Properties of Adenovirus Messenger Ribonucleic Acid Synthesized In Vitro

RICHARD J. WARREN¹

Department of Microbiology, Saint Louis University School of Medicine, Saint Louis, Missouri 63110

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Adenovirus deoxyribonucleic acid (DNA) was used as template for the in vitro synthesis of viral-specific ribonucleic acid (RNA). When the kinetics of the reaction were compared by using native and heat-denatured DNA templates, the latter synthesized RNA at a slower rate. The fate of the DNA after acting as template and physical characteristics of the RNA product were studied. The DNA template, according to its sedimentation rate, was not significantly degraded by the *Micrococcus lysodeicticus* RNA polymerase. The products of the RNA polymerase reaction had the following properties. (i) Hybridization experiments revealed a high degree of complementarity (50 to 70%) for its homologous DNA. (ii) A very low complementarity (6 to 7%) was found for its heterologous DNA. (iii) The sedimentation rate of the synthetic RNA in a sucrose gradient was 5 to 10S when native DNA was used as the template. When heat-denatured DNA was used, the resulting RNA product, free of the template, sedimented at a rate of 3 to 16S. A rapidly sedimenting (>30S) DNA-RNA complex resulted when denatured DNA was the template. The DNA moiety of the complex was sensitive to 125 μ g of deoxyribonuclease per ml. The RNA of the complex, however, was fully refractory to 50 μ g of ribonuclease per ml. When the adenovirus DNA was sonically treated and then used as template, the RNA product sedimented at 3 to 9S. The heat-denatured sonically treated DNA template yielded a DNA-RNA complex that also sedimented at an unusually fast rate (>18S).

Ribonucleic acid (RNA) synthesized in vitro utilizing as template the deoxyribonucleic acid (DNA) from mammals, bacteria, and viruses has been studied intensively (5-8, 10, 14, 15, 23, 27, 28). Of particular interest is DNA from the tumorigenic and nontumorigenic (17, 26) human adenoviruses which differ in base composition (12, 13) and degree of genetic relatedness (19). In this study, DNA from adenovirus types 2 and 12 was used as template for the in vitro synthesis of RNA utilizing the purified nucleosidetriphosphate: RNA nucleotidyltransferase (EC 2.7.7.6) from *Micrococcus lysodeicticus* (22). The characteristics of the reaction and the physical properties of the RNA product are described here to add to the accumulating information on the genetics of animal viruses.

MATERIALS AND METHODS

Chemicals. ¹⁴C-adenosine triphosphate (ATP; 7.5 mc/mmole) and ³H-ATP (1.3 c/mmole) were obtained from Schwarz BioResearch Corp.; uridine triphos-

phate (UTP) was from Pabst Laboratories; cytosine triphosphate (CTP), guanosine triphosphate (GTP), and bovine pancreatic ribonuclease "A" (Type XII-A) were from Sigma Chemical Co.; calf thymus DNA and deoxyribonuclease were from Worthington Chemical Co.; and Sephadex G-50 (medium) was from Pharmacia Fine Chemicals, Ltd. Phenol and anhydrous ether were products of Mallinckrodt Chemical Co.

DNA template. The methods for virus growth and purification have been described previously (11, 13). The DNA was extracted from adenovirus types 2 and 12 (12, 19) and dialyzed against several changes of buffer (0.1 M NaCl-0.05 M sodium phosphate) before use. Purity and molecular integrity of the DNA were ascertained by sedimentation velocity, buoyant density, and melting point determinations.

Enzyme assays. RNA polymerase was extracted from spray-dried *M. lysodeicticus* cells (Miles Chemical Co.) by the procedure A of Nakamoto et al. (22). Fraction VI enzyme was used. The components of the reaction mixture were as described by Nakamoto et al., except that 0.25 mM spermidine-HCl was substituted for spermine (15), and ¹⁴C- or ³H-labeled ATP was the radioactive precursor. The reaction was terminated by addition of ice-cold trichloroacetic acid to a final concentration of 10% in the presence of 100 μ g of herring sperm DNA. After 30 min at 0 C, the

¹ Present address: Division of Medical Genetics, Department of Pediatrics, Washington University School of Medicine, St. Louis, Mo.

trichloroacetic acid-insoluble polynucleotides were collected on a membrane filter (Millipore Corp., Bedford, Mass.). After the filters were dried, the radioactivity was determined as described previously (19). One unit of enzyme activity is defined as the amount catalyzing the incorporation of 1 nmole of labeled nucleotide triphosphate into acid-insoluble material during 10 min of incubation at 30°C (22).

