# Mode of Chromatin Reconstitution. Elements Controlling Globin Gene Transcription†

Robert A. Gadski and Chi-Bom Chae*

ABSTRACT: The mode of reconstitution has been determined for the factors that control globin gene transcription in chicken reticulocyte chromatin. During chromatin reconstitution, elements in the nonhistone protein fraction that control the globin gene reassociate with the DNA before the histones. From our results, it appears that it is necessary for these elements to reassociate with the DNA before the histones for the globin gene to be transcribed.

Reports on the similar properties of native and reconstituted chromatin (Paul & Gilmour, 1968; Bekhor et al., 1969; Huang & Huang, 1969; Gilmour & Paul, 1975; Spelsberg et al., 1971; Axel et al., 1974; Roti et al., 1974; Oudet et al., 1975) encouraged the study of the mode in which the process of chromatin reconstitution occurs for elements controlling specific gene transcription. Chicken reticulocyte chromatin was selected for this study since, when reconstituted, this chromatin appears to have similar transcription properties to native chromatin, with respect to transcription of DNA sequences that code for the globin gene (Barrett et al., 1974). It also has been reported that the nonhistone protein fraction is responsible for tissue specific gene transcription (Gilmour & Paul, 1975; Spelsberg et al., 1971; Paul et al., 1973; Barrett et al., 1974) though recently there is some skepticism of these results due to the possibility of endogenous RNA contaminating the in vitro transcribed RNA. In a previous report (Gadski & Chae, 1976) we determined that, during reticulocyte chromatin reconstitution, fractions of nonhistone proteins reassociate with DNA before, at the same time, and after the bulk of the histones reassociate with DNA. Also, the sequence of reassociation of different histone fractions with DNA during chromatin reconstitution was established (Gadski & Chae, 1976). Thus, insight into the manner in which globin genes are regulated may be obtained by determining at which point in the reconstitution process the constituents of the nonhistone protein fraction (which regulate globin gene transcription) associate with the DNA. This information would indicate whether elements in the nonhistone protein fraction can alter the reassociation of histones to specific sequences of DNA, or whether these same elements can alter an already-formed DNA-histone complex.

## Experimental Procedures

*Preparation of Chromatin and DNA.* Chicken reticulocytes were prepared by daily 1.0-mL injections of phenylhydrazine (10 mg/mL) for a 6-day period. On the 8th day, the animals were decapitated, and the blood was collected into an ice-cold solution of 0.14 M NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, and 0.1% heparin (Pemberton et al., 1972). Packed erythrocytes were purchased from Pel-Freez Biologicals Incorporated (Rogers, Ark.). The erythrocytes were washed twice with 0.15 M NaCl. Both reticulocyte and erythrocyte chromatin as well as erythrocyte DNA were prepared as previously described (Gadski & Chae, 1976).

*Preparation of Histone and Nonhistone Proteins.* Erythrocyte chromatin was dissociated in 2 M NaCl–5 M urea and 10 mM Tris-HCl (pH 7.5). The chromatin was stirred for 4 h at 4 °C and then centrifuged at 200 000*g* for 15 h. Histone and nonhistone proteins, in the high-speed supernatant, were separated by SP Sephadex column chromatography as described by Graziano & Huang (1971). Reticulocyte nonhistone proteins were obtained using the same procedure as described for erythrocyte nonhistone proteins, except that the reticulocyte chromatin was incubated for 30 min before dissociation with 1 mM phenylmethanesulfonyl fluoride and 1% isopropyl alcohol to prevent proteolysis (Gadski & Chae, 1976). Protein concentrations were determined by turbidity formed after adding trichloroacetic acid to a final concentration of 20%.

*Chromatin Reconstitution.* All chromatin reconstitution experiments were carried out at an initial concentration of 500 μg of chromatin DNA in a volume of 2.0 mL of 2 M NaCl–5 M urea and 10 mM Tris-HCl (pH 7.5). This fraction was then sonicated for 1 to 2 min with a Kontes sonifier at power setting 3. Except where otherwise stated, chromatin was reconstituted by a modification of the gradient dialysis procedure of Bekhor et al. (1969). The salt concentration was reduced to 0.1 M NaCl in the presence of 5 M urea–10 mM Tris-HCl (pH 7.5) over a 15-h period. Following further dialysis against 5 M urea–10 mM Tris-HCl (pH 7.5) for 4 h, the chromatin was dialyzed against 10 mM Tris-HCl (pH 7.5) for 12 h.

