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 28S, 18S, and 4S 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 10S. The DNA hybridizes with globin 10S mRNA but not with 28S, 18S, or 4S RNA, nor does it hybridize with an 8 to 12S 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.

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 10S 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 8.0S by Verma et al. (1), 8.3S by Kacian et al. (2), and 5.8S and 6.3S 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-ml reactions. Of each column fraction, 10 ml were added to 10 ml of the following reaction mixture which contained, per ml: 500mMoles of Tris-HCl, pH 7.9; 30mMoles of KCl; 10mMoles of MgCl₂; 5mMoles of dithiothreitol; 2 A₅₀₀ units of poly(A)·oligo(dT₃₁) (20 adenine residues to 1 thymine residue, P-L Bio Sciences, Inc., Milwaukee, Wisconsin).

1 The abbreviation used is: AMV, avian myeloblastosis virus.
Deoxyribonuclease and Ribonuclease Assays—The presence of a deoxyribonuclease activity was assayed by measuring the stability of \([\text{3H}]\text{poly(dA).poly(dT)}\) and \([\text{3H}]\text{poly(dA)}\) and \([\text{3H}]\text{poly(dT)}\) (25.6 mCi per mmole of phosphorus; Miles Laboratories, Inc.) in a reaction mixture containing, per ml: 50 pmol of Tris-HCl, pH 7.9, 50 pmole of KCl, 5 pmole of MgCl₂, 8 pmole of dithiothreitol; and 2 \(\mu\)Ci of \([\text{3H}]\text{poly(U)}\). Reaction mixtures (15 \(\mu\)l) containing various components of the transcription system were incubated for 30 min at 37°C and then processed for radioactivity determinations.

Preparation of 10 S Globin mRNA—Rabbit reticulocyte globin mRNA was prepared according to the methods of Nienhuis et al. (13). Blood cells obtained from rabbits made anemic with phenylhydrazine were lysed with 0.005 \(M\) hydrazine. The dissociated RNA was precipitated with 235 volumes of 95% ethanol at -20°C. 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 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. 1. DEAE-cellulose chromatography of the AMV RNA-directed DNA polymerase. The viral extract was prepared as described by Kacian et al. (12) and chromatographed on DEAE-cellulose. The RNA-directed DNA polymerase activity was assayed using a poly(rA)-oligo(dT)₉ system (see "Methods"). Ribonuclease was detected as described under "Methods" by measurement of the stability of \([\text{3H}]\text{poly(U)}\) in the presence of the various fractions.

Fig. 2 (left). Zonal sucrose gradient fractionation of rabbit reticulocyte RNA in which 3500 \(A_{260}\) 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 \(A_{260}\) profile of the gradient was monitored with an ISCO ultraviolet analyzer. Fractions were collected as follows: 28 S (600 to 720 ml), 18 S (980 to 1080 ml), 10 S (1100 to 1350 ml), and 4-S (1450 to 1650 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 \(A_{260}\) unit of the indicated fractions was analyzed by 2.2% polyacrylamide gel electrophoresis. The gels were prepared and run as described under "Methods." The \(A_{260}\) 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 mRNA 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° for 5 min. The RNA was fractionated in 30-ml convex exponential sucrose gradients (150 A260 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 34 hours at 5°. The RNA fractions were collected, pooled, made 0.25 M in NaCl, and the RNA was precipitated by the addition of 2.5 volumes of ethanol at −20°. The precipitate was collected by centrifugation, dissolved in 0.01 M Tris-HCl, pH 7.5, and stored in liquid nitrogen. The fractions were assayed for template activity in transcription (as described above) and hybridization reactions (as indicated in figure legends; and Table I).

