Analysis of in Vitro Transcription of Duck Reticulocyte Chromatin Using Mercury-Substituted Ribonucleoside Triphosphates†

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ABSTRACT: We have employed mercury-substituted UTP to study the transcription of duck reticulocyte chromatin in vitro by *Escherichia coli* RNA polymerase. We find that the use of this method results in large overestimates of the amount of de novo synthesis of globin-specific RNA sequences. The artefact arises because endogenous globin RNA can serve as a template for the RNA polymerase, resulting in the formation of a duplex product in which one strand is the endogenous message, and the other is the mercury-labeled complementary strand. Subsequent purification of the mercury-substituted RNA on thiol-agarose results in copurification of endogenous globin sequences. We document the details of this mechanism and describe methods which will eliminate the artefact.

The study of the transcription process in eukaryotic systems has been stimulated greatly by the recent introduction of methods which make use of mercury-substituted ribonucleoside triphosphates as substrates for RNA polymerase (Dale et al., 1975; Dale and Ward, 1975). In principle, the presence of the mercury derivative should make it possible to separate newly synthesized RNA from endogenous, unsubstituted RNA. Such a separation would be of great value for studies of transcription in nuclei and chromatin, where a limiting factor in the detection of newly transcribed RNA chains of specific sequence is the presence of large quantities of endogenous RNA with the same sequence.

In a previous paper (Zasloff and Felsenfeld, 1977), we pointed out that the use of the mercury-substitution method can lead to erroneous conclusions about the extent of transcription from DNA templates. We used the method in an attempt to study transcription by *E. coli* RNA polymerase of globin genes in duck reticulocyte chromatin. Transcription led to the appearance of globin-specific RNA sequences that contained mercury as judged by their ability to bind to thiol-agarose columns. However, the newly synthesized sequences were not transcribed from the DNA template.

In this paper, we examine the transcription process in detail and show that *E. coli* RNA polymerase is using endogenous globin message as a template to make the complementary RNA strand, which can be detected in the transcript population. This newly synthesized, mercury-containing strand forms a duplex with the globin message and, therefore, the purification of mercury-substituted sequences results in copurification of endogenous message. The mercury-substitution method as presently used is thus capable of giving rise to large false positive results, which make it unsuitable for detection of de novo RNA synthesis.

We describe modified methods which eliminate the effects of this artefact, and which should make it possible to use mercury substitution as a tool for detection of newly synthesized RNA.

Materials and Methods

Duck Reticulocyte Chromatin. Pekin ducks (2–4 kg) were made anemic by administration of 1-acetyl-2-phenylhydrazine (11 mg per kg per day, intramuscular injection) in single daily doses for 7–8 days. Following sedation of the birds with sodium pentobarbital (10 mg/kg), blood was collected by cardiac puncture, and immediately stored on ice in a heparinized container at a final heparin concentration of about 10 units/mL. Prior to chromatin preparation a differential count of the erythroid cells in the sample was made from a Wright's stained smear. The routine preparation contained 10–15% early polychromatophilic erythrocytes, 70–80% mid to late polychromatophilic erythrocytes, and about 5% mature erythrocytes, following the histological descriptions of avian erythroid cells by Lucas and Jamroz (1961). Cells are stored at 4 °C, for between 2 and 5 h prior to chromatin preparation.

All operations are conducted at 0–4 °C. Cells were harvested by centrifugation for 5 min at 5000 rpm in the Sorvall SS34 rotor and suspended in 5 volumes of ice-cold PBS[1] buffer (0.14 M NaCl, 0.003 M KCl, 0.001 M $CaCl_2$, 0.01 M sodium phosphate (pH 7.5)). The washing step was repeated four to five times. Cells were resuspended in 5 volumes of 0.25 M sucrose, 10 mM Tris-HCl (pH 8.0), 1 mM magnesium acetate, 5 mM β-mercaptoethanol (buffer A) with a Dounce homogenizer (B-pestle). Nuclei were collected by centrifugation at 10 000 rpm for 5 min in the SS34 rotor. This washing step was repeated until no further significant hemolysis was evident. The nuclear pellet was then resuspended (Dounce A-pestle) in buffer A containing 0.5% Triton X-100 (Packard), harvested by centrifugation as above, and the washing repeated once. The light brown nuclear pellet was resuspended in 5 volumes of 0.25 M NaCl, 25 mM sodium acetate (pH 6.0), 5 mM β-mercaptoethanol, and then centrifuged as above. The pellet was drained, the surface washed gently with 1 mM Tris-HCl (pH 8.0), 5 mM β-mercaptoethanol, and then gently resuspended (Dounce A-pestle) in this buffer. From about 15 mL of packed cells, about 50 mL of chromatin was obtained at a concentration of about 20 A_{260} units/mL. All transcription reactions were run within 2 days of the preparation. The chromatin was stored at 4 °C and used without further shearing. The typical preparation had a protein/DNA ratio of 1.5 g/g.

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[1] Abbreviations used: HgUTP, 5-mercuriuridine 5′-triphosphate; HgRNA, RNA containing HgUMP; R_0t, product of RNA concentration (mol of nucleotide/L) and time (s); $R_0t_{1/2}$, R_0t at which input RNA has undergone 50% hybridization; C_0t, product of DNA concentration (mol of nucleotide/L) and time (s); $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl)aminomethane.

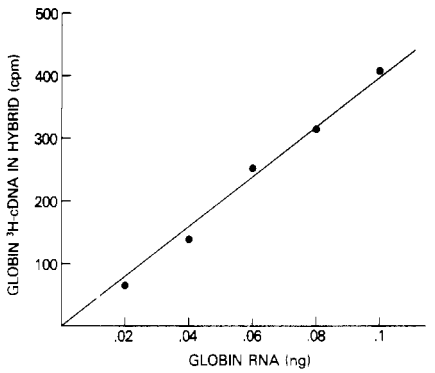

FIGURE 1: Titration of globin cDNA probe with RNA. Hybridization conditions are as described in Materials and Methods. A background of 160 cpm, corresponding to the self-annealing fraction of the cDNA probe (6.7%), has been subtracted.

Duck Globin RNA. Duck globin RNA was isolated by a modification of methods described by Williamson et al. (1971), and Gould and Hamlyn (1973).

Annealing of the purified globin RNA preparation to its complementary [^{3}H]DNA, under conditions of RNA excess, demonstrated a single major kinetic component with a $R_0t_{1/2}$ of 1.2×10^{-3} mol of ribonucleotide $\times$ s L^{-1}.

Synthesis of [^{3}H]DNA Probes. [^{3}H]DNA complementary to globin RNA (cDNA) and to the globin anti-strand sequence ($cDNA^{anti}$) was synthesized with RNA-dependent DNA polymerase under conditions permitting synthesis of double-stranded complementary DNA (Young et al., 1974). The synthetic reaction contained, in 100 μL: 50 mM Tris-HCl (pH 8.3), 10 mM magnesium acetate, 5 mM dithiothreitol, 0.1 mM each of dGTP, dATP, dTTP, 0.1 mM [5-^{3}H]dCTP (New England Nuclear, 25 Ci/mmol), 0.25 μg of oligo(dT)(12–18) (Collaborative Research, Waltham, Mass.), 2 units of RNA-dependent DNA polymerase isolated from avian myeloblastosis virus, and 2 μg of globin RNA. Actinomycin D was omitted from the reaction. The reaction was incubated at 37 °C for 2 h and then quenched by addition of $\frac{1}{20}$ volume of 20% sodium dodecyl sulfate. T7 DNA (100 μg) was added as carrier and the reaction was made 0.3 N in NaOH. Following incubation at 37 °C for 12 h to hydrolyze RNA, the reaction was neutralized with concentrated HCl and applied to a 0.7 $\times$ 30 cm bed of Sephadex G-50 (coarse mesh) in 1 mM Tris-HCl (pH 8.0), 0.1 mM EDTA. Fractions eluting in the excluded volume were pooled. About 300 ng of [^{3}H]DNA was recovered from 2 μg of globin RNA. Self-annealing of the [^{3}H]DNA at 68 °C, under conditions of the standard hybridization assay to C_0t = 0.01, resulted in protection of about 50% of the radioactivity from S1 nuclease digestion.

