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Is there specific transcription from isolated chromatin?

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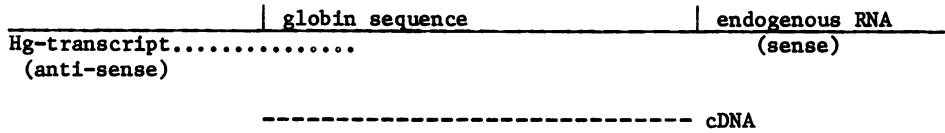
ABSTRACT

Hg-UMP-containing transcripts made from chick erythroid chromatins with E. coli RNA polymerase hybridize to chick globin cDNA. Contamination with endogenous globin RNA has been largely removed by purification on SH-agarose columns at 55°C. Some endogenous globin mRNA sequences remain, probably as hybrids with "anti-sense" Hg-transcripts produced by RNA-dependent RNA synthesis. Heating to 115°C before SH-agarose chromatography eliminates these contaminants. Hg-transcripts from adult and embryonic erythroid chromatins purified by this method are hybridized to globin cDNA; they contain a 4- to 6-fold higher proportion of globin-specific sequences (10-13 ppm) than do transcripts from brain chromatin. Dissociation of erythroid chromatins in salt and urea, followed by reconstitution using standard methods, destroys even this low degree of specificity.

In vitro transcription of chromatin offers an attractive method of examining control factors regulating eukaryotic gene expression, since the conditions can be modulated more easily than with intact cells or nuclei. Specific transcription of globin genes from erythroid chromatin using E. coli RNA polymerase has been reported[1-11]. Most of these studies cannot be interpreted, because contaminating endogenous globin RNA sequences are indistinguishable from newly synthesized transcripts by the usual hybridization assays. A solution to these difficulties uses mercuri-nucleotides to label the *in vitro* transcripts; the mercury-substituted transcripts can be purified by chromatography on SH-agarose[10-13]. We have previously reported, however, that the standard methods for isolating transcripts allow new transcripts and endogenous RNA to aggregate, which causes significant contamination to persist even after SH-agarose chromato-

graphy[14-15]. Appropriate disaggregation procedures substantially reduce the problem.

Zasloff and Felsenfeld[16] have recently reported that endogenous RNA can act as a template for E. coli RNA polymerase and hybridizes to the resulting Hg-substituted anti-sense sequences. These hybrids could contain "full sequence" endogenous globin RNA, hybridized to short anti-sense transcripts. Isolated Hg-RNA would include such stable complexes in which the non-hybridized portion of the endogenous globin mRNA is available to hybridize to the cDNA probe. Therefore estimates of the frequency of globin-specific transcripts may be too high.



We wished to examine the extent to which this problem affects our investigations of preferential transcription of globin sequences in chick erythroid chromatin. After the elimination of artifacts we find a small but consistent level of specific transcription from chromatins of both the primitive embryonic erythroid line and the definitive line adult reticulocyte, which is at least 4-fold higher than from brain chromatin. Dissociation of chromatin and reconstitution by gradient dialysis from high salt and urea did not preserve transcriptional specificity.

MATERIALS AND METHODS

Scintillants and ^{3}H- and ^{14}C-labelled nucleotides were purchased from New England Nuclear (Boston, MA). γ-^{32}P-ATP and γ-^{32}P-GTP, and ^{35}S-methionine were from Amersham (Chicago, IL).

Preparation of chromatin -- Chromatin from circulating 5 day embryonic erythroid cells was prepared as previously described[14]. Adult chickens were rendered anemic by injection of acetylphenylhydrazine (Eastman,

Rochester, NY). Blood cells were lysed in 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.6 (TKM), and nuclei were washed twice in 0.25 M sucrose-TKM before storage in 0.25 M sucrose, 33% (v/v) glycerol, 20 mM Tris-HCl, at -20°C for up to 9 months. Before use nuclei were mixed with 1% Triton-X100, 2.0 M sucrose, 3.3 mM $MgCl_2$, layered over 1.8 M sucrose, 3.3 mM $MgCl_2$, and pelleted in an IEC SB-283 rotor (Damon-IEC, Needham, MA) at 40,000 g for one hour. Chromatin was then prepared as described[14], except that the lysis buffer contained 20 mM EDTA.

