

## RNA aggregation during sulfhydryl-agarose chromatography of mercurated RNA

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ABSTRACT

Isolation of newly synthesized mercurated RNA transcripts by chromatography on sulfhydryl-agarose has recently been used to reduce contamination by endogenous RNA derived from the chromatin template. We show that substantial RNA aggregation occurs during standard isolation procedures, causing significant retention of endogenous (unmercurated) RNA on sulfhydryl-agarose. We describe methods to reduce substantially this problem and discuss the implications of our findings for interpretation of previous hybridization and transcription experiments.

INTRODUCTION

In vitro transcription of chromatin offers an attractive method for examining control factors involved in the regulation of eukaryotic gene expression, since the conditions of transcription can be modulated more readily than with intact cells or nuclei. Many groups have reported transcription of globin genes from erythroid chromatin.<sup>1-10</sup> Interpretation of many such studies is hindered by contamination of the transcript with endogenous globin RNA sequences indistinguishable from newly made transcripts by nucleic acid hybridization. Accurate determination of the hybridization background caused by such sequences is difficult and open to question.<sup>6,24</sup> A simple solution to these difficulties uses mercurated nucleotides for in vitro transcription; newly synthesized transcripts are isolated by chromatography on sulfhydryl-agarose.<sup>11,12</sup> We now report that standard isolation methods allow RNA aggregation, causing significant contamination of mercurated transcript with endogenous RNA even after SH-agarose chromatography. We describe disaggregation procedures leading to greatly reduced levels of contamination which can now be determined accurately.

### MATERIALS AND METHODS

All radiochemicals and scintillants were purchased from New England Nuclear Corp., Boston, MA.

Preparation of chromatin -- Circulating 5 day embryonic chick erythroid cells<sup>25</sup> were washed in Hanks-BSS-1%<sup>26</sup> bovine serum albumin, then lysed in 0.15% Triton X-100 in 0.25 M sucrose TKM (0.05 M Tris-HCl, pH 7.6, 0.025 M KCl, 0.005 M MgCl<sub>2</sub>) by three strokes with the Dounce homogenizer, B Pestle. Nuclei were sedimented, washed in 0.25 M sucrose-TKM and in 0.14 M NaCl - 5 mM MgCl<sub>2</sub> - 0.05 M Tris-HCl, pH 7.2, and pelleted through 1.0% Triton X-100 - 3.3 mM MgCl<sub>2</sub> - 1.6 M sucrose at 40,000 x g in an IEC SB-283 rotor. The pellet was lysed<sup>28</sup> in 1 mM EDTA - 1 mM Tris, pH 7.9-12.5% glycerol with a Potter-Elvehjem homogenizer, centrifuged at 15,000 x g, swelled in glass distilled water and sheared for 1 minute at setting 6 in a Sorvall Omnimix. All steps were carried out at 4°C.

Our chromatin isolation method avoids the usual extensive washing, since we and others have found that substantial amounts of chromosomal protein are lost in the exhaustive washings required to give "clean" chromatin.<sup>21,27</sup> Many of these lost proteins may well play an important role in regulation.<sup>22</sup> However, our chromatin consequently retains more endogenous RNA.

Preparation of globin mRNA and cDNA -- Globin mRNA from adult chicken reticulocytes was purified as described by Chan et al.<sup>13</sup> except that an oligo-DT cellulose affinity chromatography step was added.<sup>23</sup> In this and all other experiments nucleic acids were precipitated by adjusting to 0.2 M NaCl adding 2.5 volumes of absolute ethanol, and precipitating at 15,000 x g in a Sorvall HB-4 rotor after 12 hours at -20°C. The cDNA mix (200 µl) contained 1 mM each of dATP, dGTP and dTTP, 0.1 mM <sup>3</sup>H-dCTP (sp. act. 8.8 Ci/mmmole), 40 µg/ml Actinomycin D, 10 µg/ml (oligo-dT)<sub>10-13</sub> (PL Biochemicals, Milwaukee, Wisconsin), 50 µg/ml globin mRNA, 50 mM Tris-HCl, pH 8.3, 60 mM NaCl, 10 mM dithiothreitol, 6 mM MgCl<sub>2</sub>, and 1450 units/ml reverse transcriptase, kindly supplied by Dr. James Beard of Life Sciences, Inc., Miami, Fla. After 2 hours at 37°C, the reaction was adjusted to 0.3 M NaOH, incubated for 30 minutes at

95°C, neutralized, and desalted on G-50 Sephadex in 0.1 M  $\text{NH}_4\text{HCO}_3$ . cDNA-containing fractions were pooled, lyophilized, and stored at -40°C in 5 mM EDTA, pH 7.0.