The complementary DNA strands were fractionated from one another by back-annealing with an excess of globin RNA, followed by separation of single-stranded DNA from DNA hybridized to globin RNA using hydroxylapatite chromatography at 60 °C (Kohne and Britten, 1971). For the back-annealing step, 15 μg of globin RNA was added to about 300 ng of [^{3}H]DNA synthesized above and dissolved in a total of 1.35 mL of 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, with 50 μg of T7 DNA as carrier. One volume of a buffer containing 0.4 M sodium phosphate (pH 6.8), 1% sodium dodecyl sulfate, 2 mM EDTA (buffer H) was added, and the reaction was heated at 100 °C for 3 min and then maintained at 68 °C for 20 h to achieve R_0t = 1.0. The hybridization mix was diluted to 0.14 M sodium phosphate and applied to a 1 $\times$ 2 cm bed of hydroxylapatite (HTP, Bio-Rad) equilibrated in 0.14 M sodium phosphate (pH 6.8), 0.4% sodium dodecyl sulfate, in a jacketed column maintained at 60 °C. Fractions not retained on the column, constituting about 40% of the input radioactivity and representing DNA complementary to globin anti-strand, were pooled. The column was washed with about 10 volumes of buffer. RNA–DNA hybrids were eluted with a buffer containing 0.5 M sodium phosphate (pH 6.8), 0.4% $NaDodSO_4$ at 60 °C. DNA fractions were made 0.3 N in NaOH, warmed at 37 °C for 12 h, neutralized with concentrated HCl, and dialyzed extensively against 1 mM Tris (pH 8.0), 0.1 mM EDTA.

Both cDNA and $cDNA^{anti}$ self-annealed to less than 8–9% at a C_0t of 0.01, demonstrating effective strand separation. Back-annealing of globin cDNA to excess globin RNA proceeded to a limit with more than 90% of the [^{3}H]cDNA in hybrid, with a single kinetic component at $R_0t_{1/2}$ of about 1.13×10^{-3}. In contrast, less than 5% of the $cDNA^{anti}$ annealed to globin RNA at a R_0t of 0.1. Specific radioactivity of the probes, as estimated from the CTP content of chick globin RNA (Williamson, 1974) and the specific radioactivity of the [^{3}H]CTP (as stated by the manufacturer), was about 0.8×10^7 cpm/μg of DNA.

RNA–DNA Hybridization. Hybridization analysis of the globin RNA content of the in vitro transcripts was performed under conditions of cDNA excess (Young et al., 1974; Williamson, 1976). The hybridization reactions contained, in 10 μL, 0.2 M sodium phosphate pH 6.8, 0.5% $NaDodSO_4$, 0.1 mM EDTA, 7.5 μg of yeast tRNA, 0.25 ng of [^{3}H]DNA, and 0.1–1.0 μg of transcript [^{32}P]RNA, or sufficient sample to hybridize up to about 20–30% of the total cDNA output. Components were mixed on a sheet of Parafilm and sealed into 10-μL capillary pipettes (Micropet, Clay Adams). Samples were heat denatured at 107 °C (boiling saturated NaCl) for 5 min, and incubated at 68 °C for 15–24 h. The reactions were diluted into 0.5 mL of S1 nuclease buffer, containing 50 mM NaCl, 30 mM sodium acetate (pH 4.5), 1 mM $ZnSO_4$, and 20 μg/mL of denatured calf thymus DNA (Worthington). Forty units of S1 nuclease was added, and the sample was incubated at 45 °C for 1 h. The digestion was terminated by addition of 50 μg of yeast tRNA, and 0.3 mL of ice-cold 30% Cl_3CCOOH. Samples were kept on ice for 10 min, filtered onto glass fiber filters (GF/C Whatman), dried, and counted using liquid scintillation spectrometry. ^{3}H is counted at about 15–20% efficiency, with crossover from ^{32}P amounting to 1.4%.

To determine the globin RNA content of the chromatin transcript from the amount of [^{3}H]cDNA detected as hybrid in the standard assay, a standard curve was constructed giving [^{3}H]cDNA detected as hybrid as a function of globin RNA in the hybridization reaction. As seen in Figure 1, titration of [^{3}H]cDNA in a standard reaction with increasing amounts of globin RNA results in proportionally more [^{3}H]cDNA detected as hybrid. From the slope of the linear relationship it can been seen that about 3800 cpm of the [^{3}H]DNA probe appears as hybrid per ng of globin RNA in the reaction. This value is used to calculate the amount of globin RNA in a transcript sample from the measured amount of [^{3}H]cDNA hybridized per μg of chromatin transcript.

In Vitro Synthesis and Isolation of Mercury-Substituted RNA. RNA was synthesized in 1.5 mL of reaction mixtures containing 10 mM Tris-HCl, pH 8.0, 20 mM β-mercaptoethanol, 0.2 mM each of ATP, GTP, and CTP, UTP, or 5-HgUTP at 0.1 mM, [α-^{32}P]UTP at a specific radioactivity of 3–5 cpm/pmol, 250 μg of chromatin in a volume of 1 mL of 1 mM Tris (pH 8.0), 1 mM $MnCl_2$, and 40 units of *E. coli* RNA polymerase. Components were added in the order listed. When included, KCl was added to the reaction mixture as a 0.75 M solution prior to addition of enzyme. The reaction was

incubated at 37 °C for 1 h with gentle rocking. The reaction was quenched by addition of 25 μg of DNase I (electrophoretically pure, Worthington) and incubated at 37 °C for 3–4 min. $NaDodSO_4$, 20%, and 5 M NaCl were added to a final concentration of 1% and 0.5 M, respectively, and the suspension was heated at 65 °C for 15 min. The reaction mixture was deproteinized by extraction with 1 volume of water-saturated phenol (distilled under nitrogen) and 0.5 volume of isoamyl alcohol–chloroform (1:24). Following low speed centrifugation, the aqueous phase was carefully removed and the RNA was precipitated overnight at −20 °C, by addition of 0.3 mL of 1 M sodium acetate pH 6.0, and 5 mL of absolute ethanol. The precipitate was collected by centrifugation at 8000 rpm for 20 min in the Sorvall HB-4 rotor. The pellet was drained and dried under a gentle stream of nitrogen. The RNA was resuspended in 1 mL of a solution (TNS buffer) containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1% $NaDodSO_4$, and heated at 65 °C for 15 min. Packed thiol-agarose (0.6 mL) was then added to the RNA solution and shaken vigorously at 24 °C for 2 h. The gel was then washed by repeated resuspension in the above buffer and centrifugation for about 5 min at 5000 rpm in the HB-4 rotor. The gel, resuspended in about 2 mL of buffer, was transferred by Pasteur pipet into a 0.7 × 15 cm column and washed with a further 30 mL of buffer. Samples which are synthesized containing less than about 50% HgU substitution do not bind efficiently under these batch conditions and were consequently applied directly to a 0.5-mL thiol-agarose bed over a 1-h period. Elution of bound RNA was obtained with 1.5 mL of TNS buffer containing 0.1 M β-mercaptoethanol, applied to the column in 0.5-mL aliquots, and collected in a single tube. Sodium acetate (0.34 mL; 1 M; pH 6.0), 300 μg of yeast tRNA, and 5 mL of ethanol were added, and the RNA was precipitated overnight at −20 °C. RNA was centrifuged as above and the pellet dissolved in about 30 μL of buffer H.

In Vitro Synthesis of φX174 RNA. RNA synthesis was carried out in 0.5-mL reactions containing: 1 mM $MnCl_2$, 10 mM Tris-HCl (pH 8.0), 0.15 M KCl, 75 μg/mL bovine serum albumin (Miles, crystallized), 20 mM β-mercaptoethanol, 0.2 mM each of ATP, GTP, CTP, HgUTP from 0.01 to 0.2 mM (the sum of UTP and HgUTP kept constant at 0.2 mM), [5-^{3}H]UTP at a specific radioactivity of about 300 cpm/pmol, 15 μg of φX174 DNA, and 40 units of *E. coli* RNA polymerase. The reaction was incubated at 37 °C for 90 min and then quenched by addition of 15 μg of DNase I. Following an incubation of 30 min at 37 °C, the mixture was made 1% in $NaDodSO_4$, and 0.5 M in NaCl. Yeast tRNA (300 μg) was added and the reaction deproteinized in the way described in the previous paragraph. The aqueous phase was applied to a 0.7 × 30 cm column of Sephadex G-50 (coarse mesh) equilibrated in 0.5 M NaCl, 0.2% $NaDodSO_4$. Fractions in the excluded volume were pooled. Approximately 10 μg of RNA was obtained from each reaction.

