

## In Vitro Synthesis of DNA Complementary to Purified Rabbit Globin mRNA

(RNA-dependent DNA polymerase/reticulocyte/hemoglobin/  
 density gradient centrifugation/oligo(dT) primer)

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**ABSTRACT** Several properties of the viral RNA-dependent DNA polymerases and of rabbit globin mRNA make it possible to consider synthesis of the globin gene *in vitro*. These enzymes copy an RNA template using a short sequence of complementary nucleotides as a primer. Furthermore, globin mRNA has a 3'-terminal sequence of adenylic acid residues that make it particularly suitable as a template, since oligo(dT) can be annealed to a specific site on the mRNA. This small primer could phase the DNA polymerase, possibly ensuring that replication is initiated from that end of the globin message. We have used this approach and find that purified mRNA is an efficient template for the polymerase enzyme. The reaction requires the RNA template and the four deoxyribonucleoside triphosphates, and it is markedly stimulated by the addition of oligo(dT). Consistent with the expectation that the oligo(dT) uniquely phases the polymerase at an adenine-rich region in the globin message, oligo(dG), oligo(dC), and oligo(dA) fail to serve as primers. The product has a density intermediate between that of DNA and RNA, and shifts to a lighter DNA density after treatment with base. Further, it is specifically complementary to globin mRNA and sediments slightly faster in an alkaline sucrose gradient than a DNA standard that has a molecular weight of 129,000. The data suggest that a major portion of the DNA product is a sequence of at least 500 bases, about 50 more than would be necessary to encode rabbit globin. The potential usefulness of this interesting product is discussed.

While the biological significance of the RNA-dependent DNA polymerases (1-7) remains to be assessed, several properties of these enzymes, together with a convenient feature of rabbit globin mRNA, permit us to consider the possibility of synthesizing a mammalian gene *in vitro*. The RNA-dependent DNA polymerase can use several natural RNAs and DNAs as templates for the synthesis of complementary DNA (8-15), and these enzymes seem to require a primer molecule (16, 17). This primer requirement is particularly useful for the synthesis of the DNA complement of rabbit globin mRNA, since the 3'-terminus of this molecule consists of a sequence of at least five or six adenylic acid residues (18, 19). By binding complementary strands of oligo(dT) to this region, these small molecules might serve as a convenient primer to phase the RNA-dependent DNA polymerase and to ensure that it copies the globin mRNA from its 3'-terminus. If the mRNA is fully copied, the resulting product would represent, in a single strand, the structural gene for globin. The strategy is summarized in Fig. 1.

While it is not clear that the polymerase will transcribe the entire globin mRNA molecule, even if it is required to start

at its 3'-end, the ability to synthesize highly labeled complements of certain mammalian mRNAs would prove extremely useful to assay gene dosage and mRNA production in higher organisms. We have directed our initial experiments towards achieving these goals. In the present work, we have used highly purified rabbit globin mRNA as a template for the efficient production of complementary DNA with the avian myeloblastosis virus RNA-dependent DNA polymerase. The product is a DNA molecule consisting of a sequence of more than 500 bases, about 50 more than would be necessary to encode rabbit globin.

### MATERIALS AND METHODS

*Purification of Avian Myeloblastosis Virus Polymerase.* Avian myeloblastosis virus was obtained from Dr. J. W. Beard (Duke University) and was purified by isopycnic banding in sucrose (6). DNA polymerase was extracted from the virion by the polyethylene glycol-dextran procedure to remove nucleic acid, and was purified by gradient elution from a phosphocellulose column (20); all buffers contained 0.01% (v/v) Triton X-100.

