Mechanism of Action of Ribonucleic Acid-directed Deoxyribonucleic Acid Polymerase

I. TRANSCRIPTION OF GLOBIN MESSENGER RIBONUCLEIC ACID

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SUMMARY

The synthesis of DNA complementary to rabbit globin mRNA by the enzyme RNA-directed DNA polymerase (from avian myeloblastosis virus) has been studied. The reaction is dependent on mRNA as template, oligo(dT) as primer, and the four deoxynucleoside triphosphates as substrates. The 28 S, 18 S, and 4 S RNA are not effective templates; oligo(dG), oligo(dC), and oligo(dA) are not effective primers. The product of the reaction is a DNA-RNA hybrid, the DNA of which has an electrophoretic mobility of 7 to 10 S. The DNA hybridizes with globin 10 S mRNA but not with 28 S, 18 S, or 4 S RNA, nor does it hybridize with an 8 to 12 S RNA fraction isolated from rabbit liver polysomes. Thus, the RNA-directed DNA polymerase is capable of synthesizing a DNA molecule complementary to globin mRNA; however, based on its size the product does not appear to be a complete copy of the RNA template.

Using rabbit reticulocyte 10 S globin mRNA or rabbit liver mRNA as template the RNA-directed DNA polymerase can synthesize poly(dT) when dTTP is the only substrate present; no reaction occurs in the presence of any other single deoxynucleoside triphosphate. A high molecular weight poly(dT) polymer is formed, presumably transcribed from the poly(A) region of the mRNA. Thus, under some conditions, the enzyme may "slip" during transcription so that parts of the RNA are transcribed more than once in the synthesis of a single DNA molecule.

The RNA-directed DNA polymerase of avian myeloblastosis virus may provide a means by which to synthesize single complete genes. Several laboratories (1-3) have shown that purified globin mRNA can serve as a template from which the AMV DNA polymerase transcribes a complementary DNA molecule (cDNA). Such a DNA product has been used as a genetic probe for localizing and quantitating gene complements (4-10). To date, however, using 10 S globin mRNA, a cDNA has not been synthesized which is of equivalent size (based on sedimentation coefficient values) to the template from which it is transcribed. The cDNA product has been estimated to be 8.0 S by Verma et al. (1), 8.3 S by Kacian et al. (2), and 5.8 S and 6.3 S by Ross et al. (3). Ross et al. (11) have also shown that the G + C content of the cDNA is similar but slightly less than the G + C content of globin mRNA.

In this study, template, primer, and substrate requirements of the AMV DNA polymerase have been examined in detail. The size and specificity of the DNA synthesized has been characterized. During these studies, a dTMP polymerization reaction was observed which is different from transcription of the globin gene proper. The poly(dT) synthesis is dependent only on the presence of the enzyme, (poly(A)-containing) template, an oligo(dT) primer, and dTTP substrate. This novel type of transcription, with both rabbit reticulocyte globin mRNA and mRNA from rabbit liver as template, results in the synthesis of poly(dT) polymers with electrophoretic mobilities ranging from 4 S to greater than 28 S.

METHODS

Preparation of RNA-directed DNA Polymerase from Avian Myeloblastosis Virus—Avian myeloblastosis virus strain BAI, partially concentrated by centrifugation of the chicken plasma fraction, was supplied by the Cancer Virus Program of the National Cancer Institute, National Institutes of Health, Bethesda, Maryland, through Dr. Joseph Beard, Life Sciences, Inc., Gulfport, Florida. The method for purification of the DNA polymerase is the procedure of Kacian et al. (12) with minor modifications. The DEAE-cellulose chromatography step of this procedure removes all detectable poly(U)-degrading ribonuclease from the enzyme preparation (Fig. 1). The final enzyme preparation is also free of poly(dA), poly(dT), and poly(dA)-poly(dT)-degrading deoxyribonucleases.