Mercury Substitution of Duck Globin RNA. Globin RNA was mercury substituted in a reaction mixture (5 μL volume) containing 0.9 μg of globin RNA, 5 mM sodium acetate (pH 6.0), and 20 mM mercuric acetate. The components were mixed on a sheet of Parafilm and sealed in a 5-μL capillary pipet. The reaction mixture was maintained at 50 °C for 10 h, at which time the reaction was diluted into 100 μL of buffer H containing 10 mg/mL of yeast tRNA, and 10 μg of Hg–[^{32}P]RNA (5 cpm/ng) transcribed from *E. coli* DNA as carrier. The solution was passed over a 0.7 × 1 cm bed of Chelex-100 equilibrated in buffer H, to remove free Hg^{2+}. The column was washed with an additional 100 μL of buffer, and this was pooled with the other fractions. The extent of pyrimidine base mercury substitution is estimated to be between 10 and 30% based on the data for RNA mercuration reported by Dale et al. (1975).

Miscellaneous. HgUTP was synthesized from UTP essentially as described by Dale et al. (1975).

Sulfhydryl-agarose was prepared as described by Cuatrecasas (1970), using Bio-Rad A-15m (100–200 mesh) as the agarose support. The final product, as determined with Ellman's reagent (Ellman, 1959), contained between 2 and 3 μmol of reactive thiol group per mL of packed gel, and was stored in 0.1 M NaCl, 10 mM dithiothreitol, under nitrogen atmosphere.

Duck DNA and erythrocyte acid-extracted histones were purified from duck erythrocyte chromatin as described previously (Camerini-Otero et al., 1976).

E. coli RNA polymerase was purified from a strain of *E. coli* K-12 lacking polynucleotide phosphorylase and RNase A (NT525); the strain was furnished by Drs. R. Bird and Junichi Tomizawa. The purification procedure of Yarbrough and Hurwitz (1974) was followed, modified by substitution of DNA-agarose chromatography for fractionation on DNA-cellulose (Dr. Sue Wickner, personal communication). RNA polymerase was purified through the DEAE-cellulose step. The final preparation was about 50% pure as determined by $NaDodSO_4$ gel electrophoresis (Laemmli, 1970) and contained the σ subunit in approximately equimolar amounts with the core components as judged by staining of the gel with Coomassie brilliant blue. The specific activity of the RNA polymerase was about 1200 units/mg of protein in the standard assay (Yarbrough and Hurwitz, 1974) using duck DNA as template. One unit of activity is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of [^{3}H]UTP into trichloroacetic acid precipitable material in 20 min under standard assay conditions.

RNA-dependent DNA polymerase was purified from avian myeloblastosis virus as described by Verma and Baltimore (1974). Viremic chicken plasma was a generous gift of Dr. French Anderson and was used as the starting material for virus isolation. One unit of activity is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of [^{3}H]TTP into Cl_3CCOOH-precipitable material in 15 min under conditions of the standard assay (assay II, Verma and Baltimore, 1974).

S1 nuclease was purified from crude α-amylase powder obtained from *A. oryzae* (Sigma) by the method of Vogt (1973), through SP-Sephadex chromatography. The final product had a ratio of single to double-stranded DNase activity exceeding 600, assayed at 45 °C under standard conditions. The standard assay for nuclease activity contained, in 0.2 mL, 30 mM sodium acetate (pH 4.6), 50 mM NaCl, 1 mM $ZnSO_4$, 150 μg/mL heat denatured calf thymus DNA (Worthington). Enzyme was added, and the reaction mixture was incubated at 45 °C for 10 min. Reactions were quenched by adding 1.5 mL of ice-cold 2 M NaCl, 2 M perchloric acid. After 10 min at 0 °C the samples were centrifuged at 10 000 rpm for 5 min in the Sorvall HB-4 rotor. Acid-soluble nucleic acid was determined from A_{260} of the supernatant (Camerini-Otero et al., 1976). One unit of S1 nuclease activity is that amount of enzyme that solubilizes 1 μg of DNA per min under conditions of the assay.

Results

Globin RNA Sequence Abundance in in Vitro Chromatin Transcripts. Prior estimates of de novo synthesis of globin sequences have relied on the detection of an increase of globin RNA above the endogenous level, by hybridization of RNA

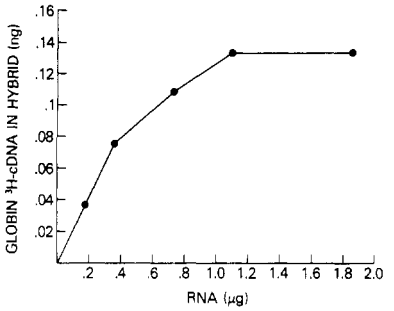

FIGURE 2: Titration of globin [^{3}H]cDNA probe with mercury-substituted in vitro transcript from reticulocyte chromatin. Hybridization conditions are as described in Materials and Methods, and background subtractions are as described for Figure 2.

isolated from a transcription reaction (Axel et al., 1973; Gilmour and Paul, 1973; Barrett et al., 1974; Steggles et al., 1974; Aviv et al., 1975). Endogenous contaminants limit the accuracy and reliability of the method. The use of mercury-substituted ribonucleoside triphosphate for in vitro synthesis of RNA (Dale et al., 1975; Dale and Ward, 1975) permits the isolation of the mercury-containing RNA product on a thiol-substituted solid support and, thus, in principle should provide a method of fractionating newly synthesized RNA from endogenous contaminants. The method has been used to study in vitro transcription from chromatin or in nuclei (Smith and Huang, 1976; Crouse et al., 1976; Beissmann et al., 1976; Beebe and Butterworth, 1976).

We have used the mercury-substitution technique in an attempt to measure the globin sequence content of a typical transcript from reticulocyte chromatin (Figure 2). In the standard transcription reaction 250 μg of chromatin, prepared with minimal shearing, was transcribed in a reaction containing 1 mM Mn^{2+}, 5 mM KCl, and 40 units of *E. coli* RNA polymerase. HgUTP fully replaced UTP in the reaction, and a sufficient excess of β-mercaptoethanol was included to provide protection of *E. coli* RNA polymerase from inhibition by HgUTP (see Materials and Methods). About 5 μg of RNA was synthesized from 250 μg of reticulocyte chromatin DNA in the standard transcription reaction, using either UTP or HgUTP.

About 70–80% of the newly synthesized RNA was capable of binding to thiol-agarose beads (see Materials and Methods). After extensive washing of the beads, 90–95% of this mercury-containing RNA was recovered by elution with β-mercaptoethanol.

The globin sequence content of this purified RNA transcript was determined by hybridization to a globin [^{3}H]cDNA probe under conditions of cDNA excess (Young et al., 1974; Williamson, 1976). As shown in Figure 2, the hybridization of globin cDNA was readily detectable. From the linear portion of the titration curve, it is seen that in the particular experiment illustrated about 0.2 ng of globin cDNA hybridized with each μg of transcript, corresponding to an apparent abundance of globin-specific sequences in the RNA transcript of 0.02%.

The detection of globin RNA sequences in the in vitro transcript was strongly dependent on the addition of *E. coli* RNA polymerase, as shown in Table I. Under the conditions outlined in Materials and Methods for the isolation of transcript RNA, nonspecific RNA aggregation of the kind reported by Crouse et al. (1976) and Konkel and Ingram (1977) was not observed. Both total RNA and the amount of globin RNA detected increased with increasing RNA polymerase concentrations between 5 and 100 units/mL.

Hybridization of DNA with RNA Synthesized from HgUTP Cannot be Detected. It has been reported recently that transcripts in which HgUTP totally replaces UTP fail to hybridize to the DNA from which they are synthesized. Beebe and Butterworth, studying the in vitro transcription of ribosomal RNA from rat liver nucleoli, were unable to detect hybridization of RNA of high specific radioactivity synthesized from HgUTP with ribosomal DNA as template. Effective hybridization was achieved only following demercuration of the RNA preparation (Beebe and Butterworth, 1976). This raised some doubt as to whether the globin sequences detected in our experiments were indeed heavily mercurated (i.e., newly synthesized).

