is recovered by reassociation of 30 to 40*S* and <10*S* RNA subunits. Recovery of the 60 to 70*S* structure is not essential to restore at least 60% of the template activity of native 60 to 70*S* RSV RNA, because 10 to 40*S* structures have the same template activity after annealing as faster-sedimenting structures. This reassociation between RSV RNA subunits appears to be specific, because <10*S* RSV RNA subunits do not affect the template activity of TMV RNA or 28*S* ribosomal RNA after incubation under annealing conditions.

TABLE 2. *Template activity for RSV DNA polymerase of 30 to 40S RSV RNA after annealing with small RNA subunits released from 60 to 70S RSV RNA at various temperatures*

RNA	Annealed with[a]	[^{3}H]dTMP incorporated (counts/min)[b]
60–70S		70,518
30–40S		5,005
30–40S	RNA component 1 released at 60 C[c]	8,588
30–40S	RNA component 2 released at 60 C[c]	4,166
30–40S	RNA component 3 released at 60 C[c]	5,894
30–40S	RNA component 1 released between 60 and 85 C[d]	32,280

[a] A 3-μg amount of 30 to 40S RSV RNA, obtained from 60 to 70S RNA heated to 85 C for 3 min, was annealed in the presence of 800 counts/min (0.08 μg) of the respective ^{32}P-labeled small RNA component. Conditions of the annealing and subsequent preparation of RNA for template activity assay are described in Table 1.

[b] A 0.4-μg amount of RNA was used for standard polymerase assay.

[c] Small RNA components were prepared after heating 60 to 70S RSV RNA in 0.1 M NaCl to 60 C for 3 min and subsequent electrophoretic fractionation, as described for Fig. 3. Elution of RNA from the gel was for 36 hr at 40 C with buffer saturated with phenol and containing 0.01 M Tris(*p*H 7.4), 0.1 M NaCl, 0.001 M EDTA, 1% mercaptoethanol, and 1% SDS.

[d] Conditions for the release of this RNA component from larger subunits are described in the text. Preparation of the RNA was as in footnote *c*.

Partial characterization of the primer molecule. ^{32}P-labeled 60 to 70S RSV RNA, heated to 60 C to remove small RNA components without primer activity (*see* Fig. 2), was fractionated by sucrose gradient sedimentation (cf, Fig. 5). The 30 to 40S fraction was recovered and heated to 85 C for 3 min to dissociate the primer, and was analyzed by polyacrylamide-gel electrophoresis as described for Fig. 3. One distinct small RNA species, which migrated similar to component 1 (Fig. 3), was detectable (*not shown*) and eluted from the gel. This RNA species, referred to as component 1(60 to 85 C), was then compared to RNA components 1, 2, and 3 (Fig. 3) released by heating 60 to 70S RSV RNA to 60 C, with regard to its ability to affect the template activity of primer-free 30 to 40S RSV RNA (prepared as described for Fig. 5). It was found that RNA component 1 (60 to 85 C) enhanced the template activity of 30 to 40S RNA, after annealing, whereas the other RNA components tested had only small effects (Table 2). We cannot determine from our data whether RNA component 1 (60 to 85 C) is the best or the sole primer, because annealing at saturating concentrations of this and the other small RNA species has not been done, due to the scarcity of the material available. Nevertheless, it may be concluded that the predominant primer molecule(s) is a distinct species of the <10S fraction of completely dissociated 60 to 70S RSV RNA. Its electrophoretic mobility is similar, but its dissociation temperature is significantly higher than that of the bulk of 60S to 70S-associated 4S RNA.

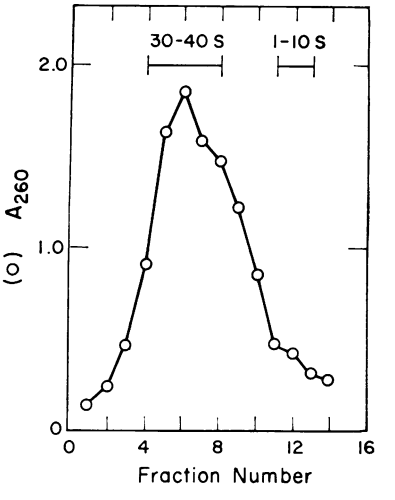

FIG. 5. *Sedimentation analysis of heat-dissociated 60 to 70S RSV RNA. The 60 to 70S RSV RNA was prepared from virus which had been released in the growth medium of infected cultures during 5-hr intervals (Materials and Methods). The 60 to 70S RNA was heated in 300 μliters of standard buffer containing 0.2% SDS for 45 sec at 100 C in a sealed ampoule. It was then analyzed by centrifugation in a 5 to 20% (w/v) sucrose gradient containing the same buffer and 0.1% SDS. Centrifugation was in a Spinco SW 65 rotor at 65,000 rev/min for 85 min at 20 C. Absorbance at 260 nm was determined for each fraction in a Zeiss PMQ II spectrophotometer.*

