Purification of Biologically Active Globin mRNA Using cDNA-cellulose Affinity Chromatography*

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A complementary DNA (cDNA) copy of mouse globin mRNA was synthesized using the RNA-dependent DNA polymerase from avian myeloblastosis virus and the oligo(dT) covalently attached to cellulose as primer. All four deoxyribonucleotide triphosphates, NaCl, the globin mRNA template, and an oligo(dT) primer were required for optimal synthesis of cDNA. By saturating the primer sites using a 3-fold excess of mRNA, sufficient concentrations of immobilized cDNA could be synthesized to allow the hybridization reactions to be performed using an excess of globin cDNA. Conditions which permitted the annealing of globin mRNA to cDNA-cellulose were selected and the sequence specificity for hybridization to cDNA-cellulose was determined when using 28 S ribosomal RNA, polyadenylic acid, and mouse L-cell RNA.

Both analytical and preparative applications of this chromatographic medium were explored. When radioactively labeled poly(A)-containing 9 S RNA isolated from nucleated erythroid cells was analyzed by affinity chromatography on globin cDNA-cellulose, 16% of the applied radioactivity hybridized to the cDNA-cellulose column. Only 1% of the labeled RNA was retained by the column during reapplication of the unbound fraction, while 96% of the bound RNA remained on the column. Hybridizations utilizing untreated RNA extracts from either mouse reticulocytes or nucleated erythroid cells provided a one-step purification method for globin mRNA sequences. The relative purity of the RNA isolated by cDNA-cellulose affinity chromatography was determined by hybridization kinetic analysis. The cDNA-bound fraction obtained from the unfraccionated RNA of either cell type was shown to have a Cr(t/2) of $2.7 \times 10^{-5}$. This represents a 60-fold purification of the globin sequences present in reticulocyte polynucleotide RNA and a 280-fold enrichment of the globin mRNA in nucleated erythroid cells. Hybridization to cDNA-cellulose did not result in any change in the sedimentation rate of globin mRNA.

Furthermore, experiments were performed which demonstrated that the globin mRNA isolated by hybridization to cDNA-cellulose retained its biological activity when assayed in a wheat germ cell-free lysate.

The ability to prepare biologically active mRNA in a highly purified form is a necessary prerequisite to the investigation of mRNA structure and function. Following the discovery that a number of eukaryotic (1-6) and viral (7-10) mRNAs contain a polyadenylate region, several affinity chromatographic techniques were developed for the purification of mRNA which utilized complementary polynucleotides immobilized to various types of matrices (11-14).

Previously, we have reported on the isolation of a poly(A)$^+$-containing 9 S mRNA fraction from nucleated erythroid cells obtained from the spleens of anemic mice which contained a mixture of globin and nonglobin mRNAs (15, 16). Since the preparation of this fraction had already involved differences in sedimentation in sucrose density gradients and the presence of a poly(A) region for chromatography on oligo(dT)-cellulose, further purification of the globin mRNA was dependent upon additional criteria for mRNA fractionation.

The synthesis of a chromatographic medium which utilizes a complementary DNA (cDNA) copy of mouse globin mRNA for the isolation of specific mRNA sequences has been previously reported (17-19). This report describes the synthesis and characteristics of mouse globin cDNA cellulose and documents the specificity of this affinity chromatographic technique for the selective purification of biologically active globin mRNA from mouse reticulocytes and nucleated erythroid cells.

**EXPERIMENTAL PROCEDURES**

Isolation of RNA—Reticulocytosis was induced in Swiss Cox mice (25 to 30 g, random sex) by six daily 0.1 ml injections of 0.9% neutralized phenylhydrazine hydrochloride (20). On the 7th day, reticulocyte polysomes were prepared as previously described (21) and the RNA extracted with phenol/chloroform/isoamyl alcohol (11, 21). Reticulocyte globin mRNA was purified from the deproteinized RNA by a combination of oligo(dT)-cellulose chromatography (11, 22, 23) and sucrose density gradient centrifugation (24).

Preparation of labeled RNA from nucleated erythroid cells was done according to the method of Merkel et al. (16). The nucleated erythroid cells were suspended at $5 \times 10^9$ cells/ml in 100 ml of RPMI-1640 (Gibco) supplemented with 10% feta calf serum, 2.0 mM glutamine, 1.0 mM sodium pyruvate, 100 units/ml of penicillin, 100 $\mu$g/ml of streptomycin, and 1 ml of 100x nonessential amino acid solution (Gibco). The cells were incubated for 4 h in the presence of 10 $\mu$Ci/ml of [2-3H]adenosine (25 Ci/mmol) at $37^\circ$C in 5% CO$_2$ in air. Isolation of RNA from nucleated erythroid cells obtained from the spleens of anemic mice and purification of the poly(A)$^+$-containing 9 S RNA fraction were performed as reported earlier (15).