For the preparation of brain chromatin 12 day chick brains were excised and dissociated in 1% BSA (Fraction V; Sigma, St. Louis, MO) in Hanks-BSS (GIBCO, Grand Island, NY) with a 40 ml Dounce homogenizer (Kontes, Vineland, NH; loose pestle). The white cerebellar material was separated from heavier red cell contaminants by centrifugation at ∿1000 g in a IEC clinical centrifuge. Nuclei were prepared by lysis in 0.2% Triton X-100 in 0.25 M sucrose-TKM with homogenization in a Sorvall Omnimix (Dupont-Sorvall, Norwalk, CT) for two minutes at setting 3. Nuclei were washed twice in 0.25 M sucrose-TKM, lysed with a Potter-Elvehjem homogenizer in 15 ml of 25 mM EDTA, 10 mM Tris-HCl, pH 7.9, and pelleted through 25 ml of 1.6 M sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl at 20,000 g for 45 minutes. The crude chromatin was purified as described for 5 day erythroid chromatin.

Preparation of globin cDNA -- Globin cDNA was synthesized as described[14], except that additional NaCl was omitted, 4 mM $Na_4P_2O_7$ and 1 mM EDTA were included, incubation was for one hour at 43-45°C and the concentration of reverse transcriptase was 177 units/ml. Boiled calf thymus DNA (Sigma) was used as a carrier and to presaturate the G-50 Sephadex column.

Acrylamide gel electrophoresis of cDNA in 98% formamide -- Acrylamide gels (10.5 x 0.6 cm, 5%) were poured as described[24] in 98% deionized formamide, 20 mM NaAc, pH 7.5 and electrophoresed at 2 mA/gel in recirculating 20 mM NaAc. Marker HaeIII fragments of SV-40 DNA (kind gift of Dr.

Bryan Roberts) were visualized by UV fluorescence after removal of formamide in water for 2-3 hours and 30-45 minutes staining in 0.5 μg/ml ethidium bromide in water.

Size fractionation of cDNA on alkaline sucrose gradients -- cDNA was fractionated on 11 ml 5-30% alkaline sucrose gradients in 0.1 M NaOH, 0.9 M NaCl, 0.01 M EDTA for 30 hours at 20°C and 40,000 rpm in the IEC SB-283 rotor. Fractions were pooled to derive preparations > 500 or < 370 bases long, as measured by acrylamide gel electrophoresis in formamide.

Preparation of 5-Hg-UTP and SH-agarose -- Hg-UTP was purchased from Calbiochem (La Jolla, CA) or prepared as previously described[14]. Sulfhydryl Bio-Gel A5M was prepared by the method of Cuatrecasas[19]; the level of substitution was determined to be 2.5 - 3.0 μmoles of SH/ml, using Ellman's reagent[20]. Columns were stored in 0.25 M 2-mercaptoethanol, 50 mM Tris-HCl, 0.1% SDS, 2 mM EDTA, pH 7.9, then washed extensively with 0.1% SDS, 10 mM Tris-Hcl, 2 mM EDTA, pH 7.9, just before use.

In Vitro transcription was conducted as described[14], with 50% of the total UTP as Hg-UTP.

Purification of Hg-transcripts on SH-agarose -- "Hot columns" were used, as previously described[14]. Reaction mixes were extracted with phenol, the aqueous phase precipitated with ethanol, the pellet treated with DNAse, dissolved in SDS buffer, dialyzed for two days at room temperature and desalted on G-50 Sephadex. The excluded pool was made up to 1% SDS, 10 mM Tris-HCl, 2 mM EDTA, pH 7.9, heated to 70°C for 20-30 minutes and then applied to an SH-agarose column equilibrated in the same buffer (but 0.1% SDS) at 55°C. The column was washed with 0.1% SDS buffer and water. Then the column was returned to room temperature, washed with 50 mM Tris-HCl, 0.1% SDS, 5 mM EDTA, and the Hg-RNA eluted in the same buffer containing 0.25 M 2-mercaptoethanol.