*Purification and Characterization of Rabbit Globin mRNA.* Crude RNA was isolated from purified rabbit reticulocyte polysomes by a modification of the procedure of Lee, Mendeki, and Brawerman (21). Globin mRNA was initially purified from this material by oligo(dT)-cellulose chromatography (22, 23, and manuscript in preparation) and sub-

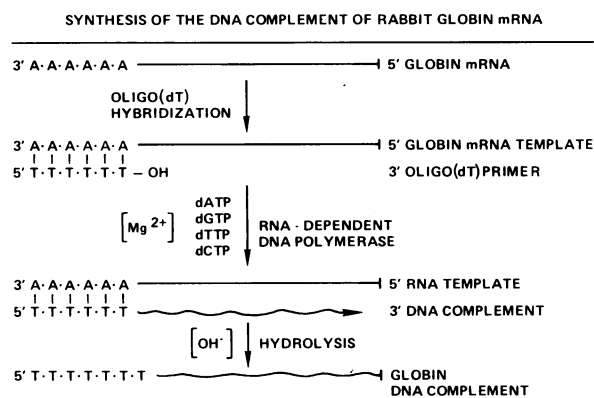


Fig. 1. Synthesis of the DNA complement of rabbit globin mRNA.

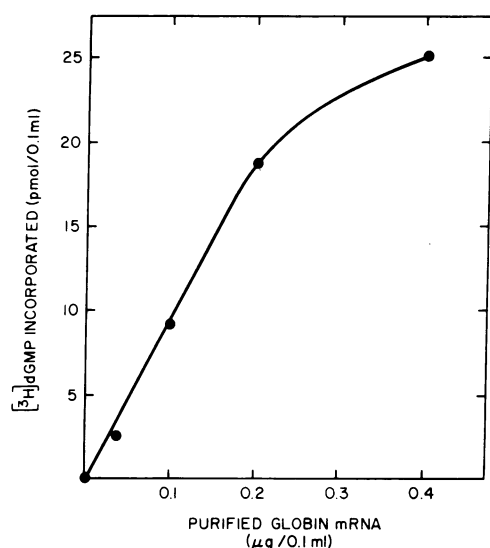


FIG. 2. DNA synthesis as a function of globin mRNA concentration. 0.2  $\mu\text{g}/\text{ml}$  of globin mRNA was annealed with a 10-fold excess of oligo(dT). Different amounts of this mixture were added to standard 0.1-ml reaction mixtures. These were incubated for 60 min at 37°C and assayed for  $\text{Cl}_3\text{CCOOH}$ -precipitable radioactivity.

sequently by sucrose gradient centrifugation. The material sedimenting at 9S, containing mRNA corresponding to rabbit globin, was concentrated by ethanol precipitation. Analysis of this material by polyacrylamide gel electrophoresis indicated that it migrated as a single band; further studies indicated that it directed the cell-free synthesis of rabbit globin.

**Polymerase Assay.** Oligo(dT), oligo(dA), oligo(dG), and oligo(dC), all 12–18 residues in length, were purchased from Collaborative Research, Inc. All other materials have been described (20). Globin mRNA was incubated with a 10-fold excess of deoxyoligonucleotide at 37°C for 15 min in 0.1 M NaCl before each reaction. The RNA-oligo(dT) mixture was then kept at 4°C until use. Each standard 0.1-ml reaction mixture contained 0.1 M Tris·HCl (pH 7.2), 0.04 M KCl, 6 mM magnesium acetate, 2 mM dithiothreitol, 0.2 mM (each) dATP, dCTP, and TTP, 0.07 mM [<sup>3</sup>H]dGTP (Schwarz-Mann, 9.3 Ci/mmol), 0.1  $\mu\text{g}$  of globin mRNA, 9  $\mu\text{g}$  of viral polymerase. Unless otherwise noted, reactions were incubated at 37°C for 60 min and the trichloroacetic acid-precipitable counts were determined (20).

