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Cell-Free Transcription of Mammalian Chromatin: Transcription of Globin Messenger RNA Sequences from Bone-Marrow Chromatin with Mammalian RNA Polymerase

(complementary DNA/DNA·RNA hybridization/RNA synthesis)

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ABSTRACT **A mammalian cell-free transcriptional system was developed in which mammalian RNA polymerase synthesizes globin messenger RNA sequences from bone-marrow chromatin. The messenger RNA sequences are detected by measurement of the ability of the transcribed RNA to hybridize with globin complementary DNA. The globin complementary DNA is synthesized by the enzyme from avian myeloblastosis virus, RNA-directed DNA polymerase, with purified globin messenger RNA as template. The specificity of the globin complementary DNA in annealing reactions was verified by preparing DNA complementary to liver messenger RNA and showing that the globin and liver complementary DNAs are specific for their own messenger RNAs. Both DNA-dependent RNA polymerase II from sheep liver and RNA polymerase from *Escherichia coli* can transcribe globin messenger RNA sequences from rabbit bone-marrow chromatin; however, the mammalian enzyme appears to be more specific in that globin gene sequences represent a higher proportion of the RNA synthesized. Neither polymerase can transcribe globin messenger RNA sequences from rabbit-liver chromatin. This cell-free assay system should be useful in searching for mammalian transcriptional regulatory factors.**

In an attempt to understand the regulation of expression of the hemoglobin genes in mammalian erythroid cells, particularly in the human genetic disease, beta thalassemia, we have developed a mammalian cell-free mRNA-synthesizing system. The approach is similar to that of Axel, Cedar and Felsenfeld (1) and of Gilmour and Paul (2) except that these two groups have used *Escherichia coli* polymerase to transcribe avian or mammalian chromatin while our approach has been to establish a fully mammalian system. The use of a mammalian cell-free system should permit a search for regulatory factors that might be involved in mRNA transcription.

In this first paper we describe the mammalian cell-free transcriptional system and its use in synthesizing globin mRNA sequences from bone-marrow chromatin; in addition, we examine the hybridization specificity of globin complementary DNA (cDNA), and we compare mammalian with *E. coli* RNA polymerase.

MATERIALS AND METHODS

Preparation of Chromatin. Rabbits (2–4 lbs; 1–2 kg) were exsanguinated, and the bone marrow and liver were removed and stored at −20° until used. The isolation of chromatin was similar to that described by Spelsberg and Hnilica (3). The rabbit tissues were defatted and/or deveined, and homogenized with a Teflon–glass homogenizer in 10 volumes of 0.5 M sucrose in TKM buffer (50 mM Tris·HCl, pH 7.9–25 mM KCl–5 mM $MgCl_2$). The 10% homogenate was filtered through two layers of cheesecloth and centrifuged at 5000 × *g* for 10 min. The 5000 × *g* pellet, which consists of nuclei contaminated with cellular debris, was suspended in 2.0 M sucrose in TKM buffer; the suspension was diluted to 1.7 M sucrose with TKM buffer and centrifuged at 25,000 × *g* for 30 min. The nuclei were washed once with 0.5 M sucrose in TKM buffer containing 0.2% Triton X-100. The purified nuclei were homogenized by hand in 80 mM NaCl, 20 mM EDTA, pH 6.3, then centrifuged at 10,000 × *g* for 10 min. The pellet was washed twice more with the NaCl–EDTA solution, once with 0.35 M NaCl, and twice with 2 mM Tris·HCl, pH 7.9–0.1 mM EDTA. The purified chromatin was stored at −20° in 2 mM Tris·HCl–0.1 mM EDTA. The chromatin was sonicated with a Branson W185 sonifier for 30 sec at 50 W before use.

Preparation of DNA-Dependent RNA Polymerase II from Sheep Liver. The isolation procedure for RNA polymerase II was similar to that described by Kedinger *et al.* for calf-thymus enzyme (4). All steps are done at 4°.