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 μl of Tris-HCl, pH 7.9; 50 μl of KCl; 6 or 10 μl of MgCl2; 8 μl of dithiothreitol; 200 to 500 μg of actinomycin D (Schwarz-Mann), as indicated in figure legends; 10 to 75 μmol of dATP, dCTP, dGTP, and dTTP, one or more of which was tritiated (specific activities are given in figure legends); 210 pmol (1 A260 unit) of 10 S globin mRNA; 1.65 to 3.3 pmol of oligo(dT12-18) or oligo(dT16) (Collaborative Research, Inc.), as indicated in figure legends; and 10 to 30 units of purified DNA polymerase. One unit of enzyme polymerized 1 nmole of dTMP in 60 min at 37° using poly(rA)-oligo(dT12-18) as template. The reaction mixtures were either 50 or 100 μl in volume. Reaction tubes were siliconized, heated to 90-95° dry heat for 24 hours, and then cooled before use. The reaction was started by addition of enzyme and incubated at 32° or 37° 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.

CspSO4 Density Equilibrium Centrifugation—In order to remove bound and free RNA associated with the DNA product, the incubated reaction mixture was heated to 100° for 2 min, quick frozen in Dry Ice, and then made 0.33 M NaOH for 18 hours, 37°) yielded identical results. This DNA solution was then mixed with CsCl 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 centrifuged in polyallomer tubes (preeaolized in 100 μg per ml of calf thymus DNA) at 35,000 rpm (SW 50.1 rotor) at 20°. 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 260 nm.

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 NaH2P04, 0.001 M EDTA, 5 × 10−6 M N,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 12 hours at 2000 V. All gels were stained with 0.2% Coomassie blue R-250 in 50% glycerol-water and then destained with 10% trichloroacetic acid.

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 RNA fractions was measured in the oligo(dT16)-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 different substrates into the oligo(dT16)-globin mRNA-directed product is shown in Table II.

### Table 1

<table>
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<tr>
<th>RNA template</th>
<th>dGMP incorporated at 37°</th>
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<tr>
<td>None</td>
<td>0.04</td>
</tr>
<tr>
<td>10 S</td>
<td>0.35</td>
</tr>
<tr>
<td>18 S</td>
<td>0.35</td>
</tr>
<tr>
<td>28 S</td>
<td>0.35</td>
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</tbody>
</table>

### Table II

<table>
<thead>
<tr>
<th>RNA template</th>
<th>dGMP incorporated at 37°</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 S</td>
<td>0.04</td>
</tr>
<tr>
<td>10 S</td>
<td>0.35</td>
</tr>
<tr>
<td>18 S</td>
<td>0.35</td>
</tr>
<tr>
<td>28 S</td>
<td>0.35</td>
</tr>
<tr>
<td>None</td>
<td>0.04</td>
</tr>
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</table>
TABLE II
Measurement of relative incorporation of substrates in dXlo-globin mRNA-directed transcription reaction

Reaction mixtures (60 μl) contained, per ml: 50 μmoles of Tris-HCl, pH 7.9; 50 μmoles of KCl; 6 μmoles of MgClt; 8 pmoles of dithiothreitol; 200 μg of actinomycin D; 200 μg of albumin, 0.2 A16 unit of the designated primer; 210 pmoles (1 AIAO unit) 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.”

<table>
<thead>
<tr>
<th>Primer</th>
<th>Labeled deoxynucleotide monophosphate incorporated at 37°</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dTMP</td>
<td>dGMP</td>
</tr>
<tr>
<td>Oligo(dT10)</td>
<td>35.5</td>
<td>14.4</td>
</tr>
<tr>
<td>Oligo(dG10)</td>
<td>1.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Oligo(dA10)</td>
<td>0.9</td>
<td>2.2</td>
</tr>
<tr>
<td>Oligo(dC10)</td>
<td>1.3</td>
<td>2.4</td>
</tr>
<tr>
<td>None</td>
<td>1.1</td>
<td>2.0</td>
</tr>
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</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 dNMPlssls were used, only oligo(dT10) 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, 4, 15 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 Crd (log RNA concentration X 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. It should be noted that this type of hybridization analysis ensures that complementary regions other than oligo(rA)-3H oligo(dT) are being measured because: (a) under the conditions employed the micrococcal nuclease degrades oligo(rA)-oligo(dT) hybrids (4), and (b) the DNA product is labeled only with dGTP, dCTP, and dATP.

