DNA-DIRECTED AND RNA-PRIMED DNA SYNTHESIS IN MICROSOMAL AND MITOCHONDRIAL FRACTIONS OF NORMAL HUMAN LYMPHOCYTES

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ABSTRACT

Nascent DNA synthesized from RNase-sensitive endogenous DNA polymerase activities of microsomal and mitochondrial fractions from phytohemagglutinin-stimulated normal human blood lymphocytes is covalently attached to a small RNA molecule (<4S in the case of the microsomal fraction). This complex is in turn originally hydrogen bonded to a much larger preexistent DNA molecule, indicating that this synthesis of DNA is directed by DNA (template) and that the RNA is utilized solely as an initiator molecule (primer). This endogenous RNase-sensitive DNA synthesis should not be confused with the endogenous RNase-sensitive DNA synthesis in type-C RNA tumor viruses and in related cytoplasmic particles from human leukemic cells catalyzed by reverse transcriptase which utilizes RNA as both template and primer. These findings are consistent with the concept that some DNA synthesis in mammalian cell organelles may be analogous to that of bacteria in utilizing RNA primers with DNA templates.

INTRODUCTION

We have previously described two types of RNase-sensitive endogenous DNA polymerase reactions from human cells; one from a high speed cytoplasmic pellet fraction of fresh leukemic blood cells (1) and the other from a similar fraction of normal blood lymphocytes stimulated by phytohemagglutinin (PHA) and grown in short term (72 hours) tissue culture (2). We have

previously shown that a major DNA polymerase purified from the leukemic pellet fraction has biochemical and immunological properties of type-C virus RNA-directed DNA polymerase. These properties include: 1) capacity to catalyze transcription of tumor virus 70S RNA (1,3,4); 2) utilization of synthetic homopolymer primer-templates in a manner characteristic of viral RNA-directed DNA polymerase (1,3,4); and 3) inhibition by antibody specific against DNA polymerase of murine and especially primate type-C viruses (5,6); and 4) molecular size (6). A small but significant fraction of the DNA synthesized in the particle purified from myelogenous leukemic pellet fractions by repeated isopycnic bandings in sucrose density gradients is complementary to 70S RNA from murine leukemia viruses (7), similar to results by Baxt, et al. (8). More significantly, we found that up to 50% of this DNA is complementary to murine and primate sarcoma virus 70S RNA (7). In addition, this DNA is originally hydrogen-bonded to a high molecular weight RNA (6-8) indicating that this DNA is transcribed on an RNA template.

In contrast, the DNA polymerases which we have extracted from the normal lymphocytes either do not catalyze transcription from any RNA template or significantly transcribe only the poly (A) regions of viral 70S RNA (2,9). They also show marked differences in response to synthetic primer-templates when compared to the viral polymerase (2, 9) and are not inhibited by antibody against murine or primate tumor virus DNA polymerase (5, 6). These results, however, do not permit a firm conclusion on the nature of the template-primer(s) of the endogenous RNase sensitive DNA polymerase activity of the normal cell cytoplasmic pellet. Although its sensitivity to RNase could be indicative of a requirement for RNA as a template (as with the endogenous DNA polymerase activity from the RNA tumor viruses [10,11], and with a component of this reaction in at least some human leukemic cell pellets [8]), it could also derive from a requirement for RNA simply as an initiator molecule (primer) particularly since RNA-primed DNA-directed DNA synthesis has been demonstrated in bacteria (12-17). Until now we have been unable to

unambiguously determine the nature of the endogenous template of the normal lymphocyte pellet (microsomal and mitochondrial) DNA polymerase activity. In this report, we show: (1) that RNA is the primer for this endogenous DNA polymerase activity from normal lymphocyte cytoplasmic particulate fractions and that the DNA product is covalently attached to this RNA, and (2) that all the demonstrable template activity is with DNA. We propose that all the RNase sensitivity of the endogenous DNA polymerase reaction from these normal cells derives from a dependency on RNA only as a primer but not as a template.

