Synthesis of Double-stranded DNA Complementary to Lysozyme, Ovomucoid, and Ovalbumin mRNAs

OPTIMIZATION FOR FULL LENGTH SECOND STRAND SYNTHESIS BY ESCHERICHIA COLI DNA POLYMERASE I

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Sequential reverse transcriptase, DNA polymerase, and S1 nuclease reactions can be employed to synthesize double-stranded DNA representing messenger RNA. Using reverse transcriptase products made from partially purified lysozyme, ovomucoid, and ovalbumin messengers from hen oviduct we have characterized the Escherichia coli DNA polymerase I reaction. We have optimized for a high yield of full length second strands under conditions which require only a small amount of mRNA. The effects of several parameters (time, enzyme levels, salt concentration, monovalent cation, and temperature) on the length of products synthesized by DNA polymerase I have been investigated. Each has a significant influence on the proportion of products which are full length. Under our conditions the three reactions are efficient in synthesizing full length duplex DNA from partially purified mRNA fractions or from total poly(A)-containing RNA.

Several laboratories have employed a series of three enzymatic reactions to synthesize double-stranded DNA copies of the entire globin structural gene (1-3). The general method used is applicable, in principle, to any messenger RNA. Avian myeloblastosis virus reverse transcriptase can be used under appropriate conditions to synthesize a single-stranded DNA complementary to the messenger RNA. Reverse transcriptase products, whether partial or complete copies of the mRNA template (4), often possess short, partially double-stranded hairpins ("loops") at their 3' termini. These hairpins can be exploited as primers for DNA polymerase from Escherichia coli (1, 3) or avian myeloblastosis virus (2, 4, 5). The products of such sequential reverse transcriptase and DNA polymerase reactions still possess a loop at one end. The loop has sufficient single-stranded character that it can be cleaved with the single-strand specific nuclease S1 to generate a duplex DNA suitable for subsequent insertion into a bacterial plasmid.

Considerable effort has been devoted to improving the yield of full length copies of various messenger templates by reverse transcriptase (6-9); much less attention has been given to similarly optimizing the DNA polymerase reaction. Since many studies require probe representing the entire structural gene, it is often necessary to purify full length species from acrylamide gels prior to insertion or to screen large numbers of transformants. Many systems provide small amounts of starting messenger RNA, putting serious constraints on preparative manipulations. In particular, the repeated organic extractions, ethanol precipitations, chromatography, and gel electrophoresis of the small amounts of DNA synthesized in vitro can lead to poor recoveries.

We have therefore examined the enzymatic reactions in double-stranded DNA synthesis with the aim of obtaining full length duplex copies in good yield using relatively small amounts of the messengers discussed in the previous paper (9). A procedure has been devised elsewhere1 in which the reverse transcriptase and E. coli DNA polymerase I reactions are carried out sequentially in the same reaction vessel with very little manipulation of the reaction products. We have used this approach as a starting point in optimizing for a high proportion of full length second strand synthesis.

The synthesis of single-stranded DNA complementary to the entire messenger by reverse transcriptase was investigated in the accompanying publication (9). In this report we examine the E. coli DNA polymerase I and S1 nuclease reactions in detail. The synthesis of full length strands by DNA polymerase I depends critically on several variables. Under our final conditions, complete, double-stranded DNA copies of lysozyme, ovomucoid, and ovalbumin messenger RNAs can be prepared in good yield, using either purified messenger fractions or total poly(A)-containing RNA as template.

EXPERIMENTAL PROCEDURES

Materials—All the experiments presented used Escherichia coli DNA polymerase I generously donated by Dr. Arthur Kornberg, Department of Biochemistry, Stanford University Medical School. It was purified as described (10) to a specific activity of 18,000 units

1 We are grateful to David Kemp for sharing his protocol and data with us prior to publication.
Double-stranded cDNA Synthesis: pol I Reaction with cDNA

Aspergillus oryzae single strand specific nuclease S1, purified by the method of Britten et al. (12), was added to a final concentration of 5 units/ml. (A unit is here defined as the amount of enzyme required to degrade completely 10 µg of denatured DNA in the presence of 10 µg of native DNA in SI buffer in a 2-h incubation at 37°C.) Digestions were incubated at 37°C for 1 h, then stopped by placing on ice and adding SDS to a final concentration of 0.1%. Five to twenty micrograms of E. coli tRNA were then added. Reactions were extracted at room temperature with an equal volume of chloroform and the organic phase re-extracted once or twice with an equal volume of SI buffer. Two and one-half volumes of ethanol were added and the mixture held at −20°C overnight or in an isopropl alcohol-dry ice bath at least 15 min.

When experiments did not require nuclease digestion, pooled void volume fractions from the Sephadex column were made 200 mM in NaCl and ethanol precipitated as described above.

Reactions using Radioactive Deoxynucleoside Triphosphate Precursors

All experiments presented involved the incorporation of radiolabeled deoxynucleoside triphosphates into either first strands by reverse transcription or second strands by polymerase I. For convenience we have termed these two types of reactions first or second strand labelings.

H or 32P-labeled deoxynucleoside triphosphate precursors were lyophilized to dryness and used within several hours. All precursors, including the labeled species, were present at the standard concentration.

In first strand labeling reactions, we wished to examine the behavior of just first strands during second strand synthesis. It was necessary to minimize the incorporation of radioactive remaining from first strand reactions during the DNA polymerase I reaction so as to avoid labeling second strands as well. This was achieved by diluting the specific activity of the precursor immediately after first strand synthesis as follows. A standard reverse transcriptase reaction mixture was prepared omitting RNA and enzyme, and divided into two aliquots. One was used to redissolve the radioactive precursor without RNA at 5 times the usual concentration, and then reverse transcriptase. To the other aliquot was added only an appropriate volume of water. (Such a reaction mixture, missing RNA, enzyme, and isotope, is abbreviated as RTM mixture.) Both were incubated at 42°C for 60 min, then chilled on ice. Four volumes of the RTM mixture were added to the labeled reaction mixture, on ice, diluting the isotope specific activity 5-fold. The entire mixture was then boiled and added to an equal volume of DNA polymerase I mixture as usual. The specific radioactivity of precursor during the DNA polymerase I reaction was thus 10% of what it had been during the synthesis of first strands.

For second strand labeling reactions, DNA polymerase I reaction mixture was used to redissolve an appropriate amount of dried radioactive precursor.

Gei Electrophoresis—Denaturing gel electrophoresis in 1.2 to 2.0% agarose gels chilling 20 mEq mercury hydroxide was carried out as described previously (9, 13).

For native gel electrophoresis, a suitable weight of agarose was dissolved in 90 mM Tris, 90 mM borate, 2.5 mM EDTA, pH 8.3 (Buffer A) by boiling. After cooling briefly, the gel was cast between glass plates (13 × 17 cm, 2-mm gel thickness) using Lucite spacers and a Teflon well former. Sample buffer was 1/10× Buffer A containing 10% glycerol and bromphenol blue.