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† Postdoctoral Fellow of the National Cancer Institute (LF22 CA-01649).

* The abbreviations used are: poly(A), polyadenylic acid; oligo(dT)-cellulose, oligodeoxythymidylylase-cellulose; cDNA, complementary DNA; SDS, sodium dodecyl sulfate; Hepes, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid.
Globin cDNA-cellulose Affinity Chromatography

Methods of Mouse Globin cDNA-cellulose—Oligo(dT)cellulose was prepared by the method of Gilham (25). Avian myeloblastosis virus was supplied by Dr. J. W. Beard, Life Sciences Inc. The virus was purified, solubilized, and chromatographed on a DEAE-Cellulose column according to the procedure of Verma and Baltz (26). Traces of nucleoside contamination were removed from the product by chromatography on CM-Cellulose (C-50) (27).

The reaction mixture for the synthesis of globin cDNA-cellulose contained: 50 mM Tris-HCl (pH 8.3), 125 mM NaCl, 5 mM magnesium acetate, 10 mM dithiothreitol, 1 mM each dATP, dGTP, dCTP, and dTTP, 50 µg/ml of actinomycin D, 20 µg/ml of mouse globin mRNA, 30 µg/ml of oligo(dT)-cellulose, and 200 units/ml of RNA-dependent DNA polymerase. 

The mixture was stirred at 37°C for 90 min. After completion of the synthesis of globin cDNA, the cellulose was transferred to a glass column and washed at room temperature with 10 volumes of 100 mM NaOH followed by 5 volumes of water. This denatures the globin mRNA-DNA hybrid and removes the mRNA while the globin complementary DNA copy remains covalently attached to the cellulose matrix through the oligo(dT) primer.

Globin cDNA-cellulose Affinity Chromatography—Hybridizations involving RNA-cellulose can be performed using an excess of RNA or cDNA to drive the reaction (28-30). All of the analytical determinations described in this report were done using an excess of globin cDNA. RNA samples were incubated with globin cDNA-cellulose, which contained 65 µg of globin cDNA/ml of packed cellulose, in a water-jacketed column at 65°C for 30 min in hybridization buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, and 0.2% SDS). The cDNA-cellulose was washed with 20 column volumes of hybridization buffer at 65°C to remove those sequences which had not been annealed to the immobilized globin cDNA. The bound RNA sequences which are hybridized to globin cDNA were then eluted with water at the same temperature. Recovery of RNA from the cDNA cellulose was routinely >95%. Stability studies indicate <1% of the cDNA covalently attached to the cellulose matrix is eluted per hybridization experiment.

Preparation of [3H]-labeled Globin cDNA—Radioactive DNA complementary to mouse reticulocyte globin mRNA was synthesized using conditions which resulted in full length copies of the globin mRNA template (31). The reaction mixture contained: 50 mM Tris-HCl (pH 8.3), 10 mM dithiothreitol, 5 mM magnesium acetate, 125 mM NaCl, 0.25 mM each dATP, dCTP, dGTP, 0.25 mM [3H]dGTP (1000 Ci/mmol), 50 µg/ml of actinomycin D, 5 µg/ml of oligo(dT) as primer, 20 µg/ml of globin mRNA, and 200 units/ml of RNA-dependent DNA polymerase (26, 27). Following a 90-min incubation at 37°C, the reaction mixture was incubated for 1 h at 60°C in the presence of 0.3 M NaOH. After neutralization with HCl, the [3H]cDNA was isolated by gel filtration on Sephadex G-100 equilibrated in 0.1 M ammonium bicarbonate. The void fractions containing [3H]cDNA were pooled, lyophilized, and then dissolved in water and stored at -70°C until use. The specific activity of the [3H]cDNA was calculated to be 4.5 x 10⁶ cpm/µg.

Sucrose Gradient Sedimentation—RNA samples were analyzed by centrifugation in aqueous 15 to 30% (w/v) sucrose gradients containing 10 mM Tris-HCl (pH 7.5) at 4°C for 19.5 h at 40,000 rpm in a Spinco SW 40 rotor. The radioactivity of the gradient fractions was assayed using 10 ml of a scintillation flour described elsewhere (32).

