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Conditions for Using DNA Polymerase I as an RNA-Dependent DNA Polymerase

(RNA-directed complementary DNA/enzyme mechanism/distamycin A/ hemoglobin complementary DNA)

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ABSTRACT **Conditions are described for using *Escherichia coli* DNA polymerase I for synthesizing complementary DNA copies of natural RNA molecules, which are suitable for use in hybridization experiments. The molar ratio of enzyme to template is critical; below a certain level, synthesis is not observed. Hybrids formed with the complementary DNA are of comparable specificity and stability to those formed with complementary DNAs synthesized by viral RNA-directed DNA polymerase. Synthesis of dA-dT polymers, a common occurrence with this enzyme, can be eliminated by including distamycin in the reaction mixture.**

RNA-directed DNA polymerase (1) from avian myeloblastosis virus (AMV) (2) lacks template specificity (3), making it useful for synthesizing highly labeled DNA complements from a wide variety of RNA species. These complementary DNAs (cDNAs) have been used as hybridization probes, affording an extremely useful and sensitive technique for detecting and quantitating nucleic acid sequences (4–6).

Only AMV is a practical source for the enzyme, and its relative unavailability has prevented many from using these techniques for resolving a variety of interesting problems. Previous attempts (7–11) to use *E. coli* DNA polymerase for copying RNA led to indifferent results until the recent reported success by Loeb and his colleagues (12). Because of the potential usefulness of the enzyme to those interested in preparing cDNA hybridization probes, we felt that further investigation of the *E. coli* enzyme was warranted. We report here that *E. coli* DNA polymerase I is able to make complementary copies from a variety of natural heteropolymeric RNAs, thus confirming the results of Loeb *et al.* (12). We also show that a high ratio of enzyme to template RNA molecules is required to elicit RNA-dependent DNA polymerase activity from the *E. coli* polymerase, and that the unbalanced incorporation of thymidine observed by others (7, 10, 13) can be eliminated.

MATERIALS AND METHODS

DNA Polymerase. Three different batches of *E. coli* polymerase I (EC 2.7.7.7; deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase) were used for these experiments. Two preparations were kindly provided by Dr. Lawrence Loeb (Institute for Cancer Research, Philadephia) and were prepared according to Slater *et al.* (14). Dr. A. Nussbaum (Hoffman-LaRoche, Inc.) generously supplied an enzyme isolated as described by Jovin *et al.* (15). The AMV polymerase was prepared as described (2).

Isolation of RNAs. Qβ virus and viral RNA were prepared by the procedure of Pace *et al.* (16). The 70S RNA of AMV was isolated as described (2). *Drosophila* 28S ribosomal RNA was kindly provided by Dr. Loeb. Rabbit-globin messenger RNA was a generous gift of Dr. A. Bank (Columbia University).

Antibiotics. Actinomycin D was obtained from Sigma Chemicals. Distamycin A hydrochloride was supplied by Farmaceutici Italia, Milan, Italy.

Preparation of DNA Products. RNA-directed DNA synthesis was carried out as described (12). A 50-μl reaction mixture contained the following: 2.5 pmol of RNA; 25 pmol of *E. coli* DNA polymerase I; 0.4 μmol of $MgCl_2$; 3 μmol of Tris·HCl (pH 8.2); 5 nmol of each deoxynucleoside triphosphate, one of which was labeled; and 5 μg of actinomycin D. Incubation was at 37°. The DNA was separated from residual substrate on a Sephadex G-50 column.

Hybridization reactions were carried out in (*a*) 50% formamide, 0.4 M NaCl, 50 mM EDTA, 0.1% sodium dodecyl sulfate at 37° or (*b*) 0.3 M NaCl, 2 mM EDTA, 0.1% sodium dodecyl sulfate, 20 mM sodium phosphate (pH 7.0) at 68°. The annealing reactions were analyzed for hybrid formation as follows: (*a*) Cesium sulfate equilibrium centrifugation was performed as described (4). (*b*) Thermal elution with hydroxylapatite was performed in 0.12 M sodium phosphate (pH 6.7). The hybridization reaction was diluted 100-fold with 0.12 M sodium phosphate and loaded onto a 2-ml bed volume hydroxylapatite column at 60°. The column was then washed with 14 ml of 0.12 M sodium phosphate buffer. The temperature was raised in small increments, and the column was eluted with 6 ml of 0.12 M sodium phosphate buffer at each temperature. The fractions were precipitated with Cl_3CCOOH and the radioactivity was measured. (*c*) *Micrococcal* nuclease was used as described (17).