Assay of Column Fractions during Enzyme Purification—Column fractions were analyzed for the presence of DNA polymerase activity in 20-μl reactions. Of each column fraction, 10 μl were added to 10 μl of the following reaction mixture which contained, per ml: 50 μmoles of Tris-Cl, pH 7.9; 30 μmoles of KCl; 10 μmoles of MgCl₂; 5 μmoles of dithiothreitol; 2 ATP units of poly(dA)-oligo(dT)n (20 adenine residues to 1 thymine residue, P-L Bio-
Deoxyribonuclease and Ribonuclease Assays—The presence of a deoxyribonuclease activity was assayed by measuring the stability of [3H]poly(dA).poly(dT) followed by rapid heat denaturation of [3H]poly(U) and [3H]poly(dA) and [3H]poly(dT). The reaction mixtures were 100 μl and contained, per ml: 50 μmoles of Tris-HCl, pH 7.5, and 0.005 M MgCl₂. Polysomes were centrifuged in a type 35 rotor at 35,000 rpm for 16 hours at 4°C. The polysomes were washed and then dissolved in 0.01 M Tris-HCl, pH 7.5, and 0.005 M MgCl₂. Next, 3500 A₂₆₀ units of purified polysomes in 50 ml were incubated in the presence of 0.5% sodium dodecyl sulfate, 0.01 M Tris-HCl, pH 7.5, and 5% sucrose at 37°C for 5 min. The precipitate was collected by centrifugation at 10,000 rpm in a Sorvall 89-34 rotor, dissolved in 0.01 M Tris-HCl, pH 7.5, and precipitated with 95% ethanol at -20°C. The precipitate was collected by centrifugation at 10,000 rpm in a Sorvall SS-34 rotor, dissolved in 0.01 M Tris-HCl, pH 7.5, at concentrations of 25 to 50 A₂₆₀ units per ml and stored in liquid nitrogen. Purity analysis of the 28 S, 18 S, and 10 S RNA fractions on 2.2% polyacrylamide gels containing sodium dodecyl sulfate is shown in Fig. 3. The 10 S globin mRNA is active in both translation (14) and transcription systems.

Preparation of 10 S Globin mRNA—Rabbit reticulocyte globin mRNA was prepared according to the methods of Nienhues et al. (13). Blood cells obtained from rabbits made anemic with phenylhydrazine were lysed with 0.005 M MgCl₂. Polysomes were purified from the lysate by centrifugation through a discontinuous sucrose gradient. The gradient, consisting of 15 ml each of 0.7 M and 1.8 M sucrose containing 0.02 M Tris-HCl, pH 7.5, 0.05 M KCl, and 0.005 M MgCl₂, was centrifuged in a type 35 rotor at 35,000 rpm for 16 hours at 4°C. The polysomes were washed and then dissolved in 0.01 M Tris-HCl, pH 7.5, and 0.005 M MgCl₂. Next, 3500 A₂₆₀ units of purified polysomes in 50 ml were incubated in the presence of 0.5% sodium dodecyl sulfate, 0.01 M Tris-HCl, pH 7.5, and 5% sucrose at 37°C for 5 min. The dissociated RNA species were separated in a 15 to 30% exponential sucrose gradient containing 0.005 M Tris-HCl, pH 7.5. The gradient was made in a Beckman Ti 15 zonal rotor and centrifuged at 30,000 rpm for 2 hours at 15°C followed by 68 hours at 8°C. Fig. 2 shows the absorption profile at 264 nm of a typical gradient. The RNA from the various pooled fractions was concentrated by centrifugation for 60 hours at 35,000 rpm in a Beckman type 35 rotor at 2°C (after this centrifugation all of the RNA had sedimented to the lower 5 to 10 ml); NaCl was then added to this fraction to a final concentration of 0.25 M and the RNA was precipitated with 2/3 volumes of 95% ethanol at -20°C. The precipitate was collected by centrifugation at 10,000 rpm in a Sorvall SS-34 rotor, dissolved in 0.01 M Tris-HCl, pH 7.5, at concentrations of 25 to 50 A₂₆₀ units per ml and stored in liquid nitrogen. Purity analysis of the 28 S, 18 S, and 10 S RNA fractions on 2.2% polyacrylamide gels containing sodium dodecyl sulfate is shown in Fig. 3. The 10 S globin mRNA is active in both translation (14) and transcription systems.