Purification of product. For phenol extraction, reaction mixtures that had been incubated for the appropriate times were diluted 10-fold with $0.1 \times \text{SSC}$ (0.15 M NaCl , 0.015 M sodium citrate). They were then dialyzed at 4°C for 18 hr against four changes of the same buffer. The dialyzed material was extracted with phenol [saturated at 0°C with $0.1 \times \text{SSC}$ (19)] followed by anhydrous ether. The extracted material was again dialyzed at 4°C for 24 hr against several changes of $0.1 \times \text{SSC}$.

For purification by gel filtration, 1 ml of 10-times diluted reaction mixture was placed on a column (1 by 10 cm) of Sephadex G-50, equilibrated with $0.1 \times \text{SSC}$, and eluted with 10 ml of the same buffer. Fractions of 1 ml were collected. Total radioactivity of samples from each of the 10 fractions was assayed in 15 ml of aqueous scintillation fluid. Radioactivity of the ribopolynucleotide product was assayed by trichloroacetic acid precipitation as described above except that the filters on which the precipitated polymer was collected were counted wet in 15 ml of Bray scintillation fluid.

Hybridization experiments. The hybridization procedure employed was that described by Lacy and Green (19) except that RNA polymerase reaction mixtures, diluted with 100 volumes of $0.1 \times \text{SSC}$, were added to the agar preparations containing the homologous or heterologous DNA. After 16 hr of incubation at 60°C, the nonhybridized RNA was eluted with $2 \times \text{SSC}$ at 60°C. The hybridized RNA was then eluted with $0.01 \times \text{SSC}$ at 75°C. The eluates were precipitated with trichloroacetic acid and assayed as described above.

Zone centrifugation. The sedimentation coefficient of the synthetic RNA was determined by zone centrifugation by use of an SW 25.1 rotor in a Spinco Model L-2 centrifuge. A linear sucrose gradient in RSB buffer [0.01 M NaCl , 0.0015 M MgCl_2 , 0.01 M tris(hydroxymethyl)aminomethane (Tris), pH 7.4] was used as the stabilizing medium. When unpurified KB cell RNA (2) was used as marker, the 5 to 20% sucrose gradients contained 0.25% sodium dodecyl sulfate (SDS). When purified RNA (9) was used as a marker, 15 to 30% sucrose gradients were used. The gradients were collected from the bottom, the effluent was passed through a Gilford multiple sample absorbance recorder (Model 2000), and the absorbancy at 260 nm was recorded automatically. Sedimentation rates were determined by the relation $D_1/D_2 = S_1/S_2$ (21).

RESULTS AND DISCUSSION

Kinetic studies. Four adenovirus DNA preparations, two type 2 and two type 12, were dialyzed against 1 mM NaCl-1 mM Tris buffer (pH

7.5), evaporated to 500 μ liters by directing a stream of filtered air into the mouth of the test tube, and adjusted to a salt concentration of 0.01 M by the addition of water and 1 M NaCl-1 M Tris (pH 7.5). Sedimentation velocity analysis of this concentrated material showed the DNA to be homogenous and to have the expected sedimentation coefficient for adenovirus DNA.

These four preparations were studied for their relative priming ability. When the amount of DNA or enzyme was varied in the reaction mixtures, a direct relationship was found between the amount of DNA or enzyme added and the amount of product formed. The kinetics of two such reaction mixtures are shown in Fig. 1. The other two reactions showed identical kinetics. During the course of the incubation, the rate of RNA synthesis decreases markedly, and the time at which this decrease occurs is a function of the amount of template or enzyme in the reaction mixture.

These four DNA preparations were also studied for their ability to serve as template after being denatured. Just prior to running the reaction, the DNA was placed in a boiling-water bath for 5 min, followed by quick cooling in ice water. When this DNA was used as template, the rate of RNA formation was about half that observed with native DNA (Fig. 2). Others have observed a similar decrease in rate with denatured templates (6, 27, 29).

