Quantitation of Labeled Globin Messenger RNA by Hybridization with Excess Complementary DNA Covalently Bound to Cellulose†

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ABSTRACT: A method is described to quantitate labeled globin mRNA by hybridization with excess cDNA which was enzymatically polymerized on oligo(dT)-cellulose. In a large excess of cDNA-cellulose the rate of RNA hybridization was dependent on DNA concentration and not on RNA concentration. Nonhybridized RNA can be digested by RNase and washed from the cDNA which is covalently bound to cellulose. This enables the detection of labeled globin mRNA even when present in a proportion as low as 0.02–0.03% of the total RNA.

The cellular concentration of a specific mRNA is a key factor in the regulation of gene expression. In prokaryotes mRNA is labile and its concentration is regulated by its synthetic rates (Adesnik and Levinthal, 1970). Eukaryotic mRNA appears to be more stable (Kafatos, 1972) and its concentration can be controlled both by alteration in its rates of synthesis and degradation. Methods to measure specific mRNA sequences have recently become available by the transcription of purified mRNA into highly labeled complementary DNA (Kacian et al., 1972; Ross et al., 1972; Verma et al., 1972). Indeed the accumulation of ovalbumin and globin mRNA in development was reported (Ross et al., 1973, 1974; Cox et al., 1974). However, since by this method of excess RNA hybridization only the concentration of mRNA can be measured, the more dynamic aspects of mRNA metabolism (synthesis, processing, and degradation) have been largely ignored. Measurements of synthesis and degradation of mRNA are possible by hybridization of labeled RNA and excess cDNA.[1] However, this approach has two major drawbacks. First, a relatively high level of nonhybridized RNA is undigestable by ribonuclease (1% is not unusual), limiting the detection of specific RNA synthesis in the range of 1% and lower. Second, large amounts of DNA are required since the DNA is not recovered after hybridization. This in particular is a problem when DNA is available in small amounts and is hard to prepare, which is the case with DNA complementary to mouse globin mRNA and many other cDNA preparations. These two problems can be solved by the use of DNA covalently bound to a solid matrix such as Sepharose or cellulose (Gilham, 1964, 1968; Shih and Martin, 1974; Venetianer and Leder, 1974; Noyes and Stark, 1975; Gilboa et al., 1975).

In this communication we describe the hybridization properties of globin cDNA enzymatically polymerized on oligo(dT)-cellulose with labeled globin mRNA. The fate of newly synthesized globin mRNA in Friend leukemic cells following treatment with DMSO will be reported elsewhere (Aviv et al., in preparation).

Materials and Methods

Enzymes and Reagents. Deoxyribonuclease I, RNase free 2400 units/mg; ribonuclease A, 4800 units/mg, and ribonuclease T1, 3×10^5 units/mg, were obtained from Worthington. Proteinase K was obtained from Merck. RNA-dependent DNA polymerase from AMV virus 3500 units/ml was supplied by Dr. J. Beard. Yeast tRNA, Actinomycin D, dATP, dCTP, dGTP, and TTP were obtained from Sigma. [^{3}H]dGTP, 6.1 Ci/mol, [^{32}P]dGTP, 1350 mCi/mmol, and ^{125}I carrier-free, 100 mCi/ml, were obtained from Amersham. Formamide was obtained from Fluka; it was deionized by stirring 3 g of Amberlite resin MP-1 (BDH) with 100 ml of formamide for 2 h. Oligo(dT)-cellulose was prepared as described (Gilham, 1964); 1 ml of packed cellulose contained 200 mg.