"Super hot columns" were treated as above, except that samples were

heated to 115-122°C in a tightly capped "Oak Ridge" 40 ml polycarbonate centrifuge tubes (IEC).

Hybridizations were conducted by titration in 25 mM HEPES, 0.5 M NaCl, 5 mM EDTA, pH 7.1, for 48 hours at 68°C as previously described[14]; hybridization was assayed by resistance to S1 nuclease (E.C.3.1.4.21; kind gift of Dr. Lillian Chan). Control hybridizations were with Hg-CT-RNA which had been coextracted with the template chromatin in the same RNA:DNA ratio as that achieved during transcription; this Hg-CT-RNA had been purified in parallel with authentic Hg-transcript. Unless indicated otherwise, this background hybridization was subtracted before plotting the data.

Estimation of transcript size -- Transcript size was estimated as the molar incorporation ratio of internal ^{14}C-AMP to terminal γ-^{32}P-GTP plus γ-^{32}P-ATP. Transcription was conducted in 250 µl reactions under the usual conditions for 30 minutes; 8 µCi each of γ-^{32}P-purine triphosphates were also included. Reactions were extracted with phenol and the aqueous phases precipitated with TCA, filtered, and counted[14]. Duplicate reactions were run in the absence of polymerase and the resulting background incorporation, due mainly to protein kinases, was subtracted.

RESULTS

Synthesis and characterization of globin cDNA -- cDNA synthesized by the method of Efstratiadis et al.[29] was still relatively short and heterodisperse (cDNA/U2, Figure 1) in our hands. Adapting the method of Kacian and Meyers[30], we found that inclusion of 4 mM $Na_4P_2O_7$ and of 1 mM EDTA, reduction of ionic strength and increased incubation temperature (43,45°C) yielded a longer product (cDNA/U3, Figure 1). However, 60% of cDNA/U3 was still < 500 bases in length.

In view of the importance of using full length cDNA to hybridize *in vitro* transcripts[32] (see Discussion), we decided to fractionate cDNA by size.