FIG. 2 (left). Zonal sucrose gradient fractionation of rabbit reticulocyte RNA in which 3500 A₂₆₀ units of sodium dodecyl sulfate-treated rabbit reticulocyte polysomes were centrifuged in a 1660-ml 10 to 30% sucrose gradient containing 0.005 M Tris-HCl, pH 7.5 (zonal Ti 15 rotor) as described under "Methods." After the centrifugation the A₂₆₀ profile of the gradient was monitored with an ISCO ultraviolet analyzer. Fractions were collected as follows: 28 S (600 to 720 ml), 18 S (930 to 1080 ml), 10 S (1100 to 1345 ml), and 4.5 S (1430 to 1550 ml). The RNA was concentrated as described under "Methods" and analyzed for purity as shown in Fig. 3.

FIG. 3 (right). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified fractions of rabbit reticulocyte RNA. The RNA was prepared as described under "Methods" (see also Fig. 2); 0.3 A₂₆₀ unit of the indicated fractions was analyzed by 2.2% polyacrylamide gel electrophoresis. The gels were prepared and run as described under "Methods." The A₂₆₀ profiles of the gels were determined using a Gilford gel scanner. Left, 28 S RNA fraction; middle, 18 S RNA fraction; right, 10 S (globin) mRNA fraction.
Preparation of RNA from Rabbit Liver—Liver RNA fractions were prepared from rabbit liver polysomes isolated by the method of Falvey and Staehelin (15). The polysomes were treated with 0.5% sodium dodecyl sulfate at 37°C for 5 min. The RNA was fractionated in 30-ml convex exponential sucrose gradients (150 A$_{260}$ units per gradient) containing 0.35 to 1.1 M sucrose and 0.005 M Tris-HCl, pH 7.5. The gradients were centrifuged at 27,000 rpm (SW 27 rotor) for 24 hours at 5°C. The RNA fractions were collected, pooled, made 0.25 M in NaCl, and the RNA was precipitated with the addition of 2½ volumes of ethanol at -20°C. The precipitate was collected by centrifugation, dissolved in 0.01 M Tris-HCl, pH 7.5, and stored in liquid nitrogen. The fractions showed the greatest activity in both systems.

Assay of Activity of RNA-directed DNA Polymerase with 10 S Globin mRNA—The basic reaction mixture for measuring globin mRNA-directed transcription consisted of the following components, per ml: 50 pmoles of Tris-HCl, pH 7.3; 50 pmoles of KCl; 6 or 10 pmoles of MgCl$_2$; 8 pmoles of dithiothreitol; 200 to 500 pmole of actinomycin D (Schwarz-Mann), as indicated in figure legends; 10 to 75 nmoles of dATP, dTTP, dCTP, and dGTP, one or more of which was tritiated (specific activities are given in figure legends); 210 pmoles (1,000 units) of 18 S globin mRNA; 1.65 to 3.3 nmoles (0.2 to 1 Aleo units) of oligo(dT)$_{15}$ or oligo(dT)$_{16}$ (Collaborative Research, Inc.), as indicated in figure legends; and 10 to 30 pmoles of purified DNA polymerase. One unit of enzyme polymerized 1 pmole of DNA per 20 min at 37°C using poly(A)$_{1}$. oligo(dT)$_{10}$ as template. The reaction mixtures were either 50 or 100 µl in volume. Reaction tubes were siliconized, heated to 90-95°C dry heat for 24 hours, and then cooled before use. The reaction was started by addition of enzyme and incubated at 32°C or 37°C as indicated in the figure legends. At various times, aliquots were withdrawn, added to 100 to 200 µg of carrier E. coli tRNA, and precipitated with approximately 2 ml of cold 10% trichloroacetic acid-0.2% pyrophosphate. The samples were processed for measurement of incorporated radioactivity as described above.