Preparation of Globin mRNA and [^{125}I]RNA. Globin mRNA (9S) was prepared from the reticulocytes of anemic Balb/c mice. RNA was extracted and poly(A)-containing RNA purified on oligo(dT)-cellulose as previously described (Aviv and Leder, 1972); the poly(A)-containing RNA was further separated on a 15–30% sucrose gradient to yield 9S globin mRNA. Poly(A)-containing RNA from rat brain was kindly supplied by I. Gozes. Iodination of mRNA was performed by modification of the Commerford procedure (1971). To 10 μl of 1-mg/ml mRNA, 10 μl of freshly prepared 10 mM $TlCl_3$ was added. These were added to a solution containing 10 μl of 1 M ammonium acetate, pH 5.0, 15 μl of 0.1 mM KI (fresh), and 10 μl of ^{125}I carrier-free (100 mCi/ml). Iodination proceeded for 30 min at 60 °C, the mixture was chilled on ice, and 50 μl of 0.1 M Na_2SO_3 was added. [^{125}I]mRNA was separated from free iodine by chromatography on oligo(dT)-cellulose as follows: To the reaction mixture were added 500 μg of yeast tRNA and an equal volume of 1.0 M NaCl, 0.1 M Tris, pH 8.0. The column was washed with 40 ml of equilibrating buffer. The poly(A)-containing [^{125}I]-mRNA was eluted with three washes of 1-ml eluting buffer containing 0.01 M Tris, pH 8.0, 1 mM EDTA and 0.1% sodium dodecyl sulfate, and then washed once with 0.5 ml of H_2O; the material was incubated at 60 °C for 20 min. Five hundred micrograms of yeast tRNA was added, and the solution was adjusted to 0.2 M NaCl and precipitated at −20 °C with two volumes of ethanol. The precipitate was allowed to stand for at least 2 h and then collected by centrifugation for 20 min at 10 000*g*. The precipitate was then dissolved in 100 μl of H_2O.

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[1] Abbreviations used are: cDNA, DNA complementary to a specific mRNA; SSC, 0.15 M NaCl, 0.015 M Na_3 citrate.

Synthesis of cDNA on oligo(dT)-cellulose. The procedure of Venetianer and Leder (1974) was followed with minor modifications. The reaction mixture contained: 0.1 M Tris-HCl, pH 7.8, 80 mM KCl, 6 mM $MgCl_2$, 2 mM dithiothreitol, 0.6 mM each of dATP, dCTP, dGTP, and TTP, 10 μg/ml of mouse globin mRNA, 70 units/ml of RNA-dependent DNA polymerase, 50 μg/ml of actinomycin D, and 25 mg/ml of oligo(dT)-cellulose in a total volume of 10 ml. In some experiments 10 μCi/ml of [^{32}P]dGTP or of [^{3}H]dGTP was incubated as well. The reaction mixture was shaken continuously at 30 °C for 4 h. It was then transferred to a column and washed with 200 ml of 0.1 M NaOH, or until all the free radioactive dGTP was eluted. The globin cDNA dT-cellulose was washed and stored in 0.01 M Tris-HCl pH 7.8, 0.1% sodium dodecyl sulfate, and 1 mM EDTA.

The amount of globin cDNA enzymatically synthesized on the oligo(dT)-cellulose was determined by the incorporation of labeled dGTP into cDNA. Several preparations of globin cDNA-cellulose were used in these experiments. The preparations varied from 0.025 μg to 0.08 μg of globin cDNA per mg of cellulose.

Hybridization Conditions and Elution of Hybridized RNA. The reaction mixture contained 50% formamide, 0.01 M Tris, pH 7.4, 0.6 M NaCl, 0.1% sodium dodecyl sulfate, 1 mM EDTA (hybridization buffer), 50 μg of poly(A), 100 μg of yeast tRNA, labeled RNA as specified in each experiment, and globin cDNA cellulose in a volume of 500 μl. The hybridization was carried out in scintillation vials in a shaking water bath at 40 °C for 18 h, or as specified. The reaction mixture was then transferred to a column and washed with 20 ml of hybridization buffer, followed by 3 ml of 2 × SSC, then transferred to a new scintillation vial in 2 ml of 2 × SSC containing 100 μg of pancreatic RNase A (Worthington) and shaken for 30 min at room temperature. The cellulose suspension was then washed with 5 ml of 2 × SSC, followed by 6 ml of 0.01 M Tris-HCl, pH 7.8, 0.1% sodium dodecyl sulfate, 1 mM EDTA. (This last buffer elutes poly(A)-containing RNA from oligo(dT)-cellulose). Hybrids were then eluted with 3 ml of 0.1 M NaOH. Alternatively, water-jacketed columns were used and the hybrids were melted by a stepwise rise in temperature.