Step 1: Whole cell homogenization—Sheep liver (500 g) (fresh or stored in liquid nitrogen) was minced and homogenized in 1 liter of TGMED buffer (50 mM Tris·HCl, pH 7.9–30% glycerol–10 mM 2-mercaptoethanol–0.1 mM EDTA–0.1 mM dithiothreitol) in two stages: first, for 1 min at medium speed in a Waring blender, then by three strokes with a Teflon-glass homogenizer. The homogenate was filtered through two layers of cheesecloth, and 80 ml of saturated $(NH_4)_2SO_4$ was added with vigorous stirring. The viscous mixture was sonicated in 60-ml batches, using three 10-sec bursts at 100 W. The sonicate was clarified by centrifugation at 16,300 × *g* for 30 min and the supernatant fraction was retained.

Step 2: Ammonium sulfate precipitation—The supernatant fraction from step 1 was brought to 50% saturation with solid $(NH_4)_2SO_4$ (313 g/liter), pH 7.0 (neutralized with NH_4OH), and stirred for 1 hr. The precipitate was collected by centrifugation at 146,000 × *g* for 1 hr and dissolved in minimal volume of TGMED buffer (roughly 500 ml final volume).

Step 3: Protamine sulfate precipitation—Thirty milliliters of 1% protamine sulfate (Calbiochem) were added to the material from Step 2. After the mixture was stirred for 1 hr, the

Abbreviations: cDNA, complementary DNA; TKM buffer, 50 mM Tris·HCl (pH 7.9)–25 mM KCl–5 mM $MgCl_2$; TGMED buffer, 50 mM Tris·HCl (pH 7.9)–30% glycerol–10 mM 2-mercaptoethanol–0.1 mM EDTA–0.1 mM dithiothreitol.

precipitate was removed by centrifugation at 16,300 × *g* for 1 hr. The supernatant fraction was stored in liquid nitrogen.

Step 4: DEAE-cellulose chromatography—Material from step 3 was diluted to 75 mM $(NH_4)_2SO_4$ (as determined by conductivity measurements) with TGMED and stirred for 1 hr with 25 g (dry weight) of fibrous DEAE-cellulose (Whatman DE-23) that had been equilibrated with 75 mM $(NH_4)_2SO_4$ in TGMED buffer. The mixture was filtered through 25 g of equilibrated DEAE-cellulose in a Buchler funnel. The DEAE-cellulose was washed with 2 liters of TGMED buffer containing 75 mM $(NH_4)_2SO_4$ and transferred to a 100 × 5-cm column. The RNA polymerase II eluted as a single peak with 300 mM $(NH_4)_2SO_4$, was precipitated with $(NH_4)_2SO_4$ as described in step 2 (roughly 10–15 ml final volume), and stored in liquid nitrogen.

E. coli K12 DNA-dependent RNA polymerase was purchased from Biopolymer Ltd. and used without further purification.

Preparation of mRNA. Globin mRNA was prepared from rabbit-reticulocyte polysomes as described by Nienhuis *et al.* (5).

Liver RNA fractions were prepared from rabbit-liver polysomes by the method of Falvey and Staehelin (6). 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 A_{260} units per gradient) containing 0.3–1.1 M sucrose and 5 mM Tris·HCl, pH 7.5. The gradients were centrifuged at 27,000 rpm (SW 27 rotor) for 34 hr at 6°. The RNA fractions were collected and pooled; the RNA was precipitated with ethanol, collected by centrifugation, and dissolved in 10 mM Tris·HCl, pH 7.5, and stored in liquid nitrogen. The fractions were assayed for template activity in transcription (7–9) and translation (10) systems. The 8S–12S RNA fractions showed the greatest activity in both systems (data not shown). In comparison with globin mRNA, the liver mRNA was roughly one-half as active in the transcriptional system ([^{3}H]dGMP incorporated into DNA) and roughly one-third as active in the translational system ([^{14}C]leucine incorporated into protein).

Preparation of cDNA. Complementary [^{3}H]DNA was synthesized (7–9) in a 0.3-ml reaction mixture that contained: 50 mM Tris·HCl, pH 7.9; 50 mM KCl; 10 mM $MgCl_2$; 8 mM dithiothreitol; 30 μg of actinomycin D; 32 μM dATP, dCTP, dTTP; 12 μM [^{3}H]dGTP (6.5 Ci/mmol; Schwarz/Mann); 0.3 A_{260} unit of mRNA (either globin or liver); 0.06 A_{260} unit oligo(dT)$_{10}$ as primer; and 10–15 units of RNA-directed DNA polymerase isolated from avian myeloblastosis virus (11). After a 60-min incubation at 37°, the reaction mixture was heated to 90° for 5 min and then frozen directly on dry ice. The RNA solution was made 0.33 N NaOH with 1.0 N NaOH and was hydrolyzed at 37° for 18 hr and neutralized with 1.0 N HCl. The reaction mixture was dialyzed against three changes of 0.5 M NH_4HCO_3 over 24 hr, followed by 2 hr of further dialysis against H_2O. After lyophilization H_2O was added to the DNA solution to give 3000–5000 cpm/μl.

