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Forms of Deoxyribonucleic Acid Produced by Virions of the Ribonucleic Acid Tumor Viruses

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The in vitro product of mouse leukemia virus deoxyribonucleic acid (DNA) polymerase can be separated into two fractions by sedimentation in sucrose gradients. These two fractions were analyzed for their content of single-stranded DNA, double-stranded DNA, and DNA-ribonucleic acid (RNA) hybrid by (i) digestion with enzymes of known specificity and (ii) equilibrium centrifugation in Cs_2SO_4 gradients. The major fraction early in the reaction contained equal amounts of single-stranded DNA and DNA-RNA hybrid and little double-stranded DNA. The major fraction after extensive synthesis contained equal amounts of single- and double-stranded DNA and little hybrid. In the presence of actinomycin D, the predominant product was single-stranded DNA. To account for these various forms of DNA, we postulate the following model: the first DNA synthesis occurs in a replicative complex containing growing DNA molecules attached to an RNA molecule. Each DNA molecule is displaced as single-stranded DNA by the synthesis of the following DNA strand, and the single-stranded DNA is copied to form double-stranded DNA either before or after release of the single strand from the RNA. Actinomycin blocks this conversion of single- to double-stranded DNA.

Virions of the ribonucleic acid (RNA) tumor viruses contain an enzyme which is able to catalyze the synthesis of deoxyribonucleic acid (DNA; 2, 17). The product consists of small fragments of DNA, most of which are complementary in base sequence to the 70*S* RNA of the virion (14, 16). To gain an understanding of the mechanism of the reaction and the biological role of the product, we have begun a study of the product of the enzyme.

Three forms of DNA might be expected to occur in the reaction: double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), and DNA-RNA hybrid (hyDNA). Other molecules involved in the reaction are probably complexes of these three basic structures. A large number of techniques for differentiating these forms of DNA are available. We have concentrated on two types of analyses: degradation with enzymes of known specificity and isopycnic banding in Cs_2SO_4. In the enzymatic methods, two types of enzymes have been used: ribonucleases A and T_1, which degrade only single-stranded RNA in medium of high ionic strength but digest all RNA (except polyadenylate) in medium of low ionic strength, and the *Neurospora* endonuclease, which digests any single-stranded nucleic acid (9, 13).

MATERIALS AND METHODS

Virus. All experiments were done with a preparation of Moloney mouse leukemia virus (6-62-8) from tissue culture purchased from Electro-Nucleonics, Inc., Bethesda, Md. The preparation contained about 10^{11} particles per ml (2 mg of protein/ml) and had been banded twice at equilibrium in sucrose gradients. It was dialyzed against 500 volumes of 0.01 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 7.5, and stored frozen at −70 C.

Buffers and enzyme reagents. Endonuclease assay buffer contained 0.1 M Tris-hydrochloride, *p*H 7.6, 0.1 M NaCl, and 0.01 M magnesium acetate; E buffer was 0.01 M Tris-hydrochloride, *p*H 7.6, and 0.01 M NaCl.

The ribonuclease reagent contained 400 μg of ribonuclease A per ml (Worthington Biochemical Corp.), 80 μg of ribonuclease T_1 per ml (Calbiochem, 5,000 units/mg), and 1 mg of bovine serum albumin per ml in E buffer. The endonuclease reagent contained 18 units of *Neurospora* endonuclease per ml (9), 0.3 M Tris-hydrochloride, *p*H 7.6, 0.3 M NaCl, and 0.03 M magnesium acetate.

Actinomycin (a kind gift of Merck, Sharp and Dohme, Inc.) was dissolved at a concentration of 1 mg/ml in water.

Radioactive DNA and DNA-RNA hybrid. P22 phage were grown on a Thy⁻ strain of *Salmonella typhimurium* in a medium containing ^{14}C-thymine. The DNA was phenol-extracted and precipitated by adding yeast transfer RNA to 10 μg/ml, NaCl to 0.4

M, and two volumes of cold ethyl alcohol. Part of it was made single-stranded by heating for 10 min at 100 C in E buffer.

DNA-RNA hybrid was made by adding heat-denatured *S. typhimurium* DNA labeled with ^{14}C-thymine to an RNA polymerase reaction mix (4) containing ^{3}H-guanosine triphosphate and 20 μg of *Escherichia coli* RNA polymerase (GG enzyme; 4). After 3.25 hr of incubation, the product was dialyzed against 0.1 M Tris, *p*H 7.6, 0.1 M NaCl, 0.01 M magnesium acetate, incubated for 1 hr with 3 units of *Neurospora* endonuclease per ml, and centrifuged to equilibrium in Cs_2SO_4 solution. The central fractions of the broad peak of radioactivity, in which the thymine to guanine ratio was about one, were pooled, and the hybrid was precipitated with ethyl alcohol.