Results

In excess cDNA, the kinetics of the hybridization were dependent mostly on the cDNA concentration, and independent of the mRNA concentration. This independence of the RNA concentration is illustrated in Figure 1. The kinetics of hybridization of 0.2 and 10 ng of RNA to 350 μg of cDNA covalently bound to cellulose were identical. On the other hand, the hybridization of a fixed amount of [^{125}I]globin mRNA (10 ng) with different concentrations of globin cDNA cellulose is shown in Figure 2, where it can be seen that the rate of hybridization varied with the concentration of cDNA. The kinetics of hybridization in the higher cDNA-cellulose concentrations had $C_0t_{1/2}$ values of 2.7×10^{-3} and 1.5×10^{-3} which are similar to the rate that we and others (Ross et al., 1974) observed for hybridization of 9S globin mRNA and its cDNA in solution, $C_0t_{1/2} = 2.8 \times 10^{-3}$. At lower cDNA cellulose concentrations (0.18 μg of cDNA/ml, 3 mg of cellulose/ml), hybridization was slower than expected; $C_0t_{1/2} = 4.5 \times 10^{-3}$. Various preparations of globin cDNA-cellulose were tested and it was found that the kinetics of hybridization of [^{125}I]globin mRNA correlated with the amount of cDNA present.

The specificity of the hybridization is illustrated in Table I. [^{125}I]Globin mRNA was hybridized to globin cDNA-cellulose, and to unsubstituted oligo(dT)-cellulose. The poly(A)-containing mRNA hybridized to the oligo(dT)-cellulose but was melted off the column by a low salt buffer, prior to the final elution (Methods). Even though RNase digestion was not performed in this experiment prior to elution of the hybrids, the nonspecific binding to the oligo(dT)-cellulose was low. The same proportion of [^{125}I]mRNA hybridized to cDNA over a range of 250 (0.2–50 ng) of RNA concentration indicating that cDNA was in large excess.

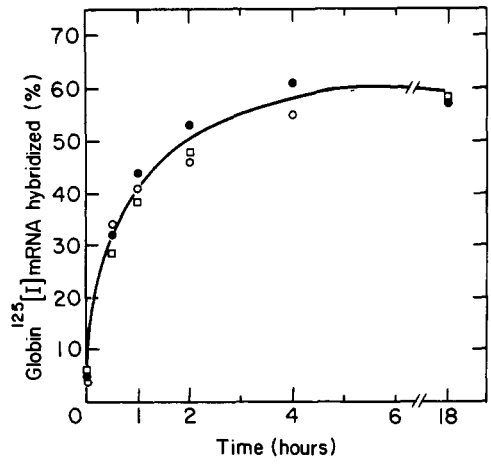

FIGURE 1: Kinetics of hybridization with different concentrations of [^{125}I]globin mRNA. Hybridization was performed as detailed in Methods: 0.35 μg of globin cDNA-(dT)-cellulose (70 μl of packed or 14 mg of dry cellulose) was hybridized for indicated time periods with 0.2 ng (○—○), 1 ng (●—●) and 10 ng (□—□) of [^{125}I]globin mRNA in a total volume of 500 μl.