To determine the efficiency of hybridization of mercury-substituted RNA with DNA under the conditions of our hybridization assay, we studied the hybridization behavior of φX174 RNA transcribed in vitro from φX174 DNA. The complexity of the φX174 genome is 1.4×10^6 (Birnstiel et al., 1972) or approximately twice that of the globin sequences being analyzed (Bishop et al., 1974). Since the viral DNA is single stranded, analysis of hybridization can be followed in reactions designed analogously to the analysis of globin RNA with cDNA. φX174 DNA was transcribed with *E. coli* RNA polymerase in reactions containing varying ratios of UTP to HgUTP. Hybridization analysis of the transcript RNA was performed in solution with a 20-fold excess of φX174 DNA under ionic conditions identical with those used in analysis of chromatin transcripts. Formation of hybrid product was determined by resistance of the input RNA to S1 nuclease digestion. In Figure 3 the kinetics of hybridization of φX174 RNA to φX174 DNA are presented. As seen, RNA synthesized using HgUTP alone hybridized at a rate less than 3% that observed with RNA containing UTP alone. Increasing the DNA concentration of the reaction by 100-fold, and the duration of hybridization to 4 h, resulted in hybridization of no more than 10% of the input RNA. When 50% of the UTP was replaced with HgUTP, the rate of hybridization was reduced to about 30% of that found with the unsubstituted RNA. In contrast, at 25% and 11% substitution with HgUTP, hybridization proceeded at a rate comparable to that for unsubstituted RNA. The effect of mercury substitution is not due to

TABLE I: Effect of RNA Polymerase Concentration on Globin RNA Content of in Vitro Transcripts from Reticulocyte Chromatin.[a]

RNA polymerase (units/mL)	Total RNA synthesized (μg)	ng of globin cDNA hybridized/ μg of RNA transcript	Total globin RNA detected (ng)
0	0.02	0	0
5	1.02	0.3	0.31
10	1.89	0.30	0.57
25	5.05	0.16	0.81
50	8.21	0.17	1.40
100	12.65	0.15	1.90
0	5.1*		0.01

[a] Mercury-substituted RNA was transcribed from duck reticulocyte chromatin with HgUTP as sole UTP source under the conditions of the standard transcription reaction described in Materials and Methods. (*) Refers to a reaction to which RNA polymerase has not been added; 5.1 μg of *E. coli* RNA, transcribed from DNA using HgUTP as described in Materials and Methods, was added following quenching of the reaction with NaCl and $NaDodSO_4$. (ng of cDNA hybridized/μg of RNA transcript) is determined as outlined in Materials and Methods. Total globin RNA is determined from the globin RNA content of the RNA and the total amount of RNA synthesized.

TABLE II: Recovery of Exogenous Globin RNA in Transcripts from *E. coli* DNA-Histone Reconstitutes.[a]

Globin RNA added (ng)	Total RNA synthesized (μg)	ng of globin cDNA hybridized/ μg of RNA transcript	Globin RNA detected (ng)[b]
0	12.8	0	0
10	11.0	0.093	1.022 (10.25)
10	10.0*	0.001	0.01 (0.1)
50	12.2	0.278	3.39 (6.8)
100	8.4	0.645	5.40 (5.4)
500	10.5	1.144	12.0 (2.4)
1000	10.0	3.049	30.4 (3.0)

[a] Conditions of the transcription reactions are as described in Materials and Methods, except that 250 μg of *E. coli* DNA-histone reconstitutes replaced reticulocyte chromatin. In addition, varying amounts of purified globin mRNA were added to the transcription reaction just prior to the addition of RNA polymerase. (*) Refers to a sample to which RNA polymerase was not added; 10 μg of *E. coli* RNA, synthesized using HgUTP, was added just following quenching of the reaction with $NaDodSO_4$ and NaCl. [b] % input in parentheses.

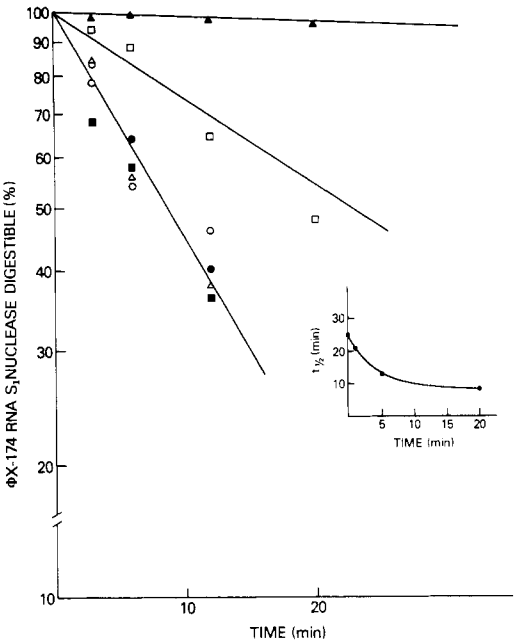

FIGURE 3: Kinetics of annealing to φX174 DNA of ^{3}H-labeled, mercury-substituted RNA transcript from φX174 DNA. Synthesis of [^{3}H]-RNA from φX174 DNA containing various proportions of Hg-UTP and UTP is described in Materials and Methods ((○) 0, (●) 11%, (■) 25%, (□) 50%, (▲) 100% Hg-UTP). Hybridization reactions contained in 200 μL: 200 ng of φX174 DNA, 25 ng of [^{3}H]cRNA (5000 cpm), 75 μg of yeast tRNA, 0.2 M sodium phosphate (pH 6.8), 0.5% $NaDodSO_4$, 1 mM EDTA. Aliquots of 20 μL were sealed into glass capillaries, heated at 107 °C for 2 min, and incubated at 68 °C for various times. Samples were quenched by dilution in 1 mL of S1 buffer and digested with S1 nuclease (84 units/mL) for 1 h at 45 °C. Prior to trichloroacetic acid precipitation, reactions were made 0.1 M dithiothreitol and heated at 45 °C for 1 h. With heavily mercury-substituted RNA, trichloroacetic acid precipitation without prior demercuration yielded highly erratic results, suggestive of nonspecific adsorption of mercury-containing, acid-soluble oligonucleotides in the S1 digest to the glass fiber filter. The effect of demercuration on the rate of annealing of RNA containing 100% Hg-UTP to φX174 DNA was determined by including 0.1 M dithiothreitol in the hybridization reaction described above. Aliquots were either incubated at 68 °C directly (zero time) or heated at 107 °C for 1, 5, or 20 min, prior to incubation at 68 °C. Analysis of the extent of hybrid formation was performed as described above, except that the S1 buffer was made 3 mM Zn^{2+}, to adjust for chelation by the additional dithiothreitol in the reaction. Full kinetic analysis of the mercury-substituted RNA heated for 20 min is presented (△). The insert shows the time required for 50% hybridization presented as a function of duration of reaction with 0.1 M dithiothreitol at 107 °C (insert).

fragmentation of the RNA during synthesis, or to the introduction of sequence error in the RNA transcript. Heating of the RNA synthesized from HgUTP at 107 °C in 0.1 M dithiothreitol prior to reannealing led to removal of mercury and a time-dependent conversion of the RNA into a product that hybridized at a rate identical with that of unsubstituted RNA (Figure 3, insert). It should be emphasized that hybrid formation is determined by entry of the RNA into a structure which should resist digestion by S1 nuclease. It is possible that fully mercury-substituted RNA hybridizes efficiently, but does not form a hybrid resistant to S1. However, when the annealed product containing fully mercury-substituted RNA was digested with S1 nuclease at concentrations as low as one-tenth that used in the routine analysis, and over incubation periods as brief as one-fourth that normally used, no differences could be demonstrated in the amount of nuclease-resistant RNA.

The experiments confirm the report of Beebe and Butterworth on the apparent inhibitory effect of mercury substitution on hybridization. These results suggested to us that the hybridization of globin cDNA observed above in the routine analysis of chromatin transcripts might not have arisen from reaction with mercury-substituted RNA, but from reaction with an unsubstituted, endogenous, globin RNA sequence.