Purification of product. The synthetic RNA was partially separated from unincorporated nucleotides, and probably other small molecules, by gel filtration. Figure 3 shows the elution profile of one preparation from a column of Sephadex G-50. Both the synthetic RNA and the DNA template are excluded from the gel bed. On the other hand, acid-soluble nucleotides were eluted from the gel with $0.1 \times \text{SSC}$. It was also possible to prepare the synthetic RNA free of enzymes, cofactors, and unincorporated nucleotides by phenol and ether extractions with 80 to 85% recovery of the trichloroacetic acid-precipitable radioactivity as ribonucleotide (Table 1). This purified RNA is completely sensitive to degradation by ribonuclease (Table 2).

Hybridization experiments. A very high complementarity between the synthetic RNA and its DNA template was observed. Homology of 50 and 70% was demonstrated between type 2 and type 12 RNA, respectively, with the homologous DNA (Table 3). This homology is the maximum obtainable under the conditions of the experiment; that is, we were working at saturated levels.

The adenoviruses contain antigenically similar proteins. Therefore some complementarity between the DNA of different types of adenoviruses

FIG. 1. Characteristics of the polymerization of adenovirus RNA in vitro. The reaction mixtures (0.4 ml each) contained 0.10 M Tris-hydrochloride, (pH 7.5), 0.4 mM each CTP, GTP, and UTP, 0.081 mM ^3H -ATP (3.3×10^8 counts per min per μmole), 2.5 mM MnCl_2 , and 0.25 mM spermine-hydrochloride; they were incubated at 35 C. A and B show the effect of varying amounts of type 2 and type 12 DNA, respectively (amounts indicated in parentheses) while holding the enzyme concentration at 10 units. C and D show the effect of varying amounts of enzyme (amounts indicated in parentheses) while holding the DNA concentration at 126 $\mu\text{g}/\text{ml}$ for type 2 (C) and 106 $\mu\text{g}/\text{ml}$ for type 12 (D). The reaction was stopped in samples at the indicated times by dilution with 200 volumes of ice-cold water containing 100 μg of carrier DNA. After thorough mixing, the polymers were precipitated with ice-cold trichloroacetic acid. After 20 min of incubation in ice water, the precipitated polymers were collected on S & S B6 filters which were dried, and then the radioactivity was assayed with a Packard Tri-Carb liquid scintillation counter. All values are the average of duplicate 5- μliter samples taken from the reaction mixtures within 5 sec of each other.

is expected, and indeed has been reported (19). In heterologous hybridization reactions (i.e., type 2 DNA with type 12 RNA), 7 and 6% of the RNA (relative to the homologous reactions) was found to the type 2 and type 12 DNA, respectively. Lacy and Green reported 20% homology between the

DNA of adenovirus types 2 and 12 (19). This discrepancy might be due to partial transcription of the template. Based on a difference of 0.29 μm in the circumference of ϕX 174 circular replicating form of DNA and the ϕX DNA-RNA hybrid made in vitro, Bassel et al. (1) suggested that the RNA polymerase might skip small segments of the template. If the adenovirus template were incompletely transcribed, the segment(s) which was skipped in our reactions represents about half of the portion of the genome held in common by adenovirus types 2 and 12. An alternative explanation is that only half the genome is being copied, assuming random distribution of the common cistrons. However, the dissimilarity in incubation mixtures (i.e., DNA-DNA versus DNA-RNA) and unknown differences in sensitivity cannot be eliminated as possible causes for the observed variation.

FIG. 2. Effect of heat denaturation of the template on the polymerization of adenovirus RNA. Reaction conditions were as in Fig. 1 except that the volume of the reaction mixture was 40 μliters and 3 μg of type 2 adenovirus DNA, and 2 units of enzyme were used in each reaction. The DNA was denatured by immersing the micro-tube containing the DNA solution in a boiling-water bath for 5 min and then immediately immersing it in an ice-water bath. Samples from the reaction mixture were handled for radioactivity assay as in Fig. 1. The plotted values are the average of duplicate 1- μliter samples. ■, Native DNA; ●, denatured DNA.