Assay for RNA Polymerase II. Each 250-μl reaction mixture contained: 30 mM Tris·HCl, pH 7.9; 8 mM KCl; 3 mM $MnCl_2$; 1.2 mM 2-mercaptoethanol; 5–15% glycerol; 2.0 mM ATP, CTP, GTP; 0.16 mM [^{3}H]UTP (12.6 Ci/mmol); 10 μg of calf-thymus DNA (Worthington), and 50–150 μl of the enzyme fraction to be tested. After incubation at 30° for 10 min, the trichloroacetic acid-precipitable radioactivity was collected on nitrocellulose filters (Millipore), washed with 10% trichloroacetic acid, and counted by liquid scintillation spectrometry.

Cell-Free Synthesis and Extraction of RNA. Each 5-ml reaction contained: 30 mM Tris·HCl, pH 7.9; 8 mM KCl; 3 mM $MnCl_2$; 1.2 mM 2-mercaptoethanol; 5–15% glycerol; 2.0 mM ATP, CTP, GTP; 0.16 mM UTP; chromatin containing 300–600 μg of DNA; and either 5–20 units of mammalian RNA polymerase II or 100–300 units of *E. coli* RNA polymerase. Reactions with mammalian polymerase were incubated at 37° for 15 min, and those with *E. coli* polymerase at 37° for 1 hr. The exact amount of enzyme used in the reactions was determined by titrating enzyme against chromatin to determine the optimum conditions for RNA synthesis; $(NH_4)_2SO_4$ was added to 0.15 M for the mammalian enzyme.

After incubation the reaction mixture was brought to 1% sodium dodecyl sulfate, 0.4 M NaCl, and 5 mM EDTA (1) and further incubated at 37° for 15 min. The solution was deproteinized by extraction with an equal volume of $CHCl_3$–isoamyl alcohol (24:1) at room temperature for 10 min. Then the aqueous phase was extracted with an equal volume of phenol (saturated with 10 mM Tris·HCl, pH 7.9–100 mM NaCl–5 mM EDTA) also at room temperature for 10 min. The final aqueous layer was mixed with 2 volumes of ethanol and left at −20° overnight. The precipitate was collected by centrifugation at 15,000 × *g* for 30 min. The precipitate was dissolved in 10 mM Tris·HCl, pH 7.4–100 mM NaCl–5 mM $MgCl_2$ and passed through a 15 × 0.9-cm Sephadex G-25 column (equilibrated with the same buffer) to remove free triphosphates and excess sodium dodecyl sulfate. The material eluting at the void volume (approximately 2 ml) was incubated with 200 μg of DNase (Worthington) at 37° for 1 hr. After a phenol extraction at 55° for 10 min, the aqueous phase was passed through a 60 × 0.9-cm Sephadex G-25 column, equilibrated with 0.1 M NH_4HCO_3. The void volume fractions were pooled, lyophilized, and stored in liquid nitrogen. When *E. coli* RNA polymerase was used, the Sephadex G-25 step before incubation with DNase was omitted.

Hybridization Assay. The hybridization procedure used was that of Kacian *et al.* (12). The reaction mixture (100 μl) contained: 20 mM sodium phosphate, pH 7.0; 0.1% sodium dodecyl sulfate; 0.3 M NaCl; 2 mM EDTA; 15,000–18,000 cpm of [^{3}H]cDNA; and RNA extracted as described above. Aliquots of 7.5 μl were sealed into individual capillary tubes (Drummond "Microcaps," 40 μl capacity). These tubes had been soaked in a solution of *E. coli* DNA (100 μg/ml; in 0.3 M NaCl–20 mM EDTA), rinsed thoroughly in distilled water, and allowed to dry in air. After a preincubation at 90° for 5 min, the sealed capillary tubes were incubated at 68° for 0.1–36 hr. Reactions were terminated by freezing the tubes on dry ice. The amount of hybridization was determined with micrococcal nuclease as described by Kacian and Spiegelman (13). The contents of each tube were added to 200 μl of 50 mM Tris·HCl, pH 8.3; 0.4 mM NaCl; 10 mM $MgCl_2$; 0.1 mM $CaCl_2$. Two 90-μl aliquots were taken; micrococcal nuclease (2 μl of a 4 mg/ml solution; Worthington) was added to one aliquot, and both aliquots were incubated at 37° for 1 hr. Cold 10% trichloroacetic acid was added to both