All samples for electrophoresis were ethanol-precipitated, collected by centrifugation (10,000 rpm, 10 to 20 min, Sorvall HH4 rotor), washed with 75% ethanol, dried briefly, and redissolved in the appropriate sample buffer.

SV40 DNA was digested with either Eco RI or HindIII restriction enzymes to generate molecular weight standards. In some experiments restriction fragments were nick-translated and viewed by autoradiography as in the accompanying report (9). In others, ethidium bromide fluorescence of nonradioactive restriction fragments was viewed using short wave ultraviolet light. The lengths used for the HindIII fragments of SV40 were those described (9). RI-treated SV40 is approximately 4970 base pairs long (14).

Analytical SI Digestions—A pair of duplicate aliquots (10 to 50 µl each) of the sample to be tested were added to 1 ml of SI buffer containing 10 µg of native and 10 µg of denatured salmon sperm DNA. One pair received 5 units of SI; the other received no enzyme. Reactions were incubated for 2 h at 37°C, and chilled on ice. They were then precipitated with trichloroacetic acid, collected on filters, and counted as described (9).

(A-T)/mg, and stored at −20°C in 50 mM potassium phosphate, pH 7, 50% glycerol. A commercial preparation was obtained from Boehringer Mannheim (Grade I) which appeared satisfactory in ovalbumin-directed reactions although it was a 5- to 10-fold less active. AM virus reverse transcriptase (Lot 1176) was obtained from Dr. Joseph Bovard. Life Sciences, Inc., St. Petersburg, Fla. SV40 DNA was provided by Steve Goff and Mike Goldberg of the Department of Biochemistry, Stanford University Medical School. Restriction endonuclease Eco RI was purchased from Miles Laboratories or from New England Bio-Labs. All other chemicals and enzymes were obtained as described in the preceding paper (9).

Messenger RNA Templates—The partially purified messenger RNA templates used were characterized in the accompanying paper (prepared in Experiment 2; see Ref. 9). Fig. 3 in that publication presents electrophoretic patterns of the preparations used. Fig. 2 and Table I in the same translation data bearing on the purity of each fraction.

Each was stored in water at −20°C.

First Strand Synthesis—Avian myeloblastosis virus reverse transcriptase was used to synthesize first strands under conditions which yield a high proportion of full length copies (9). Reverse transcriptase was carried out in autoclaved 1.5-m1 micro test tubes (Bio-Rad) and varied in total reaction volume from 5 to 100 µl. Standard reverse transcriptase reaction mixture was 50 mM Tris/Cl, pH 8.3 at 42°C, 140 mM KC1, 10 mM MgCl2, 50 mM β-mercaptoethanol, 500 µM concentration of each deoxynucleoside triphosphate, and 100 µg of oligo(dT),-actinomycin D was not included since it reduces incorporation substantially in subsequent second strand synthesis. Messenger RNAs were added to a final concentration of 10 to 40 µg/µl. Reverse transcriptase was used at 800 units/ml, a level saturating for all messenger RNA concentrations used. After being assembled on ice, mixed, and centrifuged briefly, reactions were incubated at 42°C for 60 min.

In some cases, as indicated, first strands were synthesized in the absence of salt. Compensating KC1 was included in the DNA polymerase I reaction mixture to bring the final concentration to 70 mM.

Second Strand Synthesis—Reverse transcriptase reactions were stopped by chilling on ice, and centrifuged briefly. Tubes were then placed in a boiling water bath for 3 min to separate mRNA-first strand hybrids (9) and plunged immediately into an ice water bath. After a short centrifugation to pellet denatured protein, the tubes were returned to ice.

The cold, boiled reverse transcriptase reaction was added to an equal volume of cold DNA polymerase I reaction mixture (200 mM Hepes, pH 7.5, 10 mM MgCl2, 50 mM dTTP, 0.1% SDS, and 20 to 50 µg of E. coli tRNA. Each reaction was incubated at 37°C for 60 min, then chilled on ice. Reverse transcriptase was used at 800 units/ml, a level saturating for the reverse transcriptase reaction during the DNA polymerase I reaction so as to avoid labeling second strands as well. This was achieved by diluting the specific activity of the precursor immediately after first strand synthesis as follows. A standard reverse transcriptase reaction mixture was prepared omitting RNA and enzyme, and divided into two aliquots. One was used to redissolve the radioactive precursor without RNA at 5 times the usual concentration, and then reverse transcriptase. To the other aliquot was added only an appropriate volume of water. (Such a reaction mixture, missing RNA, enzyme, and isotope, is abbreviated as RTM mixture.) Both were incubated at 42°C for 60 min, then chilled on ice. Four volumes of the RTM mixture were added to the labeled reaction mixture, on ice, diluting the isotope specific activity 5-fold. The entire mixture was then boiled and added to an equal volume of DNA polymerase I mixture as usual. The specific radioactivity of precursor during the DNA polymerase I reaction was thus 10% of what it had been during the synthesis of first strands.

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All samples for electrophoresis were ethanol-precipitated, collected by centrifugation (10,000 rpm, 10 to 20 min, Sorvall HH4 rotor), washed with 75% ethanol, dried briefly, and redissolved in the appropriate sample buffer.

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Table I

<table>
<thead>
<tr>
<th>Type of reaction</th>
<th>dATP or dCTP incorporated into second strands (% of complete reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100.0 (C, A)</td>
</tr>
<tr>
<td>Reverse transcriptase</td>
<td>0.4 (C)</td>
</tr>
<tr>
<td>Polymerase I</td>
<td>1.0 (C)</td>
</tr>
<tr>
<td>Reverse transcriptase, polymerase I</td>
<td>0.3 (C)</td>
</tr>
<tr>
<td>oligo(dT)</td>
<td>1.3 (A)</td>
</tr>
<tr>
<td>RNA</td>
<td>0.1 (C, A)</td>
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RESULTS

General Properties of Reaction Sequence

A detailed standard protocol for double-stranded cDNA synthesis is presented under "Experimental Procedures." Avian myeloblastosis virus reverse transcriptase is used to synthesize first strands from sucrose gradient-fractionated messenger RNAs under conditions shown previously to yield a high proportion of full-length cDNA (9). The total reverse transcriptase product is boiled briefly in order to free first strands from the messenger template to which they are hybridized (9). The boiled first strand mixture is added directly to an equal volume of DNA polymerase I reaction mixture containing fresh deoxynucleoside triphosphates, a new buffer selected to minimize exonuclease (11), and E. coli DNA polymerase I. After a 2-h, 15°C incubation, the reaction is stopped, RNA carrier added, and the entire mixture extracted with chloroform and passed over Sephadex G-50 in 20 mM NaCl. The pooled void volume is then adjusted to appropriate salt and buffer concentrations and digested with Aspergillus single-strand-specific S1 nuclease.