Cs$_2$SO$_4$ Density Equilibrium Centrifugation—In order to remove bound and free RNA associated with the DNA product, the incubated reaction mixture was heated to 100°C for 2 min, quick frozen in Dry Ice, and then made 0.3 N in NaOH by addition of 1 N NaOH. The solution was then incubated at 37°C for 18 to 20 hours, after which it was neutralized with 1 N HCl. The free nucleotides were then removed by dialysis in the cold against three to four changes (each 1 liter) of 0.5 M NaHCO$_3$ over a period of 16 to 20 hours followed by dialysis against 1 liter of distilled water for 2 hours. Other hydrolysis conditions (i.e., 0.33 M NaOH, 10 min, 95°C; 0.67 M NaOH, 18 hours, 37°C) yielded identical results. This DNA solution was then mixed with Cs$_2$SO$_4$ solution (ρ = 1.66 g per cc) containing 0.01 M Tris-HCl, pH 7.2, and 0.1 M NaCl-0.001 M EDTA to a final density of 1.55 g per cc. The gradients were then centrifuged in polyallomer tubes (precoated in 100 µg per ml of calf thymus DNA) at 25,000 rpm (SW 50.1 rotor) at 20°C. Rabbit reticulocyte 18 S RNA and native calf thymus DNA were used as markers. After centrifugation, fractions were collected and their refractive indices measured using a Bausch & Lomb Abbe 3L refractometer. The samples were then either precipitated with trichloroacetic acid for determination of their radioactivity or measured spectrophotometrically to determine their absorbance at appropriate wavelengths.

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis was carried out according to the method of Loening (16) and was used to determine the relative sizes of the RNA and DNA molecules. The gels were 5 × 100 mm and consisted of 2.2% acrylamide, 0.11% bisacrylamide, and the following gel buffer: 0.036 M NaH$_2$PO$_4$, 0.601 M EDTA, final pH 6.4, N,N',N'-tetramethylethylenediamine (0.033 ml) and 10% (w:v) ammonium persulfate (0.33 ml) were added per gram of acrylamide present. The gels were then subjected to electrophoresis for 30 min at 5 mA per gel in the gel buffer containing 0.2% sodium dodecyl sulfate (w:v). Samples containing 5,000 to 50,000 cpm of radioactivity or 0.3 to 0.5 A$_{260}$ units of nucleic acid in electrophoresis buffer containing 20% glycerol were applied to the gels. The gels were then subjected to electrophoresis for 75 min at 5 mA per gel with cold water cooling. After electrophoresis the gels were dialyzed against water for 10 min. (This procedure reduces the background radioactivity and absorption of the gels.) Gels containing sufficient A$_{260}$ material were then scanned using a Gilford gel scanner. For analysis of the radioactivity profiles of the gels, 2-mm slices were prepared with a Miccel gel slicer. The gel slices were incubated in 0.5 ml of NCS solution (9 parts NCS: 1 part H$_2$O) (Amersham-Searle Co.) for 2 hours at 50°C and radioactivity was measured in a scintillation system consisting of 0 parts LSC complete and 1 part Hydromix (Yorktown Research, Inc.).

DNA-RNA Hybridization—The hybridization procedure used was that of Kacian et al. (4). The reaction mixture (100 µl) contained per ml: 20 µmoles of NaHPO$_4$, pH 7.0; 300 µmoles of NaCl; 2 µmoles of EDTA; 1 mg of sodium dodecyl sulfate; 150,000 to 180,000 cpm of [H]$^3$DNA, and various concentrations of mRNA. Aliquots of 7.5 µl were sealed into individual capillary tubes (Drummond "Microcap", 40-µl capacity). These tubes had been presoaked in a solution of E. coli DNA (100 µg per ml, in 0.3 M NaCl and 20 mM EDTA), rinsed thoroughly in distilled water, and allowed to air dry. After 10 min preincubination at 90°C, the sealed capillary tubes were incubated at 68°C for 0.1 to 36 hours. Reactions were terminated by placing the tubes on Dry Ice. The amount of hybridization was determined using micrococcal nuclease (17). The contents of each tube were added to 200 µl of 50 mM Tris-HCl, pH 8.3; 0.4 M NaCl; 10 mM MgCl$_2$, 0.1 mM CaCl$_2$. Two 90-µl aliquots were taken; micrococcal nuclease (2 µg of a 4 µg/ml solution; Worthington Chemical Corp.) was added to one aliquot, and both aliquots were incubated at 37°C for 1 hour. Both aliquots were precipitated with cold 10% trichloroacetic acid and the precipitates were collected and washed with 10% cold trichloroacetic acid on nitrocellulose filters (Millipore). Radioactivity was measured by liquid scintillation spectrometry. The ratio of the tritium in the sample treated with nuclease to that in the untreated sample is a measure of the amount of hybridization.