*Preparation of Globin [$^3H$]cDNA.* A reticulocyte polysomal fraction was obtained by using the procedure of Pemberton et al. (1972). Polysomal RNA was prepared from this fraction by the procedure of Perry et al. (1972). After two chloroform–phenol (1:1) extractions, the aqueous phase was brought to 0.3 M LiCl and 2 vol of 95% ethanol was added; the fraction was allowed to precipitate overnight at −20 °C. The solution was then centrifuged at 10 000*g* for 10 min, and the pellet was taken up in 0.5 M NaCl–10 mM Tris-HCl (pH 7.5). Poly(A) containing RNA was fractionated on an oligo(dT)-cellulose column according to the procedure of Aviv & Leder (1972) with the omission of the 0.1 M KCl intermediate salt wash. Reaction conditions for the preparation of globin [$^3$H]cDNA were 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, 8 mM dithiothreitol, 320 μM each of dATP, dCTP, and TTP, 50 μg of actinomycin D, 12 μg of $(dT)_{10}$, 250 μCi of [$^3$H]dGTP (12 Ci/mol), 18.1 μg of reticulocyte poly(A) containing polysomal RNA (globin mRNA), and 25 units of RNA-dependent DNA polymerase purified from avian myeloblastosis virus

† From the Department of Biochemistry and Nutrition, University of North Carolina, Chapel Hill, North Carolina 27514. *Received July 21, 1977.* Supported by a grant (CA-17390) from the National Cancer Institute, and a grant (AG00103) from the Institute on Aging, National Institutes of Health.

0006-2960/78/0417-0869$01.00/0 © 1978 American Chemical Society

(a gift of Dr. J. W. Beard of the Life Sciences Research Laboratories, St. Petersberg, Fla.) in a volume of 0.5 mL. After 3 h at 37 °C, the fraction was extracted with phenol-chloroform (1:1) and the aqueous phase was treated with 0.3 M NaOH at 37 °C for 18 h and applied to a Sephadex G-75 column (1 × 30 cm). The excluded peak was pooled and lyophilized to dryness. At least 40% of the [$^3$H]cDNA was close to full length, that is, greater than 550 nucleotides; the rest was greater than 200 nucleotides in length. The specific radioactivity of the [$^3$H]cDNA was $1 \times 10^7$ cpm/$\mu$g.

*Chromatin Transcription. Escherichia coli* RNA polymerase was isolated according to the procedure of Burgess & Jendrisak (1976). RNA was transcribed from chromatin in a 4-mL reaction mixture containing 50 mM Tris-HCl (pH 7.9), 0.15 M KCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.8 mM each of ATP, GTP, CTP, and UTP, and 400 units of *E. coli* RNA polymerase per mg of chromatin. Following incubation at 37 °C for 1 h, the reaction mixture was centrifuged at 12 000*g* for 15 min, and to the supernatant was added 20 $\mu$g/mL of DNase I (RNase free, Worthington Biochemical Corporation). The fraction was incubated at 37 °C for 30 min. The solution was then brought to 20 mM EDTA and 0.5% sodium dodecyl sulfate, vortexed and allowed to stand for 10 min at room temperature, and then extracted twice with equal volumes of phenol, twice with phenol-chloroform (1:1), and once with chloroform. The fractions were then brought to 0.15 M LiCl and precipitated overnight with 2 vol of 95% ethanol at −20 °C. The precipitate was centrifuged at 12 000*g* for 10 min and dried, then taken up in 1.0 mL of water and applied to a Sephadex G-75 column (1 × 30 cm). The excluded peak was pooled and lyophilized to dryness.

*Hybridization Experiments.* Hybridization of [$^3$H]cDNA to transcribed RNA was carried out by a modification of the procedure of Barrett et al. (1974). Our hybridization buffer contained 0.1% sodium dodecyl sulfate, 10 mM Tris-HCl (pH 7.9), and 0.4 M NaCl. Hybridization was carried out at 60 °C and [$^3$H]cDNA-RNA hybrids, which were resistant to $S_1$ nuclease, were measured.

*Isolation of Chromatin Subunit DNA.* The digestion of reconstituted chromatin into subunits was carried out according to the method of Noll (1974) by adding 1500 units of micrococcal nuclease (Worthington) to 8 mL of reconstituted chromatin (1.27 mg/mL) in 1 mM Tris-HCl (pH 7.9) and 0.1 mM $CaCl_2$. After incubating for 3 min at 37 °C, the reaction was arrested by the addition of 0.2 mL of 0.1 M EDTA (pH 7.0). After chilling on ice for 20 min, the fraction was centrifuged at 10 000*g* for 10 min and the supernatant was layered onto a 5–30% isokinetic sucrose gradient (Noll, 1967). The chromatin subunit fraction was pooled, made 0.1% sodium dodecyl sulfate, and extracted four times with an equal volume of phenol-chloroform (1:1) and one time with an equal volume of chloroform. The aqueous phase was extensively dialyzed against water and then lyophilized. Polyacrylamide gel electrophoresis of samples of the DNA fractions was carried out in the presence of 98% formamide (Staynov et al., 1972).