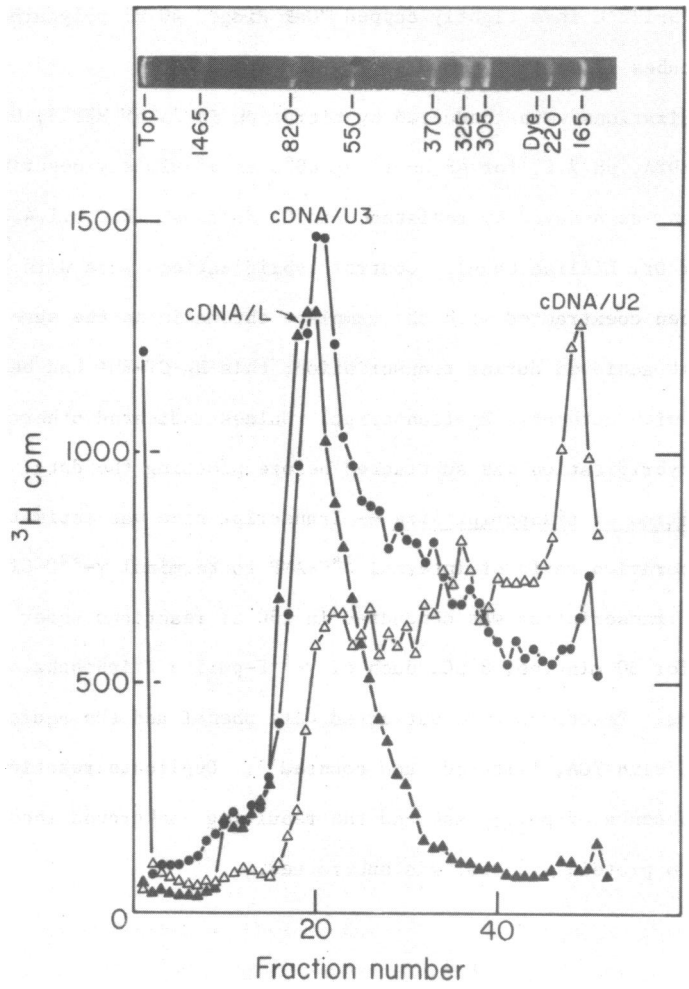


Figure 1. Electrophoresis of cDNA in 5% polyacrylamide gels containing 98% formamide. Parallel gels are not loaded with equal counts. cDNA/U2, synthesized by the method of Efstratiadis et al.[29]; cDNA/U3, made as described in Methods; cDNA/L, derived from cDNA/U3 by alkaline sucrose gradient fractionation.

Since cDNA purified on formamide gels was unusable because it contained substantial amounts of visible, brown impurities, we purified cDNA on alkaline sucrose gradients as described in Methods. Figure 1 shows that

> 80% of the molecules of the resulting cDNA/L are at least 500 bases long. The sensitivity of cDNA/L as a probe for globin sequences is increased (Figure 2A), as was also reported by Weiss et al.[32]

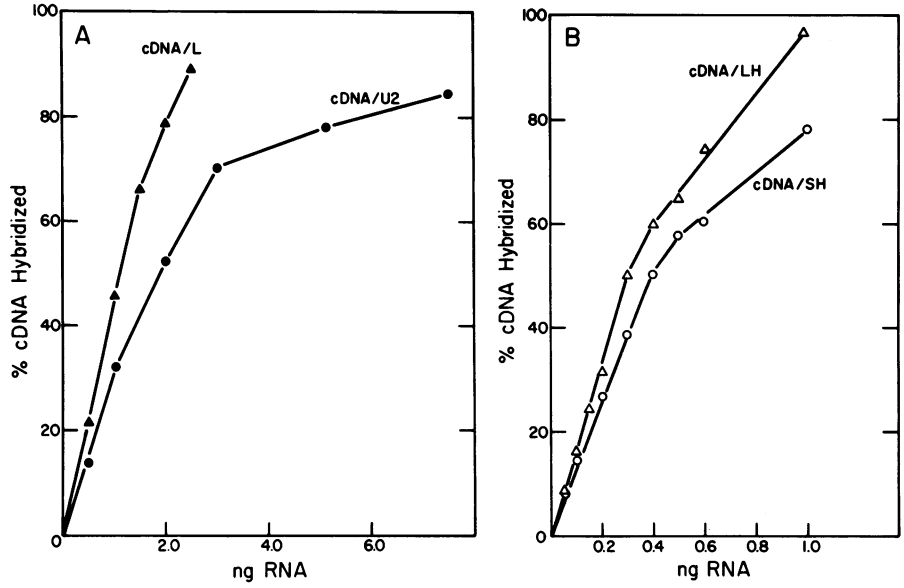


Figure 2. Hybridization titration curves of pure globin mRNA with A) 0.8 ng of cDNA/U2, cDNA/L; B) 0.2 ng of cDNA/LH (3.7 x higher specific activity than cDNA/L), cDNA/SH.