MATERIALS AND METHODS

Preparation of Microsomal and Mitochondrial Fractions

Fresh blood lymphocytes were collected from normal donors and stimulated with PHA in culture for 72 hours as described previously (18). Approximately 3 g (wet weight) of fresh cells were harvested by centrifugation (1,000 x g; 15 minutes), washed three times by suspension in 2 volumes 0.9% NaCl followed by centrifugation (1,000 x g; 15 minutes), and finally suspended in 1 1/2 volumes buffer [0.01 M Tris-HCl, pH 7.4; 0.01 M NaCl; and 1.5 mM Mg $(OAc)_2$]. All subsequent operations were performed at 4°. The cells were gently homogenized with 20 strokes in a tight fitting glass Dounce homogenizer and the degree of rupture determined under a 100 X phase contrast microscope. Cell breakage was virtually complete (>90%) and no ruptured nuclei or free strands of DNA were observed. Washed bentonite (2 mg/ml) was added and removed with the nuclei and cell debris by centrifugation (1,000 x g; 15 minutes). The mitochondrial and microsomal fractions were collected by successive centrifugations at 10,000 x g for 15 min. (mitochondrial) and then 100,000 x g for 2 hours (microsomal). These fractions were used to synthesize DNA from nucleic acid template-primers associated with the polymerase in the particle (endogenous DNA synthesis).

Preparation and Purification of Endogenous Microsomal and Mitochondrial DNA Polymerase Products

Microsomal and mitochondrial fractions were suspended in 1 ml of a solution containing 50 µmoles Tris-HCl, pH 8.3; 60 µmoles KCl; 10 µmoles Mg $(OAc)_2$; 20 µmoles

dithiothreitol; 50 μg/ml actinomycin D (Calbiochem) (where indicated); 0.015% Nonidet P-40 (Shell); and 1 mC each of ^{3}H-dATP (18.4 C/mmole), ^{3}H-dCTP (22.4 C/mmole), ^{3}H-dGTP (12.4 C/mmole), and ^{3}H-TTP (47 C/mmole) and incubated for 15 minutes at 37°C. Labelled substrates were from New England Corporation and were flash evaporated just before use. Reactions were terminated by addition of sodium dodecyl sulfate to 1% and KCl to 0.12 M. Nucleic acids were purified from these solutions by phenol:m cresol and diethyl ether extractions followed by precipitation with cetyltrimethylammonium bromide, then ethanol:salt as described previously (19) and dissolved in 0.25 ml 2 x SSC (0.015 M sodium citrate, pH 7.0; 0.15 M NaCl).

Analysis of DNA Products

Cs_2SO_4 equilibrium density gradient centrifugation was performed as described previously (1, 19). A single strand-specific (S1) nuclease (20) was purified from Aspergillus oryzae (Sanzyme R, Calbiochem) as described by Sutton (21). Pancreatic ribonuclease A and ribonuclease T1 were purchased from Worthington Biochemicals.

RESULTS

Analysis of Endogenous Microsomal DNA Polymerase Product by Cs_2SO_4 Density Gradient Centrifugation

DNA product from the RNase-sensitive endogenous microsomal DNA polymerase reaction was synthesized as described in Materials and Methods. As shown in Figure 1, in agreement with our previous report (2), DNA synthesized in this reaction bands entirely as DNA; little material with a density greater than that of DNA alone is evident. Furthermore, heat-denatured product also bands primarily as pure DNA.

In contrast, DNA product synthesized in the presence of actinomycin D bands in part (∿65%) in the density regions of RNA and in part in the DNA density region (Fig. 2). After heat treatment, however, the product bands nearly entirely (∿95%) as RNA. Treatment of the original DNA product with S1 nuclease results in the total disappearance of labelled material banding

