USE OF MERCURY-SUBSTITUTED RIBONUCLEOSIDE TRIPHOSPHATES CAN LEAD TO ARTEFACTS IN THE ANALYSIS OF IN VITRO CHROMATIN TRANSCRIPTS

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Summary: Serious complications arise in the use of mercury-substituted nucleoside triphosphates to study chromatin transcription in vitro. In studying the transcription of duck reticulocyte chromatin by E. coli RNA polymerase, we find that endogenous globin RNA contaminates the "purified" de novo transcript, giving rise to false high levels of globin sequence abundance. The anomaly arises because the endogenous globin RNA acts as template for the polymerase, and forms a duplex with the Hg-substituted complementary strand that is made. Subsequent selection of Hg-containing RNA results in purification of the endogenous globin sequences.

Mercury-substituted ribonucleoside triphosphates have recently been used as substrates for RNA polymerase in studies of transcription in nuclei and chromatin in vitro (1-4). It has been supposed that mercury substitution permits ready fractionation of newly synthesized RNA from endogenous RNA. We wish to report here, however, that in transcription experiments using duck reticulocyte chromatin, endogenous globin RNA in fact co-purifies with mercury-substituted RNA, leading to gross overestimates of the extent of de novo synthesis of the globin sequence. The anomaly arises because the endogenous globin RNA acts as a template for E. coli RNA polymerase, leading to a duplex in which one strand is mercury labelled. Subsequent procedures to purify mercury-labelled RNA also result in purification of endogenous globin RNA.

Materials and Methods: 5-Hg-UTP was synthesized from UTP (5). Thiol agarose (6) was prepared using Bio-Rad A15 as support. Duck globin RNA was isolated from reticulocytes as described by Gould and Hamlyn (7). Duck DNA and acid extracted histones were made from erythrocyte chromatin (8). RNA polymerase (1200 units/mg was purified (9) from E. coli K12 (strain furnished by Drs. R. Bird and J. I. Tomizawa). S1 nuclease was prepared by the method of Vogt, through SP-Sephadex chromatography (10). ^{3}H-DNA complementary to globin RNA was synthesized as described (11), and had an activity of 0.8 X 10^{7} cpm/µg DNA. Reticulocytes and chromatin were prepared as described previously (11), except that the chromatin (protein:DNA ratio 1.5 g/g) was not sheared.

Globin RNA content of transcripts was determined in cDNA excess (12,13) by hybridization in 10 µl samples containing 0.2 M Na phosphate pH 6.8, 0.5% SDS,[1]

[1] Abbreviations: SDS, sodium dodecyl sulfate; EDTA, ethylene diamine tetraacetic acid; cDNA, DNA complementary in sequence to globin RNA.

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TABLE 1

Recovery of Globin RNA in *in vitro* Transcripts from Duck Reticulocyte Chromatin

Transcription Reaction	Total RNA Synthesized (μg)	ng Globin cDNA hybridized/ μg RNA Transcript	Total Globin RNA Detected (ng)
1. Complete	5.05	0.16	0.81
2. Minus RNA Polymerase[a]	0.02	0.0	0.0
3. Complete + actinomycin D (10 μg/ml)	0.59	1.19	0.70
4. Complete, + rifampicin (0.16 μg/ml)	0.75	0.14	0.10

Mercury-substituted RNA was transcribed from duck reticulocyte chromatin with Hg-UTP as sole UTP source under the conditions of the standard transcription reaction described in Methods. (ng cDNA hybridized/μg RNA transcript) was determined after adsorption on thiol-agarose as outlined in Methods. Total globin RNA is calculated from the globin RNA content of the RNA and the total amount of RNA synthesized.

[a] In a similar experiment, the reaction mixture was incubated in the absence of polymerase, but 5.1 μg of Hg-RNA (transcribed from *E. coli* DNA) were added just before the thiol-agarose step. 0.01 ng of globin RNA was detected.