For simplicity we refer to the products of the reverse transcriptase reaction as first strands and to material synthesized by DNA polymerase I as second strands. The products of the combined reactions are called double-stranded cDNA. Sequential reverse transcriptase and DNA polymerase I reactions in which only first strands are radiolabeled are called first strand labelings; when only second strands are radiolabeled, the combined reactions are called second strand labelings.

Various omissions were made from a complete reaction protocol using ovalbumin mRNA and the net incorporation of either [3H]dCTP or [3H]dATP during second strand synthesis determined as a per cent of a complete reaction control (Table I). DNA polymerase I does not copy the mRNA (even though oligo(dT) still is present at 50 µg/ml), since without reverse transcriptase one finds less than 0.5% of control incorporation. It also does not copy the oligo(dT) itself, since no labeling with [3H]dATP is found in the absence of mRNA. Both reverse transcriptase and DNA polymerase I are required for second strand synthesis.

DNA polymerase I synthesizes between 65 and 130% of the DNA mass presented to it as first strand substrate. Uncertainty about what percentage of the boiled, reverse transcriptase reaction deoxynucleoside triphosphates still are suitable DNA polymerase I substrates makes a precise determination difficult. The lower end of the range is consistent with the extent to which first strands are protected from S1 nuclease digestion by second strands (about 80%; see Fig. 1).

Time Course of Second Strand Synthesis

The extent to which first strands are copied by DNA polymerase I can be monitored by the S1 nuclease resistance of labeled first strands. For this and subsequent experiments in which radioactivity was to be incorporated into first strands only, our standard protocol was modified so that radioactive precursor remaining from the reverse transcriptase reaction was only negligibly incorporated into second strands by DNA polymerase I (see "Reactions using Radioactive Deoxynucleoside Triphosphate Precursors" under "Experimental Procedures"). Approximately 18,000 acid-precipitable cpm were used in each assay. A blank of [3H]dCTP alone (200 cpm) has been subtracted from each point.

Fig. 1. Kinetics of the acquisition of first strand S1 resistance during second synthesis. A single reverse transcriptase reaction (36 µl) using 100 µg of ovalbumin mRNA/ml and [3H]dCTP at 20.7 Ci/mmol was carried out as described under "Experimental Procedures." Four volumes (104 µl) of RTM mixture were then added, the mixture boiled, and 110 µl added to an equal volume of DNA polymerase I reaction mixture. This solution was then split into two 108-µl aliquots; one (+ pol I, 0) received 20.3 units of DNA polymerase I (18.8 units/µg of mRNA); the other (- pol I, 0) received the same volume of water. Zero time points were removed before the addition of enzyme. Reactions were incubated at 15°C. At 30-min intervals, four 5-µl aliquots were removed; two were used to determine total trichloroacetic acid-precipitable radioactivity, and two to determine S1-resistant radioactivity as described under "Experimental Procedures" (see "Analytical S1 Digestions"). Approximately 18,000 acid-precipitable cpm were used in each assay. A blank of [3H]dCTP alone (200 cpm) has been subtracted from each point.
Double-stranded cDNA Synthesis: pol I Reaction with cDNA

Fig. 2. Denaturing gel electrophoresis of first strand-labeled products formed after different times of second strand synthesis. A, a reverse transcriptase reaction (8.5 μl) using 100 μg of ovalbumin mRNA/ml and [3H]dGTP at 6.8 Ci/mmol was carried out as described under "Experimental Procedures." Four volumes (34 μl) of RTM mixture was added and the mixture boiled. Ten microliters was removed and kept on ice to be prepared as an unreacted first strand marker for electrophoresis. Twenty-six microliters of the boiled reaction was then added to an equal volume of DNA polymerase I reaction mixture and DNA polymerase I added to a final concentration of 13 units/μg of mRNA. At various times thereafter, 10-μl aliquots were removed and added to 100 μl of ice-cold inactivation mixture and immediately extracted with chloroform. Each sample was processed and prepared for electrophoresis as described under "Experimental Procedures." Electrophoresis was on 1.5% agarose gels containing 20 mM CH₃HgOH as denaturant. Approximately equal amounts of radioactivity (about 12,000 cpm) were applied to each slot; each sample was greater than 95% acid-pelletable.

The ovalbumin reverse transcriptase product, initially about 13% S1-resistant, acquires additional protection from S1 digestion in a DNA polymerase I-dependent process (Fig. 1). Plateau resistance generally is about 80%. Most of the reaction appears complete in 1 h by this criterion. mRNA, first strands, and oligo(dT) all are present at significant concentrations during second strand synthesis. Some protection of first strands by messenger or oligo(dT) might be expected. However, in the absence of DNA polymerase I, no RNA-first strand hybrids stable to S1 digestion at 37°C reform after 2 h incubation at 15°C.

Aliquots of a single first strand labeling were removed at various times during second strand synthesis and electrophoresed on 1.5% agarose, 20 mM methylmercury hydroxide denaturing gels (Fig. 2A). Full length first strands disappear rapidly during the incubation, accompanied by the appearance of higher molecular weight products; very little remains at the unreacted position after 2 h. Between 1 and 2 h a prominent band emerges at the position predicted for full length second strand synthesis (3730 bases, average of nine experiments). Combined with Fig. 1, this suggests that after 1 h, most second strands terminate at different distances near but not yet at the 5’ end of the first strand. It seemed possible that some of the 2-h products between about 2500 bases and the complete duplex band might also ultimately be converted to full length with prolonged incubation. In a second experiment, we examined first strand labelings at much longer incubation times. No significant change in size distribution (Fig. 2B) was seen between 2 and 24 h.

All deoxynucleoside triphosphates polymerized by DNA polymerase I are S1-resistant. In the experiment shown in Fig. 3, [3H]dCTP was introduced at the start of second strand synthesis and aliquots removed at various times thereafter. Greater than 95% of the radioactivity in second strands is protected from S1 nuclease digestion at each time point. Ovomucoid and lysozyme second strands synthesized under standard conditions also are more than 95% resistant. The kinetics of incorporation in Fig. 3 are compatible with the time course in Figs. 1 and 2.

DNA Polymerase I Dependence

It is conceivable that total reverse transcriptase products made from different messenger templates might be utilized by DNA polymerase I with different efficiencies with respect to both second strand length and net synthesis. We therefore evaluated how much DNA polymerase I was required to saturate lysozyme, ovomucoid, ovalbumin, or conalbumin first
reverse transcriptase products required for saturation is similar for saturation suggests that the molar ratio of enzyme to ovomucoid and lysozyme first strands may contain slightly fewer inputs are 0.2 (conalbumin):1.0 (ovalbumin):1.7 (ovomucoid):2.7 (lysozyme). Reverse transcriptase synthesizes roughly the same mass of first strands using ovomucoid, ovomucoid, or lysozyme mRNA since no quantitative data are available. However, the accompanying paper (9) reports that, relative to the ovalbumin distribution, ovomucoid and lysozyme first strands may contain slightly fewer partial copies, and conalbumin more. These corrections would tend to improve the agreement between the amount of enzyme required to saturate and the molar yield of first strand.