## Results

*Endogenous RNA.* There have been reports of endogenous globin mRNA sequences present in reticulocyte chromatin (Barrett et al., 1974; Gilmour & Paul, 1975). Recently it has been reported that endogenous RNA can act as a template for RNA polymerase (Zasloff & Felsenfeld, 1977) and also that RNA initiated in vivo can be elongated in vitro (Shih et al., 1977). Therefore, the possibility of endogenous RNA being present in our reconstituted chromatin must be given serious consideration because, if present, the endogenous RNA could alter the experimental results.

For our studies on chromatin reconstitution, we decided to use chicken erythrocyte DNA, erythrocyte histones, and reticulocyte nonhistone proteins. Barrett et al. (1974) reported that reticulocyte nonhistone proteins are responsible for transcription of the globin gene. Thus, since the globin gene is turned off in chicken erythrocyte the use of erythrocyte DNA and histones will eliminate the possible effect of these fractions on the transcription of the globin gene. The only potential source of contamination by endogenous globin messenger RNA sequences would, therefore, be the reticulocyte nonhistone protein fraction.

Several experiments were conducted to ensure that our measurements of transcription of the globin gene in reconstituted chromatin were not due to endogenous globin messenger RNA sequences. The nonhistone protein fraction was extracted with phenol-chloroform in the presence of carrier tRNA and the RNA was hybridized with highly labeled globin cDNA beyond the time the cDNA could have completely hybridized, if there were any endogenous globin messenger RNA sequences. We did not detect any endogenous globin messenger RNA in the nonhistone protein fraction by this test. When chromatin was reconstituted with *E. coli* DNA instead of chicken erythrocyte DNA the same amount of total RNA was transcribed form the chromatin as that normally transcribed from the chromatin reconstituted with chicken erythrocyte DNA. However, no globin mRNA sequences could be detected by hybridization with globin [$^3$H]cDNA. This suggests that the transcription of the globin gene is dependent on the template, that is, chicken DNA. As will be shown later in this report the transcription of globin gene from reconstituted chromatin depends on the order of binding of nonhistone proteins and histones to DNA. It is very unlikely that we would see this effect if the transcription of globin gene is due to endogenous RNA. Also in the presence of heat-denatured *E. coli* RNA polymerase no detectable amounts of RNA could be isolated from a transcription reaction mixture when native chromatin was used as a template.

The possibility exists of very low undetected amounts of endogenous globin mRNA serving as a template for *E. coli* RNA polymerase. Our results indicate that globin mRNA can act as a template for *E. coli* RNA polymerase but the contribution of globin mRNA sequences to the in vitro transcribed RNA would be negligible (R. A. Gadski and C.-B. Chae, manuscript in preparation). For example, the amount of RNA synthesized from pure globin mRNA by *E. coli* RNA polymerase under our reaction conditions is 5% of the globin mRNA. If the RNA synthesized from the globin mRNA has the same strand polarity as the globin mRNA there will be only a 5% increase in the concentration of globin mRNA sequences. However, if the result presented in this report is due solely to the synthesis of RNA from endogenous globin mRNA there should be a 50-fold increase in the concentration of globin mRNA sequences from the endogenous RNA assuming that there was 1 pg of endogenous globin mRNA which is our lower limit of detection (10 cpm). From the hybridization of globin cDNA to globin mRNA and the transcript of chromatin it can be estimated that the amount of globin mRNA sequences in the total chromatin transcript is in the range of 0.01–0.001% (Figure 1). This value is close to the amount of the globin mRNA sequence present in the total reticulocyte nuclear RNA (Wilson et al., 1975).

*Optimizing DNA Protein Ratios for Globin Gene Transcription.* Although over 95% of the labeled globin [$^3$H]cDNA hybridized to its own globin mRNA, only 60% of the globin

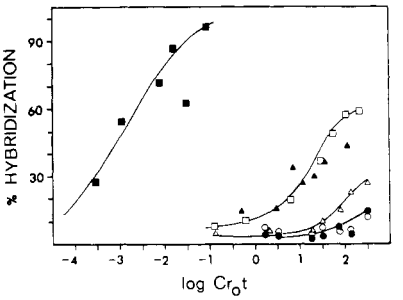


FIGURE 1: Kinetics of hybridization of RNA transcribed from reconstituted chromatin to globin [$^3$H]cDNA. Erythrocyte DNA, histones, and reticulocyte nonhistone proteins were reconstituted by gradient dialysis, as described in the Experimental Procedures with DNA:histone:nonhistone protein ratios (w/w) of 1:1:0.5 (○), 1:1:0.8 (●), 1:1:1 (△), and 1:1.2:1 (▲). RNA transcribed from native reticulocyte chromatin (□), as well as globin mRNA (■), were also hybridized to globin [$^3$H]cDNA.