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 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. Crd, moles of ribonucleotides X s per liter.
Fig. 6. Analysis of the requirements for poly(dT) synthesis. The reaction mixture (100 μl) contained, per ml: 50 μmoles of Tris·HCl, pH 7.9; 50 μmoles of KCl; 6 μmoles of MgCl₂; 8 μmoles of dithiothreitol; 100 μg of actinomycin D; 210 pmoles of (1 A₁₆₅ unit) of globin mRNA; 3.3 μmoles (0.2 A₁₆₅ unit) of oligo(dT₁₀); and 8.5 units of AMV DNA polymerase. In both reaction mixtures 96 nmoles per ml of dTTP (442 cpm per pmole) were included. The reaction mixtures were incubated at 37°C for 60 min. The reaction mixtures were incubated at 37°C for 60 min. The concentrations were as follows: 500 μg of actinomycin D; 420 pmoles (2 A₁₆₅ units) of globin mRNA; 3.3 μmoles (0.2 A₁₆₅ 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 amoles of [H]dATP (10,845 cpm per pmole); (b) 20 amoles of [H]dCTP (2,785 cpm per pmole); (c) 20 amoles of [H]dATP (2,470 cpm per pmole); and (d) 20 amoles of [H]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 was measured as described under “Methods.”

Table III shows that using oligo(dT₁₀) as primer and globin mRNA as template, only dTMP is incorporated into trichloro-acetic acid-precipitable product when a single base is present as substrate. The homogeneity of dTMP incorporation in this reaction was further measured by using 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(d₁) 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 Ca₅₀ 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, assuming a stretch of 75 to 125 nucleotides (18). Identical analyses of the alkali-treated DNA product from the reaction directed by oligo(dT₁₀)-globin mRNA have given similar results (Fig. 8, A and B).

Table IV

<table>
<thead>
<tr>
<th>Substrates present in reaction mixture</th>
<th>Unlabeled</th>
<th>[H]-Labeled</th>
<th>Labeled deoxynucleoside monophosphate incorporated</th>
<th>pmoles/50 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>dTTP + [H]dTTP</td>
<td></td>
<td></td>
<td></td>
<td>314</td>
</tr>
<tr>
<td>dTTP + [H]dCTP</td>
<td></td>
<td></td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>dTTP + [H]dATP</td>
<td></td>
<td></td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>dTTP + [H]dGTP</td>
<td></td>
<td></td>
<td></td>
<td>0.8</td>
</tr>
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</table>

Homogeneity of dTMP incorporation during poly(dT) synthesis

Table IV further substantiates that dTMP is the only significantly incorporated substrate in this reaction. The primer requirements for poly(d₁) 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.

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 pmole of (1 A₁₆₅ unit) of globin mRNA; 3.3 pmole (0.2 A₁₆₅ 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 nmoles of [H]dTTP (10,845 cpm per pmole); (b) 20 nmoles of [H]dCTP (2,785 cpm per pmole); (c) 20 nmoles of [H]dATP (2,470 cpm per pmole); and (d) 20 nmoles of [H]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 was measured as described under “Methods.”

Table III

<table>
<thead>
<tr>
<th>Primer</th>
<th>Deoxynucleoside monophosphate incorporated at 37°C</th>
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<tbody>
<tr>
<td></td>
<td>dTTP only</td>
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<tr>
<td></td>
<td>pmoles/50 μl/60 min</td>
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<tr>
<td>Oligo(dT₁₀)</td>
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</tr>
<tr>
<td>Oligo(dG₁₀)</td>
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</tr>
<tr>
<td>Oligo(dA₁₀)</td>
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<tr>
<td>Oligo(dC₁₀)</td>
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<tr>
<td>None</td>
<td>0.6</td>
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</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 trichloro-acetic acid-precipitable product when a single base is present as substrate. The homogeneity of dTMP incorporation in this reaction was further measured by using 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(d₁) 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.
Fig. 7. A, C540 equilibrium density gradient analysis of the product of oligo(dT10)-globin mRNA-directed poly(dT) synthesis. A poly(dT) synthesis system using the oligo(dT10-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 500 μg of calf thymus DNA, and analyzed in CsC140 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."

dTMP than the reactions with dTTP plus dGTP or with all four substrates present.