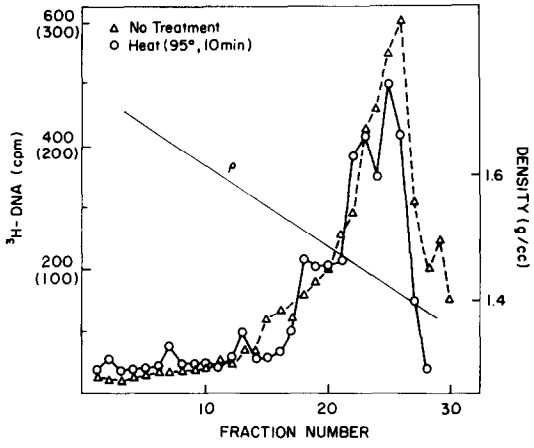


Figure 1. Cs_2SO_4 density gradient analysis of DNA product of endogenous microsomal normal human blood lymphocyte DNA polymerase. DNA product was prepared and purified from endogenous reactions of the microsomal DNA polymerase and analyzed by Cs_2SO_4 density gradient centrifugation as described in Materials and Methods after no treatment (Δ---Δ) or heat denaturation (95°, 10 min.) (o——o). Numbers in parentheses refer to the heat denatured product.

in the RNA region, indicating that all the DNA banding in the RNA region is single stranded, while the labelled material in the DNA region is almost entirely unaffected, indicating that it is primarily composed of hydrogen-bonded DNA. If, however, the apparently double stranded DNA which remains after nuclease treatment is heat denatured, once again almost all the labelled material appears in the RNA regions, indicating that a covalently joined product DNA-primer RNA complex is initially hydrogen-bonded to a much larger strand of DNA, presumably the endogenous template. After alkali treatment, which results in a loss of ∿ 80% of acid-precipitable radioactivity, only a peak of labelled material in the DNA region is seen. (In contrast, DNA product synthesized in the absence of actinomycin D is >90% alkali-stable).

These results suggest: 1) the product DNA is nearly entirely covalently attached to RNA and is very small relative to that RNA, and 2) some of the RNA to which labelled DNA is covalently attached is in part originally hydrogen bonded to a much larger piece of unlabelled DNA, since heat denaturation shifts most of the labelled material originally banding as

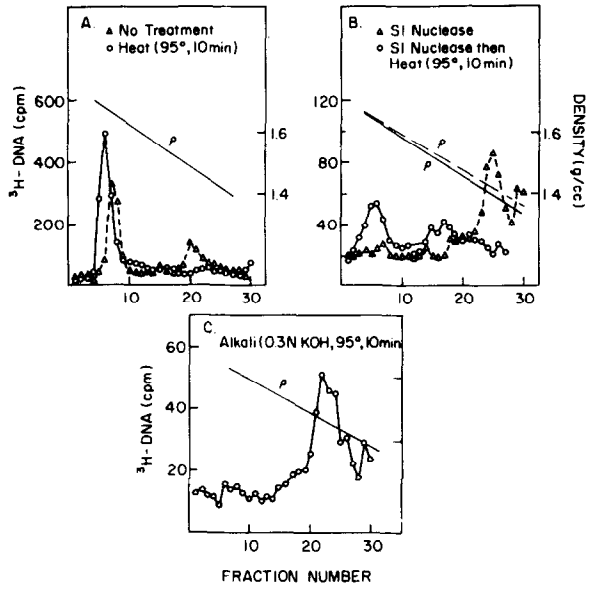
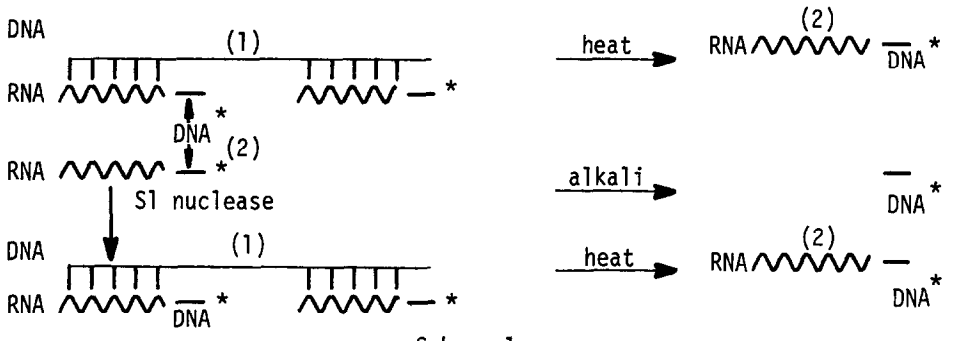