RESULTS

To avoid confusion by poly(dT) or poly(dA-dT) formation, the response of *E. coli* DNA polymerase I to RNA templates was tested by following the uptake of [^{3}H]dGTP into acid-precipitable material. With AMV 70S RNA, significant time-dependent incorporation of radioactivity was observed (Table 1), which was stimulated by oligo$(dT)_{10}$ and partially inhibited

Abbreviations: cDNA, complementary DNA; AMV, avian myeloblastosis virus; $T_{e^1/_2}$, temperature at which half the complexed DNA is eluted from hydroxylapatite columns.

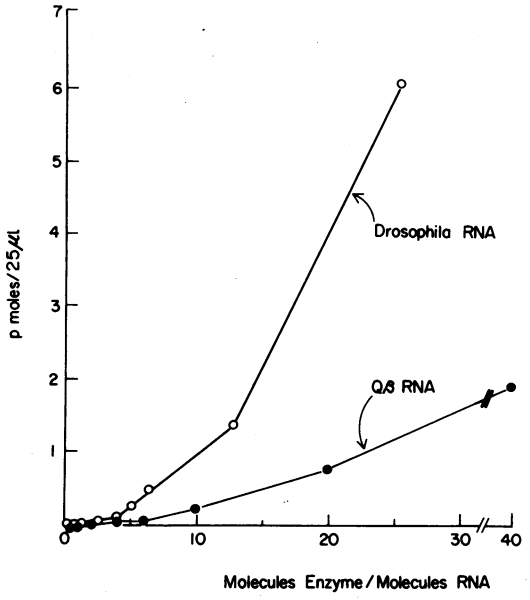

FIG. 1. Dependence of cDNA synthesis upon enzyme concentration. Standard reaction mixtures (25 µl) were prepared containing [^{3}H]dCTP (26 Ci/mmol). *Drosophila* 28S RNA (1.6 pmol) (molecular weight 1.6 × 10^6) or 1 pmol of Qβ RNA (molecular weight 1.2 × 10^6) were present in each reaction. Various amounts of purified *E. coli* polymerase I (molecular weight 110,000) were added. After a 10-min incubation, acid-precipitable radioactivity was determined. Reaction kinetics (not shown) were linear to at least 30 min.

by actinomycin D. Prior incubation with RNase A eliminated the reaction. Since the enzyme can use RNA as a primer for DNA-templated DNA synthesis (9, 18–20), RNase sensitivity could be due to destruction of primer molecules. To rule out this possibility, a portion of the RNA was heated at 100° for 2 min, quickly chilled in ice, diluted to 5.6 µg/ml, and centrifuged to equilibrium in cesium sulfate. Material banding at 1.64–1.68 g/ml was removed, dialyzed extensively against 1 mM EDTA, and used as template.

TABLE 1. *Template activity of AMV RNA with* E. coli *DNA polymerase I*

Experiment	pmol of [^{3}H]dGTP incorporated
(*1*) AMV RNA alone	11.1
+ 0.4 µg of oligo(dT)$_{10}$	25.2
+ 0.4 µg of oligo(dT)$_{10}$ and 100 µg/ml of actinomycin D	15.3
+ 50 µg/ml of RNase A	<0.1
(*2*) Unfractionated AMV RNA	15.2
Cs_2SO_4-purified AMV RNA	0.3
+ 0.5 µg of oligo(dT)$_{10}$	5.5
+ 0.5 µg of oligo(dT)$_{10}$ and 50 µg/ml of RNase A	0.3
0.5 µg of oligo(dT)$_{10}$ alone	<0.1

Reaction mixtures were prepared as described in *Methods*. [^{3}H]dGTP was present at 3600 cpm/pmol. Incubation was at 37° for 10 min. Incorporation is expressed per 0.1-ml reaction volume. In Exp. 1, 2 µg of AMV RNA was present; in Exp. 2, 1 µg of AMV RNA was used. For determination of RNase sensitivity (50 µg/ml), reactions were incubated at room temperature or 10 min before addition of the DNA polymerase.