DISCUSSION

Three types of experiments suggested that small <10S subunits, rather than 30 to 40S subunits of 60 to 70S RSV RNA are the major sources of primers for the viral DNA polymerase. (i) The T_m of most of the template activity of 60 to 70S RSV RNA is 15 C higher than that of the 60 to 70S RNA structure. (Similar results were obtained

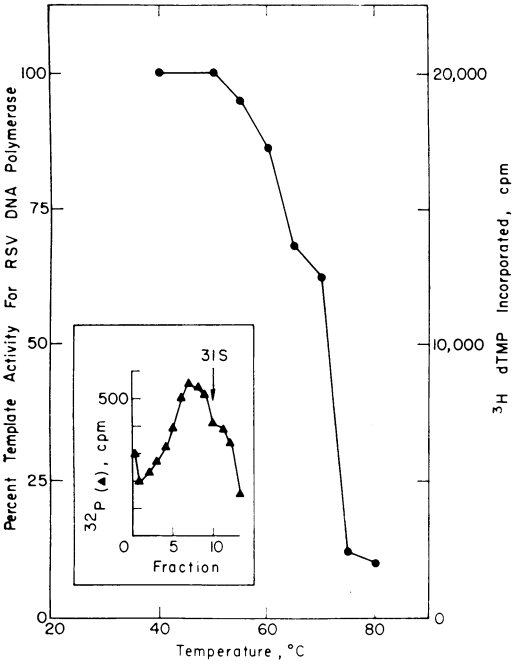

FIG. 6. *Thermal transition of template activity of reannealed PR RSV RNA. Heat-dissociated* ^{32}P*-labeled 60 to 70S RSV RNA was reannealed as described for Table 1, experiment 5. Samples (20 μliters) containing 0.15 μg of RNA in standard buffer were incubated at the temperatures indicated for 3 min. The template activity of RNA samples was subsequently tested in a standard polymerase assay (10). Sedimentation analysis of the reannealed* ^{32}P*-RSV RNA is shown in the insert. Sedimentation conditions were those described in Fig. 1. Radioactivity of the fractions was determined after the addition of 6 ml of 20% NCS in toluene-based scintillation fluid as described for Fig. 4A.*

by G. T. Parsons, P. A. Bromley, R. K. Harvey, and C. Weissmann, *personal communication*.) Consequently, we suppose that the T_m of most of the template activity is that of linkages between small subunits and 30 to 40*S* RNA subunits, although this has not been directly demonstrated. (ii) Reassociation experiments indicated that 60% of the template activity of heat-dissociated RSV RNA could be recovered only by annealing of both 30 to 40*S* and <10S RSV RNA subunits. (iii) According to several reports (2, 15, 17, 21), the viral DNA polymerase links DNA product covalently to RNA or DNA primers. Analysis of the primary DNA products made with viral DNA polymerase, with the use of 60 to 70*S* RSV RNA as template, demonstrated that RNA covalently linked to DNA product was much smaller than the bulk of the viral template RNA. This is compatible with the view that small RNA subunits served as major primers for DNA synthesis. In addition, some of the DNA synthesized in the presence of 60 to 70*S* RSV RNA appeared to be linked to very little or no RNA. This could be due either to DNA synthesis without RNA primer or to DNA synthesized on very small RNA primers.

It was proposed by Verma et al. (21) that the 4*S* subunit species of 60 to 70*S* tumor virus RNA are responsible for the high template activity of the 60 to 70*S* complex. However, since the T_m of most of the 4*S* subunits of 60 to 70*S* RSV RNA and the T_m of its high template activity for the enzyme do not coincide, it is unlikely that all 4*S* subunits of RSV RNA function as primers. It appears, however, that the 4*S* species is heterogeneous. This is indicated by its broad melting profile which suggests that it consists of a major fraction without primer activity, dissociating from large RNA subunits below 60 C, and a minor fraction with primer activity, dissociating from larger RNA subunits above 60 C. Our finding that 4*S* cellular RNA did not affect the template activity of 30 to 40*S* RSV RNA after incubation under annealing conditions is compatible with the view that most of the viral 4*S* RNA, which is thought to be cellular transfer RNA (12; M. Bishop, *personal communication*), is not the primer.

The efficiency of the <10*S* subunits of 60 to 70*S* RSV RNA in enhancing the template activity of 30 to 40*S* RNA subunits after annealing is similar to that of oligo-deoxythymidine or oligo-deoxycytidine (10), but it appears to be specific for RSV RNA. It remains to be determined whether DNA synthesis primed by the oligo-deoxynucleotides occurs at the same sites of the viral RNA template as DNA synthesis primed by natural <10*S* RNA primers.

If RSV replication in vivo can be proven to involve RNA-dependent DNA synthesis (20), the small RNA subunits of 60 to 70*S* RSV RNA may function as specific primers for DNA synthesis in the host cell. Enzymatic synthesis of hybrid molecules containing both ribo- and deoxyribonucleotides in bacteria has been described by Hurwitz (14). More recently, the use of RNA primers to initiate DNA synthesis in prokaryotic systems has been postulated in several laboratories (4, 16).

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