Recovery of Endogenous Globin RNA in Mock Chromatin Transcripts. It seemed necessary to determine whether endogenous globin RNA would copurify with newly synthesized mercury-substituted RNA under our transcription conditions. We carried out experiments in which nucleoprotein complexes were transcribed in the presence of added globin RNA (containing no Hg). In this experiment the nucleoprotein was a complex of histones with *E. coli* DNA, and thus contained no globin-specific sequences.

E. coli DNA and erythrocyte histones were reconstituted into a 1:1 complex (w/w) by gradient dialysis from 2 M NaCl, 5 M urea (Camerini-Otero et al., 1976). To samples of the reconstitute containing 250 μg of DNA, varying amounts of purified globin RNA were added. The mock chromatin preparations were transcribed with HgUTP replacing UTP under conditions standard for the transcription of native chromatin. Isolation of RNA followed the usual methods. As shown in Table II, endogenous globin RNA is readily detected in the in vitro transcript. Between 3 and 10% of the input globin RNA was recovered following purification of the mercury-substituted RNA on thiol-agarose. Addition of 10 ng of globin RNA to 250 μg of chromatin DNA led to the recovery of about 1 ng of globin sequence. The abundance of globin RNA in the final RNA varied from 0.009% to 0.3%, entirely dependent on the amount of globin present in the "chromatin" sample transcribed. In the absence of added RNA polymerase only 0.01 ng of globin RNA was detected. Thus, the recovery of the globin RNA sequences was also dependent on RNA polymerase activity.

In our transcription experiments with reticulocyte chromatin (see above), approximately 1 ng of globin RNA is generally recovered with the newly synthesized RNA for each 250 μg of chromatin template. This is a response comparable to that derived from mock reconstitutes containing 10 ng of added globin RNA. Rabbit reticulocyte chromatin preparations are reported to contain about 2 ng of globin RNA per 250 μg of DNA (Wilson et al., 1975a), and we find about 5 ng of globin RNA per 250 μg DNA in our chromatin preparations. These

TABLE III: Effects of Actinomycin D and Rifampicin on Globin RNA Content of in Vitro Transcripts from Reticulocyte Chromatin.[a]

	Total RNA Synthesized (μg)	Inhibition (%)	ng of globin cDNA hybridized/ μg of RNA transcript	Total globin RNA detected (ng)
Act D concn (μg/mL)				
0	5.05		0.16	0.81
1	2.41	52	0.33	0.79
3	1.49	70	0.60	0.42
10	0.59	88	1.19	0.70
100	0.23	95	2.49	0.57
Rifampicin concn (μg/mL)				
0	5.44		0.172	0.94
0.16	0.75	86	0.140	0.10
0.32	0.49	91	0.160	0.08

[a] All conditions are as described for the standard reaction in Materials and Methods, except that actinomycin D or rifampicin, in the amounts stated above, was added prior to addition of RNA polymerase.

are probably underestimates, since nuclease action would lead to losses of RNA during its isolation. It is thus possible that a major fraction of the globin RNA recovered from in vitro mercury-substituted transcripts of reticulocyte chromatin derives from endogenous RNA sequences.

Effect of Inhibitors of RNA Synthesis on Globin RNA Recovery in in Vitro Transcripts. What is the origin of this polymerase-dependent copurification of endogenous globin RNA sequences with newly synthesized mercury-substituted RNA? A plausible explanation is that the endogenous RNA is functioning as a primer or template for the enzyme: either it serves directly as a primer, which is extended with HgUTP at the 3′ terminus, or it acts as a template for the synthesis of a mercury-containing complementary strand (anti-strand), with resultant formation of an RNA duplex. In either case, mercury-labeled product would be associated with, and might copurify with, endogenous globin sequences.

The synthesis of RNA by prokaryotic RNA polymerase from single-stranded RNA templates has been well documented for the enzyme from *M. luteus,* the predominant reaction being the synthesis of the complementary RNA strand and formation of a template–product duplex (Fox et al., 1964). Indeed, this reaction has been used recently to synthesize RNA of high specific radioactivity complementary to globin RNA (Melli and Pemberton, 1972; Wilson et al., 1975b). Since the priming of RNA synthesis with single-stranded RNA is relatively insensitive to inhibition by actinomycin D (Fox et al., 1964), the effect of this antibiotic on the abundance of globin RNA in the in vitro transcript from reticulocyte chromatin was studied. As seen in Table III the globin RNA content of in vitro transcripts is only weakly affected by the presence of actinomycin D. Even at concentrations of actinomycin D as high as 100 μg/mL, sufficient to inhibit greater than 95% of total RNA synthesis, less than a 30% reduction in the yield of globin RNA is observed.

In contrast to the effect of actinomycin D on the synthesis of RNA and the recovery of globin sequences in the in vitro transcript, rifampicin (Table III) inhibits both processes to the same extent. This result is to be expected, since rifampicin acts by direct inhibition of the polymerase (Chamberlin, 1975).

The experiments with actinomycin suggest that the presence of globin RNA in the mercury-substituted RNA fraction results from formation of a duplex between globin RNA and newly synthesized Hg-RNA complementary to it. Further evidence to this effect is presented below.

Properties of the in Vitro System. The time course of synthesis of total RNA (utilizing HgUTP) was compared with the time dependence of globin RNA abundance. Incorporation of [α-^{32}P]UTP into RNA under standard ionic conditions (1 mM Mn^{2+}, 5 mM KCl) progressed almost linearly over a period of 1 h. Recovery of globin RNA followed identical kinetics: the fraction of total RNA appearing as globin sequence remained almost constant over the course of the reaction (data not shown).

We have studied the effect of varying ionic conditions on the measured abundance of globin RNA in transcript. Fox et al. (1964) have reported that the metal ion requirement for transcription of natural RNA can be satisfied either by Mn^{2+} or Mg^{2+}, but that transcription of certain synthetic ribopolymers can be observed only in the presence of Mn^{2+}. In Table IV the abundance of globin RNA in transcript is reported for experiments performed at various Mn^{2+} and Mg^{2+} concentrations, representative of the ranges employed in most reports, both at high (150 mM) and low (5 mM) KCl concentrations. In all the transcription reactions HgUTP fully replaced UTP, and thus all globin RNA sequences detected in the transcript following purification of the RNA were of endogenous origin. As shown in Table IV, globin RNA was carried into the newly synthesized fraction under all conditions tested. The variation in abundance of globin sequence in the in vitro transcript is principally a result of the effect of ionic conditions on bulk RNA synthesis from the chromatin template, rather than on the amount of endogenous globin RNA trapped.

E. coli Polymerase Can Transcribe Globin RNA. We can demonstrate directly that the *E. coli* RNA polymerase will utilize globin RNA as a template. We find that under conditions similar to those used in the in vitro transcription of chromatin, *E. coli* RNA polymerase synthesizes about 20 ng of [^{3}H]RNA in 1 h from 0.8 μg of globin RNA template (using nonmercury containing ribonucleoside triphosphates). As shown in Figure 4, when the radioactive RNA product is treated with S1 nuclease more than 85% of the sample is resistant to digestion, consistent with the formation of a product-template RNA duplex. If, however, the RNA product is heated prior to S1 digestion under conditions leading to RNA duplex denaturation, less than 15% of the globin RNA-templated product resists S1 nuclease, as expected if strand separation of product and transcript has occurred. This experiment demonstrates directly that globin RNA-templated synthesis of complementary RNA can be catalyzed by *E. coli* RNA polymerase and that the product is recoverable as a duplex with its template. It should be stressed that the tran-

TABLE IV: Effect of Ionic Composition on Globin RNA Content of in Vitro Transcripts from Reticulocyte Chromatin.[a]

Mg^{2+} (mM)	Mn^{2+} (mM)	KCl (mM)	Total RNA synthesized (μg)	ng of globin cDNA hybridized/ μg of RNA transcript	Total globin RNA detected (ng)
1		5	1.53	0.63	0.96
1		150	10.46	0.13	1.36
5		5	1.70	0.55	0.94
5		150	3.72	0.28	1.04
10		5	1.82	0.56	1.02
10		150	2.37	0.26	0.62
	1	5	5.44	0.15	0.82
	1	150	22.16	0.088	1.95
	3	5	1.22	0.82	1.00
	3	150	8.78	0.14	1.23
	5	5	1.28	0.5	0.64
	5	150	6.54	0.18	1.18

[a] All conditions are as described for the standard reaction in Materials and Methods, except for presence of varying concentrations of Mn^{2+}, Mg^{2+}, and KCl. Prior to addition of enzyme, a solution of $MnCl_2$ or magnesium acetate, alone or in combination with KCl, was added. All salts were added in a volume of 0.3 mL (20% of the final reaction volume) to reduce transient exposure of the chromatin to much more concentrated salt solutions.

scription of globin RNA proceeds under our standard chromatin transcription conditions whether or not the ribonucleoside triphosphate precursors are mercury substituted.