Hybridization of Hg-transcripts purified on "hot column" -- Figure 3A shows the titration curves for cDNA/L with Hg-transcripts purified by the "hot column" method, which eliminates most of the aggregated endogenous sequences[14]. Although our cDNA probe was prepared from globin mRNA of adult cells, it hybridizes extensively (80% protected) with globin mRNA for embryonic cells (data not shown). Significant hybridization above background was obtained with Hg-transcripts from adult and embryonic chicken erythroid chromatins, but not with those from brain chromatin

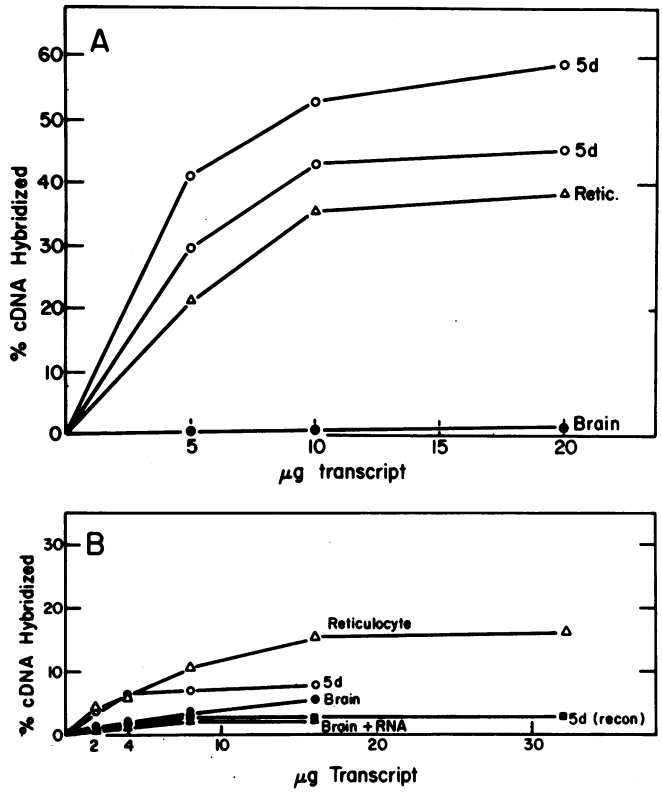


Figure 3. Hybridization titration curves of: A) purified Hg-transcripts from the following chromatins: 5 day embryonic (2 preparations), adult reticulocyte, brain. [0.8 ng cDNA/L]; Hg-transcripts were purified on "hot columns". B) 0.2 ng cDNA/LH (a probe with higher specific activity than the one used in A); Hg-transcripts were purified on "super hot columns". In addition to the chromatins of panel A, reconstituted chromatin from 5 day embryonic cells and brain chromatin plus globin mRNA were also used.

(Figure 3A). This result appeared to confirm previous reports of specific transcription of Hg-RNA globin-specific sequences from chick erythroid chromatin[10].

Purification of Hg-transcripts on "super-hot columns" -- Even with the above precautions the results of figure 3A might be due to contaminating endogenous globin RNA, because Zasloff and Felsenfeld[16] recently reported that RNA-dependent RNA synthesis could cause endogenous contamination to persist. This can be eliminated by heating samples to at least 107°C

before SH-agarose chromatography. We therefore compared hybridization with duplicate aliquots of 5 day embryonic erythroid Hg-transcripts purified on "hot" and on "super-hot" columns. "Super-hot column" purification (see Methods) caused a 3- to 16-fold reduction in the degree of hybridization (data not shown). Hybridization of the fraction not bound to SH-agarose was not reduced (data not shown), so that degradation cannot explain the observed reduced hybridization.

Hybridization of highly purified Hg-transcripts — We wished to determine whether erythroid chromatin preferentially directs globin-specific transcription or whether the difference in the degree of hybridization obtained after "hot column" purification of Hg-transcripts from erythroid vs. non-erythroid chromatins was entirely artifactual. Figure 3B compares net hybridization curves for highly purified ("super hot column") Hg-transcripts from adult reticulocyte, native and reconstituted 5 day embryonic erythroid chromatins, as well as from 12 day brain chromatin with or without added globin mRNA. In each case background hybridization (see Methods) has been subtracted. A small but consistent increase in the degree of hybridization is seen with the native erythroid Hg-transcripts, as compared to the curves for brain transcripts. The proportion of the total transcripts representing globin-specific sequences (Table I) was calculated by comparison of the linear portion of these curves with the titration curve for globin mRNA (Figure 2B, cDNA/LH). Globin-specific sequences represent 0.0009-0.0013% (9-13 ppm) of the Hg-transcripts from adult reticulocyte chromatin, and 0.0010-0.0011% (10-11 ppm) of those from 5 day embryonic erythroid chromatin. The values are at least 4- to 6-fold higher than the 0.00025% (2.5 ppm) in brain transcripts. However, chromatin dissociated in high salt and urea and then reconstituted by the standard gradient dialysis method, failed to show preferential transcription of globin-specific sequences above the level seen with non-erythroid chromatins.