RNA-DNA Hybridization—Hybridization reactions were performed in a final volume of 100 µl in sealed tubes containing 10 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 0.2 mM EDTA, 0.5 mg/ml of carrier yeast RNA, 50% (v/v) formamide (Fisher, stabilized) 20 ng/ml of [3H]cDNA (680 cpm/ng), and variable amounts of the RNA being analyzed. The tubes were heated to 75°C for 5 min and then incubated at 45°C for the appropriate times. Salt solutions used in the hybridization assays were passed through Chelex 100 resin (Bio-Rad Laboratories) and then autoclaved. The extent of hybridization to globin [3H]cDNA was determined using S. single-stranded specific nuclearase prepared from Aspergillus oryzae by the method of Vogt (33).

RESULTS

Synthesis of Globin cDNA-cellulose—Mouse reticulocyte globin mRNA which had been rigorously purified by a combination of oligo(dT)-cellulose chromatography and sucrose density gradient centrifugation was used in the preparation of globin cDNA-cellulose. Utilizing the oligo(dT) covalently attached to a cellulose matrix as primer, RNA-dependent DNA polymerase from avian myeloblastosis virus catalyzed the synthesis of a complementary DNA copy of the globin mRNA template. Conditions were employed which had been previously reported to yield complete copies of the globin template using an oligo(dT) primer which was not immobilized on cellulose (31).

The effect of omitting various reaction components on the incorporation of [3H]dGTP into globin cDNA-cellulose is shown in Table I. Sodium chloride and all four deoxyribonucleotide triphosphates were required for optimal incorporation. While no significant change was observed in the absence of actinomycin D, [3H]dGTP incorporation was totally dependent on the presence of oligo(dT)-cellulose. Pure cellulose could not substitute as primer for the synthesis of cDNA. The incorporation of label into cDNA-cellulose was linear for 45 min with the reaction reaching completion by 60 to 75 min (data not shown).

The oligo(dT)-cellulose used in this study has a binding capacity of 65 µg of globin mRNA/ml of packed cellulose. Synthesis of globin cDNA using oligo(dT)-cellulose as primer was completely dependent upon the addition of globin mRNA and required a 3-fold excess of mRNA to saturate the primer sites (Table II). The capacity of the globin cDNA-cellulose was determined by hybridization using an excess of reticulocyte globin mRNA to drive the reaction. Using the conditions described above, routine preparations of cDNA-cellulose had a globin mRNA binding capacity of 65 µg/ml of packed cellulose.

Specificity of Globin cDNA-cellulose—Purification of globin mRNA by hybridization to cDNA-cellulose is contingent upon this chromatographic medium possessing a high degree of
Characteristics of synthesis of globin cDNA cellulose

The composition of the complete reaction mixture, final volume 0.5 ml, was as described under "Experimental Procedures" except for the substitution of 0.5 mM $[^{1}H]dGTP$ (specific activity 50 mCi/mmol). Reactions were incubated at 37° for 60 min and terminated by the addition of 25 mM NaOH. The cellulose was collected on a cellulose nitrate filter (Bactiflex B6, Schleicher and Schuell), washed with 5.0 ml of $H_{2}O$, and the radioactivity determined (32). A picomole of $[^{1}H]dGMP$ is equivalent to an incorporation of 55 cpm.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>[$^{1}H]dGMP$ incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>1135</td>
</tr>
<tr>
<td>-NaCl</td>
<td>306</td>
</tr>
<tr>
<td>-Actinomycin D</td>
<td>1128</td>
</tr>
<tr>
<td>-dATP</td>
<td>46</td>
</tr>
<tr>
<td>-Oligo(dT)-cellulose + Cellulose</td>
<td>0</td>
</tr>
</tbody>
</table>

Table II

Effect of varying concentration of globin mRNA on incorporation of $[^{1}H]dGMP$ into globin cDNA cellulose

Composition of the reaction mixture, final volume 0.20 ml, was identical with the complete reaction mixture described in Table I except the globin mRNA concentration varied as indicated. The 6.0 pmol of oligo(dT)-cellulose present in each reaction is equivalent to 20 μl of packed cellulose. Reactions were incubated at 37° for 60 min and the radioactivity determined as in Table I.