The reaction now exhibited an absolute requirement for added primer (Table 1). When oligo(dT)$_{10}$ was offered, incorporation was again observed. The primer alone stimulated no synthesis. Pretreatment with RNase A inhibits the oligo(dT)$_{10}$-primed reaction; therefore, the RNase sensitivity cannot be ascribed to a DNA-templated synthesis primed by RNA.

Since previous attempts to obtain RNA-directed DNA synthesis with *E. coli* polymerase I failed (8–11), it was of interest to identify the feature that leads to a successful reaction. As

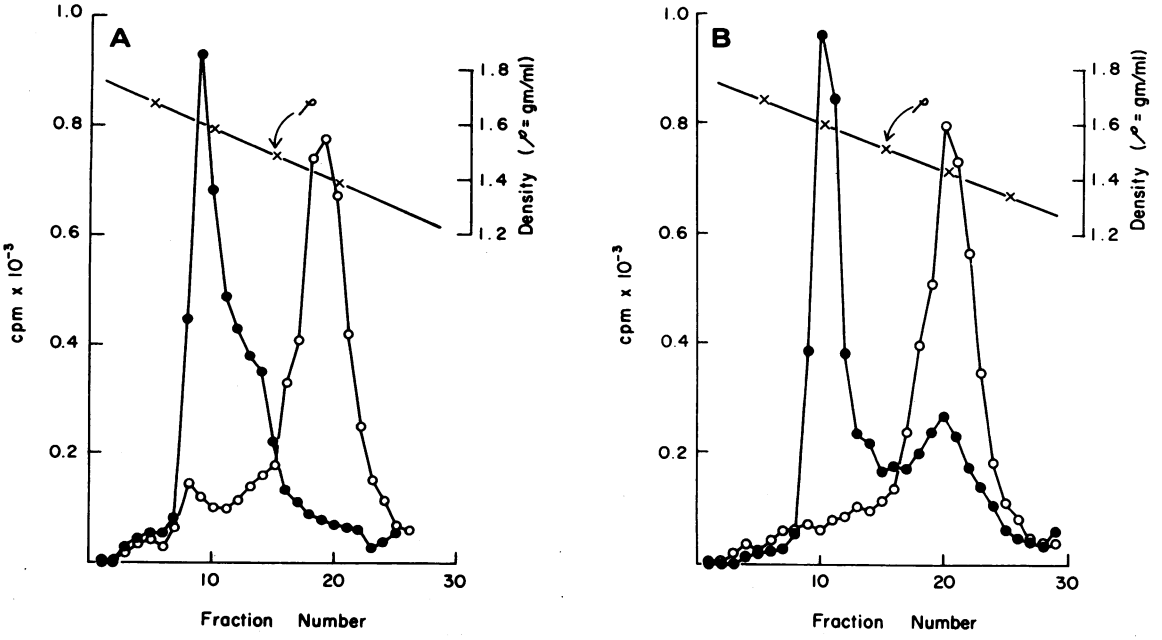

FIG. 2. Cs_2SO_4 density gradient analysis of hybridization with (*A*) *Drosophila* 28S ribosomal cDNA, and (*B*) Qβ cDNA. All cDNAs except hemoglobin were prepared without added primer. Annealing was performed in 50% formamide in 50 µl as described in *Methods*. The *Drosophila* cDNA was annealed for 16 hr with 1 µg of *Drosophila* 28S RNA (●——●) or AMV RNA (○——○). The Qβ cDNA was annealed with 2.5 µg of Qβ RNA (●——●) or AMV RNA (○——○).