## RESULTS

### Characteristics of enzymatic reaction

As shown in Fig. 2, DNA synthesis is fully dependent upon the addition of small amounts of globin mRNA, the reaction approaching saturation at concentrations of less than 5.0  $\mu\text{g}/\text{ml}$  of RNA. In addition to being dependent upon the addition of globin mRNA, synthesis (Table 1) depends upon the addition of oligo(dT) and each of the unlabeled deoxyribonucleoside triphosphates. The reaction is also sensitive to prior incubation with ribonuclease. Consistent with the expectation that oligo(dT) uniquely phases the polymerase at an adenine-rich region in the globin mRNA, oligo(dA), oligo(dG), and oligo(dC) fail to serve as primers (Table 2). These compounds were fully active as primers in reactions in

TABLE 1. Characteristics of the viral DNA polymerase reaction with globin mRNA as template

Reaction mixture	[ <sup>3</sup> H]dGMP, pmol incorporated
Complete	8.70
– oligo(dT)	0.42
– RNA	0
– TTP	0.10
– dCTP	0.87
– dATP	0.27
+ RNase	0

RNase treatment was performed as follows: RNase A (50  $\mu\text{g}/\text{ml}$ ), which had been heated to 95°C for 15 min, was incubated with nonannealed mRNA in 0.1 M NaCl for 30 min at 37°C. Oligo(dT) in a 10-fold excess to RNA was then added, and the mixture was incubated 15 min at 37°C, cooled to 4°C, and used as such. Control experiments indicated that the RNase had no effect on a DNA template.

which their respective complementary polyribonucleotides were used as template (not shown). Other experiments (not shown) indicate that the synthetic reaction is rapid for about 30 min, and is virtually complete in 120 min. It has optimal  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  concentrations of about 7.5 and 0.5 mM, respectively. Product synthesized in 120 min at optimum  $\text{Mg}^{2+}$  concentration was characterized.

### Characterization of template and product

The globin mRNA used in these studies was isolated by chromatography on oligo(dT)-cellulose (22, 23), a procedure that we find quite useful in freeing globin mRNA from ribosomal RNA. In addition, the material was further purified by sucrose density gradient centrifugation. The resulting RNA migrates as a single band on polyacrylamide gel electrophoresis (Fig. 3) and efficiently directs the synthesis of rabbit globin in a cell-free system derived from Krebs II ascites tumor cells (manuscript in preparation).

The DNA product was characterized in several ways. Before treatment with alkali, the product density, as determined by  $\text{Cs}_2\text{SO}_4$  equilibrium centrifugation, is approximately 1.500  $\text{g}/\text{cm}^3$  (Fig. 4). This density is less than that of single-stranded RNA, but is greater than that of single-stranded DNA, suggesting that the product, as isolated from reaction mixtures, is a DNA-RNA hybrid. Consistent with this interpretation, the product density shifts to that of DNA, approximately 1.440 (Fig. 5A), after alkaline hydrolysis. The

TABLE 2. Primer specificity of the viral DNA polymerase reaction with globin mRNA template

Addition	[ <sup>3</sup> H]dGMP pmol incorporated
None	0
+ globin mRNA, oligo(dT)	2.53
+ globin mRNA, oligo(dA)	0
+ globin mRNA, oligo(dG)	0.26
+ globin mRNA, oligo(dC)	0.17
+ globin mRNA	0.07

Each reaction mixture contained only 50 ng of globin mRNA.