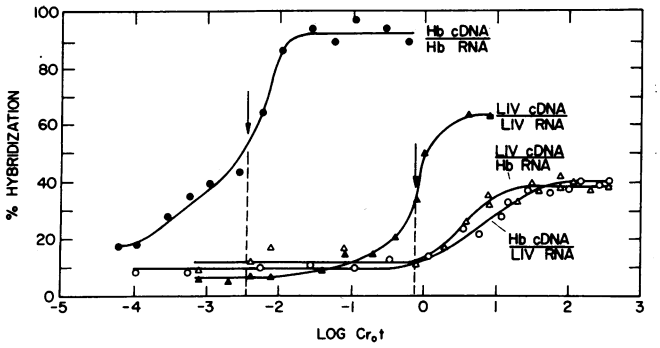

FIG. 1. Hybridization of globin and liver cDNAs to globin and liver mRNAs. Reaction mixtures contained: (*1*) 1500 cpm of globin (Hb) cDNA and 0.01–180 ng of globin (Hb) mRNA (●); (*2*) 1500 cpm of Hb cDNA and 0.01 ng to 15 μg of liver mRNA (○); (*3*) 1200 cpm of liver cDNA and 0.1 ng to 2.7 μg of liver mRNA (▲); or (*4*) 1200 cpm of liver cDNA and 0.1 ng to 18 μg of Hb mRNA (△). Reactions were incubated at 68° for 0.1 to 36 hr. Cr_0t = mol of ribonucleotides × sec/liter. 1 μg of RNA = 0.02 A_{260} unit.

aliquots, and the precipitates were collected and washed with cold 10% 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 taken to calculate the percentage of hybridization (13).

RESULTS

Specificity of the Globin cDNA. In determining the validity of the hybridization assay, it is important to verify that the globin cDNA is specific for globin mRNA sequences. In order to test this, liver cDNA was prepared in a manner similar to that of the globin cDNA. The results of experiments hybridizing globin cDNA and liver cDNA against globin mRNA and liver mRNA are shown in Fig. 1. When globin cDNA and globin mRNA are hybridized, the $Cr_0t_{1/2}$ value is around 3×10^{-3} mol·sec/liter. When liver cDNA is hybridized to the liver RNA fraction from which it was synthesized, the $Cr_0t_{1/2}$ value is approximately 9×10^{-1} mol·sec/liter. The higher (less specific) value for the liver/liver hybrid probably reflects the multiple species of mRNA present in the liver fraction as opposed to the two (α and β globin) mRNAs found in reticulocytes. When, however, the liver cDNA is hybridized with the globin mRNA or the globin cDNA with the liver RNA, low levels of hybridization with high $Cr_0t_{1/2}$ values are obtained. Thus, the globin cDNA does appear to hybridize specifically with globin mRNA sequences.

Transcription of Mammalian Chromatin with Mammalian Polymerase. The ability of sheep-liver RNA polymerase II to transcribe rabbit bone-marrow and liver chromatin is shown in Fig. 2. When the RNA synthesized *in vitro* from bone-marrow chromatin is annealed with globin cDNA, hybridization takes place with a $Cr_0t_{1/2}$ value of 3.8×10^0. No hybridization occurs between globin cDNA and the RNA synthesized *in vitro* from liver chromatin. Thus, globin mRNA sequences can be detected in the RNA transcribed from the bone-marrow chromatin but not from the RNA from liver chromatin.