Preparation and fractionation of DNA polymerase product. Reaction mixtures were prepared as described previously (2), but contained 0.01% Nonidet P-40. The concentrations of virus, thymidine triphosphate (TTP), and actinomycin D used were as shown in Table 1. After the period of incubation shown, an equal volume of 3% sodium dodecyl sarcosinate was added, and the mixture was layered on a 15 to 30% sucrose gradient in 0.01% sodium dodecyl sulfate in 0.1 M NaCl, 0.01 M Tris-hydrochloride, *p*H 7.5, 0.001 M ethylenediaminetetraacetate (EDTA). After centrifugation (195 min at 38,000 rev/min in an SW40 rotor), fractions were collected by puncturing the bottom of the tube; portions of the fractions were pooled, ethyl acohol-precipitated, and redissolved in E buffer.

Enzymatic analysis. A 10-μliter amount of the sample to be tested was transferred to a Parafilm sheet with an Eppendorf microliter pipette; 1 μliter of the ribonuclease reagent (or E buffer) was added to the sample drop, and the mixture was sealed in a 50-μliter capillary tube. After incubation for 1 hr at 37 C, the capillary was opened. The sample was added to a 5-μliter drop of endonuclease reagent (or salt mixture without endonuclease), resealed in the same capillary, and again incubated for 1 hr at 37 C. The sample was precipitated in cold 10% trichloroacetic acid with 0.1 mg of yeast RNA carrier, filtered, and counted (3). The percentage resistant to digestion with ribonuclease is interpreted as dsDNA. The difference between this and the percentage resistant to endonuclease alone is hyDNA. The remainder is ssDNA.

To confirm the specificity of the *Neurospora* endonuclease and ribonuclease digestion procedures (9, 13), we have tested their ability to degrade dsDNA from phage P22, ssDNA (heat-denatured) from P22, and hyDNA produced by RNA polymerase on ssDNA from *Salmonella*. Table 2 shows representative data indicating that endonuclease digests less than 10% of dsDNA, less than 15% of hyDNA and more than 90% of ssDNA to acid-soluble fragments. If samples of the three forms of DNA were first treated with ribonuclease (at low ionic strength) and then digested with *Neurospora* endonuclease, over 90% of hyDNA was digested because the ribonuclease converted the hyDNA to ssDNA. Use of these enzymes, therefore, allows discrimination among the three forms of DNA. The sensitivity of the assay is such that values of 10% or less are not meaningful.

Cs_2SO_4 centrifugation. Samples were treated as follows. Heat denaturation: samples in E buffer were heated for 10 min at 100 C. Ribonuclease: samples in 0.3 M NaCl-0.03 M sodium citrate were treated for 10 min at 37 C with 10 μg of pancreatic ribonuclease per ml. Endonuclease: samples in endonuclease assay buffer were treated for 60 min at 37 C with 3 units of *Neurospora* endonuclease per ml. Samples treated as indicated above were then mixed with the following: 1.40 ml 0.01 M Tris-hydrochloride, *p*H 7.4, and 0.001 M EDTA saturated (at 24 C) with Cs_2SO_4; 100 μg of sonically treated, denatured salmon sperm DNA; and enough Tris-EDTA buffer to make 3.0 ml. These solutions were centrifuged for 65 to 70 hr at 33,000 rev/min and 20 C in a Beckman SW39 rotor. Nitrocellulose tubes were used which had been soaked for 1 day in 1% sodium dodecyl sulfate with 5×10^{-3} M EDTA, followed by 1 day in 0.9 M NaCl-0.09 M sodium citrate with 25 μg of sonically treated, denatured DNA per ml and overnight drying. (This method was suggested by G. Attardi to reduce adsorption of nucleic acids.)

Three- to five-drop fractions were collected from the bottom of the tube, trichloroacetic acid-precipitated, and counted. Recovery of product from Cs_2SO_4 gradients was greater than 70%.

TABLE 1. *Reaction mixtures for preparation of DNA polymerase product*

Time of incubation	Actinomycin	Virions	TTP	TTP specific activity
min	*μg/ml*	*particles/ml*	*nmoles/ml*	*counts/min/pmole*
120	--	3×10^{10}	48	840
120	5	3×10^{10}	48	840
120	20	2×10^{10}	48	840
20	—	3×10^{10}	10	5,000

TABLE 2. *Digestion of standard samples by neurospora endonuclease and ribonuclease*[a]

Sample	Per cent resistance	
	Endonuclease only	Ribonuclease and endonuclease
Double-stranded DNA	99	85
	91	92
	100	99
DNA-RNA hybrid	82	1
	85	1
	90	0
	102	9
Single-stranded DNA	3	1
	1	4
	6	6

[a] The data represent selected results from a series of experiments and were chosen to include samples which gave the widest divergence.