0.1 mM EDTA, 7.5 μg yeast tRNA, 0.25 ng ^{3}H-cDNA, and 0.1-1.0 μg transcript RNA. The mixture was sealed in capillaries, heat denatured, incubated at 68° for 15-24 hours, then diluted into 0.5 ml buffer (50 mM NaCl, 30 mM sodium acetate pH 4.5, 1 mM $ZnSO_4$, 20 μg/ml denatured calf thymus DNA) and treated with S1 nuclease (40 U, 1 hr, 45°). The mixture was acid precipitated, filtered (Whatman GF/C), dried and counted.

RNA was synthesized in 1.5 ml reaction mixtures containing: 10 mM Tris, pH 8, 20 mM β-mercaptoethanol, 0.2 mM each ATP, GTP and CTP, 0.1 mM 5-Hg-UTP, [α-^{32}P]UTP (3-5 cpm/pmol), 250 μg chromatin, 1 mM $MnCl_2$ and 40 units *E. coli* RNA polymerase. After incubation (1 hr, 37°), 25 μg DNase I (Worthington, electrophoretically pure) were added and incubation continued for 4 minutes. The mixture was then made 1% in SDS and 0.5 M in NaCl, heated (65°, 15 min), and deproteinized with phenol-chloroform-isoamyl alcohol (1:0.5:0.02). RNA was then ethanol precipitated at 20°. After collection and drying, the RNA was dissolved in 1 ml of TNS buffer (50 mM Tris, pH 8, 50 mM NaCl, 1% SDS) and heated at 65° for 15 min. 0.6 ml thiol-agarose was added and shaken at 24° for 2 hrs. The gel was repeatedly washed in TNS buffer by centrifugation and resuspension, then packed in a 0.7 cm diameter column, washed again, and the RNA eluted with 1.5 ml TNS containing 0.1 M β-mercaptoethanol. After addition of 0.34 ml of 1 M sodium acetate, pH 6, and 300 μg yeast RNA, the RNA was ethanol precipitated as above.

TABLE 2

Recovery of Exogenous Globin RNA in Transcripts from Reconstituted Chromatin

Globin RNA Added to Reconstitute (ng)	Total RNA Synthesized (µg)	ng Globin c DNA hybridized/ µg RNA Transcript	Globin RNA Detected (ng)
0	7.0	0.005	0.035
3	6.0	0.04	0.24
3*	6.5*	0.00	0.00

0.25 mg duck DNA was combined with 0.25 mg duck erythrocyte acid-extracted histones in 1 ml containing 2 M NaCl, 5 M urea, 10 mM Tris·HCl pH 8.0, and 20 mM β-mercaptoethanol. 3 ng globin RNA were added where shown, and the mixtures then reconstituted following the standard procedure (8). * Refers to a transcription reaction from which RNA polymerase was omitted and 6.5 µg of *E. coli* Hg-RNA added as carrier as in footnote a, Table 1.

Results and Discussion: In our first experiments, we transcribed duck reticulocyte chromatin with *E. coli* RNA polymerase, using Hg-UTP instead of UTP as substrate, isolated the RNA product on thiol-agarose, and determined the globin RNA content of this product by hybridization in cDNA excess (Methods). Globin-specific sequences are readily detected in the RNA that adheres to thiol-agarose (Table 1). The appearance of globin RNA sequences is dependent on the action of RNA polymerase (Table 1). If rifampicin is added to the reaction mixture, the abundance of globin RNA sequences is markedly reduced; if the enzyme is omitted, no globin RNA sequences are detected.

Although these experiments might appear to indicate that a large amount of globin RNA is being transcribed from DNA *de novo*, that is not the case: the globin RNA being detected is in fact endogenous message. To demonstrate that endogenous message contaminates our "purified" RNA product, we carried out transcription studies using reconstituted chromatin (8) made from duck DNA and acid-extracted erythrocyte histones. Prior to reconstitution globin mRNA was added to the mixture When the reconstitute was transcribed using Hg-UTP, and the transcript purified on thiol-agarose, globin RNA sequences appeared in the product (Table 2). If globin mRNA was not added before transcription, globin sequences were absent from

the product. This appearance of globin RNA sequences also depends upon RNA polymerase activity. We have recently repeated these experiments (data not shown) using reconstituted "chromatin" in which E. coli DNA replaces duck DNA. Again, if globin mRNA is added before transcription, it is detected in association with mercury-containing transcript. In this case, it is clear that none of the globin RNA could have been transcribed from DNA. It appears likely that the same process occurs when normal chromatin is transcribed using Hg-UTP: as the data in Table 1 show, actinomycin does not significantly reduce the amount of globin RNA in thiol-agarose-bound product, even when overall rates of transcription are reduced by 95%. This suggests that DNA is not the template.