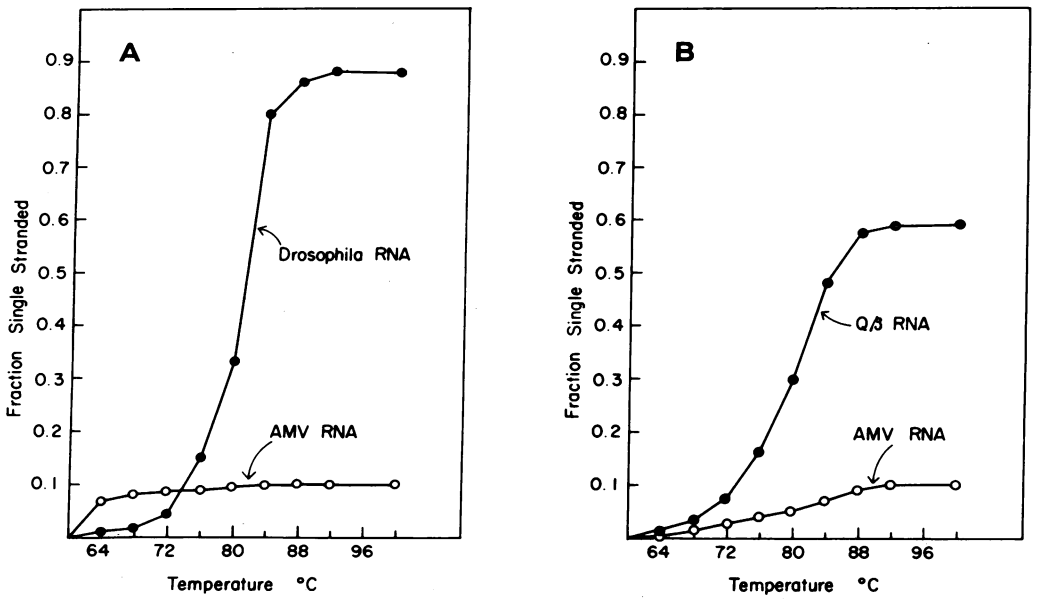

FIG. 3. Thermal elution chromatography of hybridizations with (*A*) *Drosophila* 28S ribosomal cDNA, and (*B*) Qβ cDNA. Annealing reactions, prepared as described in the legend to Fig. 2, were diluted to 4 ml with 0.12 M sodium phosphate (pH 6.7) and loaded onto a 2-ml hydroxylapatite column at 60°. Four 2-ml washes of 0.12 M sodium phosphate (pH 6.7) were collected at the indicated temperatures and counted in Aquasol as described. (*A*) *Drosophila* cDNA annealed to *Drosophila* 28S RNA (●——●) or AMV RNA (○——○). (*B*) Qβ cDNA hybridized to Qβ RNA (●——●) or AMV RNA (○——○).

shown in Fig. 1, the critical factor is an excess of enzyme molecules. With *Drosophila* 28S ribosomal RNA and Qβ RNA (also hemoglobin mRNA, data not shown), almost no incorporation is observed until the ratio of enzyme to RNA molecules exceeds four. Above that value, the rate of incorporation rises exponentially.

To provide more rigorous proof that an RNA-instructed reaction was being observed, hybridizations were performed with cDNAs copied from several RNA templates, and the hybrids were analyzed by various methods to show that a high-melting hybrid was formed with the template RNA and not with heterologous RNAs. Fig. 2 shows the results obtained when the reactions were analyzed by Cs_2SO_4 density gradient centrifugation. Approximately 90% of the *Drosophila* cDNA annealed to its template RNA. With AMV RNA, only about 15% of the counts were found outside the DNA region. With the Qβ cDNA, 68% of the counts annealed to Qβ RNA and about 15% to AMV RNA. Results obtained with DNAs synthesized from AMV 70S RNA and rabbit-globin messenger RNA (not shown) were similar.