Fractionation of Globin RNA Sequences on Thiol-Agarose Following Heat Denaturation. We sought further direct evidence for the presence in chromatin transcripts of a duplex containing endogenous globin message and a complementary, mercury-substituted strand. If the newly synthesized Hg-RNA is a separate strand rather than an extension of existing globin RNA strands, then heat denaturation of the chromatin transcript, under conditions designed to melt RNA duplex structures, should result in a clear partitioning on thiol-agarose of globin RNA sequences from mercury-substituted de novo RNA transcripts.

Reticulocyte chromatin was transcribed with HgUTP replacing UTP. RNA was purified over thiol-agarose as described in Materials and Methods. One-half of the transcript was then reapplied to the thiol-agarose support in the usual manner, and the other half was heated to denature RNA duplex structures prior to passage over the thiol support. The globin RNA content of the transcript RNA not retained on thiol-agarose was compared with that retained in each experiment. As shown in Table V, when the undenatured RNA transcript was passed over thiol-agarose about 80% of the RNA was bound, representing the efficiency of capture of the mercury-substituted RNA under the conditions of analysis. The abundances of globin sequence in the RNA fraction retained and in that which passed through were virtually identical, 0.026-0.024%, demonstrating the identical partitioning of globin sequences with the bulk transcript. When the RNA transcript was heat denatured prior to chromatography, about 75% of the total sample bound to thiol-agarose, demonstrating that, under the conditions designed to melt RNA duplex structures, no significant decrease in the affinity of the bulk mercury-substituted product for the thiol-agarose bed was observed. In the heat denatured sample, however, globin-specific sequences were no longer adsorbed to the column with the bulk mercury-substituted RNA. Less than 5% of the globin RNA detected in the RNA sample applied to the thiol-agarose bed was retained, and over 90% could be recovered in the pass-through fraction.

No Newly Synthesized Globin RNA Can Be Detected in These Preparations. We have demonstrated that RNA synthesized in vitro from reticulocyte chromatin with *E. coli* RNA polymerase contains globin RNA sequences of endogenous origin. Clearly, the identification of newly synthesized globin-specific sequences requires more than the demonstration that globin RNA copurifies with mercury-substituted RNA by affinity procedures. Specifically, it must be shown directly that the globin RNA sequences detected in the chromatin transcript in fact contain mercury-substituted bases, or, in other words, that hybridization of the globin cDNA probe has occurred with mercury-substituted RNA. A simple, rapid approach, suitable for use as a routine assay in the analysis of hybridization reactions containing mercury-substituted RNA, is based on the principle that globin cDNA in hybrid with mercury-substituted globin RNA should bind to a thiol-agarose support. The utility of this approach has been discussed by Dale and Ward (1975) previously.

Before making use of this method, we first demonstrated

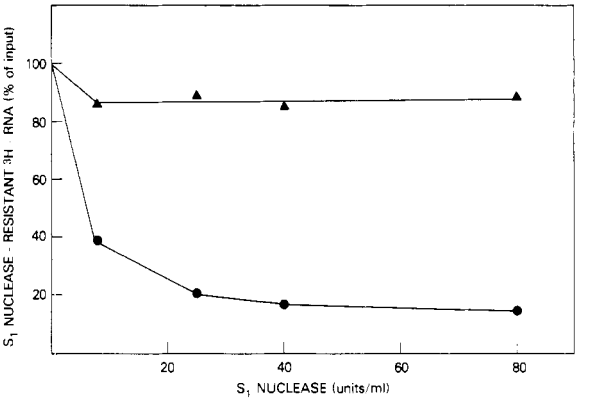

FIGURE 4: Globin RNA was transcribed in a reaction (100 μL) containing 50 mM Tris-HCl (pH 8.0), 7.5 μg of bovine serum albumin, 10 mM β-mercaptoethanol, 1 mM $MnCl_2$, 150 mM KCl, 0.2 mM each of ATP, UTP, GTP, 0.05 mM CTP, [^{3}H]CTP (specific radioactivity 5000 cpm/pmol), 1 μg of globin RNA, and 8 units of *E. coli* RNA polymerase. The reaction was incubated for 1 h at 37 °C, and the RNA purified as described for ϕX174 RNA (Materials and Methods). [^{3}H]RNA (1500 cpm) samples were either digested directly (▲) or first heated at 107 °C for 5 min in 50 mM NaCl and 0.05% $NaDodSO_4$ (●). RNA samples were digested with varying concentrations of S1 nuclease under conditions described in Materials and methods, except that the reactions were run at 37 °C for 20 min.

TABLE V: Fractionation of Globin RNA on Thiol-Agarose following Heat Denaturation of RNA Transcripts.[a]

RNA sample	Fractions on thiol-agarose	RNA (μg)	ng of globin cDNA hybridized/ μg RNA transcript	Total globin RNA detected (ng)
RNA transcript	Retained	2.0	0.26	0.52
	Pass through	0.5	0.24	0.12
RNA transcript	Retained	1.8	0.01	0.02
(heat denatured)	Pass through	0.51	1.02	0.52

[a] The standard transcription reaction was scaled up threefold. RNA was isolated as described in Materials and Methods. Purified transcript (6 μg) was dissolved in 2 mL of 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1% sodium dodecyl sulfate. One-half the sample was heated at 107 °C for 10 min, the other half untreated. Each sample was then applied at 22 °C to a 0.7 × 3 cm bed of SH-agarose, previously washed with 50 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% $NaDodSO_4$ (TNS), and allowed to react with the beads for about 1 h. The columns were washed with two 1.5-mL portions of TNS buffer and the washes pooled with the pass-through fraction. Each column was then washed with an additional 20 mL of TNS, and these fractions were discarded. Bound RNA was eluted with 2 mL of TNS containing 0.1 M β-mercaptoethanol. RNA was collected from the pools by ethanol precipitation as described in Materials and Methods.

TABLE VI: Hybridization Analysis of Chromatin Transcript with cDNA Probes Complementary to Messenger and Anti-Messenger Sequences.[a]

RNA	cDNA	$cDNA^{anti}$	[^{3}H]DNA probe in hybrid (cpm) Cl_3CCOOH precipitable	[^{3}H]DNA probe in hybrid (cpm) Thiol-agarose bound	Retained (%)	Corrected (%)
Transcript 1.18 μg	+	−	512 (131)	0 (18)	0	
	−	+	76 (113)	26 (20)	34	64
Transcript 3.54 μg	+	−	940	8	1	2
	−	+	238	60	25	45
Globin Hg-RNA 0.01 μg	+	−	744	408	55	100

[a] Conditions for synthesis and isolation of RNA transcripts were as described in Materials and Methods, except that reactions contained 0.1 mM UTP and 0.035 mM HgUTP. About 20 μg RNA was dissolved in 2 mL and heated at 107°C for 15 min. The sample was cooled to 22 °C, applied to a 0.7 × 3 cm bed of SH-agarose beads equilibrated in TNS buffer, and allowed to react with the beads for about 1 h. The column was washed with 30 mL of TNS buffer, the bound RNA eluted with 1.5 mL of TNS containing 0.1 M β-mercaptoethanol and the RNA isolated by ethanol precipitation. The RNA was hybridized to 0.5 ng (about 5000 cpm) of either [^{3}H]DNA complementary to globin RNA (cDNA) or to globin anti-strand ($cDNA^{anti}$), in the standard hybridization reaction described in Materials and Methods scaled-up twofold. A control reaction containing 10 ng of globin RNA mercury-substituted in vitro was carried through the analysis to provide an estimate of efficiency of sequence detection. Samples were denatured at 107 °C for 2 min and then hybridized at 68 °C for 24 h. Reactions were diluted in 1 mL of S1 buffer (see Materials and Methods). S1 nuclease (84 units) was then added, and the reactions were incubated for 60 min at 45 °C. Digestions were quenched with addition of 0.2 volumes of 20% $NaDodSO_4$. One-half of the digest was precipitated with trichloroacetic acid and analyzed as described in Materials and Methods. The other half was applied to a 0.7 × 2 cm bed of SH-agarose overlaid with about 0.1 mL of Chelex-100; both materials were equilibrated with TNS buffer (Chelex-100 was included to remove Zn^{2+} contained in the S1 buffer, which would otherwise compete for thiol groups on the SH-agarose). The gel was contained in a 0.7 × 15 mL disposable column (Bio-Rad, Econo-column). The solution was kept in contact with the gel bed for about 1 h at 22 °C, after which the column was washed with about 45 mL of TNS buffer. The glass column was cut just above the head of the gel, and the gel evacuated into a standard 15-mL scintillation vial containing 1 mL of water. β-Mercaptoethanol (0.2 mL) was added, and the mixture incubated at 45 °C for 15 min to free bound RNA from the gel. Aquasol (NEN) (15 mL) was added and the retained radioactivity determined by liquid scintillation counting. Backgrounds obtained in the absence of added RNA are included in parentheses. The corrected values have been adjusted for the retention efficiency of globin RNA-cDNA hybrids as shown in the bottom line.