RESULTS

Template Activity of Rabbit Reticulocyte RNA Fractions—Rabbit reticulocyte 28 S, 18 S, 10 S, and 4 S RNA fractions were prepared as described under "Methods" and as shown in Figs. 2 and 3. The template activity of these RNAs was measured in the oligo(dT)$_{18}$-primed RNA-directed DNA polymerase transcription system (Table I). The 10 S fraction is the only RNA species with significant template activity. The slight activity of the 18 S and 28 S fractions might result from a minor contamination with 10 S RNA.

Characterization of Globin mRNA-Directed Reaction—The relative incorporation of the four different substrates into the oligo(dT)$_{16}$-globin mRNA-directed product is shown in Table II.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Template activity of rabbit reticulocyte RNA fractions</th>
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<tr>
<td>RNA template</td>
<td>dGMP incorporated at 37°C</td>
</tr>
<tr>
<td>4 S</td>
<td>0.04</td>
</tr>
<tr>
<td>10 S</td>
<td>0.07</td>
</tr>
<tr>
<td>18 S</td>
<td>0.33</td>
</tr>
<tr>
<td>28 S</td>
<td>0.35</td>
</tr>
<tr>
<td>None</td>
<td>0.04</td>
</tr>
</tbody>
</table>
TABLE II

Measurement of relative incorporation of substrates in dX12-globin mRNA-directed transcription reaction

Reaction mixtures (50 μl) contained, per ml: 50 μmoles of Tris-HCl, pH 7.9; 50 μmoles of KCl; 6 μmoles of MgCl₂; 8 μmoles of dithiothreitol; 200 μg of actinomycin D; 200 μg of albumin, 0.2 A₄₅₀ unit of the designated primer; 210 pmol of globin mRNA; 32 nmoles of dATP, dCTP, dGTP, and dTTP. For measurements of dTMP incorporation the specific activity of [³H]dTTP was 707 cpm per pmole; for dAMP incorporation, [³H]dATP was 648 cpm per pmole; for dGMP incorporation, [³H]dGTP was 1560 cpm per pmole; and for dCMP incorporation, [³H]dCTP was 1363 cpm per pmole. The reactions were incubated at 37° for 60 min. After the incubation, 100 μg of carrier E. coli tRNA were added and the samples were prepared for measurement of radioactivity as described under “Methods.”

Labeled deoxynucleoside monophosphate incorporated at 37°

<table>
<thead>
<tr>
<th>Primer</th>
<th>dTMP</th>
<th>dGMP</th>
<th>dAMP</th>
<th>dCMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo(dT₁₀)</td>
<td>35.5</td>
<td>14.4</td>
<td>10.4</td>
<td>8.0</td>
</tr>
<tr>
<td>Oligo(dG₁₀)</td>
<td>1.4</td>
<td>2.4</td>
<td>3.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Oligo(dA₁₀)</td>
<td>0.9</td>
<td>2.2</td>
<td>3.9</td>
<td>1.4</td>
</tr>
<tr>
<td>Oligo(dC₁₀)</td>
<td>1.3</td>
<td>2.4</td>
<td>3.6</td>
<td>1.6</td>
</tr>
<tr>
<td>None</td>
<td>1.1</td>
<td>2.0</td>
<td>3.8</td>
<td>1.4</td>
</tr>
</tbody>
</table>

The extent of dTMP incorporation is 2 to 4 times that of any other single substrate. The data in Table II also demonstrate that oligo(dT₁₀) is the only effective primer for the globin mRNA directed reaction. Even when primers of longer length dNMP₁₀-₁₈ were used, only oligo(dT₁₀-₁₈) was an efficient primer for the reaction (data not shown). The NaOH-treated product from the oligo(dT₁₀)-globin mRNA-directed reaction has a buoyant density in CsSO₄ characteristic of pure DNA (data not shown). Electrophoresis on sodium dodecyl sulfate-polyacrylamide gels demonstrated that this DNA product migrates as 7 to 10 S species relative to 18 S, 16 S, 10 S, 5 S, and 4 S RNA markers. The radioactivity profile of cDNA products after incubation of a complete reaction mixture at 37° for 0, 2, 6, 10 and 60 min is shown in Fig. 4. Based on this analysis the size of the cDNA product remains relatively constant throughout the incubation. Some of the product migrates as a large molecule (i.e., greater than the 10 S globin mRNA template). This fraction may result from a novel type of reaction involving transcription of the poly(A) region of the globin mRNA as described in detail later.