FIG. 3. Elution of synthetic adenovirus RNA from Sephadex G-50. Reaction conditions were as in Fig. 1 except that the reaction mixture was 80 μ liters; 5.2 μ g of adenovirus type 2 DNA- 32 P (1,700 counts per min per μ g) and 4 units of enzyme were used. The reaction was stopped after 45 min by the addition of 10 volumes of mM ethylenediaminetetraacetate. A 100- μ liter amount of the diluted reaction mixture was placed in 1 ml of 0.1 $\times$ SSC; this was carefully layered on the top of the prepared Sephadex G-50 column. The sample was allowed to enter the gel bed, and 10 ml of 0.01 $\times$ SSC was carefully pipetted onto the column; 1.5 psi was utilized to keep the flow rate constant. Ten 1-ml fractions were collected and trichloroacetic acid-precipitable radioactivity was assayed as in Fig. 1. ■, Ribopolynucleotide; ○, DNA template; ●, unin-corporated nucleotides.

TABLE 1. Recovery of polymerized RNA after purification^a

Recovery time	Counts/min	
	Type 2	Type 12
Before extraction	1,088	486
After extraction	887	400

^a The reaction mixtures (0.4 ml each) were similar to those described in Fig. 1 except that 12.2 and 11.5 μ g of type 2 and type 12 DNA, respectively, and 80 units of enzyme were used. After 180 min, the reactions were stopped by chilling; 0.1 ml of the mixtures was diluted with 10 volumes of ice-cold 0.1 $\times$ SSC. After dialysis for 10.5 hr against two 1,000-ml volumes of 0.1 $\times$ SSC, they were extracted with phenol and ether. The values are the average of duplicate 1- μ liter samples which were assayed as in Fig. 1.

Ribonuclease sensitivity. Another parameter for checking the integrity of the synthetic polymer of the described enzyme reaction involves the utilization of specific degradative enzymes. When

phenol-purified synthetic RNA was subjected to hydrolysis by pancreatic ribonuclease under various salt concentrations, it was completely degraded (Table 2). It is clear that resistance to hydrolysis is augmented by higher salt concentrations (Fig. 4). Additional evidence of the salt effect can be seen by comparing Table 2 with Fig. 4. The presence of 10mM SSC protects 25% of the synthetic RNA against digestion by ribonuclease. These results are similar to those of Hayashi et al. (16), who discussed the relation between salt molarity and ribonuclease digestion.

TABLE 2. Effect of ribonuclease on adenovirus RNA synthesized *in vitro*^a

Incubation time (sec)	Type 2		Type 12	
	Counts/min	% Soluble	Counts/min	% Soluble
0	648	0	649	0
30	43		124	
60	39		169	
120	33		75	
900	16	98	42	95

^a A 10- μ liter amount of the purified RNA described in Table 1 was placed in 1 ml of 0.01 M Tris (pH 8.1) and incubated at 25 C with 6 μ g of bovine pancreatic ribonuclease "A" Type XII-A (Sigma). The values are the averages of 0.1-ml samples which were precipitated immediately with ice-cold trichloroacetic acid, as in Fig. 1.

TABLE 3. Complementarity between adenovirus DNA and synthetic adenovirus DNA^a

Temp	SSC concn	Fraction no.	Counts/min	
			Type 2	Type 12
60 C	2 $\times$	1	900	600
		2	35	35
		3	0	0
75 C	0.01 $\times$	1	880	1,390
		2	50	100
		3	0	0

^a A 30-mg amount of type 2 (1,800 counts/min) and a 50-mg amount of type 12 (2,100 counts/min) DNA-agar, were each incubated with 50 μ liters of homologous synthetic RNA solution. The RNA was from a sample which was withdrawn at 30 min from the reaction mixtures of Fig. 1 and placed in 100 volumes of ice-cold 0.1 $\times$ SSC. The ratio of RNA to DNA was 1/100 for type 2 and 1/30 for type 12. After 16 hr at 60 C, the nonhybridized RNA was eluted at 60 C with 6-ml amounts of 0.01 $\times$ SSC. Eluates were assayed for trichloroacetic acid-precipitable radioactivity as in Fig. 1.