In order to insure that the globin mRNA sequences measured were synthesized in response to the added RNA polymerase and were not endogenously present, an identical

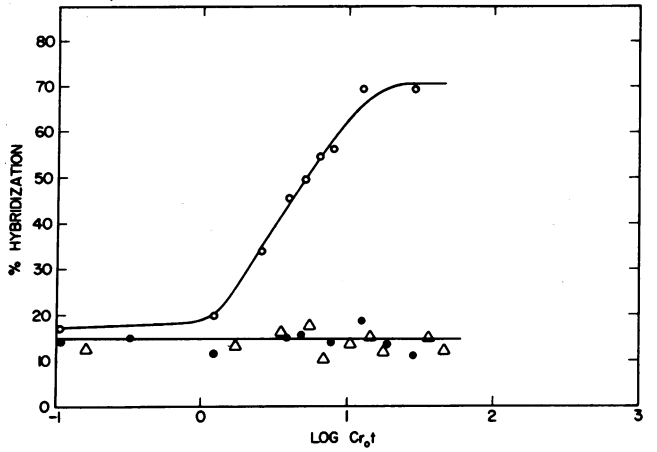

FIG. 2. Hybridization of globin cDNA to RNA transcribed from rabbit bone-marrow and rabbit liver chromatin with sheep liver RNA polymerase II. The preparation of chromatin, the purification of RNA polymerase II (*step 4*), and the isolation of the *in vitro* RNA transcription product are described in *Methods*. Each reaction mixture (7.5 μl) contained 1000–1500 cpm of globin cDNA and 1 μg (0.02 A_{260} unit) of RNA transcribed from rabbit-marrow chromatin (○), 1 μg of RNA transcribed from rabbit-liver chromatin (●), or 1.5 μg (0.03 A_{260} unit) of RNA extracted from rabbit-marrow chromatin incubated without RNA polymerase (△). The reaction mixtures were incubated at 68° from 0.1 to 24 hr.

reaction mixture to that described above with bone-marrow chromatin was incubated in the absence of added polymerase. As seen in Fig. 2, no globin mRNA sequences were detected. Thus, the mRNA sequences measured were newly synthesized material.

Comparison of Mammalian and E. coli RNA Polymerase. The identical reaction mixture to that described for Fig. 2 was incubated but with the mammalian enzyme replaced by *E. coli* RNA polymerase (Fig. 3). Globin mRNA sequences can be detected in the RNA transcribed from the bone-marrow chromatin but not from the RNA from liver chromatin. However, the $Cr_0t_{1/2}$ value obtained when the bacterial enzyme was used is higher than that obtained with the mammalian enzyme. Several experiments are summarized in Table 1. The mammalian enzyme, therefore, appears to be more specific than the bacterial enzyme, although the enzyme from either source can be used to transcribe chromatin into mRNA sequences.

The RNA transcribed from bone-marrow chromatin, which recognizes globin cDNA, will not hybridize with liver cDNA (Fig. 3); thus (at least a portion of) the mRNA sequences are specific for globin cDNA.

DISCUSSION

A cell-free transcriptional system has been developed that uses mammalian chromatin and partially purified mammalian DNA-dependent RNA polymerase II. This system, just as those described by Axel *et al.* (1) and Gilmour and Paul (2) that use *E. coli* polymerase, uses globin cDNA as a probe for detecting globin mRNA sequences. Axel and his coworkers demonstrated that globin mRNA sequences could be detected in the RNA transcribed from duck-reticulocyte chromatin but not from duck-liver chromatin. Gilmour and his coworkers obtained similar results using fetal-mouse liver (a hematopoietic organ) and mouse brain.