The complementarity between the DNA product and globin mRNA was established by annealing reactions. In these reactions the species formed had a buoyant density characteristic of DNA-RNA hybrid molecules (data not shown). The extent of hybridization was determined by measuring the amount of [³H]DNA product which became resistant to micrococcal nuclease under conditions where this enzyme acts as a single strand specific nuclease (17). The percentage of hybridization versus log Cr₄ (log RNA concentration × time) plot of this reaction is shown in Fig. 5. The annealing of globin cDNA to globin mRNA is over 1000 times more specific than the annealing of globin cDNA to mRNA isolated from liver. The hybridization assays were performed as described under “Methods.” Each reaction mixture contained 1500 cpm of globin (Hb) DNA and either 0.01 to 180 ng of globin (Hb) mRNA (●—●) or 0.01 ng to 15 μg of liver mRNA (O—O). Reactions were incubated at 68° for 0.1 to 36 hours. Cr₄, moles of ribonucleotides × s per liter.

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of oligo(dT₁₀)-globin mRNA-directed cDNA products. The 2.2% gels were prepared and electrophoresed as described under “Methods.” Applied to each gel were 10,000 to 50,000 cpm of alkali-treated product.

Fig. 5. Hybridization of rabbit reticulocyte globin cDNA with rabbit reticulocyte 10 S mRNA and rabbit liver 8 to 10 S RNA. The hybridization assays were performed as described under “Methods.” Each reaction mixture contained 1500 cpm of globin (Hb) DNA and either 0.01 to 180 ng of globin (Hb) mRNA (●—●) or 0.01 ng to 15 μg of liver mRNA (O—O). Reactions were incubated at 68° for 0.1 to 36 hours. Cr₄, moles of ribonucleotides × s per liter.

Characterization of Poly(dT) Synthesis Directed by Globin mRNA—The requirements and time dependence of poly(dT) synthesis were analyzed using two different templates, rabbit reticulocyte 10 S globin mRNA and 8 to 12 S mRNA from rabbit liver. The dependence of the reaction on enzyme, mRNA (globin 10 S mRNA) and oligo(dT) primer is shown in Fig. 6. The rate of the reaction with only dTTP present as substrate appears to be approximately 2 to 3 times faster than the rate of the reaction with all four deoxyribonucleoside triphosphates present. With some preparations of enzyme, the rate of poly(dT) synthesis remains linear for 90 min of incubation.
Tris-HCl, pH 7.9; 50 pmoles of KCl; 6 pmoles of MgCl₂; 8 pmoles and processed as described under "Methods" for measurement of radioactivity. Incorporation is normalized to 0.1 ml aliquots. were incubated at 32°C and aliquots were removed at various times.

In the reaction mixture with all four deoxynucleoside triphosphates present, dATP, dCTP, and dGTP were present at a concentration of 40 nmoles of each per ml. The reaction mixtures were incubated at 37°C for 60 min. The reaction mixtures were incubated at 37°C for 60 min. After this incubation, the reactions were stopped and the incorporated radioactivity measured as described under "Methods." Substrates present in reaction mixture

Table III

<table>
<thead>
<tr>
<th>Deoxynucleoside monophosphate incorporated at 37°C</th>
<th>dTTP only</th>
<th>dGTP only</th>
<th>dATP only</th>
<th>dCTP only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer</td>
<td>pmoles/50 μl/60 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligo(dT₁₀)</td>
<td>114.2</td>
<td>0.9</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Oligo(dG₁₀)</td>
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<td>0.7</td>
<td>0.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Oligo(dA₁₀)</td>
<td>0.6</td>
<td>1.4</td>
<td>0.6</td>
<td>1.2</td>
</tr>
<tr>
<td>Oligo(dC₁₀)</td>
<td>0.6</td>
<td>0.8</td>
<td>0.4</td>
<td>1.2</td>
</tr>
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<td>None</td>
<td>0.6</td>
<td>1.1</td>
<td>1.0</td>
<td>0.4</td>
</tr>
</tbody>
</table>