^b Hybridization was 50% for type 2 and 70% for type 12.

FIG. 4. Effect of salt concentration on ribonuclease digestion of purified adenovirus RNA. The RNA used was that which remained of the reaction mixtures of Fig. 1. It was treated with 200 μ g of electrophoretically purified deoxyribonuclease (Worthington), phenol-extracted, and dialyzed to the appropriate SSC concentration. The ribonuclease digestion was conducted at 25 C for 1 hr with 40 μ g of pancreatic ribonuclease per ml. Assay for trichloroacetic acid-precipitable radioactivity was as described. ○, Type 2; ●, type 12.

Zone centrifugation. Sedimentation analyses, with sucrose as a stabilizing medium, were performed on the synthetic RNA transcribed on native, denatured, and fragmented templates. Attempts were made to detect changes in size of the RNA with time during the course of the reaction and the formation of template-enzyme-product complexes utilizing double labeling.

Effect of purification. The sedimentation profiles of the synthetic RNA were the same for phenol-extracted, Sephadex-filtered, and unpurified reaction mixtures. Therefore, it was not considered necessary to extensively purify the synthetic RNA before studying its sedimentation characteristics in subsequent sucrose gradient studies.

Effect of incubation time on the size of the synthetic RNA. Bremer and Konrad (3) observed an increase proportional to incubation time in the sedimentation coefficient of bacteriophage T4 synthetic RNA. Others (4, 29) have shown an increase with time in the buoyant density of ϕ X 174 DNA-RNA complexes, suggesting an increase in the size of the RNA as the RNA polymerase reaction progresses.

The effect of the incubation period on the size of the adenovirus synthetic RNA was determined. The polymerization was stopped by dilution of samples of the reaction mixture with 100 volumes of ice-cold 0.1 $\times$ SSC at 10-min intervals. Zone centrifugation of these diluted samples revealed no change in sedimentation rate of the RNA syn-

thesized at 10, 30, or 60 min during the reaction utilizing as template the DNA from either adenovirus type 2 or type 12.

Molecular interaction in the gradients. Experiments in which the DNA template or synthetic RNA was centrifuged with and without marker RNA showed no effect on sedimentation rate due to macromolecular interaction. Also, none was observed when 32 P-labeled DNA, 14 C-labeled type 2 RNA, and 3 H-labeled type 12 RNA were mixed and centrifuged in a sucrose gradient with marker RNA (Fig. 5); each molecule moved at the same rate observed when sedimented alone.

FIG. 5. Sedimentation of synthetic RNA in a sucrose gradient. Adenovirus type 2 32 P-DNA (1,100 counts/min), type 2 14 C-RNA (2,000 counts/min), and type 12 3 H-RNA (4,000 counts/min) were mixed with unpurified marker RNA from 3×10^7 KB cells, and carefully layered on a 5 to 20% sucrose gradient. The gradient was centrifuged at 24,000 rev/min at 20 C for 15 hr. Simultaneously centrifuged gradients containing only the type 2 14 C-RNA or type 12 3 H-RNA showed identical sedimentation profiles for these synthetic polymers. ●, 32 P; ■, 3 H; ○, 14 C.

Effect of DNA integrity. The sedimentation characteristics were measured of the synthetic RNA synthesized with native, denatured, and sonically treated and sonically treated-denatured DNA as template. The differences noted were as follows.

(i) **Native DNA template.** The sedimentation rate of the synthetic RNA was 5 to 10S with type 2 or type 12 native DNA as template. A similar sedimentation rate has been observed for the synthetic RNA of *Azotobacter vinelandii* (10) and bacteriophage T2 (29). The adenovirus template was not degraded during the RNA polymerase reaction, as revealed by the constancy of trichloroacetic acid-precipitable radioactivity throughout the course of the reaction, when ^{32}P -labeled DNA was used as template. Also, the sedimentation rate of this labeled DNA remained the same before and after it was used as template (Fig. 6). When reaction mixtures containing native helical adenovirus types 2 or 12 DNA as template were centrifuged in sucrose, they sedimented separately, both in the presence and absence of 0.5% SDS (Fig. 6). These findings are different from some reported in bacteriophage systems in which the template appeared to complex with the product (4, 20, 25). Such a complex was seen in the present study when denatured adenovirus DNA was the template. Thus the reported complexes might be caused by the presence of denatured DNA in the reaction mixture. Another possibility is that animal virus DNA is less likely to form complexes with the RNA product than is bacteriophage DNA. Before this can be definitely established, further studies are needed with adenovirus or other animal virus systems. However, other workers employing bacteriophage systems also were unable to demonstrate any complex formation either in vitro (8) or in vivo (18). Therefore, the presence of contaminating denatured DNA may be the more plausible explanation for the conflicting reports.