Figure 2. Cs_2SO_4 density gradient analysis of DNA product of endogenous microsomal normal human blood lymphocyte DNA polymerase synthesized in the presence of actinomycin D. DNA product was prepared and purified from an endogenous microsomal DNA polymerase reaction containing actinomycin D and analyzed by Cs_2SO_4 density gradient centrifugation as described under Materials and Methods. A) DNA product was analyzed after no further treatment (Δ---Δ) or after heat denaturation (95°, 10 min) (o—o); B) DNA product was analyzed after digestion with S1 nuclease (~10 μg protein) in 0.2 ml buffer (0.025 M NaOAc, pH 4.3; 0.3 M NaCl, 0.006 M $ZnSO_4$) for 60 min. at 37° without further treatment (Δ---Δ) or after subsequent heat denaturation (95°, 10 min) (o—o). (S1 nuclease activity is abolished by heating at 95° for 10 min.) C) DNA product was analyzed after treatment with 0.3 N KOH (95°, 10 min.). All gradients were performed with equal radiolabelled DNA inputs measured prior to S1, heat, or alkali treatment.

DNA to the density regions of RNA. We interpret these results to mean that this RNA is serving as a primer for a reaction involving a DNA template. These treatments and our interpretation of the template, primer, and product are schematically illustrated below: Product (1) is a complex of template-primer and a newly made DNA. Product (2) consists only of primer and covalently attached newly synthesized DNA.



Scheme 1

Actinomycin D, added in some experiments to prevent synthesis of a second product strand, presumably also inhibits the synthesis of the initial strand so that only very small pieces of DNA are formed. In its absence, DNA chain elongation may proceed far enough so that the product DNA is large enough to determine the density of the product-primer complex. Alternatively, beyond some point in the chain elongation reaction the RNA primer may be degraded. The latter case has its precedent in a bacterial system (12).

The DNA moiety of this primer-product complex, as shown by the above data, would be expected to contribute only minimally to its size. Consequently, a velocity gradient analysis of heat-denatured product should give an estimate of the size of the primer. As shown in Figure 3, the peak fraction from such a gradient sediments somewhat more slowly than a 4S RNA marker, indicating that the size of the primer is probably less than 4S.

Characterization of Endogenous Microsomal Pellet DNA Polymerase Product by Nuclease Treatment

Endogenous microsomal DNA polymerase product synthesized in the presence of actinomycin D was also analyzed by a series of nuclease treatments as detailed in the legend to Table 1. Unfractionated DNA product is approximately 60% susceptible to S1 nuclease (single stranded DNA). This microsomal DNA product is only about 20% resistant to RNase A under conditions which degrade single and double stranded RNA, suggesting that the bulk of the newly synthesized DNA is of a very small size and is attached to RNA. Pre-treatment with RNase exposed no add-

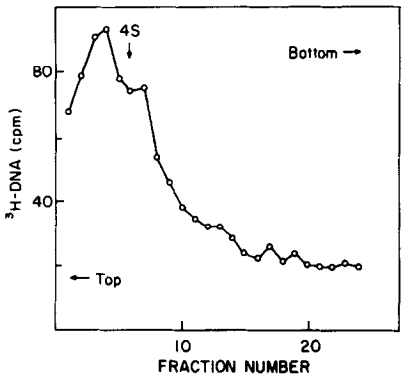


Figure 3. Velocity sedimentation gradient of heat-denatured normal lymphocyte microsomal DNA polymerase product. DNA product was prepared and purified from an endogenous microsomal DNA polymerase reaction in the presence of actinomycin D as described under Materials and Methods and then heat denatured (95°, 10 min.). This was layered in 0.2 ml on top of an 11 ml gradient of 10-30% glycerol in 0.01 M Tris-HCl buffer, pH 7.4, which contained 0.1 M NaCl and 1 mM EDTA, and centrifuged in an SW-41 rotor for 16 hours at 40,000 rpm. The gradient was collected in 24 fractions from the top with a Buchler Auto Densi-Flow and the absorbance at 260 nm (A_{260}) or acid-precipitable radioactivity determined for each fraction. The vertical arrow indicated the position of the peak of an external marker of E. coli 4S RNA (Worthington Biochemicals) as measured by A_{260}.