TABLE I

Hybridization of Globin cDNA/LH by Highly Purified Hg-Transcripts

	2 µg of Transcript				4 µg of Transcript[a]			
Chromatin	Net	Back ground	Globin Sequences		Net	Back ground	Globin Sequences	
Brain	0.9%	(0.1%)	5.5pg[b]	2.7ppm	1.3%	(0.4%)	8.1pg	2.0ppm
Brain + RNA	0.8	(1.1)	5.0	2.5	1.2	(0.7)	7.5	1.9
5 day (recon.)	0.4	(1.1)	2.5	1.3	1.0	(1.8)	6.3	1.6
5 day	3.5	(0.8)	21.9	11.0	6.5	(1.2)	40.6	10.2
Adult Retic.	4.0	(0.7)	25.0	12.5	6.0	(1.3)	37.5	9.4

[a]Curves may be starting to plateau at 4 µg, lowering the proportion of globin sequences calculated.
[b]Calculated from the value 16% = 100 pg.

Hg-transcripts were purified by the "super-hot column" procedure and hybridized to cDNA/LH. Values are close averages of duplicate determinations which showed good linearity.

A feature of the hybridization curves in figure 3B is the low level at which they plateau, relative to the 85 to 90% level achieved using globin mRNA. Destabilization of hybrids by mercury substitution was ruled out by the very similar hybridization behavior of duplicate aliquots of erythroid Hg-transcripts before and after demercuration. Increasing the hybridization time had no effect, thus eliminating incomplete hybridization reactions as a possible explanation (results not shown). Another cause could be short transcript size relative to cDNA (see Discussion). To test this hypothesis, transcript size was determined as the molar incorporation ratio of internal ^{14}C-AMP to terminal (γ-^{32}P-ATP and γ-^{32}P-GTP) label (Table II). The average size of Hg-transcripts from 5 day erythroid and adult reticulocyte chromatins is only 25-40% that of globin cDNA.

To test whether the "super-hot column" method eliminates all endogenous contamination resulting from RNA-dependent transcription, Hg-RNA was synthesized from brain chromatin with and without addition of 50 ng of globin mRNA before transcription. The Hg-transcripts were purified in parallel on "super-hot columns", along with the appropriate controls. When these Hg-transcripts and controls were hybridized to the higher specific activity cDNA/LH (with 3.7-fold higher senstivity than cDNA/L), brain Hg-

TABLE II

Estimated Transcript Lengths

Template		
5 day erythroid chromatin	355 268 221	} 281 bases
5 day erythroid chromatin (reconstituted)	257	
Adult reticulocyte chromatin	205 183	} 194
Chick DNA	560	

Transcriptions were conducted for 30 minutes in 250 µl reactions including γ-^{32}P-labelled ATP and GTP. The estimated Hg-transcript lengths (the molar incorporation ratios of internal:external label) was calculated as in Methods.

transcripts were found to contain ∿2.5 ppm of globin-specific sequence, not increased by the presence of globin mRNA during transcription (Figure 4).