(ii) **Heat-denatured DNA template.** DNA-RNA hybrids have been observed in RNA polymerase reaction mixtures containing denatured or single-stranded templates from calf thymus and bacteria (10) and from bacteriophages (1, 4, 10, 24, 29). The use of heat-denatured adenovirus DNA template results in a fast-moving complex in addition to RNA that sediments at a rate of 3 to 16S (Fig. 7).

Of particular interest is the rapidly sedimenting species ($> 30\text{S}$) which, as revealed by double labeling, is probably in DNA-RNA complex (Fig. 7A). This very high sedimentation rate cannot be accounted for simply on the basis of a 1:1

DNA-RNA hydrogen-bonded complex both on the basis of specific activity and molecular weights. It apparently is the result of a drastic configurational change to a faster sedimenting species. The complex is not broken by SDS (0.5%); therefore, the enzyme probably is not holding it together (3). Heat denaturation of this complex results in a shift in its sedimentation rate to approximately 15S (Fig. 7B). Since DNA itself treated similarly exhibits identical sedimentation behavior, the shift toward the slower-sedimenting species suggests a change in the size of the complexed DNA, probably caused by breakage of phosphodiester bonds.

The nature of the DNA-RNA complex was further studied by its response to deoxyribonuclease and ribonuclease (Fig. 8). The trichloroacetic acid-precipitable radioactivity of the com-

FIG. 6. Sedimentation of synthetic RNA and its labeled template in a sucrose gradient. Reaction conditions for the synthesis of the ^3H -RNA were as in Fig. 2 except that 4 μg of adenovirus type 2 ^{32}P -DNA (850 counts per min per μg) was used as template. The reaction was stopped after 45 min by the addition of 10 volumes of 1 mM ethylenediaminetetraacetate. The diluted reaction mixture was mixed with unpurified marker RNA from 4.2×10^7 KB cells, carefully layered on a 5 to 20% sucrose gradient, and then centrifuged as in Fig. 5, except at 12 C for 17 hr. ●, ^{32}P ; ■, ^3H .

plexed DNA is completely obliterated by deoxyribonuclease releasing the complexed RNA which now sediments at about 4S (Fig. 8A). This sedimentation rate suggests the release of a particle of size similar to the uncomplexed 3 to 16S RNA mentioned above and illustrated in Fig. 7.

The trichloroacetic acid-precipitable radioactivity of the complexed RNA is refractory to ribonuclease digestion; however, only 50% is converted to an acid-soluble form. The ribonuclease-resistant portion of the RNA is found in the gradient with the DNA, implicating the double-stranded nature of the complex (Fig. 8B). Its double strandedness is also suggested by the fact that heat denaturation of the DNA-RNA complex increases the proportion of radioactive RNA made acid-soluble by ribonuclease to 89% (Fig. 8C). Therefore, approximately 10% of the radioactive ribonucleotides are associated with the DNA template in a manner which is insensitive to enzymatic hydrolysis (ribonuclease), denaturation, and SDS. Thus, under the conditions employed, 50% of the synthetic RNA is free of the template, 40% is probably hydrogen bonded (10, 27), and about 10% is refractory to ribonuclease digestion even after heating.