Fig. 3. Kinetics of second strand synthesis and S1 resistance of second strands. A single reverse transcriptase reaction (100 μl) using 40 μg of ovalbumin mRNA/ml was carried out as described under “Experimental Procedures.” After boiling, 96 μl was added to an equal volume of DNA polymerase I reaction mixture containing [3H]dCTP at 0.8 Ci/mmol. Sixty-eight units of DNA polymerase I were added (16 units/μg of mRNA) and the reaction incubated at 15°. Zero time points were removed before the addition of enzyme. At various times after starting the incubation, four 5-μl aliquots were removed; two were used to determine total acid-precipitable radioactivity and two to determine S1-resistant radioactivity as described under “Experimental Procedures” (see “Analytical S1 Digestions”). The total acid-precipitable counts per min in a 5-μl aliquot; O, S1-resistant counts per min in a 5-μl aliquot. A blank of 260 cpm (determined using the DNA polymerase I reaction mixture alone) has been subtracted from each point.

Conalbumin, ovalbumin, ovomucoid, and lysozyme mRNAs were each used as templates for first strand synthesis by reverse transcriptase. After boiling, each reaction was added to an equal volume of DNA polymerase I reaction mixture containing radioactive precursor, and divided into several aliquots. Different amounts of DNA polymerase I were added to catalyze the second reaction and the total incorporation into second strands determined. The results are summarized in Fig. 4. For each template, with increasing enzyme, net synthesis approaches a plateau and then declines slightly. The ratios of enzyme required to saturate first strand products made from equal masses of mRNA are approximately 0.3 (conalbumin):1.0 (ovalbumin):1.7 (ovomucoid):2.7 (lysozyme). Reverse transcriptase synthesizes roughly the same mass of first strands using ovomucoid, ovomucoid, or lysozyme mRNA fractions under our conditions; the reaction is about 4-fold less productive with the conalbumin mRNA fraction (9). Correcting for relative sizes of the messengers (9), the calculated molar ratios of first strands produced from identical RNA inputs are 0.2 (conalbumin):1.0 (ovalbumin):2.3 (ovomucoid):3.0 (lysozyme). The correlation between the relative molar yials of first strand and the amount of DNA polymerase I required for saturation suggests that the molar ratio of enzyme to reverse transcriptase products required for saturation is similar with each template. (In each case, that ratio is roughly 5 to 1.) It might be anticipated that subsaturating enzyme levels would leave most first strands unreacted while producing a small proportion of completed second strands. This was not the case with ovalbumin (Fig. 5). To obtain complete second strand synthesis, enzyme had to be present near the saturating level determined in Fig. 4. Below this amount, very little full length was seen. The addition of enzyme beyond the amount required for saturation (Track 5) produced no detectable difference on denaturing gels.

Effects of Salt, Temperature, and Prior Boiling of First Strand Products on Second Strand Synthesis

Several possible problems unique to DNA polymerase I can be anticipated (branch migration, slippage of the enzyme upon reaching the terminal oligo(T) region, etc.) which might be eliminated by judicious choice in the parameters affecting secondary structure or hybridization rate. We found that salt concentration, monovalent cation, and incubation temperature each has marked effects on the size distribution of DNA polymerase I products.

Salt Concentration—Unlabeled ovalbumin first strands...
A single reverse transcriptase reaction (45 μl) using 83 μg of ovalbumin mRNA/ml and [32P]dGTP at about 3 Ci/mmol was carried out as described under "Experimental Procedures." After incubation, 10 μl was removed and kept on ice to be used as unreacted first strand marker (not shown on gel). To the remainder was added 4 volumes of RTM mixture. After boiling, this mixture was added to an equal volume of DNA polymerase I reaction mixture and divided into five equal aliquots. Different amounts of DNA polymerase I were then added and reactions incubated, inactivated, extracted, chromatographed, and prepared for electrophoresis as described under "Experimental Procedures." Electrophoresis was on 1.5% agarose, 20 mM CH3HgOH gels. SV40 DNA restriction fragments were run on a parallel track and viewed by ethidium bromide fluorescence; the positions of the single 5' fragment and the largest HindIII fragment are indicated. Unreacted ovalbumin first strand was also run in a track not displayed, and co-migrated with the band in Track 1, Track 1, no DNA polymerase added; Track 2, 2.5 units of DNA polymerase/μg of mRNA; Track 3, 5.6 units/μg of mRNA; Track 4, 13.5 units/μg of mRNA; Track 5, 45 units/μg of mRNA.

were synthesized in the absence of salt. First strands made in 0 and 140 mM KCl appear identical upon denaturing gel electrophoresis and display no material larger than the full length band. After boiling, aliquots were removed and added to DNA polymerase I reaction mixtures containing [32P]dGTP and various KCl concentrations. After a 2-h incubation with saturating polymerase (based on Figs. 4 and 5 at 70 mM KCl), the products were analyzed on denaturing gels (Fig. 6). Without added salt, material longer than double-stranded full length was found on long autoradiographic exposures. Approaching 70 mM, the size of this product progressively decreased, until between 70 and 125 mM KCl, the full length band was the slowest migrating species. Beyond 125 mM KCl, no complete second strands are made. (In repeating this experiment several times we found that 125 mM KCl was too close to the point at which no full length synthesis was found to be used routinely. We therefore used 70 mM KCl in standard reactions.) Data in the accompanying paper make it unlikely that the ovalbumin mRNA used is significantly contaminated by messengers of higher molecular weight. Therefore, the slowly migrating material produced in low salt concentrations is likely to represent aberrant ovalbumin second strand synthesis.

**NaCl versus KCl**—DNA polymerase I products significantly different in size are synthesized if NaCl is used instead of KCl during second strand synthesis. Ovalbumin-directed first strand synthesis was carried out using either KCl or NaCl at 140 mM. The standard protocol was followed thereafter, so that 70 mM salt (judged optimal from Fig. 6) was present during second strand synthesis. With either strand labeled, no material of full length was found upon electrophoresis of the NaCl product on denaturing gels (Fig. 7). Instead the largest species made was not a discrete band, but a distribution of lengths between about 2800 and 3300 bases. The addition of more enzyme (up to 4 times the level needed for full length synthesis in standard KCl reactions) did not yield larger products. The monovalent cation effect is probably on the DNA polymerase I reaction per se, since first strands made in either salt at 140 mM do not differ substantially in yield, S1 resistance, or size distribution on denaturing gels. We have not determined whether there is a NaCl concentration which would enhance full length second strand synthesis.