that hybrids of cDNA with mercury-substituted RNA are retained on thiol-agarose. We employed for this purpose globin mRNA that had been mercury-substituted by reaction with mercuric acetate (see Materials and Methods). As seen in Table VI, when [^{3}H]cDNA was hybridized to an excess of this modified globin RNA, about 55% of the probe detected in S1 nuclease-resistant hybrid was retained on thiol-agarose.

We next carried out in vitro transcription using reticulocyte chromatin, and an HgUTP:UTP molar ratio of 1:3. At this low concentration of HgUTP, efficient hybridization of mercury-substituted RNA with globin [^{3}H]cDNA can be expected (see Figure 3). The RNA transcript was heat denatured, prior to purification over thiol-agarose, as described above. The purified RNA was hybridized with [^{3}H]cDNA in the usual manner and the mixture digested with S1 nuclease to hydrolyze all cDNA not in stable hybrid with RNA. One-half of the digest was precipitated with Cl_3CCOOH and thus represented the total amount of globin specific sequence in the transcript. In 1.18 μg and 3.54 μg of RNA transcript, about 512 and 940 cpm, respectively, of [^{3}H]cDNA complementary to globin RNA were detected. This reflects the total globin sequence content of the sample, independent of mercury substitution. The second half of the S1 digest was applied to a bed of thiol-agarose, to determine the fraction of [^{3}H]cDNA hybridized with mercury-substituted RNA. When this was done, less than 1% of the radioactivity originally detected in the Cl_3CCOOH precipitate was bound to the thiol support. An identical analysis was performed on transcripts synthesized at 1 mM Mn^{2+} and 150 mM KCl, with similar results. The minimum amount of globin RNA detectable by our assay amounts to a response of 10 cpm above background. Since we were unable to detect 0.007 ng of globin RNA in 1.18 μg of RNA transcript, the maximal abundance of de novo globin-specific RNA synthesized that would go undetected would be about 0.0007%. This represents a detection limit on the order of 3–5 gene transcripts of each of the three globin genes (Weintraub and Groudine,

1976) actively transcribed in the reticulocyte per haploid duck genome.

By means of the thiol-binding assay, however, we can demonstrate unequivocally the de novo synthesis of the globin anti-strand in the purified RNA transcribed in vitro from reticulocyte chromatin. This experiment is identical with the preceding one, except that the probe is the anti-cDNA strand synthesized by reverse transcriptase (see Materials and Methods). We find that the chromatin transcript forms stable hybrids with this probe, and after S1 nuclease treatment, between 45 and 60% of the Cl_3CCOOH-precipitable radioactive material (corrected for the efficiency of the control) could be bound to thiol-agarose (Table VI).

Thus, by direct analysis of the RNA transcribed from reticulocyte chromatin with *E. coli* RNA polymerase, both globin sense and anti-sense sequences can be detected, but in these experiments only the anti-strand sequences are newly synthesized.

Discussion

The transcription of duck reticulocyte chromatin in vitro, utilizing HgUTP as a substrate, permits the ready isolation of the mercury-substituted RNA by means of its affinity for thiol–agarose, and the fractionation of newly synthesized, mercury-containing RNA from sequences of endogenous origin (Dale et al., 1975; Dale and Ward, 1975). The basic concept underlying this approach was devised by Dale and coworkers and recently applied to the analysis of in vitro transcripts from preparations of nuclei and chromatin from various sources (Smith and Huang, 1976; Crouse et al., 1976; Beissmann et al., 1976; Beebe and Butterworth, 1976). Indeed, using this method, we can also readily detect globin RNA sequences in purified mercury-substituted in vitro transcripts from our duck reticulocyte chromatin preparations. However, from all evidence that we have presented, it appears that the globin sequences detected in the purified transcript are of endogenous origin: the globin RNA sequences are not in fact newly synthesized, but rather copurify with bulk mercury-substituted RNA. This anomalous fractionation is highly dependent on the addition of RNA polymerase to the transcription reaction, yet insensitive to inhibition by actinomycin D. We believe that globin RNA sequences of endogenous origin appear in the mercury-substituted transcript as a result of globin RNA-directed transcription catalyzed by RNA polymerase, leading to the formation of a duplex structure in which one strand is the globin RNA template and the other is a mercury-substituted complementary sequence. The RNA-dependent synthesis of RNA by prokaryotic RNA polymerase has been described (Krakow and Ochoa, 1963; Fox et al., 1964; Maitra et al., 1967; Melli and Pemberton, 1972; Wilson et al., 1975b), but never previously reported to occur under the conditions of the in vitro transcription of chromatin. As we have shown above, *E. coli* RNA polymerase will utilize globin RNA as a template for the synthesis of a radioactive RNA product. The product is found in a duplex structure with its template.

A summary of the reactions observed in the in vitro transcription of reticulocyte chromatin by *E. coli* RNA polymerase, and our approach to analysis of the product, are presented in Figure 5. The bulk of newly synthesized RNA is transcribed from chromatin DNA, and, as demonstrated above, in these experiments contains no detectable globin sequences. Endogenous RNA, including globin-specific sequences, provides a template for RNA polymerase, resulting in a template–product duplex structure. Purification on thiol–agarose of the RNA in the transcription reaction partitions the nucleic acids on the

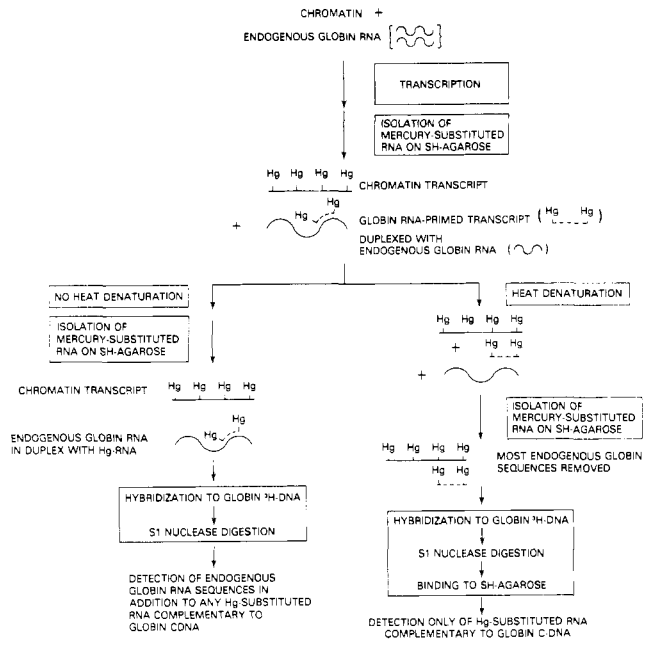

FIGURE 5: The experimental scheme for the analysis of mercury-substituted chromatin transcripts.

basis of mercury substitution, so that the template–product duplex is retained, while all non-mercury-containing RNA passes through. Elution of the thiol–agarose bound RNA and subsequent denaturation and hybridization with globin [^{3}H]cDNA leads to the detection of globin sequences carried as duplex, with the erroneous interpretation that these detected sequences represent de novo product. If, instead, the RNA transcript is heat denatured before passage over thiol–agarose, a considerable fraction of the globin-specific sequences no longer partition with the bulk mercury-subsituted RNA. The appearance of some globin specific sequences in the thiol-bound RNA fraction following the heat denaturation of partially mercury-substituted RNA transcripts probably reflects the partial rehybridization of complementary sequences during the purification of the transcript over the thiol column.