Preparation of 5-Hg-UTP and sulfhydryl agarose -- HgUTP<sup>26</sup> was prepared as described by Dale *et al.*<sup>11</sup> and purified past the Sephadex G-10 desalting step. The product was characterized as 5-Hg-UTP by its increased  $\text{OD}_{\text{max}}$  and its behavior on thin layer cellulose chromatography in ethanol-1 M ammonium acetate (1:1) with and without mercaptoethanol.<sup>11</sup> Sulfhydryl Bio-Gel A5m was prepared by the method of Cuatrecasas<sup>14</sup>; the level of substitution was 2.45  $\mu\text{mol/ml}$  using Ellman's reagent.<sup>15</sup> Columns were stored in 0.25 M 2-mercaptoethanol - 50 mM Tris-HCl, pH 7.9 - 0.1% SDS - 2 mM EDTA, then washed extensively with 0.1% SDS - 0.01 M Tris-HCl, pH 7.9 just before use.

In vitro transcription -- *E. coli* RNA polymerase was prepared by the method of Burgess<sup>16</sup> using the double agarose column procedure to retain sigma factor activity. Two additional high salt sucrose gradients were used, yielding phosphorylase-free enzyme with a specific activity >1000 units/mg. 750  $\mu\text{g}$  of chromatin (DNA as  $\text{OD}_{260}$ :1 mg/ml = 22) was transcribed for one hour at 37°C in 6 ml reactions containing 0.4 mM each of UTP, GTP, CTP, 5-Hg-UTP, and 8-<sup>14</sup>C-ATP (sp. act. 1  $\mu\text{Ci/ mole}$ ) or <sup>3</sup>H-ATP (sp. act. 0.42 mCi/mmol); 0.05 M Tris-HCl, pH 7.9; 0.15 M NaCl; 0.01 M 2-mercaptoethanol; 6 mM  $\text{MgCl}_2$ ; 1.6 mM  $\text{MnCl}_2$ ; and 300 units RNA polymerase. The ratio of polymerase to chromatin was kept low to reduce transcription of the minus strand.<sup>17,18</sup> In some experiments native calf thymus DNA (Sigma Chemical Co., St. Louis, MO) was used as template to prepare mercurated or unmercurated calf thymus transcripts.

Purification of transcripts by SH-agarose affinity chromatography -- The following procedure gave maximal binding to the SH-agarose with minimal aggregation. The reaction mix was brought to 1% SDS - 0.2 M NaCl - 5 mM EDTA and extracted once with phenol-chloroform-isoamyl alcohol (1:1:0.01) and once with chloroform-isoamyl alcohol alone. The second nonaqueous phase was re-extracted with 2.0 ml of the same buffer without SDS. The pooled aqueous phases were precipitated and dissolved in 0.9 ml of 15 mM  $\text{MgCl}_2$  - 2.5 mM EDTA - 0.025 M Tris-HCl, pH 7.5