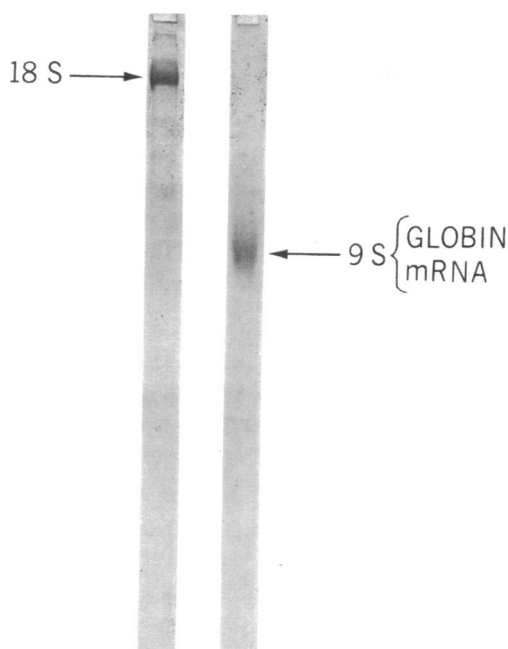


FIG. 3. Polyacrylamide-agarose gel electrophoretic analysis of globin mRNA. Electrophoresis was in a 4% polyacrylamide-0.5% agarose gel at 75 V for 240 min, according to the procedure of Peacock and Dingman (24). A 3- $\mu$ g sample was applied and detected by the "Stains all" technique (24).

intermediate density of the "native" product also suggests that the hybrid consists of equivalent amounts of DNA

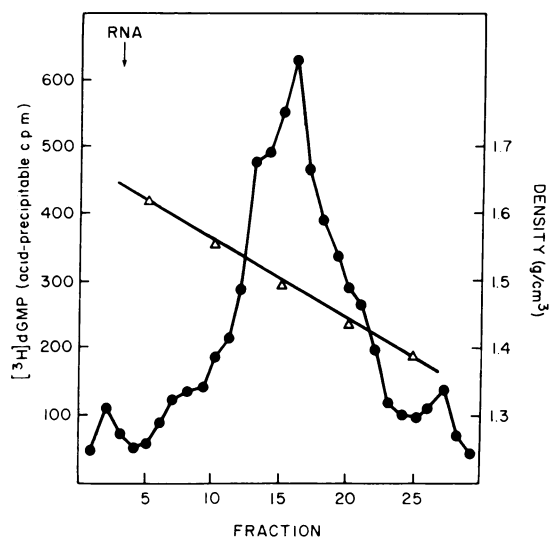


FIG. 4. Equilibrium centrifugation in  $\text{Cs}_2\text{SO}_4$  of the reaction product. A reaction mixture, incubated for 120 min, was made 1.0% in sodium dodecyl sulfate and extracted with a mixture of 10% (v/v) *m*-cresol in phenol. The product was precipitated from the aqueous phase by the addition of 2 volumes of ethanol, and was then dissolved in 4.8 ml of 0.01 M Tris·HCl (pH 7.2)-1 mM EDTA. To this, an equal volume of a solution of saturated  $\text{Cs}_2\text{SO}_4$  in the above buffer was added. The sample, in polyallomer tubes, was centrifuged in the Spinco 65 rotor at 38,000 rpm for 70 hr at 10°C. About 0.32-ml fractions were collected from the bottom of each tube. The density of every fifth fraction was measured (25), and the radioactivity of each fraction was determined after precipitation by the addition of 10%  $\text{Cl}_3\text{CCOOH}$ .

product and RNA template. That this is probably the case is indicated by alkaline sucrose gradient analysis (Fig. 6), which resolves the single-stranded DNA product into two peaks, with  $s_{20,w}$  values of about 5.8 and 6.3. These  $s_{20,w}$  values correspond to molecular weights of 127,000 and 155,000, respectively (26). The materials in the heavier peak should contain about 500 bases, 50 more than necessary to encode rabbit globin. The material in the lighter peak should contain about 410 bases.

In order to determine the specific complementarity of the single-stranded DNA product, it was tested for its ability to hybridize to the globin mRNA template and to other reticulocyte RNA fractions (Fig. 5, Table 3). DNA-RNA hybrid formation was assayed in two ways: by isolation of material of hybrid density in  $\text{Cs}_2\text{SO}_4$  equilibrium density gradients (Fig. 5) and by specific elution of DNA-RNA hybrids from hydroxyapatite (Table 3). By both assays, hybrid formation was very efficient in the presence of globin mRNA, whereas little or no hybrid could be detected when annealing was done

TABLE 3. Specific complementarity of DNA product as measured by DNA-RNA hybridization