itional single stranded DNA to S1 nuclease digestion, again suggesting that no detectable DNA product is hydrogen bonded to RNA.

Analysis of Microsomal DNA Product Fractionated with Hydroxylapatite

Further evidence that the DNA product which bands as RNA is single stranded was provided by preparing DNA products in the presence of actinomycin D and fractionating the single-stranded and double-stranded material on hydroxylapatite (22-24). The DNA product eluting at 0.15 M phosphate was ascertained to be totally single-stranded by treatment with S1 nuclease (Table 1). This material bands entirely as RNA (Figure 4) and is totally sensitive to ribonuclease (Table 1) and alkali. The nascent DNA moiety of this primer-product complex averages only a few nucleotides in length as determined by electrophoresis on DE-81 paper in the presence of 7 M urea at pH 7.5. DNA product which elutes at 0.5 M phosphate is entirely double-stranded as measured by S1 nuclease (Table 1) and bands as DNA (Figure 3).

Table 1. Enzymatic Analysis of Endogenous Microsomal DNA Polymerase Product

Exp.	Product	Enzyme Treatment	Acid Precipitable radioactivity (cpm)
1	Unfractionated	none	781
		S1 nuclease	333
		RNase	160
		RNase, then S1 nuclease	180
2	low salt eluate from hydroxylapatite	none	862
		S1 nuclease	124
		RNase	105
3	high salt eluate from hydroxylapatite	none	248
		S1 nuclease	251

DNA product was purified from an endogenous microsomal DNA polymerase reaction containing actinomycin D as described in Materials and Methods, or was further purified (where indicated) on hydroxylapatite (Bio Gel HT, BioRad Laboratories, DNA grade) as follows: 0.25 ml DNA product was diluted with 1 ml 0.01 M sodium phosphate (pH 7.8) and mixed with 1 ml bed volume hydroxylapatite equilibrated with 0.01 M sodium phosphate (pH 7.8) and centrifuged in an International Model CL centrifuge for 5'. The supernatant solution was discarded and the pellet resuspended in 2 ml of 0.05 M sodium phosphate (pH 7.8) at room temperature for 5 min., then pelleted as before. The pellet was next resuspended in 2 ml of 0.15 M sodium phosphate (pH 7.8) for 8-10 min. at 60°, and again pelleted as before. This was repeated twice and the supernatant solutions were pooled dialyzed overnight at 4° against 100 vols 0.25 M NaOAc (pH 4.5), precipitated overnight at -20° in 67% ethanol: 0.3 M NaCl, and dissolved in 0.25 ml 2X§§C. The yield of acid-precipitable radioactivity was about 60% of input. Elution of double stranded product was then accomplished by resuspension of the pelleted hydroxylapatite in 2 ml 0.5 M sodium phosphate (pH 7.8) for 8-10 min. at 60° and then centrifuged at room temperature as above (three times), followed by pooling, dialysis, and ethanol precipitation