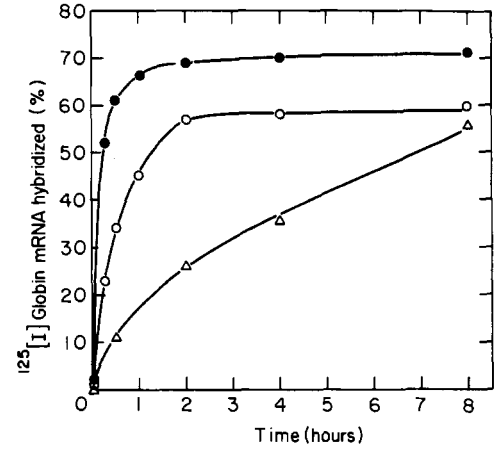

FIGURE 2: Hybridization with different concentrations of globin cDNA-cellulose. Hybridization was performed as detailed in Methods: 10 ng of [^{125}I]globin mRNA was hybridized for the indicated time periods with different dilutions of globin cDNA-cellulose in a total volume of 500 μl. The cellulose matrix dry weight is indicated in parentheses. 0.09 μg of globin cDNA-cellulose (1.6 mg) (△—△), 0.27 μg of globin cDNA, cellulose (4.8 mg) (○—○), and 0.8 μg of globin cDNA-d(T) cellulose (14 mg) (●—●).

Hybridization of ^{125}I mouse globin mRNA could be competed by excess nonlabeled mouse globin mRNA and not by chicken globin mRNA (Figure 3). Increasing amounts of unlabeled globin mRNA were hybridized to saturation with 0.27 and 0.8 μg of globin cDNA on cellulose; 15 ng of ^{125}I-labeled globin mRNA was then added and the RNA allowed to hybridize for another cycle of hybridization. The concentration of unlabeled RNA required to inhibit 50% of the hybridization capacity of DNA was about equal to the concen-

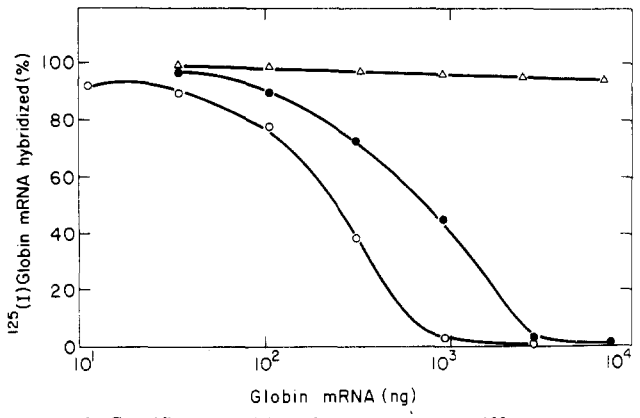

FIGURE 3: Specific competition of hybridization of [^{125}I]mouse globin mRNA with mouse globin cDNA-cellulose by unlabeled mRNA. Hybridization was performed as detailed in Methods: 0.27 μg of mouse globin cDNA-cellulose (○—○) and 0.8 μg of globin cDNA-cellulose (●—●) were hybridized for 18 h with increasing amounts of nonlabeled mouse globin mRNA as indicated in a total volume of 500 μl. 15 ng of [^{125}I]globin mRNA was then added and hybridization continued for 3.5 h when 0.27 μg of globin cDNA-cellulose and for 2 h when 0.8 μg of globin cDNA-cellulose was used. In a control experiment, chicken globin mRNA was used to compete for the hybridization of [^{125}I]mouse globin mRNA to 0.27 μg of cDNA (△—△).

TABLE I: Hybridization of Different Concentrations of [^{125}I]9S Globin mRNA to cDNA-Cellulose and to Oligo(dT)-cellulose.[a]

Input RNA		% Hybridized	
ng	cpm	cDNA-Cellulose	d(T)$_n$-cellulose
0.2[b]	1 600	58	ND
1.0[c]	4 000	57	2.2
3.0[b]	23 500	63	0.02
5.0[b]	37 500	60	0.22
10.0[b]	72 000	65	0.08
12.5[c]	50 000	57	0.17
50.0[c]	200 000	60	0.016