Since the presence of dGTP inhibits poly(dT) synthesis in the oligo(dT10)-globin mRNA-directed system (Fig. 9), the size of the products from the dTTP reaction with and without added dGTP were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The product of the oligo(dT10)-globin mRNA-directed reaction in the presence of dTTP and dGTP as substrates is fairly homogeneous and approximately 5 S in size, whereas, with the same reaction, but with dTTP as the only substrate, the product is large and heterogeneous (Fig. 10).

Effect of Addition of dATP, dGTP, and dCTP during Poly(dT) Synthesis—Experiments were conducted to determine whether the system was still capable of synthesizing cDNA by the conventional transcription reaction utilizing all four deoxynucleoside triphosphate substrates after poly(dT) synthesis had begun. An oligo(dT10) globin mRNA-directed reaction containing [3H]dTTP as the only substrate was supplemented with unlabeled dATP, dCTP, and dGTP after 2 min and 4 min of incubation. Upon addition of the three deoxynucleoside triphosphates to the reaction, the rate of incorporation of dTMP decreases to a level identical with that of the oligo(dT10)-globin mRNA-directed reaction with all four substrates present throughout the reaction (Fig. 11, A and B).

Experiments conducted to determine the length of time that the oligo(dT10)-globin mRNA-directed poly(dT) synthesizing system is capable of incorporating all four substrates show that this competence decreases exponentially with incubation time.

For these experiments an enzyme preparation was used which supports linear incorporation of dTMP as a function of time for longer than 60 min at 32°. After 10 min of incubation at 32° in the presence of only dTTP, the system is capable of incorporating all four substrates at a level of 50% of the original capacity, and after 60 min this level is 8%.

DISCUSSION

The work presented here is in agreement with the data from several laboratories (1-3) and demonstrates that a DNA product complementary to rabbit reticulocyte 10 S globin mRNA can be synthesized using the AMV RNA-directed DNA polymerase. This system is dependent on the 10 S globin mRNA, deoxy-nucleoside triphosphates, oligo(dT), and the AMV DNA polymerase. In this system the dependence on oligo(dT) primer and the high incorporation of dTMP into product (Table II) is consistent with the presence of a poly(A) region at the 3' terminus of the globin mRNA. The product obtained from the transcription reaction is DNA which reanneals with the rabbit reticulocyte globin mRNA (Fig. 5). However, from the studies in this and other laboratories, it is clear that much of the DNA product is smaller than the template from which it is synthesized. Sodium dodecyl sulfate-polyacrylamide gel analysis shows that 7 to 10 S product is synthesized (Fig. 4).

A novel type of transcription reaction has been observed using AMV DNA polymerase. The reaction is dependent on dTTP as the only substrate, oligo(dT) as primer, and mRNA (containing poly(A) region(s)) as template. The reaction proceeds
Fig. 8. A, CsSO₄ equilibrium density gradient analysis of the product of oligo(dT₁₀)-liver mRNA-directed poly(dT) synthesis. Alkali-treated product from an oligo(dT₁₀)-liver mRNA reaction was incubated at 68° in 50% formamide and analyzed in CsSO₄ 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°. B, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the product of oligo(dT₁₀)-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; 5 µmoles of dithiothreitol; 200 µg of actinomycin D; 22 nmoles of dTTP (1284 cpm per pmole); 20 nmoles of unlabeled dATP, dCTP, or dGTP as indicated; 483 pmole (2.3 X 10⁶ units) of globin mRNA; 3.3 pmole (0.2 X 10⁶ units) of oligo(dT₁₀); and 30 units of AMV DNA polymerase. The reaction mixtures were incubated at 32° and 20 µl aliquots were withdrawn at the indicated times and processed as described under "Methods" for measurement of the radioactivity incorporated into product. O-O, dTTP only; ·--·, dTTP + dGTP; Ω--Ω, dTTP + dCTP; Δ--Δ, dTTP + dGTP + dCTP; △--△, dTTP + dCTP + dGAP; O-----O, dTTP, dCTP, dGTP, dATP. 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(dT₁₀)-globin mRNA-directed reaction mixture containing [³H]dTTP as the only substrate (Ο--Ο) or [³H]dTTP + unlabeled dCTP (Ο--Ο) 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) products. The poly(A) region is transcribed faithfully. 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.

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