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<tr>
<td>μg/reaction</td>
<td>pmol</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>923</td>
</tr>
<tr>
<td>4</td>
<td>429</td>
</tr>
<tr>
<td>6</td>
<td>425</td>
</tr>
<tr>
<td>10</td>
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</tr>
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</table>

sequence specificity. To establish the specificity of the globin cDNA-cellulose, several RNAs were examined for their ability to hybridize to the immobilized cDNA (Table III). The homopolymer polyadenylic acid did not hybridize to globin cDNA-cellulose, thus demonstrating that the existence of a poly(A) region alone is insufficient base homology for an RNA to be retained by the column. Furthermore, no significant level of hybridization was observed when either mouse erythroid cell 28 S ribosomal RNA or mouse L-cell RNA was applied to the cDNA-cellulose column. These results demonstrate the specificity exhibited by globin cDNA-cellulose.

Globin cDNA-cellulose Affinity Chromatography of Nucleated Erythroid Cell Poly(A) containing 9 S RNA—Previously, we have reported on a poly(A)-containing 9 S RNA fraction isolated from nucleated erythroid cells obtained from the spleens of anemic mice (15). This RNA fraction was shown to be 67% as active as reticulocyte globin mRNA when equivalent amounts of mRNA were added to a wheat germ cell-free lysate. A similar poly(A)-containing 9 S RNA fraction was prepared from mouse nucleated erythroid cells following a 4-h incubation in the presence of $[^{1}H]adenosine$. Analysis by affinity chromatography on globin cDNA-cellulose showed that 40% of the radioactivity applied to the column was eluted in the bound (65% $H_{2}O$) fraction (Table IV). Reapplication of the unbound RNA to the cDNA-cellulose column resulted in only 1% of the radioactively labeled RNA being hybridized to globin cDNA. This demonstrates the efficiency of this chromatographic medium for the selective isolation of globin RNA sequences. Following reapplication of the bound RNA fraction, 96% of the radioactivity was found to reanneal to globin cDNA-cellulose. These observations confirm the existence of a poly(A)-containing 9 S nonglobin mRNA fraction in RNA preparations from nucleated erythroid cells.

To establish that globin mRNA was not degraded during hybridization to cDNA-cellulose, an aliquot of the radioactively labeled RNA which had been annealed to cDNA-cellulose was analyzed by sucrose gradient centrifugation (Fig. 1). A comparison of the sedimentation profile of the hybridized RNA with that of the original poly(A)-containing 9 S RNA used in the preparation of the cDNA-bound fraction shows no change in sedimentation, indicating the absence of degradation of the RNA during hybridization to globin cDNA-cellulose.

Purification of Globin mRNA from Unfractionated RNA Extracts by cDNA-cellulose Affinity Chromatography—The usefulness of globin cDNA cellulose as a preparative technique for the isolation of globin mRNA directly from RNA preparations which had not undergone any prior purification was investigated (Table V). Following the incubation of mouse nucleated erythroid cell RNA with globin cDNA-cellulose, 0.5% of the RNA applied to the column was eluted as a bound fraction. Similar experiments using mouse reticulocyte RNA demonstrated that 1.4 to 1.5% of this RNA preparation annealed to globin cDNA. Replication of the bound RNA fractions showed that 95% of this RNA reannealed to globin cDNA, while less than 1% of the unbound RNA was retained by the column. Since the concentration of cDNA was not limiting during these determinations, the percentage of the RNA which hybridized to globin cDNA-cellulose represents the percentage of globin RNA sequences present in the respective RNA preparation.

The relative purity of globin mRNA sequences isolated by cDNA-cellulose affinity chromatography was determined using hybridization kinetic analysis (28-30). Globin $[^{1}H]cDNA$ was hybridized in the presence of excess RNA from each of the various stages of RNA purification (Fig. 2). The $C_{rt}^{4}$ was plotted against the corresponding extent of RNA hybridization to globin $[^{1}H]cDNA$ as determined by resistance to S, nuclease digestion. Reticulocyte poly(A)-containing 9 S globin mRNA was used in this study as a standard representing the hybridization of globin cDNA to a population of pure globin mRNA (Fig. 2, a and b). Since the $C_{rt,d}$ ($C_{rt}$ value at which 50% of the $[^{1}H]cDNA$ is hybridized) is inversely proportional to the relative concentration of globin sequences present in the RNA

$^{4}C_{rt}$ is defined as the concentration of RNA in an annealing reaction times the time of incubation and is expressed as moles of nucleotide per liter × s (calculated as $A_{260}$ units/ml x t/2).