**Incubation Temperature**—A single ovalbumin reverse transcriptase reaction (in the absence of radioactive precursors) was added to an equal volume of DNA polymerase I reaction mixture containing [32P]dGTP. After the addition of DNA polymerase I, the mixture was divided into four aliquots and incubated at 4°, 15°, 25°, and 42° for 2 h. The ratios of total DEAE chromatography, and prepared for electrophoresis as described under "Experimental Procedures." Electrophoresis was on 1.5% agarose, 20 mM CH3HgOH gels. SV40 DNA restriction fragments were run on a parallel track and viewed by ethidium bromide fluorescence; the positions of the single 5' fragment and the largest HindIII fragment are indicated. Unreacted ovalbumin first strand was also run in a track not displayed, and co-migrated with the band in Track 1, Track 1, no DNA polymerase added; Track 2, 2.5 units of DNA polymerase/μg of mRNA; Track 3, 5.6 units/μg of mRNA; Track 4, 13.5 units/μg of mRNA; Track 5, 45 units/μg of mRNA.

**FIG. 6.** KCl dependence of second strand synthesis. First strands were synthesized in the absence of KCl using 33 μg of ovalbumin mRNA/ml. After boiling, aliquots were added to equal volumes of DNA polymerase reaction mixtures containing different concentrations of KCl and [32P]dGTP at about 5 Ci/mmol. DNA polymerase I was added to a final concentration of 27 units/μg of mRNA. Electrophoresis was on 1.2% agarose, 10 mM CH3HgOH gels. About 85% of each electrophoresis sample was acid-precipitable. Differences in autoradiograph intensities between tracks reflect differences in applied radioactivities; an especially long autoradiographic exposure was used to improve the detection of material larger than the full length band. Track 1, 0 mM KCl during second strand synthesis; Track 2, 25 mM KCl; Track 3, 50 mM KCl; Track 4, 70 mM KCl; Track 5, 125 mM KCl; Track 6, 170 mM KCl.
incorporation were roughly 0.5:1:0.2:0.2:0.2.0, respectively.

Since about 100% of the input first strand mass is synthesized during a 15° polymerase I reaction, a 42° synthesis probably generates products not directly copied from first strands. When the products were electrophoresed on denaturing gels, a dramatic trend toward increased length with elevated temperature was found (Fig. 8). At 4°, no full length second strand synthesis occurs, as corroborated by first strand labelings in which most first strand is found unreacted (data not shown). At 25° material co-migrating with the 4000-base-long 15° product is superimposed on a wide distribution of sizes extending almost to the top of the gel. Even this residual 4000-base-long band disappears at 42°, most of the product barely enters the gel. Analogous second strand labeled ovomucoid (Fig. 8B (Track 1)); and lysozyme (Fig. 8B (Track 3))-directed 42° products were electrophoresed on denaturing gels; they too migrated very slowly. Upon native gel electrophoresis all three behaved as if either very long or extensively single-stranded, not entering 1.5% agarose gels (data not shown). We considered the possibility that the 42° products might be polyphosphate, although it seemed unlikely since no such products are found under nearly identical reaction conditions during the reverse transcriptase incubation. The ovalbumin product is not, since it is 100% phosphatase-resistant, 100% trichloroacetic acid-precipitable before and after boiling, binds ethidium bromide, and does not behave like the non-DNA material described by Efstratiadis et al. (6) in that it is excluded on Sephadex G-150 and is acid-precipitable.

The slowly migrating DNA polymerase I products may contain hairpins as judged by their behavior upon digestion by SI nuclease. Each second strand labeled 42° product is 80 to 100% SI-resistant; boiling prior to digestion decreases the SI resistance by only 10%. After digestion, however, each enters a denaturing gel as a broad size distribution near the size of the appropriate SI-cleaved 15° product. Fig. 8B (Tracks 2 and 4) displays these results with ovomucoid and lysozyme.

**Boiling versus not Boiling after First Strand Synthesis**

We established the importance of boiling the reverse transcriptase reaction prior to second strand synthesis in the following experiments. A single ovalbumin-directed reverse transcriptase reaction was carried out as described under "Experimental Procedures," then divided in half. One part was kept on ice, while the other was denatured in a boiling water bath as usual. An equal volume of DNA polymerase I reaction mixture containing [32P]dGTP was then added to each tube, and the reactions begun by the addition of DNA polymerase I.

When the boiling step was omitted from an otherwise standard protocol, DNA polymerase I directed the incorporation of [32P]dGTP into acid-precipitable material. Net synthesis was approximately 50% of that found in a standard reaction. However, when analyzed on methylmercury-containing agarose, only labeled second strand products longer than full length first strands (1900 bases) were found. Instead a broad distribution of radioactivity was observed, with an average length of roughly 500 bases, extending nearly up to 1900 bases. The length distribution suggests that in an unboiled reaction, DNA polymerase I does not extend the first strand from a hairpin primer, but instead copies the messenger itself. Messenger RNA-oligo(dT) hybrids would not have been denatured; any such hybrids which had not been used as templates by reverse transcriptase might provide suitable template-primer complexes for DNA polymerase copying of mRNA. When reverse transcriptase reactions are boiled prior to second strand synthesis to separate mRNA-oligo(dT) and mRNA-first strand hybrids, DNA polymerase I does not copy the messenger significantly (see Table I). These data indicate that the denaturation step is necessary for efficient second strand synthesis.