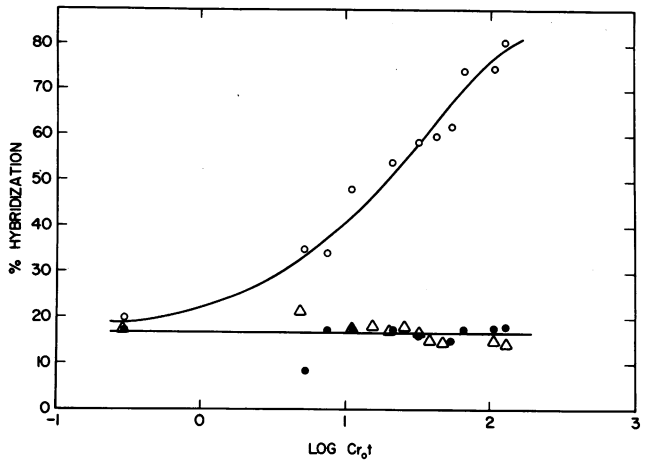

FIG. 3. Hybridization of globin cDNA and liver cDNA to RNA transcribed from rabbit bone-marrow and liver chromatin with *E. coli* RNA polymerase. The preparation of chromatin and the isolation of the *in vitro* RNA transcription product are described in *Methods*. Each reaction mixture (7.5 μl) contained: (*1*) 1000 cpm of globin cDNA and 4.2 μg (0.084 A_{260} unit) of RNA transcribed from rabbit-marrow chromatin (○); (*2*) 1000 cpm of globin cDNA and 4.2 μg of RNA transcribed from rabbit liver chromatin (●); or (*3*) 1500 cpm of liver cDNA and 4.2 μg of RNA transcribed from rabbit marrow chromatin (△). The reaction mixtures were incubated at 68° from 0.1 to 24 hr.

TABLE 1. *Comparison of RNA polymerase from sheep liver and E. coli in transcription of bone-marrow chromatin*

Polymerase source		$Cr_0t_{1/2}$ value
Mammalian		3.8×10^0
		4.4×10^0
		7.0×10^0
		4.3×10^0
	Average:	4.9×10^0
E. coli		1.3×10^1
		2.1×10^1
		1.3×10^1
		1.2×10^1
		1.7×10^1
	Average:	1.5×10^1

For experimental details see legends to Figs. 2 and 3.

The use of a mammalian rather than bacterial DNA-dependent RNA polymerase in transcribing mammalian chromatin has two advantages. First, although the enzyme from either source will transcribe unique mRNA sequences from mammalian chromatin, the mammalian enzyme appears to do so with more specificity. The average value for the Cr_0t (concentration of RNA × time) at which 50% of the possible hybridization takes place is 4.9 when mammalian enzyme is used. When *E. coli* is the source of the enzyme, the $Cr_0t_{1/2}$ value increases to 15. The higher value indicates that there are fewer globin mRNA molecules in the total RNA transcribed. An estimate of the amount of globin RNA in the two chromatin-directed transcripts can be made as follows. Since purified rabbit-globin mRNA (assumed to be 80% pure mRNA) has a $Cr_0t_{1/2}$ of 3×10^{-3}, the $Cr_0t_{1/2}$ value of 4.9 for rabbit-marrow chromatin transcript is higher by $4.9/(3 \times 10^{-3} \times 0.8) = 2000$; i.e., 1 RNA base in 2000 (or 0.05% of the total RNA) is capable of annealing to the rabbit-globin cDNA when mammalian enzyme is used. In contrast, a value of only 1 RNA base in 6000 (or 0.016% of the total RNA) is capable of annealing when bacterial enzyme is used. The latter estimate (using *E. coli* polymerase) agrees with the results of others (1, 2, 14).

Secondly, in searching for transcriptional regulatory factors, a fully mammalian system is probably essential. In *E. coli*, a number of regulatory factors have been identified (15). It is likely that similar factors exist in the mammalian nucleus, but a cell-free assay for synthesis of unique (and identifiable) mRNA sequences has not previously been available.

The specificity of the present assay is shown by the fact that globin cDNA, but not liver cDNA, will hybridize with the RNA synthesized from bone-marrow chromatin. Globin cDNA is specific for globin mRNA sequences since liver cDNA very poorly hybridizes to globin mRNA (at 68°); likewise, globin cDNA hybridizes very poorly to liver mRNA. Thus, the cell-free transcriptional system, using globin cDNA as a specific probe, appears well suited for use in analyzing nuclear protein (and nucleic acid) fractions in search of transcriptional regulatory factors.

Nuclear regulatory factors probably play crucial roles in the expression of the hemoglobin genes in normal and diseased erythroid cells. Beta thalassemia is a human genetic disease that is manifested by a decrease in the number of beta-globin chains synthesized in erythroid cells. Previous studies using an initiating cell-free protein-synthesizing system from rabbit and human reticulocytes (16–18) revealed that the defect in thalassemia is specifically in the mRNA (18–21). Other workers have shown that the beta globin mRNA in beta thalassemia is present in decreased amount (12, 22, 23). It should be possible to utilize human chromatin in the cell-free transcriptional system to measure the relative amounts of α- and β-globin mRNA sequences that can be transcribed from thalassemic chromatin.

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