(iii) Fragmented DNA template. Uniformly sized 9S fragments are produced when native adenovirus DNA is subjected to sonic disruption (Raytheon 10 kc, 1.25 amp, 15 min; Fig. 9). Therefore, the DNA is broken into about 30 pieces. When this fragmented DNA is used as template, 3 to 9S RNA is produced (Fig. 9A). However, when this fragmented DNA is heat-

denatured before being used as template, double labeling reveals a DNA-RNA complex which has the characteristic broad peak and accelerated sedimentation rate (>18S) observed when un-

FIG. 8. Sedimentation of synthetic RNA and its labeled denatured template in a sucrose gradient after enzyme digestion. The DNA used in these reactions was labeled with ^3H -thymidine, and the synthetic RNA was labeled with ^{14}C -ATP. A 2- μg amount of DNA (5,800 counts per min per μg) was used as template. After a 45-min incubation at 30 C, the reaction mixture was divided into three equal parts. One portion (A) was treated with 125 μg of electrophoretically purified deoxyribonuclease (Worthington) per ml; another (B) was treated with 50 μg of pancreatic ribonuclease per ml; and the third (C) was heated to 100 C for 10 min, followed by rapid cooling in ice water, and then incubated with ribonuclease as in B. The deoxyribonuclease and ribonuclease digestions were conducted simultaneously at 24 C for 60 min; the reaction was stopped by adding 5% SDS to a final concentration of 0.25%. The sucrose gradients were run as in Fig. 6, and samples were assayed for trichloroacetic acid-precipitable radioactivity as described. $\bullet$, ^3H ; $\blacksquare$, ^{14}C .

FIG. 7. Sedimentation of synthetic RNA and its labeled denatured template in a sucrose gradient. Reaction conditions for the synthesis of the ^3H -RNA were as in Fig. 6 except that the ^{32}P -DNA was heat-denatured. The DNA used in these reactions is identical with that used in Fig. 6, and the reactions were run simultaneously, as were the centrifugations. (A), Purified RNA from 7×10^7 KB cells was used as marker in 15 to 30% sucrose gradient; (B), unpurified RNA from 4.2×10^7 KB cells was used as marker in a 5 to 20% sucrose gradient. Collection of the gradients and assay of trichloroacetic acid-precipitable radioactivity were as in Fig. 5. The difference between A and B is that the reaction mixture in B, after being stopped with ethylenediaminetetraacetate, was immersed in a boiling-water bath for 5 min and then fast-cooled in ice water before being mixed with the marker RNA. $\bullet$, ^{32}P ; $\blacksquare$, ^3H .

FIG. 9. Sedimentation of synthetic RNA and its labeled, sonically treated, and denatured template in a sucrose gradient. A sample of the ^{32}P -DNA (15 μg) of Fig. 7 was subjected to sonic oscillation (Raytheon 10 kc, 1.25 amp, 10 min). The sonically treated DNA was used in three reactions as in Fig. 7. Reaction A contained helical sonically treated DNA, B and C contained denatured sonically treated DNA. Reaction mixture C was heat-denatured after being stopped with ethylenediaminetetraacetate as in Fig. 8. All three diluted reaction mixtures were centrifuged at 15 C for 19.5 hr with unpurified marker RNA from 4.2×10^7 normal KB cells and collected as in Fig. 6. ●, ^{32}P ; ■, ^3H .

fragmented, denatured templates are utilized (Fig. 9B). After heat denaturation the fragmented DNA-RNA complex exhibits a shift toward a lower sedimentation rate analogous to that observed with unfragmented DNA (Fig. 9C).

The sedimentation rates of the RNA made on native and fragmented DNA (5 to 10S and 3 to 9S, respectively) revealed a similarity in size. The size of a messenger RNA, be it monocistronic or polycistronic, according to current hypotheses, is proportional to the size of the cistron(s) being copied. If there is a message termination signal at the end of the cistron or polycistron, then it is the size-limiting factor in the synthesis of RNA and might be considered the functional unit. The similarity in the size of the RNA made on intact and fragmented DNA suggests that this termination signal remains functional in the latter. It would be particularly interesting if the break caused by sonic oscillation occurred at this signal preferentially, owing to a difference in configuration or bond strength. One could then hypothesize that this configuration change might be responsible for the signal indicating the completion of a message and thus defines the functional unit. The present data, however, allow one to say only that this functional unit apparently remains intact under the experimental conditions. Also, each DNA fragment is equal to or larger than the synthetic RNA ordinarily transcribed in one piece. Nothing beyond pure conjecture can be offered about any chain-terminating signal in the present system.

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