We also investigated the effects of not boiling on the second strand labeled ovalbumin products synthesized by DNA polymerase I at 42°. Total incorporation of [32P]dGTP into second strands in a 42° reaction which had not been boiled was 3 times higher than that found with boiling. Upon methylmercury-agarose electrophoresis, second strands from the unboiled reaction were indistinguishable from those of a boiled reaction (see Fig. 8), barely entering the gel. Thus, eliminating the denaturation step does not prevent the synthesis of anomalous products under suboptimal conditions.
FIG. 8. Temperature dependence of second strand synthesis. A, a single reverse transcriptase reaction using 40 µg of ovalbumin mRNA/ml was incubated, boiled, and added to an equal volume of DNA polymerase reaction mixture containing [32P]dGTP at 3.2 Ci/mmol. DNA polymerase I was then added to a final concentration of 13.5 units/µg of mRNA. The reaction was then divided into four aliquots incubated at 4°C (not shown), 15°C, 25°C, or 42°C for 2 h. Reactions were processed, prepared for electrophoresis, and electrophoresed on 1.2% agarose, 20 mM CH3HgOH gels as described under "Experimental Procedures." Approximately 250,000 cpm are present on each track; greater than 95% of the radioactivity applied was acid-precipitable in each case. o designates the origin. Track 1, 15°C product; Track 2, 25°C product; Track 3, 42°C product. B, Tracks 1 and 2, a single reverse transcriptase reaction using 37 µg of ovomucoid (Mu) mRNA/ml was incubated, boiled, and added to an equal volume of DNA polymerase I reaction mixture containing [32P]dGTP at 16 Ci/mmol. DNA polymerase I was then added to a final concentration of 50 units/µg of mRNA. The reaction was incubated at 42°C for 2 h, inactivated, extracted, and passed over Sephadex G-150 in 20 mM NaCl as described under "Experimental Procedures." The pooled void volume was divided into two parts. One was ethanol-precipitated and prepared for electrophoresis directly (Track 1). The other part (Track 2) was digested with 5 units of S1 nuclease/ml as described under "Experimental Procedures." (S1 buffer contained endogenous DNA). Samples were prepared for electrophoresis and electrophoresed on 2.5% agarose, 20 mM CH3HgOH gels as described under "Experimental Procedures." HindIII-cut SV40 DNA ([32P]dGTP, labeled by nick translation) and S1-treated ovomucoid double-stranded cDNA synthesized at 15°C were run on tracks not shown; the position of the largest HindIII fragment and that of S1-cleaved double-stranded cDNA are indicated. o indicates the origin. Both tracks contained 30,000 cpm. Track 1, ovomucoid-directed DNA polymerase I product synthesized at 42°C, prior to S1 treatment; Track 2, the same product as in Track 1 after digestion with S1 nuclease. Tracks 3 and 4, all procedures and manipulations were as described for Tracks 1 and 2, except that the template was lysozyme (Lys) mRNA, and DNA polymerase I was used at a final concentration of 66 units/µg of mRNA. Markers were as described for Tracks 1 and 2. Both tracks contained 30,000 cpm. Track 3, lysozyme-directed DNA polymerase I product synthesized at 42°C, prior to S1 treatment; Track 4, the same product as in Track 1 after digestion with S1 nuclease.

S1 Nuclease Digestion of DNA Polymerase I Products

Earlier reports (1, 3) demonstrated that S1 is capable of cleaving the hairpin loop to generate duplex DNA suitable as a terminal transferase substrate. In examining this cleavage with each of our DNA polymerase I products, we first removed deoxynucleoside triphosphates by Sephadex G-150 chromatography, since concentrations of deoxynucleoside triphosphates as low as 1 µM are reported to inhibit S1 activity substantially (15). RNA carrier was added to minimize losses. Fig. 9 shows the results of an experiment in which different S1 nuclease concentrations were used in the digestion of double-stranded cDNA. Ovalbumin double-stranded cDNA was synthesized with [32P]dGTP in the first strand and [3H]dGTP in the second, and prepared for S1 digestion as usual. Half the product was boiled to denature any non-hairpin duplexes while the other half was left untreated. The S1 resistance of both strands was then determined over a range of nuclease concentrations. Without denaturation, S1 resistance approaches a plateau of 80% for the first strand and 100% for the second (as shown in Figs. 2 and 4). After denaturation the level of resistance of each strand, while remaining high, drops by about 30%. Thus roughly 70% of the double-stranded molecules after DNA polymerase I reaction are indeed the expected hairpin structures, while the remainder do not snap back after denaturation. Non-hairpin duplexes may be derived from priming by messenger fragments or by oligo(dT) (see "Discussion").

In preparing material for transformation, the addition of exogenous DNA is undesirable. Therefore, in experiments analogous to those described in Fig. 9, 10, and 12C, we carried out S1 nuclease digestions in the absence of exogenous DNA carrier using lysozyme and ovalbumin double-stranded cDNAs as substrates. The plateau S1 resistance of ovalbumin first strands was 78% and of second strands 90% (as opposed to...
moving material on native gels which disappears with nuclease digestion. This slower moving species are completely double-stranded before digestion (probably full length first strands synthesized on partial first strands) and disappears. The most obvious interpretation of these data is that the band itself and most lower molecular weight material fore migrate at about 1600 bases long on denaturing gel electrophoresis. Similarly, lysozyme (about 650 bases long) should yield a full length DNA polymerase I product 1200 nucleotide bases is converted to one-half that size co-migrating with full length ovomucoid DNA polymerase I products should there be double-stranded DNA polymerase I reaction mixture containing [3H]dCTP at 20.5 Ci/mmol on ice, and DNA polymerase I added to a final concentration of 13.5 units/μg of mRNA to start the reaction. The incubation was stopped after 3 h at 15°, inactivated, extracted, and chromatographed over Sephadex G-150 in 20 mM NaCl as described under “Experimental Procedures.” Half the pooled void volume was then denatured in a boiling water bath for 5 min; the other half was not treated. Each portion was added to 25 volumes (6.2 ml) of SI buffer containing 19 μg of native and 10 μg of denatured salmon sperm DNA/μl and divided into six 1-ml aliquots. Each was assayed for SI resistance as a different amount of SI nuclease as described under “Experimental Procedures” (see SI Nuclease Digestion”). The input radioactivity in each digestion was 12,000 [3P] cpm and 1300 [3H] cpm. •, [3H]-labeled SI resistance, sample not denatured prior to nuclease digestion; △, [3P]-labeled SI resistance, sample not denatured prior to nuclease digestion; O, [3H]-labeled SI resistance, sample denatured prior to nuclease digestion; □, [3P]-labeled SI resistance, sample denatured prior to nuclease digestion.

81% and 97%, respectively, in parallel reactions containing salmon sperm DNA). The denaturing gel distributions of double-stranded cDNAs digested in the presence or absence of salmon sperm DNA were indistinguishable over the range of nuclease concentrations tested (up to 5 units of SI/μl).

If second strand synthesis is primed by a hairpin loop at the 3′ end of the first strand, then SI cleavage full length double-stranded cDNA should reduce its single-stranded length by half while leaving the duplex length nearly unchanged. Either first or second ovalbumin strands were labeled, digested with different amounts of SI, and electrophoresed under denaturing conditions (Fig. 10). As SI is added the band at about 3800 bases is converted to one-half that size co-migrating with full length ovalbumin first strands. When the same samples are electrophoresed on native gels (Fig. 11), the 1900 base pair band and faster moving species do not change significantly upon SI treatment; slower moving material progressively disappears. The most obvious interpretation of these data is that the band itself and most lower molecular weight material are completely double-stranded before digestion (probably full length second strands synthesized on partial first strands) and relatively unaffected by cleavage of their hairpins. The slower moving material on native gels which disappears with nuclease digestion is likely to be derived from incomplete second strand synthesis, since single-stranded DNA probably migrates more slowly than double-stranded DNA on this gel system (16). We estimate that with either strand labeled about 25% of the total radioactivity on native gels is found in full length ovalbumin double-stranded cDNA after SI cleavage (Fig. 11, A (Track 4) and B (Track 4)).