[a] Hybridization was performed as detailed in Materials and Methods for 18 h. 0.35 μg of globin cDNA-cellulose (14 mg) and oligo(dT)-cellulose (14 mg) were hybridized with increasing amounts of [^{125}I]9S globin mRNA as indicated in a total volume of 500 μl. *b* and *c* indicate different experiments.

tration of cDNA. Thus when 0.27 μg of cDNA-cellulose was used for hybridization, 0.25 μg of globin RNA was required to reduce the hybridization of [^{125}I]globin RNA to 50% and about 0.8 μg of RNA was required to reduce to 50% the hybridization of [^{125}I]RNA when 0.8 μg of cDNA-cellulose was used. In addition, about threefold excess nonlabeled RNA was required to block 90–95% of the hybridization capacity of the cDNA-cellulose (Figure 3).

The nature of the hybrid formed between [^{125}I]globin mRNA and cDNA-cellulose was determined by its melting property (T_m). The melting temperature in 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.4, was 89 °C and had a sharp transition, indicating a perfect RNA:DNA hybrid (Figure 4). This temperature is similar to T_m of globin RNA:DNA hybrids in solution (Ross et al., 1974). The effects of formamide and salt concentration on the T_m were those expected for nucleic acid hybrids (McConaughy et al., 1969). Fifty percent formamide reduced the T_m by about 25 °C. Lowering the salt concentration in 50% formamide also reduced the melting temperature: the T_m was 72 °C in 0.6 M NaCl, 64 °C in 0.1 M NaCl, and 52 °C in 0.01 M Tris.

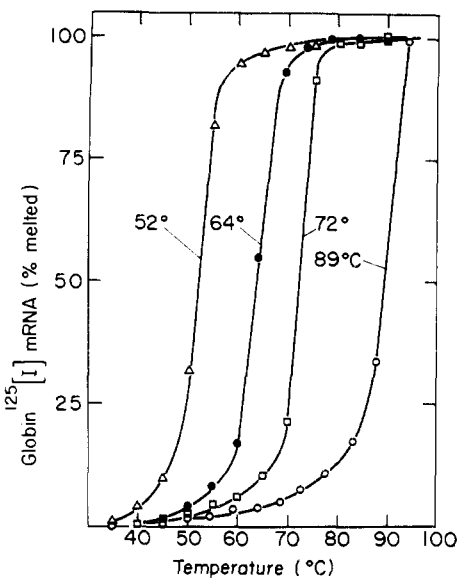

FIGURE 4: Melting properties of hybrids of [^{125}I]globin mRNA and globin cDNA-cellulose: 15 ng of [^{125}I]globin mRNA and 0.6 μg of globin cDNA-cellulose were hybridized in a volume of 500 μl as detailed in Methods. Hybridized RNA was eluted by a 5 °C stepwise rise in temperature. 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.4 (○—○); 50% formamide, 0.6 M NaCl, 0.01 M Tris-HCl, pH 7.4 (□—□); 50% formamide, 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.4 (●—●); 50% formamide, 0.01 M Tris-HCl (△—△).

Globin cDNA-cellulose was stable under the hybridization and elution conditions used. When labeled cDNA-cellulose was used, not more than 1–2% of the DNA was lost in one cycle of hybridization and elution. Moreover, very little loss of cDNA could be detected even after heating cDNA-cellulose to 90 °C for a short period in the presence of 50% formamide. Thus, a single preparation of cDNA-cellulose could be reused at least ten times without significant changes in its hybridization properties.

The sensitivity of this detection method for hemoglobin messenger RNA synthesis and degradation depends on its background, that is, the amount of labeled RNA not containing globin RNA sequences which will bind to the globin cDNA-cellulose. We found that the nonspecific binding was reduced two- to threefold by the inclusion of tRNA and poly(A) in the hybridization mixture, whereas inclusion of poly(U) had no effect. A further reduction of the background was accomplished by the use of RNase. When rat brain [^{125}I]mRNA was used to measure nonspecific binding, 0.1–0.2% bound to globin cDNA- cellulose or to the oligo(dT)-cellulose matrix. This level was further reduced to 0.02–0.04% by treating the hybrid with RNase (Table II). In other experiments we noticed that the background level was indirectly related to the amount of input RNA. As more non-globin labeled RNA was used, a lower proportion of counts bound nonspecifically, thus reducing the practical background level. Thus, if the input of labeled RNA was 10^5 cpm, the background was about 0.1–0.2% (100–200 cpm), and if the input was 10^6 cpm, the background was 0.02–0.03% (200–300 cpm).