Rabbit RNA added to annealing mixture	Input [ $^3\text{H}$ ]DNA eluted from hydroxyapatite in 0.23 M sodium phosphate at 60°C, % (DNA-RNA hybrid)
None	25
globin mRNA, 1 ng	35
globin mRNA, 5 ng	55
globin mRNA, 50 ng	89
unabsorbed ribosomal RNA, 1 ng	26
unabsorbed ribosomal RNA, 5 ng	32
unabsorbed ribosomal RNA, 50 ng	30

Hybridization was performed as indicated in the legend to Fig. 5, except that 2 ng of [ $^3\text{H}$ ]DNA was added to annealing mixtures. Unabsorbed RNA is the fraction that does not bind to oligo(dT)-cellulose during globin mRNA purification. It is largely ribosomal RNA. The double-stranded hybrid of DNA and RNA was assayed by a slight modification of the hydroxyapatite chromatography technique developed by Kohne (27). The assay takes advantage of the fact that single-stranded DNA is eluted from hydroxyapatite at one ionic strength, while the DNA-RNA hybrid is eluted at a higher ionic strength. Both fractions are precipitated and their radioactivity is determined. Results are expressed in terms of percent of the total applied material recovered as hybrid. Total recovery in these assays was >90%. Reaction mixtures were diluted to 3 ml in 0.025 M sodium phosphate (pH 6.8) and added to a 2-ml suspension of 0.5 g hydroxyapatite in the same buffer to adsorb nucleic acids. The mixtures were kept at room temperature for 5 min with occasional shaking and the hydroxyapatite was removed by centrifugation. The nonhybridized single-stranded DNA was eluted with two 6-ml washes at 60°C with buffer containing 0.12 M sodium phosphate (pH 6.8)-0.4% sodium dodecyl sulfate. The material eluted in this way was then precipitated by the addition of 3 ml of 60%  $\text{Cl}_3\text{CCOOH}$  and isolated on nitrocellulose filters for counting. The DNA-RNA hybrid was eluted in an identical manner, except that the buffer contained 0.23 M sodium phosphate. About 8000 cpm of [ $^3\text{H}$ ]DNA were applied to the hydroxyapatite, with the proportion isolated in the higher ionic strength eluate indicated in the Table.

in the presence of reticulocyte ribosomal RNA. Additional experiments (not shown) indicate that the DNA product does not hybridize to total polysomal RNA derived from mouse myeloma tumor cells.