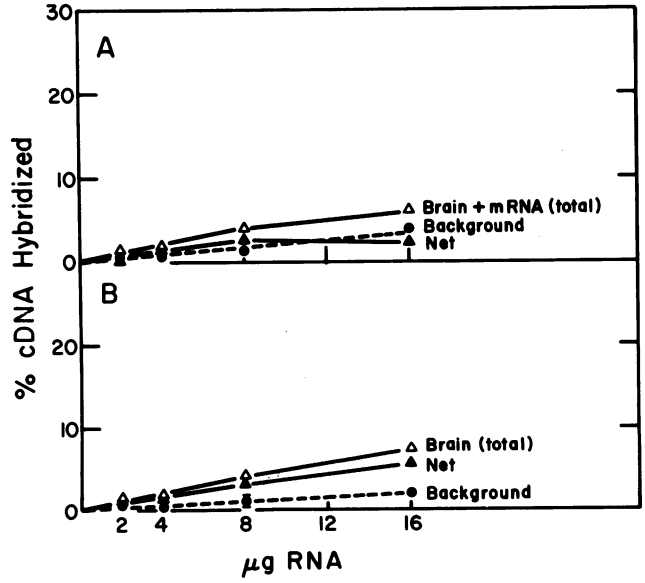


Figure 4. Hybridization titration curves (0.2 ng cDNA/LH) of "super-hot column" purified Hg-transcripts made from (A) 750 µg brain chromatin in the presence of 50 ng globin mRNA, (B) 750 µg brain chromatin.

A similar experiment with "hot column" purified Hg-transcripts gave a value of 17 ppm of globin sequences in Hg-transcripts made with the added mRNA.

DISCUSSION

Hybridization of Hg-transcripts from native chromatin -- Our findings of a 4- to 6-fold higher level of globin-specific sequences in "super-hot column" purified Hg-transcripts from erythroid chromatins than in brain transcripts give only weak support to the notion that transcriptional control is an important mechanism regulating synthesis of specialized proteins in differentiated cells. However, some explanations of the "plateau phenomenon" (see below) could cause underestimation of the level of specific transcription. Further, hybridization between sense and anti-sense globin-specific Hg-transcripts could reduce the observed hybridization of cDNA[11], but this is probably not a major factor. Accurate assessment would require hybridization in cDNA excess.

Our conclusions differ at least quantitatively from those of Zasloff and Felsenfeld[17] in the chick reticulocyte system. They detected some hybridization by the S1 nuclease assay and attempted to bind Hg-transcript:cDNA hybrids to SH-agarose. Finding no such binding, they concluded that globin-specific sequences remaining in highly purified (107° melted) Hg-transcripts contain no Hg and are artifacts. However, their limit of detection was 7 ppm. Our results (10-13 ppm) are only 1.3 to 1.9-fold higher than their limit. Since they estimate that Hg-RNA:cDNA duplexes bind to SH-agarose with only 25 to 35% efficiency, they might not have detected specific transcription at the levels we observe (0.35 x 13 ppm = 4.5 ppm). We did not attempt to bind our hybrids to SH-agarose, since our Hg-RNA, once purified on SH-agarose, binds to a second similar column with only ∿20% efficiency (result not shown). Our experiment with brain Hg-RNA made in the presence of globin mRNA (Figure 4) shows that the "super-hot column" method eliminates all endogenous contamination due to RNA-

dependent transcription and also rules out the possibility of multiple rounds of RNA-dependent transcription.

In addition, our method of chromatin preparation is more gentle, with fewer washes. Lower synthesis of globin sequences by Zasloff and Felsenfeld[17] might reflect loss of regulatory factors. Although our chromatin probably has a greater endogenous polymerase activity, inclusion of 0.4 μg/ml of α-amanitin during transcription did not appreciably reduce our level of globin-specific sequences. This eliminates the possibility that endogenous RNA polymerase II produces globin-specific Hg-RNA transcripts (results not shown).