Ovomucoid messenger RNA is about 850 bases long. Full length ovomucoid DNA polymerase I products should therefore migrate at about 1600 bases long on denaturing gel electrophoresis. Similarly, lysozyme (about 650 bases long) should yield a full length DNA polymerase I product 1200

![Figure 9](http://www.jbc.org/)

**Fig. 9.** Digestion of ovalbumin double-stranded cDNA with different concentrations of SI nuclease. Ovalbumin double-stranded cDNA containing [3P]dGTP in the first strand and [3H]dCTP in the second were synthesized as follows. A reverse transcriptase reaction (10.5 μl) using 100 μg of ovalbumin mRNA/μ1 and [3P]dGTP at 45 Ci/mmol was prepared and incubated as described under “Experimental Procedures.” Four volumes (42 μl) of RTM mixture was added after the reaction, and the mixture boiled. Fifty microliters of the boiled reaction was added to an equal volume of DNA polymerase I reaction mixture containing [3H]dCTP at 20.5 Ci/mmol on ice, and DNA polymerase I added to a final concentration of 13.5 units/μg of mRNA to start the reaction. The incubation was stopped after 3 h at 15°, inactivated, extracted, and chromatographed over Sephadex G-150 in 20 mM NaCl as described under “Experimental Procedures.” Half the pooled void volume was then denatured in a boiling water bath for 5 min; the other half was not treated. Each portion was added to 25 volumes (6.2 ml) of SI buffer containing 19 μg of native and 10 μg of denatured salmon sperm DNA/μl and divided into six 1-ml aliquots. Each was assayed for SI resistance using a different amount of SI nuclease as described under “Experimental Procedures” (see SI Nuclease Digestion”). The input radioactivity in each digestion was 12,000 [3P] cpm and 1300 [3H] cpm. •, [3H]-labeled SI resistance, sample not denatured prior to nuclease digestion; △, [3P]-labeled SI resistance, sample not denatured prior to nuclease digestion; O, [3H]-labeled SI resistance, sample denatured prior to nuclease digestion; □, [3P]-labeled SI resistance, sample denatured prior to nuclease digestion.

![Figure 10](http://www.jbc.org/)

**Fig. 10.** Denaturing gel electrophoresis of ovalbumin double-stranded cDNA with either strand labeled after cleavage with different concentrations of SI nuclease. Reverse transcriptase reaction mix was prepared using 100 μg of ovalbumin mRNA/μ1 and divided into two aliquots (15 μl each). One (for first strand labeling) was used to redissolve [3P]dGTP to a specific radioactivity of 7.5 Ci/mmol; the other (for second strand labeling) received no isotope. Reverse transcriptase was then added to both aliquots, and the reactions incubated at 42° for 60 min. Five microliters of the labeled reaction was removed and kept on ice to be used as untreated first strand marker in electrophoresis. Four volumes of RTM mixture were added, and the mixtures boiled. Each was then combined with an equal volume of DNA polymerase I mixture. The second strand labeling reaction received DNA polymerase I mixture containing [3P]dGTP at 7.5 Ci/mmol; the first strand labeling received no additional isotope. DNA polymerase I was then added to a final concentration of 13.5 units/μg of mRNA. Reactions were incubated, inactivated, extracted, chromatographed over Sephadex G-150 in 20 mM NaCl, and the pooled void volume adjusted to contain CH3OH gel sample buffer by the addition of a 5-fold concentrated stock solution. Electrophoreses were on 1.5% agarose, 20 mM CH3OH gels. The position of unreacted ovalbumin first strand (ov cDNA) determined on a parallel track on the same gel, is indicated. Approximately equal radioactivity was applied to each track. A, first strands labeled. Track 1, no SI nuclease added; Track 2, 0.13 unit of SI nuclease/μl; Track 3, 1.9 units/μl; Track 4, 5 units/μl. B, second strands labeled. Track 1, no SI nuclease added; Track 2, 0.13 unit of SI nuclease/μl; Track 3, 1.9 units/μl; Track 4, 5 units/μl.
The template for the synthesis of double-stranded cDNA. First and second strand labeled ovalbumin reactions were identical with those described in the legend to Fig. 10. Samples for electrophoresis were dissolved in 5 mM NaCl after ethanol precipitation and adjusted to Buffer A gel sample buffer by the addition of a 5-fold concentrated stock solution. Electrophoresis was on a 1.5% agarose, Buffer A gel as described under "Experimental Procedures." SV40 DNA restriction fragments were electrophoresed on the same gel and viewed by ethidium bromide fluorescence; the positions of marker bands are indicated. Equal radioactivity was applied to each track. A, first strands labeled. Track 1, no SI nuclease added; Track 2, 0.13 unit of SI nuclease/ml; Track 3, 1.9 units/ml; Track 4, 5 units/ml. B, second strands labeled. Track 1, no SI nuclease added; Track 2, 0.13 unit of SI nuclease/ml; Track 3, 1.9 units/ml; Track 4, 5 units/ml.

bases long. When first strand labeled ovomucoid and lysozyme products were electrophoresed on denaturing gels, two prominent bands were observed in each case (Fig. 12, A and C). One co-migrated with full length first strand; the other was found at approximately the predicted full length second strand position. (Second strand labelings did not display a band at the unreacted position.) Higher amounts of enzyme did not alter the distribution. When treated with SI nuclease, the higher molecular weight band disappeared and an increase in messenger-sized material was observed, as predicted from hairpin cleavage. When these same ovomucoid and lysozyme products were electrophoresed on native gels (Fig. 12, B and D), an overall pattern similar to that observed with ovalbumin (Fig. 11) was found. A prominent band at approximately the predicted full length position is unaffected by SI digestion, while some trailing radioactivity disappears.

Total Poly(A)-containing RNA as Template

We have also examined total poly(A)-containing RNA as the template for the synthesis of double-stranded cDNA. First strand-labeled DNA polymerase I products were prepared using an enzyme concentration estimated to be sufficient for the synthesis of full length second strands. A portion was digested with SI nuclease. The reverse transcriptase, DNA polymerase, and SI-treated DNA polymerase products were electrophoresed under denaturing conditions (Fig. 13). The pattern of SI-treated double-stranded cDNA is very similar to that of the unreacted reverse transcriptase products themselves. With the exception of ovalbumin, which serves as a poor template for the synthesis of full length first strand, each messenger RNA is efficiently converted to opened double-stranded DNA.

DISCUSSION

Some of the drawbacks of previous protocols for sequential reverse transcriptase, DNA polymerase, and SI nuclease reactions were presented in the introduction. To limit difficulties due to E. coli DNA polymerase I inefficiency, we have examined in detail many reaction conditions with respect to the synthesis of second strands. Each parameter investigated had a significant effect on product length. A suboptimal choice in any one variable reduced the proportion of complete products substantially. In addition, the method we have used considerably reduces the handling of the in vitro products and thereby minimizes losses.