The Cs_2SO_4 gradient analyses showed that the cDNAs were able to form specific complexes with the template RNA but gave no information concerning the type of hybrids formed. Strong hybrids formed between cDNAs made with the AMV DNA polymerase, and their corresponding RNAs have been found to elute from hydroxylapatite columns at above 80° in 0.12 M phosphate buffer. Hybrids between adenine and thymidine homopolymers and poorly matched hybrids are released at somewhat lower temperatures. Single-stranded DNA is not retained on the column at 60° (5). The cumulative fraction of denatured double-stranded nucleic acid (DNA eluted from the column at temperatures above 60°) is plotted against the temperature. The plateau value is the maximum fraction of the total input cDNA hybridized, whereas the temperature at which half the complexed DNA is eluted, $T_{e1/2}$, is a measure of the stability of the hybrid.

Fig. 3*A* shows the results obtained when hybrids with *Drosophila* cDNA product were analyzed. It is apparent that a strong hybrid ($T_{e1/2}$ = 81°) is formed with *Drosophila* 28S ribosomal RNA, whereas hybrid formed with AMV 70S RNA melts at a considerably lower temperature ($T_{e1/2}$ < 63°). Fig. 3*B* shows that similar findings are obtained with Qβ cDNA. These results strongly indicate that the *E. coli* DNA polymerase is making a faithful copy of at least a portion of these RNA templates.

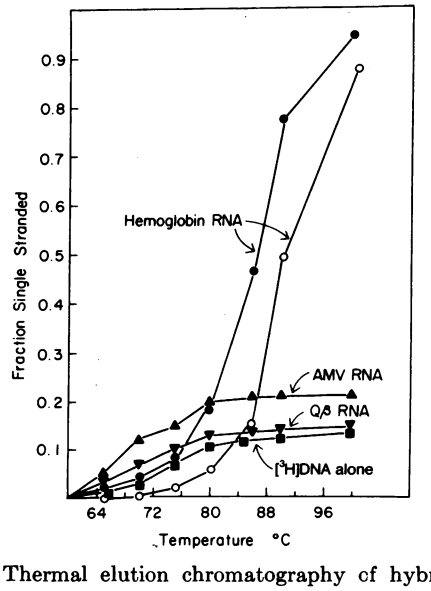

FIG. 4. Thermal elution chromatography of hybridizations with rabbit-globin cDNAs. Hybridizations were performed in 25 μl with 0.2 μg of rabbit-globin mRNA (●——●, ○——○), 0.5 μg of AMV RNA and 6 μg of poly(A) (▲——▲), or 2.5 μg of Qβ RNA (▼——▼), or alone (■——■) for 12 hr. *Closed symbols:* hybrids with *E. coli* polymerase product; *open symbols:* hybrids with AMV polymerase product. Hydroxylapatite chromatography was performed as described in the legend to Fig. 3, except that the buffer contained 0.4% sodium dodecyl sulfate.

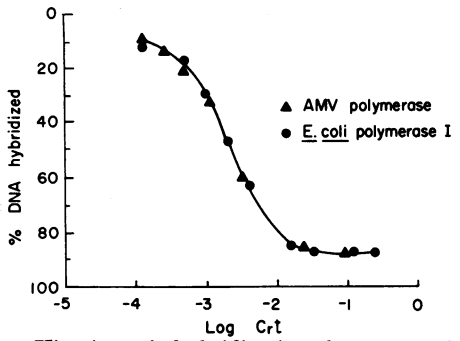

FIG. 5. Kinetics of hybridization between rabbit-globin cDNAs and rabbit-globin mRNA. Hybridization reaction mixtures (20 μl) contained 0.3 M NaCl, 2mM EDTA, 0.02 M sodium phosphate (pH 7.0), 0.1% sodium dodecyl sulfate, 0.1 pmol of globin cDNA, and various amounts of globin mRNA. After incubation at 68° for 8 hr, two aliquots of 8 μl each were removed and treated as described (26).