Discussion

This communication describes a useful method to study different aspects of globin mRNA metabolism, even when labeled globin mRNA is present as a small proportion of other

TABLE II: Specific Hybridization of [^{125}I]Globin mRNA to cDNA-Cellulose.[a]

RNA	%, Hybridized: Globin cDNA-Cellulose −RNase	Globin cDNA-Cellulose +RNase	$d(T)_n$-Cellulose +RNase
[^{125}I]Globin mRNA	67	60.2	0.016
[^{125}I]Rat brain RNA	0.17	0.04	0.04

[a] Hybridization was performed as detailed in Materials and Methods for 18 h. 0.35 μg of globin cDNA-cellulose (14 mg) and oligo(dT)-cellulose (14 mg) were hybridized with 10 ng of [^{125}I]globin mRNA and 10 ng of ^{125}I-poly(A)-containing rat brain RNA in a total volume of 500 μl.

mRNA sequences. Hybridization of labeled RNA with cDNA using cDNA covalently bound to cellulose has several advantages over other approaches. In contrast to hybridization in solution, the solid-phase technique allows removal of RNase-resistant nonhybridized RNA by a simple washing step, whereas in solution reduction of the background is possible only by a more complicated procedure (Coffin et al., 1974). Covalent immobilization of the cDNA to the cellulose is an improvement over previous solid-phase techniques in which DNA has been noncovalently attached to filters. Immobilization of denatured DNA on filters is not suitable for small-molecular-weight DNA such as globin cDNA. Moreover, noncovalently bound DNA tends to detach and compete in hybridization with the material remaining on the filter (Haas et al., 1972).

When cDNA is enzymatically synthesized onto oligo(dT)-cellulose, the cDNA-cellulose product is stable both to the usual conditions of hybridization, 50% formamide at 40 °C, and to the conditions of elution, 0.1 M NaOH. Even heating to 90 °C resulted in a maximum loss of 2% of radioactively labeled DNA from the cellulose. In earlier preliminary studies we have also used globin cDNA covalently bound to Sepharose (Gilboa et al., 1975); however we found the enzymatic preparation of cDNA-cellulose more convenient.

Hybridization of nucleic acids immobilized on filters is slower than hybridization in solution (Gillespie and Spiegelman, 1965; Spiegelman et al., 1973; Flavell et al., 1974). The data presented in this paper suggest that the kinetics of hybridization of globin mRNA and cDNA-cellulose can be as rapid as hybridization in solution (Figure 2). The theoretical basis for these differences between hybridization using DNA immobilized on filters and cDNA covalently bound to cellulose is not completely understood.

The kinetics of hybridization in a large excess of cDNA is dependent on the cDNA concentration and not on RNA concentration. This enables the detection of very low amounts of labeled RNA. In application of this technique to in vivo studies, the detection of low concentrations of a specific mRNA can be hindered by the nonspecific binding of other labeled nucleic acids. The use of RNase increases the sensitivity of the method by reducing background due to nonspecific hybrids to a level of 0.02–0.03%.

The technique described here is a general method useful for studies of mRNA metabolism in eukaryotic cells. The major requirement is the availability of pure RNA of the species one wishes to study. Once cDNA-cellulose is generated, it can then be used repeatedly to measure the synthesis, processing, and degradation of one specific RNA. We have applied this approach to study the metabolism of globin mRNA in differentiating erythroleukemia cells. These results will be published elsewhere (Aviv et al., in preparation).

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