We believe that endogenous globin RNA is carried into the thiol-agarose bound product because it has formed a duplex with a newly synthesized RNA strand containing mercury. This newly synthesized RNA strand must be complementary to the globin RNA sequences, and cannot have arisen from transcription of DNA, as indicated by experiments described in the preceding paragraph. It seems reasonable to suppose that the template for the new strand is the endogenous globin sequence: it is well known (14) that RNA polymerase can use RNA as a template, and indeed this process has been used recently to synthesize RNA complementary to globin RNA (15,16). Although the chromatin transcription experiments reported here were carried out in the presence of Mn^{++}, similar results (data not shown) were obtained when Mg^{++} replaced Mn^{++}, and under a variety of ionic strength conditions. Our results are thus unlikely to have arisen from an accidental choice of unique conditions that favor the use of RNA templates.

If globin RNA sequences are trapped on thiol-agarose only because they have formed duplex structures with Hg-substituted complementary strands, it should be possible to abolish the effect by denaturing the duplex before the transcript is applied to thiol-agarose. The results of such an experiment are shown in Table 3. Compared to an unheated control, only about 1% of the globin RNA sequences are retained on thiol-agarose after heating, while in both control and experiment about 80% of the mercurated RNA is retained, showing that heating does not

TABLE 3

Fractionation of Globin RNA Sequences on Thiol-Agarose Following Heat Denaturation of Transcript RNA

RNA Sample	% Total Input RNA Retained on Thiol-Agarose	% Globin-specific RNA Retained on Thiol-Agarose
RNA Transcript	80	82
RNA Transcript, heat denatured	78	1

RNA was isolated as described in Methods. 6 μg of purified transcript were dissolved in 2 ml 50 mM Tris·HCl pH 8.0, 50 mM NaCl, 1% SDS. One half the sample was heated at 107° for 10 minutes, the other untreated. Each sample was then purified on thiol-agarose (Methods).

affect the latter process. In a subsequent paper (Zasloff and Felsenfeld, in preparation) we will show that the globin RNA is present in the unbound fraction (if the transcript is heated) and that the newly synthesized, Hg-substituted RNA contains sequences complementary to globin RNA. The synthesis of the "anti-sense" strand, and its pairing with globin RNA sequences, are processes that will presumably occur in the absence of Hg-substituted precursors, and could complicate interpretation of *in vitro* transcription experiments, whether or not mercury label is used.

We also wish to call attention to another difficulty in the use of the mercury substitution method: fully mercurated RNA is quite inefficient in the formation of S1 nuclease-resistant hybrid with its complementary DNA. Beebe and Butterworth (3) have already reported that ribosomal RNA transcribed from rat liver nucleoli does not hybridize efficiently to rat DNA if UTP is fully replaced by Hg-UTP. We have confirmed this observation using Hg-UTP-substituted RNA transcribed from phage ϕX 174 DNA. Such RNA hybridizes to ϕX 174 DNA only at a very low rate. If the RNA is demercurated, or if Hg-UTP replaces less than 25% of the UTP in the transcription reaction, normal rates of hybridization are restored (Zasloff and Felsenfeld, in preparation).

We have presented evidence in this report that serious misinterpretations

can occur in the analysis of purified in vitro transcripts synthesized using Hg-substituted ribonucleoside triphosphates. Under the usual conditions of the assay, endogenous message is carried into duplex structures with newly synthesized, Hg-substituted RNA, and is detected as though it were itself newly made. In a subsequent paper (in preparation) we will discuss methods for eliminating this potential source of major errors.

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