RESULTS

Sucrose gradient analysis. The size of the product of the mouse leukemia virus DNA polymerase was examined by sedimentation through a sucrose gradient after deproteinization with sodium dodecyl sarcosinate. Two fractions were obtained (Fig. 1): one that sedimented at the same rate as native viral RNA (60 to 70*S*), which we call fraction F (for "fast"), and a second that sedimented at less than 15*S*, which we call fraction S (for "slow"). Fraction F contained most of the product after 20 min of reaction (Fig. 1A). Fraction S was of variable extent, but it contained most of the product by 120 min of reaction (Fig. 1B). These results are comparable to observations made in other laboratories with different RNA tumor viruses (6, 14, 16).

These two fractions were isolated from sucrose gradients and analyzed further by nuclease digestion and centrifugation to equilibrium in Cs_2SO_4.

Content of different forms of DNA. Enzymatic analysis showed that fraction S from a 120-min reaction consisted of approximately equal amounts of ssDNA and dsDNA with small amounts of hyDNA (Table 3). Analysis on Cs_2SO_4 gradients has confirmed this result (see below). Fraction S from a 20-min reaction had more ssDNA, less dsDNA, and possibly a little more hyDNA than the equivalent fraction after 120 min (Table 3).

Fraction S from a 120-min reaction was centrifuged to equilibrium in a Cs_2SO_4 gradient which clearly separated native and denatured P22 marker DNA. The DNA in fraction S had a density intermediate between the two markers (Fig. 2A). The broadness of the band of product DNA is consistent with the low molecular weight indicated by its slow sedimentation rate in sucrose gradients. Since heat-denatured product DNA is only slightly denser than the denatured marker (Fig. 2B), the base composition of the product is probably close to that of P22 DNA (50% guanine plus cytosine; 11), and P22 DNA is an appropriate marker for such gradients. This was confirmed by the finding that the portion of the product which resists digestion by *Neurospora* endonuclease has about the same density as P22 native DNA (Fig. 2D). Since ribonuclease had no effect on the density of the product (Fig. 2C), the product is not associated with single-stranded RNA.

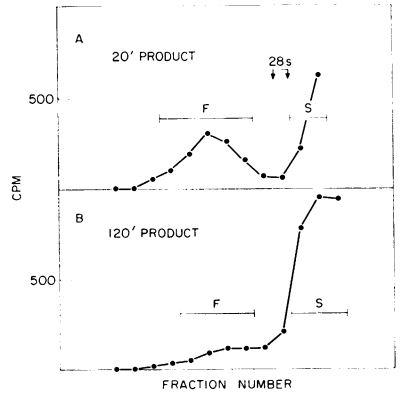

FIG. 1. *Sucrose velocity gradient analysis of polymerase product from reactions stopped at 20 and 120 min.*

TABLE 3. *Enzyme digestion studies on DNA polymerase product*[a]

Product	Time	Actinomycin D	ssDNA	hyDNA	dsDNA
	min	*μg/ml*	%	%	%
S fractions	120	—	50	10	40
	120	5	75	10	15
	120	20	85	10	5
	20	—	70	15	15
F fractions	120	—	40	35	25
	120	5	50	40	10
	120	20	60	35	5
	20	—	45	45	10

[a] Polymerase products formed in the presence or absence of actinomycin were separated into two fractions as shown in Fig. 1 and analyzed with ribonuclease and *Neurospora* endonuclease as described in Materials and Methods. The values shown are averages of two determinations.

Fraction S, therefore, appears to be a mixture of ssDNA and dsDNA. The fact that it has a unimodal density distribution between ssDNA and dsDNA markers (Fig. 2A) suggests that the double- and single-stranded products are linked to each other.

The secondary structure of the DNA in fraction F appeared to be more complex than that in fraction S (Table 3). Fraction F had more hyDNA than fraction S but was not wholly hyDNA. Furthermore, after both 20 min and 120 min of reaction, fraction F contained detectable amounts of dsDNA.