TABLE I

Globin cDNA-cellulose Affinity Chromatography

The composition of the complete reaction mixture, final volume 0.5 ml, was as described under "Experimental Procedures" except for the substitution of 0.5 mM $[^{1}H]dGTP$ (specific activity 50 mCi/mmol). Reactions were incubated at 37° for 60 min and terminated by the addition of 25 mM NaOH. The cellulose was collected on a cellulose nitrate filter (Bactiflex B6, Schleicher and Schuell), washed with 5.0 ml of $H_{2}O$, and the radioactivity determined (32). A picomole of $[^{1}H]dGMP$ is equivalent to an incorporation of 55 cpm.

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<tr>
<td>-Oligo(dT)-cellulose + Cellulose</td>
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TABLE II

Effect of varying concentration of globin mRNA on incorporation of $[^{1}H]dGMP$ into globin cDNA cellulose

Composition of the reaction mixture, final volume 0.20 ml, was identical with the complete reaction mixture described in Table I except the globin mRNA concentration varied as indicated. The 6.0 pmol of oligo(dT)-cellulose present in each reaction is equivalent to 20 μl of packed cellulose. Reactions were incubated at 37° for 60 min and the radioactivity determined as in Table I.

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<td>6</td>
<td>425</td>
</tr>
<tr>
<td>10</td>
<td>430</td>
</tr>
</tbody>
</table>

TABLE III

Specificity of globin cDNA-cellulose

Globin cDNA-cellulose affinity chromatography was performed as described under "Experimental Procedures" using a 0.25-ml column of cDNA-cellulose. The specific activities of the RNAs used in this experiment were: poly(A), $5 \times 10^{4}$ cpm/μg; 28 S ribosomal RNA, $2 \times 10^{6}$ cpm/μg; L-cell RNA, $8 \times 10^{5}$ cpm/μg. The radioactivity present in each elution fraction was determined using 10 ml of a scintillation flour described elsewhere (32).

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<thead>
<tr>
<th>Table III</th>
<th>Specificity of globin cDNA-cellulose</th>
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<tbody>
<tr>
<td>RNA sample</td>
<td>RNA applied to column</td>
</tr>
<tr>
<td></td>
<td>cpm</td>
</tr>
<tr>
<td>Poly(A)</td>
<td>47,000</td>
</tr>
<tr>
<td>28 S ribosomal RNA</td>
<td>395,000</td>
</tr>
<tr>
<td>L-cell RNA</td>
<td>173,000</td>
</tr>
</tbody>
</table>
Globin cDNA-cellulose Affinity Chromatography

Table IV

Table IV
Globin cDNA-cellulose affinity chromatography of nucleated erythroid cell poly(A)-containing 9 S RNA

Nucleated erythroid cells (5 x 10⁶ cells/ml) were incubated for 4 h in the presence of [2-¹⁴C]adenosine (25 Ci/mmol) and the poly(A)-containing 9 S RNA fraction (5 x 10⁶ cpm/μg) isolated and analysed on a 0.5-ml cDNA-cellulose column as described under "Experimental Procedures." The radioactivity present in each of the 1.0-ml elution fractions was determined as in Table III.

<table>
<thead>
<tr>
<th>Sample</th>
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<th>RNA bound</th>
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<tbody>
<tr>
<td>9 S poly(A) + RNA</td>
<td>295,000</td>
<td>135,000</td>
</tr>
<tr>
<td>Reapplication of unbound RNA</td>
<td>96,100</td>
<td>1,070</td>
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<tr>
<td>Reapplication of bound RNA</td>
<td>69,400</td>
<td>66,400</td>
</tr>
</tbody>
</table>

Fig. 1. Sucrose gradient centrifugation of the [¹⁴C]adenosine-labeled nucleated erythroid cell poly(A)-containing 9 S RNA before and after hybridization to globin cDNA-cellulose. Sucrose gradient centrifugation was performed as described under "Experimental Procedures." The gradients were fractionated from top to bottom and the radioactivity present in each 550-μl aliquot determined. ○, poly(A)-containing 9 S RNA; ●, cDNA-bound poly(A)-containing 9 S RNA.

Table V

Table V
Isolation of globin mRNA using globin cDNA affinity chromatography

RNA extracts from mouse reticulocytes and nucleated erythroid cells were analyzed on a 2.0-ml column of cDNA-cellulose as described under "Experimental Procedures." The absorbance at 260 nm was determined and converted to micrograms of RNA assuming 23 A₂₆₀ units/μg.