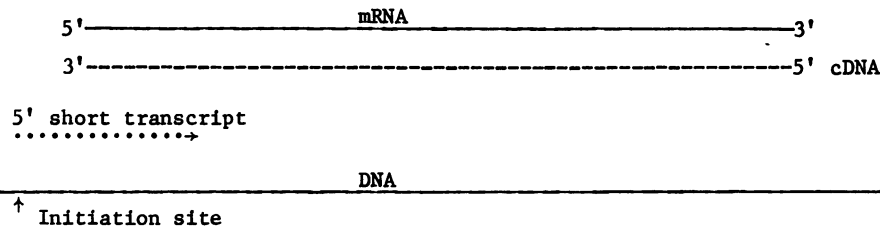
Hybridization of highly purified Hg-transcripts from reconstituted chromatin -- Hg-transcripts from reconstituted embryonic erythroid chromatin shows even less hybridization of globin cDNA than do transcripts from native brain chromatin and at least 9-fold lower than transcripts from native erythroid chromatin. Thus, in our experiments most if not all specificity of transcription is lost during the dissociation and reconstitution process.

Submaximal hybridization plateaus -- A feature of figure 3B is the low hybridization level at which the titration curves plateau, compared with the 85 to 95% achieved using purified globin mRNA. The fact that a real plateau is reached in most cases indicates that the actual hybridization reactions are always carried to completion.

Plateaus may result either because some cDNA molecules are completely hybridized, while others remain unhybridized or because all cDNA molecules are only partially hybridized. Possible explanations of the low plateau values are based on the idea that only a portion of each cDNA molecule is protected by hybridization with mRNA transcript. From our experiments we know that while the cDNA probes used are full length (mostly 700 bases), the RNA transcripts are only about 1/3 of that length. This explanation assumes that all transcripts are initiated at a single site, although that

site could be anywhere within the general area of the globin gene. The actual plateau values are considerably lower than the 33% expected on the basis of these arguments (see Diagram). It is therefore possible that the site for initiation of transcription is either well to the "left" of the DNA sequences which correspond to the globin mRNA, perhaps including control or leader sequences, or near the "right" end of that DNA sequence. In either case only a portion of the RNA transcript would be represented by the globin mRNA sequences and therefore by the cDNA sequences. Another assumption implicit in this explanation is a 1:1 relationship between DNA sequences and globin mRNA sequences, which in view of recent reports[38] on the processing of primary transcripts may not be correct.

Sequence Relationship Between Short Transcripts and Full Length cDNA



If plateaus represent partial hybridization of all cDNA molecules, rather than complete hybridization of a few, analysis of these cDNA:transcript hybrids on hydroxylapatite would show a much higher amount of radioactive cDNA in the DNA:RNA hybrid than was found in the nuclease resistant fraction of the hybrids.

It is also possible that one class of globin genes is inactive in isolated chromatin, perhaps due to the loss of control factors. In that case only those cDNA molecules corresponding to transcribed globin genes would be protected by the transcripts. This explanation assumes that there is no cross hybridization between the cDNA sequences corresponding to different globin mRNA. The use of purified α and β globin cDNA probes

could help to assess this possibility.

The low level of specific transcription in our experiments and its loss under the conditions generally used for chromatin reconstitution make the chromatin-E. coli RNA polymerase system unattractive for the study of factors controlling specificity of transcription, at least for single-copy genes. New conditions must be found allowing synthesis of transcripts approaching the length of the in vivo products. The use of homologous enzymes, less purified chromatin, addition of nucleoplasmic and/or cytoplasmic factors and transcription in isolated nuclei should be considered.

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39 Abbreviations:

BSA	bovine serum albumin
EDTA	ethylenediaminetetraacetic acid
Hg-UMP	5-mercuri-UMP
Hg-RNA	RNA containing Hg-UMP
SDS	sodium dodecyl sulfate
cDNA/U2 cDNA/U3	} two different preparations of unfractionated cDNA
cDNA/L	preparation of full-length cDNA
cDNA/LH	preparation of full-length cDNA of higher specific activity
cDNA/SH	dtto, short cDNA
Hg-CT-RNA	Hg-transcript from calf thymus chromatin.