The results from Cs_2SO_4 gradients reinforce the impression of complexity in fraction F. The native fraction F product banded at the density of single-stranded RNA, presumably because it is associated with a large amount of viral RNA (Fig. 3A; 6, 14, 16). After ribonuclease digestion at high ionic strength, the product had a broad range of densities from about 1.41 (near dsDNA) to 1.53 (near hyDNA) with a mode at about 1.45 (Fig. 3B). Thus, the ribonuclease-treated product

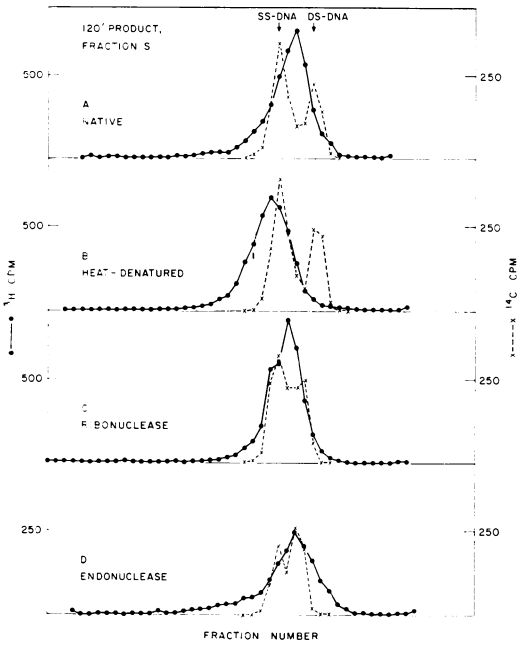

FIG. 2. *Cs_2SO_4 equilibrium gradient analysis of fraction S shown in Fig. 1B. Native and heat-denatured ^{14}C-labeled P22 DNA have densities of 1.42 and 1.44 g/ml as determined by refractometry in this laboratory. In these gradients, single-stranded RNA would appear at or near the bottom of the tube. Endonuclease (part D) digested 40% of the product in this preparation.*

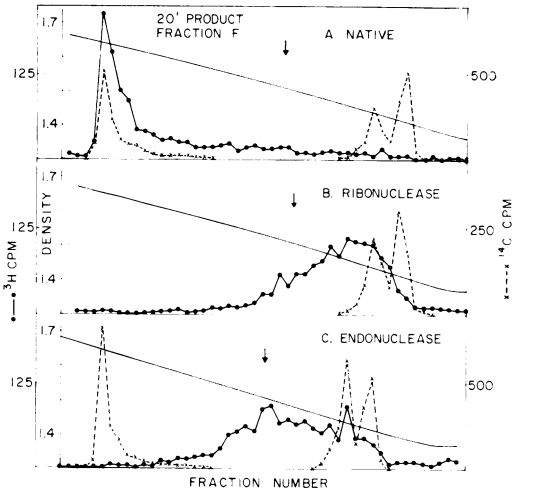

FIG. 3. *Cs_2SO_4 equilibrium gradient analysis of fraction F shown in Fig. 1A. These gradients differ from those in Fig. 2 and 4 in that they were made in polyallomer tubes, the carrier DNA was omitted, ^{14}C-labeled cytoplasmic marker RNA was added, and an additional 0.15 ml of saturated Cs_2SO_4 solution was layered at the bottom of the gradient just before centrifugation. The arrows show the expected position (5) of DNA-RNA hybrid (1.51 g/ml). Endonuclease (part C) digested 40% of the product in this preparation.*

seems to consist of a collection of structures with varying ratios of DNA to RNA and an average ratio greater than 1. Treatment with *Neurospora* endonuclease left a product with a similar broad density heterogeneity (Fig. 3C) but a distinctly higher average density than the ribonuclease-treated product. The difference in average density between the two nuclease-resistant fractions presumably reflects the digestion by endonuclease of ssDNA which is present in the ribonuclease-treated sample.

These experiments indicate that fraction F consists of a complex mixture of ssDNA, dsDNA, and hyDNA, all of which are linked to a much larger amount of viral RNA so that the mixture has the density of RNA.

Effect of actinomycin. Actinomycin at 20 μg/ml produces a partial inhibition of the DNA polymerase reaction. This sensitivity amounts to about 50% and extends through the course of the reaction (12). Higher concentrations of actinomycin cause no greater inhibition, and 20 μg/ml is necessary to obtain maximal inhibition (*unpublished data*).