To permit a better comparison between the *E. coli* and AMV polymerases, we characterized cDNA products synthesized from rabbit-globin messenger RNA by both enzymes. Globin cDNA made with the AMV enzyme has been examined thoroughly (21–23). Fig. 4 shows that globin cDNA made with the *E. coli* enzyme forms a high-melting ($T_{e1/2}$ = 86°) hybrid only with the template RNA and not with RNAs from AMV or Qβ. A higher percentage of lower melting duplexes is formed between the cDNA and AMV RNA than with Qβ RNA, probably due to poly(dT) and poly(rA) interactions. A melting curve of hybrid formed with globin mRNA and the cDNA synthesized by AMV RNA-dependent DNA polymerase is also shown in Fig. 4. Both cDNA–template RNA duplexes show relatively sharp elution profiles; however, those made with the *E. coli* polymerase have a slightly lower $T_{e1/2}$ (86°) than those made with the AMV enzyme (89°).

We next compared the kinetics of reannealing of the two products with the template RNA. Hybridization was monitored with micrococcal nuclease under conditions whereby the enzyme specifically degrades single-stranded regions of nucleic acids (17). We found (Fig. 5) that the hybridization kinetics with the two cDNAs are indistinguishable.

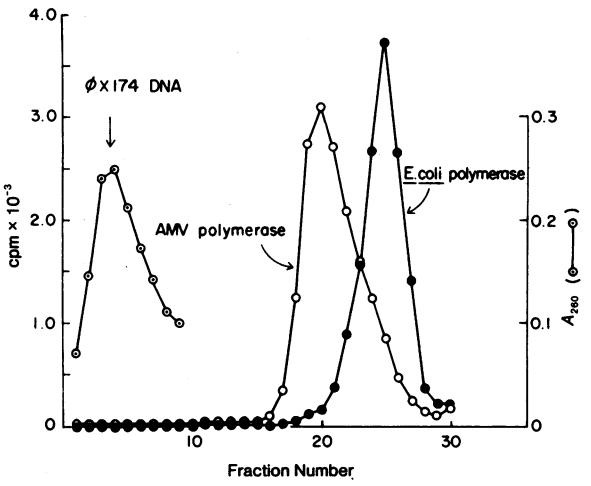

FIG. 6. Alkaline sucrose gradients of rabbit-globin cDNAs. Globin cDNAs labeled, respectively, with ^3H and ^{32}P were synthesized with the AMV and *E. coli* polymerases, mixed with φX174 DNA, and sedimented through a 5–20% sucrose gradient containing 0.1 M NaOH, 0.9 M NaCl in a Spinco SW 50.1 rotor at 50,000 rpm, 0°, for 7.5 hr. Fractions were collected from the bottom and the acid-precipitable radioactivity was determined.

The sizes of the two cDNAs were also compared. DNA products, one labeled with ^3H and the other with ^{32}P, were synthesized in parallel reactions with the same template RNA. After extraction with phenol–cresol, the reaction mixtures were combined with unlabeled φX174 DNA and sedimented in an alkaline sucrose gradient. Fig. 6 shows the sedimentation profiles of the three DNA species. Taking the value of φX174 DNA to be 18 *S*, the cDNA synthesized with AMV polymerase sediments at approximately 7 S, corresponding to a molecular weight of 200,000 (24). The molecular weight of the globin messenger RNA has been found to lie between 170,000 and 220,000 (25, 26). The product made with the *E. coli* enzyme is about 4 S. The corresponding molecular weight is about 50,000.

Since others (7, 10, 13) had previously shown that *E. coli* polymerase I almost exclusively incorporated dATP and dTTP with RNA templates, we intentionally used radioactive dGTP and dCTP to monitor cDNA synthesis. Our hybridization results with the *Drosophila* and Qβ RNAs (Fig. 3) rule out the possibility that lengthy stretches of poly(dT) were covalently linked to the [^3H]dCTP-labeled cDNA, a feature which would have been revealed by significant hybridization to AMV RNA. Nevertheless, it was of interest to determine whether or not any thymidine-containing homopolymers were being synthesized in the reaction mix.