containing 20 µg of Worthington DNase I (code DPFF), incubated with stirring for 30 minutes at 37°C, and brought to 1% SDS. The sample was dialysed for 48 hours against 100 volumes of 25 mM Tris-HCl - 2 mM EDTA - 0.1% SDS at room temperature; the dialysate was changed once to fresh buffer. The dialysis tubing (8000 MW cut off from Arthur H. Thomas, Philadelphia, PA) was boiled in several changes of 1 mM EDTA over at least 4 hours. Dialysis improves binding efficiency to the SH-agarose column from 50-70% to >90%. Unincorporated nucleotides were removed by chromatography on G-50 Sephadex in the same buffer. The excluded material was precipitated with ethanol and dissolved in 1% SDS - 2 mM EDTA - 10 mM Tris-HCl, pH 7.9, at a concentration of about 25 µg RNA/ml, heated for 30 minutes at 70°C, and applied to a jacketed SH-agarose column equilibrated in the same buffer at 55-60°C. Sample was applied at about 0.2 ml/min. The column was washed with 0.1% SDS - 10 mM Tris - 2 mM EDTA at 55-60°C. The temperature was reduced to ambient and washing continued with water, 50% DMSO - 5 mM Tris, and water. The bound fraction was eluted in about two column volumes of 0.25 M 2-mercaptoethanol - 50 mM Tris - 0.1% SDS - 2 mM EDTA and precipitated in ethanol without added carrier.

Hybridization reactions -- were conducted by the titration method.<sup>7</sup> Mixtures of RNA and cDNA were lyophilized in 6 x 60 mm siliconized glass tubes, dissolved in 6 µl of hybridization buffer (0.5% SDS - 25 mM HEPES, pH 7.1 - 0.5 N NaCl - 5 mM EDTA) and sealed into 5 µl Rochester Boralex microcaps. These were incubated at 68°C for 48 hours and then emptied into 1 ml of nuclease buffer (0.1 M sodium acetate, pH 4.5, 1 mM ZnSO<sub>4</sub>, and 10 µg/ml boiled calf thymus DNA) in 12 x 75 mm polypropylene tubes. One half of this sample was added to 0.5 ml of the same buffer containing 310 units/ml S1 nuclease (E.C. 3.1.4.21) and 20 mM 2-mercaptoethanol. After incubation of both samples at 45-50°C for one hour, each tube was brought to 0.5 M NaOH - 5 mM EDTA and incubated at 50°C for two hours to hydrolyze RNA. Tubes were then chilled and 200 µl of 1 mg/ml tRNA added, followed immediately by 3.5 ml of 10% TCA containing 5 mM sodium pyrophosphate. Samples stood on ice for 15-30 minutes before filtration on GF/C glass fiber filters (Whatman, Clifton,

NJ) and washing with 15 ml of 2% TCA-0.5 mM pyrophosphate and 95% ethanol. Filters were dried and incubated with 0.31 ml Protosol in glass vials capped with polyethylene discs for two hours at 50°C; 10 ml of 0.4% Omnifluor-toluene was added. Percentage hybridization was measured as  $\frac{\text{dpm incubated} + \text{S1}}{\text{dpm incubated} - \text{S1}} \times 100\%$ , after subtraction of the appropriate reagent blanks. This laborious procedure produced virtually 100% solubilization of cDNA with 37% counting efficiency in a Beckman LS-230 liquid scintillation counter. Use of BSA or crude yeast RNA as carrier reduced the counting efficiency considerably due to decreased solubilization.

Hybridizations were performed in 5 mM EDTA, as suggested by Crouse *et al.*<sup>10</sup> to reduce the thermal stability effects of mercury on the hybridization reaction. We found it necessary to include 10 mM 2-mercaptoethanol in the S1 nuclease buffer to eliminate inhibition of S1 nuclease by mercury. However, each lot of mercaptoethanol must be tested in a control reaction, as we found that four out of five lots from various suppliers inhibited the nuclease reaction (and also transcription).

## RESULTS

### Binding of Hg-RNA transcripts to sulfhydryl agarose --

Table I shows that mercurated transcripts bind well to the column and are quantitatively eluted with 0.25 M mercaptoethanol, while unmercurated RNA is apparently not bound. However, if Hg-<sup>3</sup>H-RNA is extracted together with unmercurated <sup>14</sup>C-RNA and the mixture applied to the column after the usual purification procedure,<sup>12</sup> about 10% of the <sup>14</sup>C-RNA is retained by the column and eluted with Hg-<sup>3</sup>H-RNA (Table I, line 3).