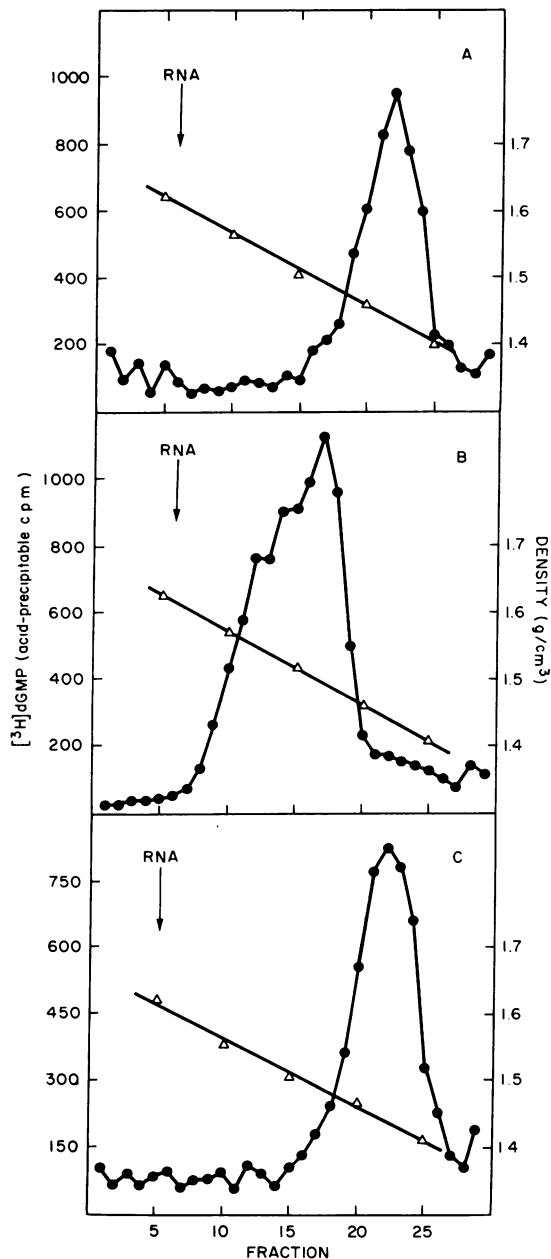


FIG. 5. Analysis of alkali-treated and rehybridized DNA product by  $\text{Cs}_2\text{SO}_4$  equilibrium centrifugation. The single-stranded DNA product, freed of template mRNA, was prepared from reaction mixtures that were made 0.3 M in NaOH, incubated for 15 hr at  $37^\circ\text{C}$ , and neutralized with HCl. Each hybridization reaction mixture contained, in 0.1 ml, 0.3 M NaCl, 0.01 M Tris·HCl (pH 7.2), 0.01 M EDTA, 4 ng of DNA product, 40 ng of unlabeled oligo(dT). Where noted, 0.1  $\mu\text{g}$  of DNA was added and the mixtures were incubated for 23 hr at  $60^\circ\text{C}$ . They were then centrifuged to equilibrium in  $\text{Cs}_2\text{SO}_4$  and analyzed as described in Fig. 4. Experiments differed as follows: (A) no RNA in hybridization reaction, (B) 0.1  $\mu\text{g}$  9S globin mRNA, (C) 0.1  $\mu\text{g}$  unabsorbed reticulocyte ribosomal RNA (see legend to Table 3).

## DISCUSSION

While our data strongly suggest that we have synthesized an extensive complementary DNA replica of rabbit globin mRNA, a major caveat must be introduced in evaluating any study of this type. This arises from the limitations involved in assessing the purity of mRNAs derived from complex mixtures of macromolecules in mammalian cells. Several points, however, argue in favor of the purity of our globin mRNA and the authenticity of its putative DNA complement. The globin mRNA is derived from purified reticulocyte polysomes involved almost exclusively in the synthesis of rabbit globin (29). The usual purification scheme yields an mRNA that has a unique "fingerprint" after degradation with specific nucleases (30). We have added a step (manuscript in preparation) to this scheme that relies upon the fact that globin message contains adenine-rich regions (18, 19), whereas ribosomal RNA does not (28). The globin mRNA purified in this way runs as a single component in sucrose gradient and polyacrylamide gel electrophoretic analyses and is biologically active for the synthesis of rabbit globin. Furthermore, synthesis of the DNA product is strongly dependent upon the addition of oligo(dT), but not upon the addition of the other three homo-oligodeoxynucleotides. This is especially interesting, and may be due to the absence of extensive sequences of the other bases in the globin mRNA or to some unique feature of the poly(A) sequence that permits it to act as a transcriptional promoter for the viral enzyme. Finally, the product hybridizes efficiently with the globin mRNA template, but not with reticulocyte ribosomal RNA. Nevertheless, the best support for the authenticity of