Unequivocal proof of the synthesis of globin-specific sequences requires the demonstration that the highly radioactive probe, used to detect the globin sequences, has indeed hybridized with mercury-substituted RNA. As shown in Figure 5, the transcripts are hybridized to globin [^{3}H]DNA, digested with S1 nuclease, and again applied to a column of thiol–agarose. Binding of the [^{3}H]DNA probe can only result from the hybridization of the probe to mercury-substituted RNA, and thus provides definitive proof of de novo synthesis. In our current series of experiments, no detectable [^{3}H]cDNA appeared in hybrid with newly synthesized RNA. In contrast, de novo synthesis of anti-messenger RNA sequences is detected in the transcript from reticulocyte chromatin. Its presence is demonstrated both by the formation of hybrid molecules of globin sense [^{3}H]DNA ($cDNA^{anti}$) with RNA in the thiol-purified transcript, and by the fact this the hybrid, after treatment with S1 nuclease, binds to thiol–agarose.

The ability of endogenous RNA contaminants in chromatin to act as template for RNA polymerase complicates the analysis of in vitro transcription of chromatin as studied by either the mercury-substitution method or by previous techniques. Since endogenous globin RNA is readily detected in the transcripts synthesized from HgUTP as substrate, an increase in the abundance of globin RNA in such a transcript synthesized from chromatin reconstituted with partially pu-

rified non-histone protein fractions is not necessarily a demonstration of specific gene activation. It must be shown, in particular, that endogenous RNA, expected to partition with the acidic non-histone chromosomal proteins by the usual fractionation schemes, has not been detected as a spurious activity by the mercury-substitution method.

Although it is difficult to generalize about reactions as complex as the in vitro transcription of chromatin, it would appear that at least one mechanism to explain the peculiar appearance of symmetric transcription in vitro but not in vivo (Astrin, 1973; Honjo and Reeder, 1974; Wilson et al., 1975a,b; Beissmann et al., 1976) follows from the RNA-directed transcription of RNA by polymerase. Thus, the appearance of globin anti-strand RNA sequences in in vitro transcripts can result from transcription of globin RNA contaminating the chromatin, rather than from transcription of the incorrect strand of the globin DNA sequence. This argument is not unrealistic when one considers that, in a chromatin preparation of the kind described here, 100 μg of chromatin DNA generally contains about 0.1 ng of globin DNA sequence available for transcription. The existence of anti-strand transcription resulting from RNA-dependent RNA synthesis catalyzed by RNA polymerase should be readily demonstrable through its expected insensitivity to inhibition by actinomycin D.

The formation of a globin RNA duplex in vitro leads us to speculate on the possibility that under certain reaction conditions transcription of this duplex might occur, with the resulting synthesis of either or both strands. Such a structure would function in vitro as a transcriptional unit, possibly competing with the structural gene for available RNA polymerase molecules. Transcription of an RNA duplex formed during the extended transcription of TYMV in vitro has been reported to occur (Fox et al., 1964).

When *E. coli* RNA polymerase is added to a chromatin transcription reaction mixture, a significant fraction of the endogenous RNA in the reaction will act as template for the synthesis of its complementary strand and will be converted into a duplex structure. In a duplex, the endogenous globin sequences would be expected to be more resistant to nuclease attack than they would be as single-stranded molecules. Thus, an apparent increase in the abundance of a gene sequence following transcription might represent protection of endogenous RNA from contaminating nuclease activity, rather than de novo synthesis. It is therefore important to demonstrate the absence of such activity in chromatin transcription reactions.

In addition, as reported by Crouse et al. (1976) and Konkel and Ingram (1977) Hg-substituted RNA will aggregate nonspecifically with endogenous RNA under certain conditions of isolation from in vitro chromatin transcription reactions and carry non-mercury-containing, endogenous sequences through the purification over thiol–agarose. Although the conclusions to be drawn from our results depend on further study of the effects of mercury-substituted nucleotides on transcription, it seems clear that great caution is necessary in interpreting all in vitro transcription experiments with chromatin templates.

We have shown that the mercury-substitution method is capable of giving false indication of de novo messenger-strand RNA synthesis. The supposed ability of the mercury-substitution technique to discriminate endogenous message from message synthesized de novo might encourage investigators to ignore high levels of endogenous RNA contamination in their preparations. This would obviously be an error. Analysis of the results is further complicated by the fact that RNA in which all UTP is replaced by HgUTP appears to be very inefficient in formation of S1 nuclease-resistant hybrids with cDNA. Thus, highly mercurated de novo transcripts, if they occur, might go undetected.

The methods we have used to detect these difficulties can also be employed to avoid them. The use of mercury-substituted derivatives should be accompanied by demonstrations that the putative de novo synthesis is actinomycin sensitive and that the specific sequences being detected are in fact mercury labeled. It is important, for the latter purpose, to show that the specific sequences are retained on thiol–agarose if the RNA transcript is heated before application to the column. It is also important to show that the hybrid between cDNA probe and RNA can be bound to thiol–agarose after treatment with S1 nuclease.

With these precautions, contributions resulting from transcription of endogenous RNA can be eliminated. In our hands, this is the major artefact arising from the use of mercury-labeled RNA precursors. We cannot, of course, rule out the possibility that other artefacts (such as extension of existing endogenous RNA sequences) exist under other conditions or in other systems. Nonetheless, with the elimination of this major difficulty, we hope that the mercury-substitution method will fulfill its promise as a technique for detecting de novo synthesis of RNA in vitro.

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Determination of the Extent of Secondary Structure in Chick Embryo Procollagen Messenger RNA†

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ABSTRACT: The secondary structure of highly purified chick embryo procollagen mRNA has been investigated by thermal denaturation and by reaction with formaldehyde. Thermal denaturation parameters of procollagen mRNA have been compared to ribosomal RNAs and to a synthetic random copolymer. It is concluded that procollagen mRNA contains considerable secondary structure. Stacking interactions, calculated from the melting of procollagen mRNA previously reacted with 1% formaldehyde, account for about 10% of secondary structure. Kinetics of reaction with formaldehyde and thermal denaturation of procollagen mRNA in 4 M guanidinium chloride, a solvent which markedly reduces stacking interactions without affecting significantly hydrogen bond formation, show that about 49% of bases are present in a double helical configuration. Contrary to what is observed in the random sequence copolymer, the rise in temperature determines melting of hairpin loops progressively richer in G·C base pairs. The number of base pairs/hairpin loop has been estimated as 6. Assuming 4500 as the minimal number of nucleotides/mRNA and neglecting the contribution of poly(A) to hairpin loop formation, a minimum of 130 such hairpin loops are present in procollagen mRNA. $\Delta A_{260}/\Delta A_{280}$ for procollagen mRNA is in good agreement with the value predicted by a linear relationship between this ratio and base composition of any RNA species.

Ribosomal RNAs, as well as transfer RNAs, present extensive hydrogen bonding and base stacking interactions, leading to a high degree of secondary structure (Fresco et al., 1963). A comparable amount of structural complexity has also been shown for viral RNAs (Min Jou et al., 1972; Billeter et al., 1969). However, since random RNA sequences can contain as much as 60% hydrogen bonding (Fresco et al., 1960; Gralla and DeLisi, 1974) it is necessary, when studying the conformation of mRNA, to exclude chance base pairing as a cause for the observed secondary structure.

Studies have indicated that in R_{17} mRNA the initiator codon AUG is enclosed in a hairpin loop (Steitz, 1969). Other studies have shown that faithful translation of polycistronic mRNA can be altered by heat denaturing the message (Lodish and Robertson, 1969). It has also been suggested that the conformation of mRNA might play a role in its stability in the cell (Klambt, 1975).

Careful analysis of the structure of hemoglobin mRNA (Holder and Lingrel, 1975) has indicated the existence of a highly ordered structure, determined by about 60% hydrogen bonds. Three distinct helical domains with respect to base composition were evident. Analysis of the structure of ovalbumin mRNA (Van et al., 1976) has also shown the presence of short hairpin loops differing from the whole molecule in

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