at 37°C. Similar results were obtained using 8 to 12 S mRNA from rabbit liver as template. The primer and substrate requirements of the poly(dT) synthesis reaction were analyzed using the globin mRNA-directed system. The top row of Table III shows that using oligo(dT₁₀) as primer and globin mRNA as template, only dTMP is incorporated into trichloroacetic acid-precipitable product when a single base is present as substrate. The homogeneity of dTMP incorporation in this reaction was further measured by unlabeled dTTP plus one at a time of each of the other labeled substrates. The results (Table IV) further substantiate that dTMP is the only significantly incorporated substrate in this reaction. The primer requirements for poly(dT₁) synthesis using globin mRNA as template are tabulated in Table III. In the presence of only one type of deoxynucleoside triphosphate as substrate, oligo(dT₁₀) is the only primer which stimulates transcription.

Physical and Chemical Nature of Product of Poly(dT) Synthesizing System—To determine its chemical nature, the product of the poly(dT) synthesizing system directed by oligo(dT₁₀)-globin mRNA was alkali-treated to remove the annealed RNA and then analyzed by C₅₀S₄ density equilibrium centrifugation. As seen in Fig. 7A, the labeled product has a buoyant density of 1.45 g per cc, which is characteristic of DNA.

The RNA-free product was also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in order to determine its electrophoretic mobility relative to 28 S, 10 S, and 4 S RNA markers. Fig. 7B shows that this DNA product is very heterogeneous ranging from 5 S to greater than 28 S. Thus, the largest DNA synthesized from the poly(dT) region of the template and approximately 100 to 150 times greater than the poly(A) region of the template, assuming a stretch of 75 to 125 nucleotides (18). Identical analyses of the alkali-treated product from the reaction directed by oligo(dT₁₀)-liver mRNA have given similar results (Fig. 8, A and B).

Transcription of Globin mRNA in Presence of Two Deoxynucleoside Triphosphates—Using the oligo(dT₁₀)-globin mRNA-directed transcription system, the time course of incorporation of [³H]dTTP into acid-insoluble product in the presence of two of the four deoxynucleoside triphosphate substrates was measured. Fig. 9 shows that dGTP inhibits the high incorporation of dTMP; the reactions with dTTP alone, dTTP plus dCTP, or dTTP plus dATP show 3 to 8 times more incorporation of

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**Table IV**

Homogeneity of dTMP incorporation during poly(dT) synthesis

Reaction mixtures (50 μl) contained the same ionic and buffer conditions as described in Table III. Albumin was not included in this reaction. The concentration per ml of other components was as follows: 500 μg of actinomycin D; 420 pmoles (2 A₂₆₀ units) of globin mRNA; 3.3 nmoles (0.2 A₂₆₀ unit) of oligo(dT₁₀); and 25 units of purified AMV DNA polymerase. The substrates were included in the following concentrations per ml of reaction mixture: 16 nmoles of unlabeled dTTP plus (a) 20 μmoles of [³H]dTTT (10,845 cpm per pmole); (b) 20 μmoles of [³H]dCTP (2,376 cpm per pmole); (c) 20 μmoles of [³H]dATP (2,780 cpm per pmole); and (d) 20 μmoles of [³H]dGTP (5,941 cpm per pmole). The reactions were incubated at 37°C for 60 min. After this incubation, the reactions were stopped and the incorporated radioactivity measured as described under "Methods."
A tRNA