<table>
<thead>
<tr>
<th>Sample</th>
<th>RNA applied to column</th>
<th>RNA bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleated erythroid cell RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>7500</td>
<td>35</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>3500</td>
<td>17</td>
</tr>
<tr>
<td>Reticulocyte RNA</td>
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<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>3800</td>
<td>57</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>2700</td>
<td>37</td>
</tr>
</tbody>
</table>

Fig. 2. Hybridization of cDNA with RNA from various stages of purification by affinity chromatography on globin cDNA-cellulose. Annealing reactions were performed as described under "Experimental Procedures" and the per cent of [³²P]cDNA resistant to S, nuclease digestion was determined. Unincubated controls containing the [³²P]cDNA probe, RNA, and all reaction components were treated with S, nuclease and the per cent cDNA resistance (5 to 8%) was subtracted from all values. a, reticulocyte RNA: reticulocyte globin mRNA standard, ●, cDNA-bound RNA; ○, total polysomal RNA; □, cDNA-unbound RNA. b, nucleated erythroid cell RNA: reticulocyte globin mRNA standard, ●, cDNA-bound RNA; ○, total RNA; □, cDNA-unbound RNA.

Translation Analysis of RNA Purified by Globin cDNA-cellulose Affinity Chromatography—Sequence selectivity only partially satisfies the requirements of a chromatographic medium designed to isolate a specific mRNA sequence. Evidence demonstrating that the purified mRNA retains its biological activity after being hybridized to cDNA-cellulose is equally important if this technique is to be applicable as a preparative procedure.

The time course of L-[⁴⁰K]leucine incorporation into total protein using a wheat germ cell-free lysate programmed with equivalent amounts of various mRNA fractions is shown in Fig. 3. Results confirming that hybridization to globin cDNA cellulose does not alter the translation capacity of globin mRNA are presented in Panel a. The kinetic data of L-[⁴⁰K]leucine incorporation using globin mRNA which had been
lysates programmed with these mRNAs is shown in Fig. 4. Approximately 85% of the $^{14}C$-labeled leucine incorporated into total additive response (Table VI). Carboxymethylcellulose chromatography of the products synthesized in wheat germ cell-free protein using equivalent amounts of either poly(A)-containing 9 S cDNA-unbound RNA to a wheat germ lysate resulted in an stimulation observed following the addition of various mixtures of reticulocyte globin mRNA and the poly(A)-containing 9 S RNA fraction was identical with the rate observed with an equivalent amount of globin mRNA, O-O; cDNA-bound nucleated erythroid cell poly(A)-containing 9 S RNA, A-A. d, cDNA-unbound nucleated erythroid cell poly(A)-containing 9 S RNA, □-□. 

Purification of the poly(A)-containing 9 S RNA fraction isolated from nucleated erythroid cells or reticulocytes provided an excellent source for the isolation of a specific mRNA which was not retained by globin cDNA-cellulose. In addition, both mRNA preparations resulted in a 175-fold stimulation of the endogenous protein synthesis activity. The poly(A)-containing 9 S RNA fraction isolated from nucleated erythroid cells was only 76% as active when added to a wheat germ lysate as an equivalent amount of reticulocyte globin mRNA prepared in an identical manner (Fig 3d). Affinity chromatography using globin cDNA-cellulose separated the poly(A)-containing 9 S RNA into two fractions and these mRNAs were analyzed in a wheat germ lysate (Fig. 3d). Incorporation of $^{14}C$-labeled leucine using the cDNA-bound RNA fraction was identical with the rate observed with an equivalent amount of reticulocyte globin mRNA, while the unbound RNA was only 20% as active. This low efficiency of incorporation was not due to an inhibition of translation, since the stimulation observed following the addition of various mixtures of reticulocyte globin mRNA and the poly(A)-containing 9 S cDNA-unbound RNA to a wheat germ lysate resulted in an additive response (Table VI). Carboxymethylcellulose chromatography of the products synthesized in wheat germ cell-free lysates programmed with these mRNAs is shown in Fig. 4. Approximately 85% of the $^{14}C$-labeled leucine incorporated into total protein using equivalent amounts of either poly(A)-containing 9 S cDNA-bound RNA from nucleated erythroid cells or reticulocyte globin mRNA was found to co-chromatograph with authentic mouse $\alpha$- and $\beta$-globin chains (Fig. 4, a and b). An additional 12 to 15% of the radioactivity migrated as an adjacent peak and shoulder to the $\beta$-globin chain, possibly resulting from premature termination of the nascent polypeptide chains (36-38). Analysis of the labeled products synthesized with the mRNA which was not retained by globin cDNA-cellulose showed that 38% of labeled total protein migrated in a region similar to authentic mouse $\alpha$- and $\beta$-globin chains (Fig. 4c). It is unknown whether this material actually represents globin.