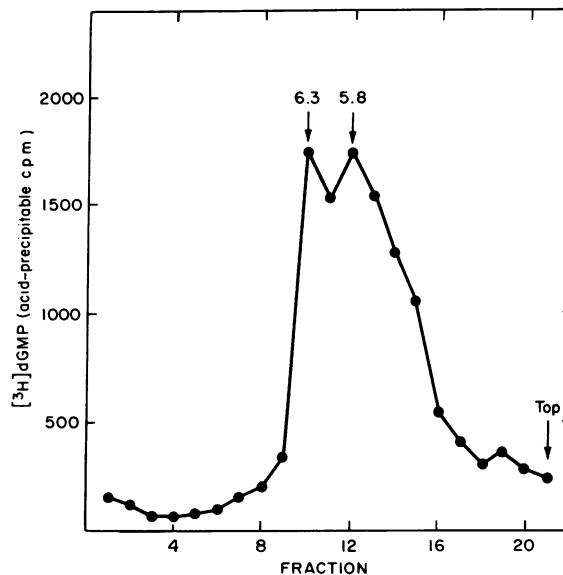


FIG. 6. Alkaline sucrose gradient analysis of DNA product. The product was obtained from a reaction mixture incubated 120 min, purified, and treated with alkali as noted in the legend to Fig. 4. The sample was applied to a 5–20% linear sucrose gradient, adjusted to pH 12.5, containing 0.9 M NaCl and 1 mM EDTA. Centrifugation was at 55,000 rpm in a Spinco SW56 rotor at  $5^\circ\text{C}$ .  $s_{20,w}$  Values as shown were calculated by the technique of Dingman (*Anal. Biochem.*, submitted), and checked with single-stranded DNA standards from *Escherichia coli* of known  $s_{20,w}$  values. Molecular weights were calculated from  $s_{20,w}$  values by the relationship described by Studier (26).

the globin DNA complement would be provided by its ability to synthesize globin mRNA and globin in a cell-free system.

The significance of the observation that the DNA product consistently forms two peaks on alkaline sucrose gradients also is not clear. This may reflect two distinct adenosine-rich regions or two terminator regions in the globin message. It may also be due to the fact that globin mRNA actually consists of two distinct molecules, one encoding the  $\alpha$  and one the  $\beta$  globin subunit. While our larger product appears to contain at least 500 nucleotides, sufficient to encode either protein, globin mRNA may be as much as 150 bases longer than this (31). If we assume there are redundant regions on either side of the structural message, and that the mRNA molecular weight estimate is correct, our product may lack the complement of the translational initiation region of the globin message. Still, the size of our DNA product and its specific complementarity suggest that large regions of the globin mRNA have been transcribed by the viral enzyme. This is an encouraging *in vitro* property of an enzyme presumed to have this role *in vivo*. Equally important, however, is the potential usefulness of the globin DNA complement itself. Together with sensitive hybridization techniques, it should prove a means of assaying gene dosage and mRNA production in higher organisms. The DNA product should also be amenable to enzymatic conversion into a double-stranded form, which may be useful for genetic transformation. Inasmuch as poly(A)-rich regions occur in various eukaryotic mRNAs (18, 21, 28, 32), the approach we have outlined may be generally applicable whenever cellular mRNAs can be obtained in sufficient purity.

#### NOTE ADDED IN PROOF

In an effort to assess the general applicability of this approach, we have recently isolated RNA from human reticulocyte polysomes using oligo(dT)-cellulose chromatography, and have transcribed this RNA into DNA with the avian myeloblastosis virus enzyme. The reaction with the human template is as efficient as that with the rabbit template. It specifically requires an oligo(dT) primer and its product (the product has an  $s_{20,w}$  greater than 6.3 in alkaline sucrose) and hybridizes with the human template and with rabbit globin mRNA, but not with human ribosomal RNA. We have also recently received manuscripts from Verma, Temple, Fan, and Baltimore and from Kacian, Spiegelman, Bank, Terada, Metafora, Dow, and Marks that describe studies similar to ours; their results are in accord with those we have obtained.

We are grateful to Dr. C. Wesley Dingman for his calculations of the  $s_{20,w}$  values used in this study and for making his manuscript available to us prior to publication. We are also grateful to Dr. Gary Felsenfeld for providing us with DNA of known  $s_{20,w}$  value and to Miss Barbara Loyd for her valuable help. This manuscript could not have been prepared without the able assistance of Mrs. K. Kunkle.

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