Fig. 7. A, Cs2SO4 equilibrium density gradient analysis of the product of oligo(dT10)-globin mRNA-directed poly(dT) synthesis. A poly(dT) synthesis system using the oligo(dT)14-globin mRNA-directed reaction was incubated for 90 min at 37° as described under "Methods." The alkali-treated product was prepared, heated to 68° in the presence of 50% formamide, mixed with 60 μg of calf thymus DNA, and analyzed in Cs2SO4 gradients as described under "Methods." Of this product, 20,000 cpm were analyzed in the gradient. The gradients were centrifuged at 33,000 rpm in an SW 50.1 rotor for 72 hours at 20°. After centrifugation, 0.2-ml fractions were collected and the radioactivity profile was determined after trichloroacetic acid precipitation as described under "Methods." B, polyacrylamide gel electrophoresis of the product of oligo(dT10)-globin mRNA directed poly(dT) synthesis. A portion of the same alkali-treated product prepared for the experiment described in Fig. 7A was treated with 0.5% sodium dodecyl sulfate and analyzed by electrophoresis on 2.2% polyacrylamide gels. The gels were subjected to electrophoresis 80 min at 5 mA per gel and then processed for measurement of radioactivity as described under "Methods."
FIG. 8. A, CsCl equilibrium density gradient analysis of the product of oligo(dT)₃₀-liver mRNA-directed poly(dT) synthesis. Alkali-treated product from an oligo(dT)₃₀-liver mRNA reaction was incubated at 68°C in 50% formamide and analyzed in CsCl gradients as described under "Methods." Approximately 20,000 cpm of product were analyzed in the gradient. The gradients were centrifuged and processed identically with that described in Fig. 7A except centrifugation was at 10°C. B, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the product of oligo(dT)₃₀-liver mRNA-directed poly(dT) synthesis. A portion of the alkali-treated product prepared for the experiment described in Fig. 8A was treated with 0.5% sodium dodecyl sulfate and analyzed by electrophoresis on 2.2% polyacrylamide gels. The gels were subjected to electrophoresis at 5 ma per gel for 80 min and then processed as described under "Methods." The product of the reaction when all four deoxynucleoside triphosphates are present is also shown.

Fig. 9. Transcription of globin mRNA by AMV DNA polymerase in the presence of two deoxynucleoside triphosphates. Reaction mixtures (100 μl) contained, per ml: 50 μmoles of Tris-HCl, pH 7.9; 50 μmoles of KCl; 10 μmoles of MgCl₂; 8 μmoles of dithiothreitol; 200 μg of actinomycin D; 22 μmoles of dTTP (1284 cpm per pmole); 20 μmoles of unlabeled dATP, dCTP, or dGTP as indicated; 483 pmole (2.3 A₂₆₀ units) of globin mRNA; 3.3 pmole (0.2 A₂₆₀ unit) of oligo(dT)₃₀; and 30 units of AMV DNA polymerase. The reaction mixtures were incubated at 32°C and 20 μl aliquots were withdrawn at the indicated times and processed as described under "Methods" for measurement of the radioactivity incorporated into product. ○—○, dTTP only; ●—●, dTTP + dCTP; ▲—▲, dTTP + dGTP; ▼—▼, dGTP. The incorporation is normalized to 0.1-ml aliquots.

FIG. 10. Effect of addition of dGTP on the size of the product produced in the poly(dT) synthesizing system. The alkali-treated products from an oligo(dT)₃₀-globin mRNA-directed reaction mixture containing [³H]dTTP as the only substrate (●—●) or [³H]dTTP + unlabeled dCTP (○—○) were analyzed on 2.2% polyacrylamide gels. Electrophoresis and processing of the gels for radioactivity were as described under "Methods."
at approximately the same rate as the conventional transcription reaction, but for a longer extent and results in the synthesis of very large molecules (Fig. 7B). Inclusion of dGTP in the reaction inhibits the synthesis of the large poly(dT) molecules (Figs. 9 and 10), suggesting that cytidine may be the first (transcribed) base following the poly(A) region on the globin mRNA. This finding is consistent with the observations of Froudfoot and Brownlee (20). Such a reaction with dTTP and dGTP present is followed by a pyrimidine and in agreement with the sequence of limiting substrate, the reaction may not always be faithful. In addition, under such conditions, theoretically it is possible to synthesize almost any size of product. This further hampers interpretations of the completeness of the transcription of mRNAs by the AMV DNA polymerase as size alone is not a definitive criterion. Although complementary DNA (which can be employed as a genetic probe) is synthesized by the AMV DNA polymerase reaction, this transcription system is not sufficiently perfected to be used for the synthesis of a complete DNA gene. Thus, in order to analyze accurately the reaction mechanism, it appears preferable to study the transcription reaction directed by artificial templates of defined lengths and base compositions. With such a system the expected product can be precisely predicted under various conditions and unfaithful transcription easily detected.

Acknowledgments—We would like to thank Dr. Dorothy Beard, Dr. Joseph Beard, and the National Cancer Institute, Special Virus Program, for their generous cooperation in supplying virus; Dr. Robert Gallo for helpful discussions; Dr. William Merrick for reading the manuscript and help with figures; and Dr. Golder Wilson for assistance with the annealing reactions.

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