Enzymatic analysis showed that the product formed in the presence of 20 μg of actinomycin per ml contains little or no dsDNA (Table 3). Actinomycin at a concentration of 5 μg/ml partially inhibited dsDNA formation. The increased percentage of ssDNA in the treated samples suggests that the drug blocks conversion of ssDNA to dsDNA. Data from Cs_2SO_4 gradients lead to the same conclusion. Fraction S from a 120-min reaction in the presence of 20 μg of actinomycin per ml had the density of ssDNA (Fig. 4A), and its density was unaffected by heat denaturation (Fig. 4B). Ribonuclease had no detectable effect on the density of the product (Fig. 4C). The small amount of DNA which was resistant to endonuclease had a density distribution which extended from dsDNA to hyDNA (Fig. 4D). It was similar to the endonuclease-resistant portion of fraction F and could result from contamination of fraction S with degradation products from fraction F.

DISCUSSION

It is clearly premature to try to draw together these data into a specific model of DNA synthesis by the virion polymerase. One aspect of the data, however, seems readily interpretable. The existence of ssDNA in fraction F, and especially its accumulation in the presence of actinomycin D,

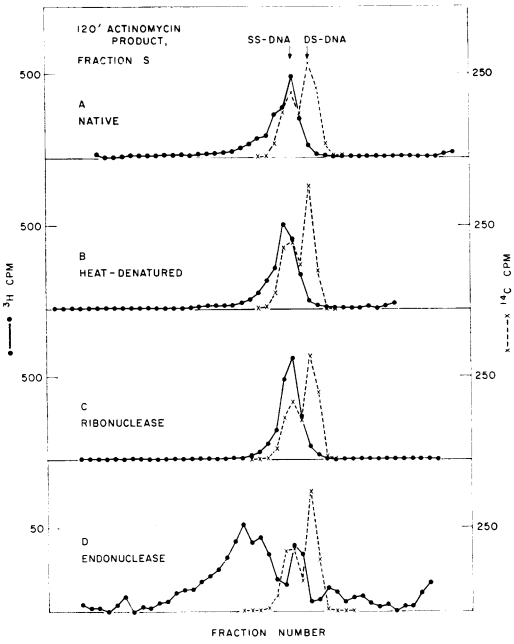

FIG. 4. *Cs_2SO_4 equilibrium gradient analysis of fraction S from a reaction product similar to that shown in Fig. 2 but made in the presence of actinomycin D (20 μg/ml). Endonuclease (part D) digested 75% of the product in this preparation.*

suggests the following sequence of events (Fig. 5). A region of viral RNA is first copied to form hyDNA. The same region is then copied again to displace a strand of ssDNA still attached to the RNA at one end. The structures formed in this way consititute fraction F. They are analogous to the poliovirus replicative intermediate and could have either configuration proposed for that molecule (1). DNA is released from fraction F to fraction S by nucleases or by some other mechanism.

Double-stranded DNA, which is found in both fractions F and S, is presumably formed by copying ssDNA. The existence of dsDNA in fraction F is easily explainable only by its synthesis as part of the complex structure which constitutes fraction F. The dsDNA in fraction S could arise by release from F, but most likely it can also be formed from ssDNA in S, because even exogenous nucleic acids can be replicated (12, 15). The dsDNA of fraction F could form if a polymerase molecule began somewhere on a displaced single strand and copied it from that point toward the free end. This would form a structure in which dsDNA and hyDNA were connected by ssDNA. After digestion with endonuclease, such a structure should yield separate dsDNA and

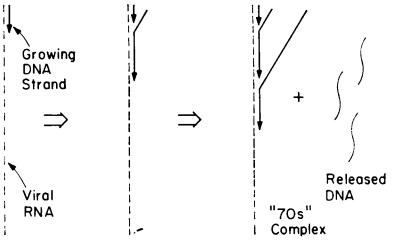

FIG. 5. *Proposed sequence of reactions by Moloney mouse leukemia virus DNA polymerase.*

hyDNA fractions, not the distribution we observed (Fig. 3C). Although this result may indicate that the formation of dsDNA occurs by some other mechanism, it could reflect the limitations of *Neurospora* endonuclease digestion. The conditions we used are sufficient to render ssDNA acid-soluble, but they do not assure the complete hydrolysis of all segments of ssDNA (*see* 10).

Our results indicate that actinomycin D inhibits the DNA polymerase by blocking the conversion of ssDNA to dsDNA. Although this could result from a direct effect on the enzyme it is more likely due to interaction between actinomycin and ssDNA (7, 8). *E. coli* RNA and DNA polymerases, when using ssDNA as template, are inhibited by concentrations similar to those required to inhibit the virion DNA polymerase (8).

Finally, we do not know the relationship between the reactions we observed in vitro and the reactions which occur in vivo. The presence of cellular factors or enzymes could drastically alter the extent of the reaction, the size of the product, or the secondary structure of the product.

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