Reactions were prepared with [^3H]dTTP and [^{32}P]dCTP and template-primer combinations as shown in Table 2. In the presence of AMV RNA, without primer, approximately 6.3 times more dTTP is incorporated than dCTP. When oligo(dT) primer is added, the ratio of dTTP to dCTP incorporated rises to over 6000. In the presence of primer alone, no incorporation of either base is observed, suggesting that end addition to the primer is not responsible for the large dTTP usage. It is likely that repeated rounds of poly(dT) and poly(dA) synthesis occur, starting with the original poly(A) regions of the RNA template. The same situation

TABLE 2. *Relative incorporation of dTTP and dCTP with* E. coli *DNA polymerase I*

Experiment	pmol incorporated/0.1 ml dTTP	pmol incorporated/0.1 ml dCTP	dTTP/dCTP
(*1*) 1.25 μg of AMV RNA	27.8	4.4	6.3
1.25 μg of AMV RNA + 0.13 μg of oligo(dT)$_{10}$	7144.0	1.1	6340.0
(*2*) 0.5 μg of rabbit-globin mRNA	1.7	<0.1	—
0.5 μg of rabbit-globin mRNA + 0.05 μg of oligo(dT)$_{10}$	460.0	19.0	24.2
0.5 μg of rabbit-globin mRNA + 0.05 μg of oligo(dT)$_{10}$ + 50 μg/ml of distamycin	51.7	24.3	2.1
0.05 μg of oligo(dT)$_{10}$	<0.1	<0.1	—

Reaction mixtures were prepared as described in *Methods*, including actinomycin D at 100 μg/ml, [^3H]dTTP at 13,700 cpm/pmol, and [^{32}P]dCTP at 20,400 cpm/pmol (Exp. 1) or 34,000 cpm/pmol (Exp. 2). Incubation was for 130 min (Exp. 1) or 100 min (Exp. 2).

obtains with globin mRNA; however, the ratio of dTTP to dCTP is less extreme.

The antibiotic distamycin has been shown to have an activity analogous to that of actinomycin D except that it binds preferentially to dA-dT-rich regions of DNA (27). It occurred to us that the biased incorporation of dTTP could be limited by including distamycin in the reaction and, as may be seen in Table 2, this prediction was realized. As a consequence, syntheses may be performed with distamycin when the presence of poly(dT) and poly(dA) in the product may be objectionable. Alternatively, by hybridization with the template RNA and isolation of the high-melting fraction from hydroxylapatite, cDNAs may be prepared with only a small percentage of low-melting homopolymer present.

DISCUSSION

It is clear from the results presented that *E. coli* DNA polymerase I is able to synthesize extensive, well-matched complementary DNA copies from a variety of natural RNAs, provided that the molar ratio of enzyme to RNA template is above a critical level. The reason for this requirement is not clear, and several possibilities can be entertained. There may exist secondary structures that do not serve as initiation points but for which the enzyme molecules have a very high affinity. Another mechanism could involve destabilization of secondary structures by bound enzyme. Finally, there may be trace amounts of another component required to elicit synthesis, perhaps a nuclease activity creating initiation sites.

An unresolved question is the extent of copying by the *E. coli* enzyme. The smaller size of the product produced by the bacterial enzyme and the lower $T_{e1/2}$ of hybrids formed with it may be the result of fragmentation of a complete copy by the exonucleases present in the *E. coli* enzyme. Additional exploration of the reaction conditions is required to optimize cDNA synthesis.

Animal cells may also contain DNA polymerases that can function as RNA-directed DNA-synthesizing enzymes under appropriate conditions. Certainly, this sort of activity can no longer be considered unique to the RNA tumor viruses, thus complicating the task of clarifying their role in oncogenesis. Other features diagnostic of these agents must be present to identify them conclusively in malignant tissues. Characteristics that have been found useful include banding density in sucrose, 70S RNA·DNA complexes (4, 28), morphology in the electron microscope, nucleic-acid homology with, and antigenic relatedness to, known tumor viruses, and response of the enzyme to various templates and antisera (29). Provided that the hybridization experiments are done under rigorous conditions, nucleic-acid homology offers the most sensitive and certain evidence.

Note Added in Proof. Modak *et al.* (30) have recently obtained similar results.

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