Both of the RNA preparations isolated by hybridization to globin cDNA-cellulose using the unfractionated RNA extracts obtained from either reticulocytes or nucleated erythroid cells also directed the synthesis of protein in a wheat germ cell-free lysate (Fig. 3, b and c). However, the rate of synthesis and the total incorporation observed using these mRNAs was only 75% of the reticulocyte globin mRNA standard. Reapplication of either preparation to globin cDNA-cellulose did not result in any further increase in the translational capacity of the RNA. A partial explanation for this inconsistency was derived from sucrose gradient analysis of the RNA isolated from nucleated erythroid cells following a 2-h incubation in the presence of $^{14}C$-adenosine (Fig. 5). While globin mRNA represents the predominant poly(A)-containing RNA, several additional size classes ranging from 7 to 23 S comprised the RNA retained by oligo(dT)-cellulose. This is to be expected since the spleen cell population is only 70 to 80% erythroid and even the erythroid cells themselves contain other mRNAs (15). Sucrose gradient analysis of the poly(A)-containing RNA which hybridized to globin cDNA-cellulose resulted in a peak of radiolabel corresponding to newly synthesized globin mRNA and a second RNA migrating at 7 S. A similar profile was obtained for the mRNA preparations isolated by hybridization to cDNA cellulose using unlabeled RNA extracts of either reticulocytes or nucleated erythroid cells (data not shown). Since the 7 S RNA is retained by both oligo(dT)-cellulose and globin cDNA-cellulose, the simplest interpretation of these results is that the 7 S RNA represents a globin mRNA minus a portion of the 5'-terminal sequence. Such a loss may alter the translation efficiency observed.

**DISCUSSION**

The analysis of the detailed mechanisms for the synthesis, processing, and fate of a particular mRNA requires a system which is actively synthesizing the mRNA and the ability to isolate this mRNA in a highly purified form. While reticulocytes provide an excellent source for the isolation of a specific mRNA, these cells contain no nuclei and, therefore, do not hybridized to globin cDNA cellulose were identical with those observed with an equivalent amount of globin mRNA which had not been annealed to cDNA-cellulose. In addition, both mRNA preparations resulted in a 175-fold stimulation of the endogenous protein synthesis activity. The poly(A)-containing 9 S RNA fraction isolated from nucleated erythroid cells was only 76% as active when added to a wheat germ lysate as an equivalent amount of reticulocyte globin mRNA prepared in an identical manner (Fig 3d). Affinity chromatography using globin cDNA-cellulose separated the poly(A)-containing 9 S RNA into two fractions and these mRNAs were analyzed in a wheat germ lysate (Fig. 3d). Incorporation of $^{14}C$-labeled leucine using the cDNA-bound RNA fraction was identical with the rate observed with an equivalent amount of reticulocyte globin mRNA, while the unbound RNA was only 20% as active. This low efficiency of incorporation was not due to an inhibition of translation, since the stimulation observed following the addition of various mixtures of reticulocyte globin mRNA and the poly(A)-containing 9 S cDNA-unbound RNA to a wheat germ lysate resulted in an additive response (Table VI). Carboxymethylcellulose chromatography of the products synthesized in wheat germ cell-free lysates programmed with these mRNAs is shown in Fig. 4. Approximately 85% of the $^{14}C$-labeled leucine incorporated into total protein using equivalent amounts of either poly(A)-containing 9 S cDNA-bound RNA from nucleated erythroid cells or reticulocyte globin mRNA was found to co-chromatograph with authentic mouse $\alpha$- and $\beta$-globin chains (Fig. 4, a and b). An additional 12 to 15% of the radioactivity migrated as an adjacent peak and shoulder to the $\beta$-globin chain, possibly resulting from premature termination of the nascent polypeptide chains (36-38). Analysis of the labeled products synthesized with the mRNA which was not retained by globin cDNA-cellulose showed that 38% of labeled total protein migrated in a region similar to authentic mouse $\alpha$- and $\beta$-globin chains (Fig. 4c). It is unknown whether this material actually represents globin.

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Globin cDNA-cellulose Affinity Chromatography

Procedures. The gradients were fractionated from top to bottom and the radioactivity present in each 400-μl aliquot determined.