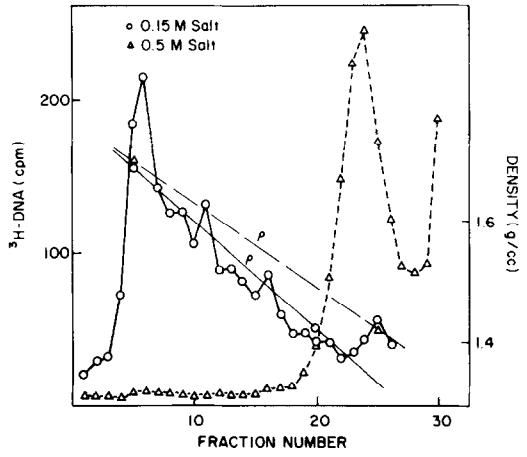


Figure 4. Cs_2SO_4 density gradient analysis of DNA product of endogenous microsomal DNA polymerase of normal blood lymphocytes after fractionation by hydroxylapatite. DNA product was prepared and purified from an endogenous microsomal DNA polymerase reaction containing actinomycin D and fractionated on hydroxylapatite as described in Table 1. Single stranded DNA product (0.15 M phosphate eluate) (o——o) and double stranded DNA product (0.5 M phosphate eluate) (Δ---Δ) were then analyzed by Cs_2SO_4 density gradient centrifugation as described under Materials and Methods.

Analysis of Endogenous DNA Polymerase Product from the Mitochondrial Fraction

DNA was prepared in the presence of actinomycin D and purified from an endogenous DNA polymerase reaction present in the mitochondrial fraction and then analyzed by Cs_2SO_4 density centrifugation as described in Materials and Methods. As shown in Figure 5, DNA product from the mitochondrial

Table 1 cont.
of the supernatant solutions as described above. The acid precipitable radioactivity of this fraction was about 15% of the input. DNA products were incubated 45 min. at 37° in the presence of 10 mM Tris-HCl, pH 7.4, with or without 100 µg/ml RNase A and 1000 U/ml RNase T1 as indicated in a total volume of 25 µl, and then incubated a further 45 min. at 37° after addition of 0.2 ml 0.025 M NaOAc, pH 4.3, containing 0.3 M NaCl and 0.006 M $ZnSO_4$ with or without 10 µg purified S1 nuclease protein. The first incubation was omitted for Experiment 3. Acid precipitable radioactivity was then determined by collection on a Millipore filter and counting in a liquid scintillation counter.

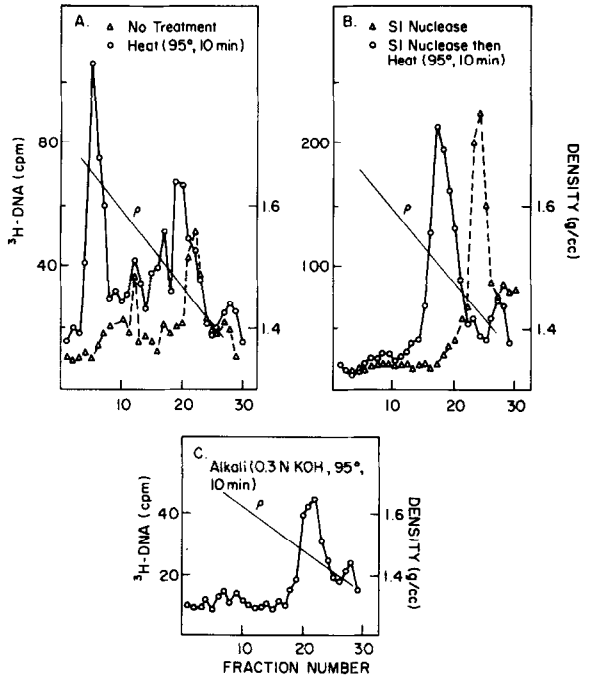


Figure 5. Cs_2SO_4 density gradient analysis of DNA product of endogenous normal lymphocyte mitochondrial DNA polymerase. DNA product was prepared and purified from an endogenous mitochondrial DNA polymerase reaction in the presence of actinomycin D and analyzed by Cs_2SO_4 density gradient centrifugation as described under Materials and Methods. A) DNA product was analyzed after no further treatment (Δ---Δ) or after heat denaturation (o—o) (95°, 10 min.). B) DNA product was analyzed after digestion with S1 nuclease as described for Figure 1 and analyzed without further treatment (Δ---Δ) or after subsequent heat denaturation (95°, 10 min.) (o—o). C) DNA product was analyzed after treatment with 0.3 N KOH (95°, 10 min.).