A rough estimate of the yield of full length double-stranded ovalbumin DNA synthesized under our conditions can be made. In a reaction using 1 μg of ovalbumin mRNA we estimate that about 0.1 μg of full length duplex is synthesized. (Some full length duplexes may be nicked or contain short gaps as judged by a comparison of native and denaturing gels of the same products.) This estimate was derived from reactions using as little as 50 ng of mRNA and so is applicable to small amounts of template.

When hen oviduct total poly(A)-containing RNA was used as a template for the three reactions, the distribution of SI-cleaved double-stranded products on denaturing gels was very similar to that of first strands themselves (Fig. 13). The only obvious, reproducible difference between the two is that the bands in the double-stranded products (especially lysozyme and ovomucoid) are less sharp than those in unreacted first strands, probably the result of variable digestion of duplex ends by SI. Their similarity suggests that the optimized DNA polymerase I and SI nuclease reactions are efficient using total mRNA and may be applied to obtain full length copies in a reaction using as little as 50 ng of mRNA and so is applicable to small amounts of template.

From a starting reverse transcriptase reaction using 1 μg of ovalbumin mRNA, roughly 0.25 μg of first strand is synthesized (9). After the DNA polymerase reaction, first strands are 80% resistant; thus 0.4 μg of double-stranded cDNA is present after SI cleavage. When electrophoresed on native gels, we estimate that 10 to 50% of this material is full length (Fig. 11, and unpublished data). 40 to 200 ng of duplex DNA representing the entire mRNA sequence would thus be made from 1 μg of messenger template.
Double-stranded cDNA Synthesis: pol I Reaction with cDNA

B and D, the indicated markers are only accurate to ±50 nucleotides due to gel edge distortion. A, ovomucoid products electrophoresed on a denaturing gel. Track 1, ovomucoid double-stranded cDNA before S1 cleavage (83,000 cpm); Track 2, unreacted ovomucoid first strand (11,000 cpm) mixed with the HindIII fragments of SV40 DNA (10,000 cpm). From top to bottom, the bands are 1700 bases, 1120 and 1010 bases (doublet not distinguished), full length ovomucoid first strand, and 520 and 420 base markers (unclear). Equal radioactivities (11,000 cpm) were applied to Tracks 3 through 6. Track 3, ovomucoid double-stranded cDNA before cleavage by S1 nuclease; Track 4, cleavage with 1.25 units of S1 nuclease/ml; Track 5, cleavage with 2.5 units/ml; Track 6, 5 units/ml. B, ovomucoid products electrophoresed on a native gel. Each track contains an equal amount of radioactivity. Track 1, no S1 nuclease added. Track 2, 1.25 units of S1/ml; Track 3, 2.5 units of S1/ml; Track 4, 5 units of S1/ml. C, lysozyme products electrophoresed on a denaturing gel. Equal radioactivities (28,000 cpm) were applied to Tracks 2 through 6. Track 1, HindIII fragments of SV40 DNA (28,000 cpm) mixed with lysozyme double-stranded cDNA before S1 cleavage (28,000 cpm). The positions of unreacted lysozyme first strands and full length double-stranded cDNA are not distinguishable from HindIII SV40 DNA fragments on this gel. Track 2, lysozyme double-stranded cDNA prior to cleavage by S1 nuclease; Track 3, 0.5 unit of S1/ml; Track 4, 1.25 units of S1/ml; Track 5, 2.5 units of S1/ml; Track 6, 5 units of S1/ml. D, lysozyme products electrophoresed on a native gel. Each track contains an equal amount of radioactivity. Track 1, lysozyme double-stranded cDNA prior to cleavage by S1 nuclease; Track 2, 0.5 unit of S1/ml; Track 3, 1.25 units of S1/ml; Track 4, 2.5 units of S1/ml; Track 5, 5 units of S1/ml.
Many systems in which only crude or slightly purified mRNAs are available.

Several lines of evidence indicate that most of the synthesis seen during the DNA polymerase I reaction is primed from a 3'-terminal hairpin on the first strand: (a) a large proportion of the duplex formed is capable of snapping back immediately after denaturation (Fig. 9); (b) examined on denaturing gels, the band found after second strand synthesis at twice the mRNA length is converted back to mRNA length upon cleavage by single-strand specific S1 nuclease; (c) on native gels, the full length band is unchanged upon digestion with increasing nuclease concentrations (Figs. 11 and 12). Some second strand synthesis probably does not begin from the hairpin primer, however, since not all duplexes snap back (Fig. 9). Oligo(dT) or messenger fragments (which might be generated by the RNAse H activity of reverse transcriptase by contaminating nucleases (9), or by thermal scission during the boiling step) could serve as primers for non-hairpin syntheses. Any hybrids formed during the DNA polymerase reaction are likely to be poorly matched. No hybrids stable to S1 nuclease digestion at 37°C are formed (Fig. 1); imperfectly base-paired hybrids formed during the DNA polymerase reaction are likely to be poorly matched. No hybrids stable to S1 nuclease digestion are formed (Fig. 1); imperfectly base-paired hybrids formed during the DNA polymerase I reaction are likely to be poorly matched. No hybrids stable to S1 nuclease digestion are formed (Fig. 1).
Double-stranded cDNA Synthesis: pol I Reaction with cDNA

Other mechanisms involving extensive hairpinning, branch migration, or the enzyme turning back to recopy its own product upon reaching an unstable dA:dT terminus are possible. While we have not rigorously attempted to distinguish among them, the 42° products probably do contain one or more hairpins (Fig. 8B).

In spite of the unresolved ambiguities, it is clear that full length double-stranded copies of lysozyme, ovomucoid, and ovalbumin mRNAs can be synthesized in reasonable yield under optimized conditions. The proportion of full length first strand synthesis by AM virus reverse transcriptase was not significantly affected by any of the reaction conditions we investigated in the accompanying publication once the problem of nuclease in the reverse transcriptase had been eliminated (9). In contrast, second strand synthesis by E. coli DNA polymerase I was very sensitive to each parameter examined. It was necessary to determine an optimal incubation time, enzyme/mRNA ratio, salt concentration, monovalent cation, and temperature, in order to obtain a high proportion of duplex DNA representing each complete structural gene.

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Note Added in Proof—We have subsequently studied other preparations of DNA polymerase I purified identically and find that they differ with respect to the incubation conditions that give full length second strand synthesis with ovalbumin mRNA. This may be due to differential stability of the nucleolytic and polymerizing activities of the enzyme. Thus, it appears necessary to optimize the reaction conditions for each preparation of DNA polymerase I.

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Synthesis of double-stranded DNA complementary to lysozyme, ovomucoid, and ovalbumin mRNAs. Optimization for full length second strand synthesis by Escherichia coli DNA polymerase I.
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