The development of globin cDNA-cellulose has provided a means for the selective purification of globin mRNA from a heterogeneous RNA population. This chromatographic technique will be extremely applicable in studies designed to determine the kinetics of globin mRNA synthesis, in establishing the half-life of globin mRNA, and in identifying various structural modifications such as the methylated nucleotides present in this eukaryotic mRNA. Furthermore, the search for a putative precursor to globin mRNA should be greatly facilitated by this isolation technique.

Acknowledgment—Avian myeloblastosis virus was supplied through the Office of Program Resources and Logistics, Viral Cancer Program, Viral Oncology Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Md. 20014.

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Globin cDNA was synthesized from a highly purified globin mRNA template, using the RNA-dependent DNA polymerase from avian myeloblastosis virus and the oligo(dT) covalently attached to a cellulose matrix as primer. Saturation of the oligo(dT) primer using a 3-fold excess of globin mRNA resulted in sufficient synthesis of cDNA to allow the hybridization reactions to be performed using an excess of the cDNA. Polyadenylc acid, ribosomal 28 S RNA, and mouse L-cell RNA were not retained by the cDNA-cellulose column using hybridization conditions which permitted the formation of stable hybrids between globin mRNA and the immobilized cDNA. This established the stringent sequence specificity required for annealing to globin cDNA-cellulose. Comparison of the Crtl₉₀ values obtained for the RNA fractions isolated by hybridization to cDNA-cellulose with the Crtl₉₀ for reticulocyte globin mRNA confirmed the purification capability of the affinity column. The absence of detectable globin RNA sequences in the RNA fractions not retained by cDNA-cellulose demonstrates the efficiency of this chromatographic medium. Hybridization of globin mRNA to cDNA-cellulose did not result in any RNA degradation nor was there any discernible loss in the biological activity of this mRNA as determined by translation in a wheat germ cell-free lysate.

The size of the cDNA copy synthesized using the oligo(dT)-cellulose primer can be estimated from the picomoles of [3H]GMP incorporated into the immobilized cDNA. Using an average molecular weight of 2.2 x 10⁶ for globin mRNA (39-45) and the nucleotide composition (46), the average size of the cDNA copy was calculated to be 40% of the globin mRNA template. While the exact size distribution has not been determined, the finding that the globin cDNA-cellulose binds the same quantity of globin mRNA as the oligo(dT)-cellulose primer used in its synthesis demonstrates that each cDNA copy is of sufficient length to selectively retain a globin mRNA using the described hybridization conditions.

Previously, we described the isolation of a poly(A) containing 9 S RNA fraction from nucleated erythroid cells which contained globin mRNA (15). In the present study, this preparation was shown to contain nearly equal proportions of globin and non-globin mRNA when analyzed by affinity chromatography on globin cDNA-cellulose. The RNA retained by the cDNA-cellulose column was identical with globin mRNA in its stimulation of a wheat germ cell-free lysate and its ability to direct the synthesis of α- and β-globin chains. An equivalent amount of the unbound mRNA was only 20% as active as the globin mRNA standard. The explanation for this observation is not yet clear; however, this reduction in translation efficiency may result from a difference in the salt requirements necessary for optimal synthesis of the two mRNA fractions (34).

The 7 S RNA which hybridized to globin cDNA-cellulose may represent the product of a specific nuclease involved in globin mRNA turnover or possibly have resulted from a more generalized degradation during phenol extraction. No evidence of RNA degradation resulting from hybridization to globin cDNA-cellulose was observed. Alternatively, this fraction may reflect RNA sequences shared by the 7 S RNA and globin mRNA.

synthesize RNA. Nucleated erythroid cells obtained from the spleens of anemic mice actively synthesize globin mRNA when incubated in tissue culture media (15). However, because these cells synthesize other mRNAs in addition to those for the α- and β-globin chains, it was necessary to develop a procedure for purifying the globin mRNAs. This report describes the preparation of a chromatographic medium which employs a complementary DNA copy of mouse reticulocyte globin mRNA for the selective purification of globin mRNA sequences.

The size of the cDNA copy synthesized using the oligo(dT) primer can be estimated from the picomoles of [3H]GMP incorporated into the immobilized cDNA. Using an average molecular weight of 2.2 x 10⁶ for globin mRNA (39-45) and the nucleotide composition (46), the average size of the cDNA copy was calculated to be 40% of the globin mRNA template. While the exact size distribution has not been determined, the finding that the globin cDNA-cellulose binds the same quantity of globin mRNA as the oligo(dT)-cellulose primer used in its synthesis demonstrates that each cDNA copy is of sufficient length to selectively retain a globin mRNA using the described hybridization conditions.

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