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fraction synthesized in the presence of actinomycin D also originally bands in part as RNA, and heat treatment again results in a significant shift of DNA label previously banding in the DNA region to RNA regions of the gradients. S1-treated DNA product bands entirely as DNA, while the product which has been first S1-treated and then heat-denatured again bands with a density greater than DNA (but less than RNA). This intermediate density is probably due to the large size of the DNA moiety of this product-primer complex relative to the primer. Indeed, this mitochondrial product is 100% RNase-stable (not shown), in contrast to the microsomal products studied. Taken

together, these data indicate that at least some of the endogenous DNA polymerase reactions of the mitochondrial fraction utilize an RNA primer and a DNA template.

DISCUSSION

We have presented an analysis of DNA products synthesized in microsomal and mitochondrial fractions from PHA-stimulated proliferating normal human fresh blood lymphocytes. A characteristic of these endogenous DNA polymerase activities is partial sensitivity to RNase (2). At least two classes of products can be isolated from reaction mixtures incubated for a relatively short time in the presence of actinomycin D, an inhibitor of this reaction (2). The first is a single stranded species in which a small RNA molecule is covalently linked to an even shorter fragment of nascent DNA. The second class consists of the above complex hydrogen bonded to much larger pieces of unlabelled, and hence pre-existent, DNA. Heat denaturation of this template-primer-product complex results in the appearance of single-stranded RNA-DNA molecules. These molecules can be isolated by hydroxylapatite chromatography. Omission of actinomycin D results in the recovery of only double-stranded DNA. These results taken together indicate that DNA serves as a template for this reaction, and that the RNase sensitivity may derive at least in part from a dependency on RNA as a primer, analogous to DNA replication in E. coli (12-14 , 17) and phage (15,16). However, we cannot state whether the free single-stranded RNA-DNA molecules observed prior to heat denaturation originated on a DNA or RNA template, nor do we know the reasons for their apparently premature dissociation from that template.

It is clear that RNase sensitivity alone does not indicate that an endogenous DNA polymerase reaction is RNA-directed even when product DNA can be isolated which is associated with RNA. To demonstrate RNA-directed synthesis of an endogenous reaction, it would appear desirable to show either 1) a strand of unlabelled DNA which is hydrogen-bonded to RNA i.e., can be shifted in Cs_2SO_4 gradients to the density region of DNA from that of RNA

by heat denaturation or is rendered susceptible to S1 nuclease by pretreatment with ribonuclease, or 2) complementarity of the product DNA to RNA but not to DNA isolated from the same fraction. A RNase-sensitive DNA synthesis which is DNA-directed might be verified by 1) preparing a product DNA chain small enough to not substantially effect the density of the primer RNA to which it is attached and showing that this product-primer complex is hydrogen bonded to unlabelled DNA, and/or 2) showing that the product DNA is complementary only to the DNA isolated from the same fraction. Because of technical difficulties encountered in the second approach, we have used the first to show a DNA-directed synthesis of DNA in the endogenous RNase-sensitive DNA polymerase reaction of these cytoplasmic particulate fractions from normal fresh blood lymphocytes.

The initiation of endogenous DNA synthesis in the mitochondrial fraction bears strong similarity to the findings with the microsomal pellets. DNA synthesis in this fraction then also appears to utilize DNA as a template and RNA as a primer. It has been recently reported that circular mitochondrial DNA contains covalently inserted ribonucleotide sequences (25,26), and although we have no evidence that the endogenous DNA polymerase reactions we have studied, in fact, represent replication of this mitochondrial DNA, it is tempting to speculate that these ribonucleotide sequences represent the primer molecules which we observe. Whether the reactions of both fractions are identical (as would be the case, if, for example, the microsomes were to include fragments of partially ruptured mitochondria) and whether in fact the activities observed originate in the microsomes and mitochondria or are a result of nuclear leakage are questions which remain to be answered. Answers are currently being pursued through molecular hybridization experiments.

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