### Aggregation of endogenous RNA with mercurated transcripts --

Mercurated calf thymus <sup>14</sup>C-RNA shows no hybridization with globin cDNA if isolated alone, but shows substantial hybridization if coextracted with 750 µg of erythroid chromatin prior to SH-agarose chromatography (Table II, lines 1 and 2). Globin RNA sequences are present in erythroid chromatin; they presumably aggregate with the Hg-<sup>14</sup>C RNA transcripts and accompany them through the SH-agarose chromatography, causing the observed hybridization. Various procedures reduce the level of contamination, as shown in Table II, where the averages of duplicate

TABLE I

Binding of RNA Transcripts from Calf Thymus DNA to SH-agarose

Experiment	RNA Transcripts	% cpm applied	
		Unbound	Bound
1	Hg-RNA- <sup>3</sup> H	9.5	90.1
2	RNA- <sup>14</sup> C	99.4	<0.3
3	RNA- <sup>14</sup> C and	85.2	10.9
	Hg-RNA- <sup>3</sup> H	9.2	94.3
4	RNA- <sup>14</sup> C and	>99.6	<0.4
	Hg-RNA- <sup>3</sup> H	23.1	83.5

Transcripts from calf thymus DNA were prepared as described in Materials and Methods until the gel filtration step. In experiments 3 and 4, the Hg-<sup>3</sup>H-DNA was mixed with half its weight of unmercurated <sup>14</sup>C-RNA transcript before extraction. In experiments 1-3, an SH-agarose column was equilibrated at room temperature with 100 mM Tris-HCl, pH 7.9 - 0.1% SDS - 2 mM EDTA and washed extensively in the same buffer before elution. In experiment 4, the "hot" column was run as described in Materials and Methods. Total percentage for <sup>3</sup>H-RNA in experiments 3-4 is >100, probably due to error in calculation of spill and quenching.

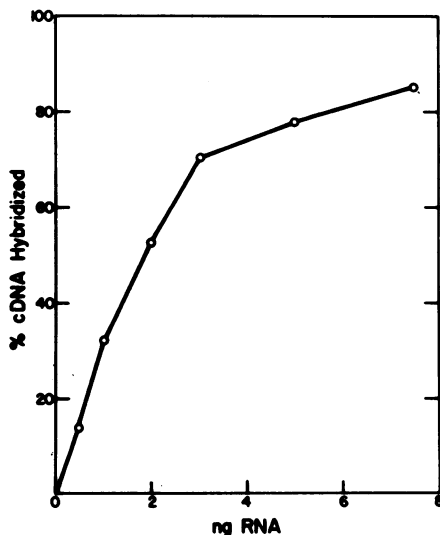


Figure 1. Saturation hybridization curve of adult chicken globin cDNA vs. mRNA from which it was made. Varying amounts of mRNA were hybridized to 0.8 ng each of cDNA and S1 nuclease resistance of the cDNA determined as described in Materials and Methods.

Table II  
Separation of Hg-RNA Transcripts from Contaminating Globin RNA Sequences

Sample	Application Buffer		Temperature Sample	Temperature Column	% cDNA Hybridized* by 5 µg of eluted <sup>14</sup> C-RNA	
	Tris	SDS				EDTA
1. CT Hg- <sup>14</sup> C-RNA**	100 mM	0.1%	50 mM	RT***	RT	0.45 ± 0.1 (range)
2. CT Hg- <sup>14</sup> C-RNA plus 5D Chromatin	100 mM	0.1%	50 mM	RT	RT	54.1
3. "	10 mM	1.0%		60°	RT	11.4 ± 0.1
4. "	10 mM	1.0%	8 M	60°	RT	7.4 ± 0.5
5. "	10 mM	1.0%	2 mM	60°	55°	3.2 ± 0.1
6. "	10 mM	1.0%	2 mM	70°	60°	1.3 ± 0.1
7. "	10 mM	1.0%	2 mM	70°	60°	1.7 ± 0.2

\*1.0 ng of purified globin mRNA gives 32% hybridization under our conditions. (See Figure 1.)

\*\*CT Hg-RNA is mercurated RNA synthesized from native Calf thymus DNA with E. coli RNA polymerase.

\*\*\*Room Temperature.

After sample application was complete, columns were washed with application buffer, water, 50% DMSO - 5 mM Tris, water, and 50 mM Tris-0.1% SDS-5 mM EDTA prior to elution in the final buffer which contained 0.25 M 2-mercaptoethanol. Where heated columns were used, the temperature was reduced to room temperature during the first water wash. Where indicated, 750 µg (as DNA) of 5 day embryonic chick erythroid chromatin was mixed with about 150 µg CT Hg-RNA, phenol extracted and treated as described in materials and methods up to the SH-agarose chromatography step.

values are given for the 5  $\mu$ g point on the hybridization titration curve of each experiment. Contamination, assayed by hybridization, was reduced about 5-fold by the procedure of Crouse *et al.* (Table II, line 3)<sup>10</sup>: the sample was heated in low ionic strength buffer before application to an SH-agarose column at room temperature and the column washed with water and 50% DMSO before elution. As expected, the unbound material (5  $\mu$ g) still saturated 0.8 ng of globin cDNA, indicating that heat degradation of RNA is not responsible for the difference (result not shown). A further small decrease in contamination resulted from the inclusion of 8 M urea during the same procedure (Table II, line 4). We obtained an acceptable (35-fold) reduction of contamination by using the Crouse procedure with a jacketed column maintained at 55-60°C until the DMSO wash (lines 5-7). This "hot column" method gave a similar reduction of contamination in the mixing experiment (Table I, line 4).

However, use of SH-agarose columns at elevated temperatures reduced binding efficiency for Hg-RNA from >90% to 70-85%, and lower if columns are reused; there are fewer available SH groups as determined with Ellman's reagent. This problem increases with resin age.

Room temperature sample application in 50 or 100% DMSO - 10 mM Tris HCl to Affigel 401 (Bio-Rad, Rockville Centre, NY) did not appreciably reduce aggregation, while application in 90% formamide abolished binding (results not shown).

Comparison of Table II with Figure 1 shows that eluted calf thymus Hg-RNA (5  $\mu$ g) carries with it 0.05 - 1.6 ng (0.001%-0.03%) of endogenous globin sequences, depending on the disaggregation method used. These results cannot be attributed to our Hg-UTP or resin, since similar results are obtained with Affigel 401 and with Hg-UTP kindly given us by Dr. Gray Crouse.

### DISCUSSION

Our experiments call into question the quantitation of results previously reported in which Hg-RNA transcripts were purified by SH-agarose chromatography. Experiments 3 and 4 of Table I are analogous to transcription reactions. The result indicates that contamination with endogenous globin sequences



contributes substantially to hybridization of mercurated erythroid transcripts isolated under standard conditions (results not shown). Some studies have checked for contamination only in terms of "non-specific adsorption" of non-mercurated RNA passed alone through the column, ignoring the possibility of aggregates "carrying" unmercurated sequences along with the mercurated RNA.<sup>20</sup> Others mix two purified RNA samples just before application, rather than prior to phenol extraction.<sup>12,18</sup>

Our initial level of contamination (Table II, line 2), as determined by hybridization, is about ten times that reported by Crouse *et al.*<sup>10</sup> for similar mixing experiments with transcripts from adult chicken reticulocyte chromatin. This does not necessarily mean that we generate higher levels of aggregation. It is probably due to the higher level of endogenous RNA in our chromatin preparations and to the higher proportion of globin sequences in embryonic erythroid nuclear RNA as compared to that from adult reticulocytes.<sup>19</sup> These considerations suggest caution when comparing experiments using transcripts from whole cells or nuclei, since the latter have available for aggregation a much higher degree of endogenous RNA, containing both globin specific and other sequences. This could partially explain the 20-fold higher level of immunoglobulin-specific sequences in transcript from MOPC 66.2 nuclei as compared to those from the corresponding chromatin.<sup>20</sup>

In order to determine the amount of RNA transcribed *in vitro*, free from contamination with endogenous RNA, various precautions must be taken. The extent of aggregation and its effect in terms of hybridization vary with the individual system. "Mixing" control experiments are required for each chromatin or nuclear preparation used for transcription with mercurated nucleotides. If necessary, appropriate disaggregation procedures should be incorporated into the SH-agarose chromatography protocol.

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