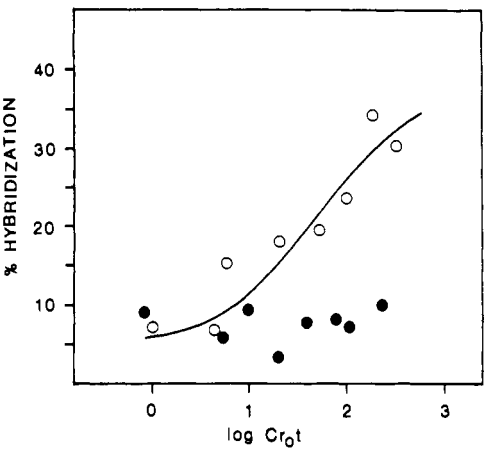


FIGURE 2: Kinetics of hybridization of RNA transcribed from reconstituted chromatin to globin [$^3$H]cDNA. Erythrocyte DNA and reticulocyte nonhistone proteins were reconstituted at ratios of 1:1 (w/w) by direct dialysis overnight against 5 M urea–10 mM Tris-HCl (pH 7.5). The fraction was then centrifuged at 200 000$g$ for 4 h. The pellet was taken up in 5 M urea–10 mM Tris-HCl (pH 7.5) and erythrocyte histones in 5 M urea–10 mM Tris-HCl (pH 7.5) were slowly added to give what would be an initial DNA:histone ratio (w/w) of 1:1.2 (○). Erythrocyte DNA and histones were reconstituted at a ratio (w/w) of 1:1.2 by direct dialysis overnight against 5 M urea–10 mM Tris-HCl (pH 7.5). The fraction was then centrifuged at 200 000$g$ for 4 h. The pellet was taken up in 5 M urea–10 mM Tris-HCl (pH 7.5) and reticulocyte nonhistone proteins in 5 M urea–10 mM Tris-HCl (pH 7.5) were slowly added to give what would be an initial DNA:nonhistone protein ratio (w/w) of 1:1 (●). The mixture was then dialyzed against 10 mM Tris-HCl (pH 7.5).

complexes using *E. coli* RNA polymerase. The RNA transcribed from the reconstituted chromatin fractions was hy-[$^3$H]cDNA hybridized to the RNA transcribed from reticulocyte chromatin under our hybridization conditions (Figure 1). The chromatin was prepared from the same cell batch from which the globin mRNA was isolated for the synthesis of labeled cDNA. Others also report the incomplete hybridization of globin [$^3$H]cDNA to the RNA transcribed from avian reticulocyte chromatin (Axel et al., 1973; Barrett et al., 1974).

For our reconstitution experiments we thought it was necessary to adjust the ratio of DNA to chromatin proteins for optimum globin gene transcription. We have determined that, in native reticulocyte chromatin, DNA, histone, and nonhistone proteins are present in ratios of 1:1:0.5 (w/w). Various ratios of erythrocyte DNA, histone, and reticulocyte nonhistone proteins were reconstituted to obtain optimum conditions for globin gene transcription. At a DNA:histone:nonhistone protein ratio of 1:1.2:1 (w/w), optimal conditions for globin gene transcription occurred as determined in Figure 1. Higher ratios of nonhistone proteins to DNA did not increase the amount of globin gene transcribed. The ratio of DNA to histones was not made greater than 1:1.2 (w/w) because we wished to simulate as closely as possible the conditions found in native chromatin. The higher ratio of nonhistone proteins to DNA required in our reconstitution mixture to obtain optimum hybridization (when compared with native reticulocyte chromatin) is most likely due to the possible, partial denaturation of nonhistone proteins during the fractionation of histones and nonhistone proteins in the presence of 7 M urea.

*Necessary Order of Reassociation of Chromosomal Proteins.* One of the goals was to determine at what point in the reconstitution process gene-specific elements in the nonhistone protein fraction reassociate with DNA. We were particularly interested in determining whether these gene-specific elements reassociate to DNA before or after the reassociation of the histones to DNA. To determine this, either reticulocyte nonhistone proteins or erythrocyte histones were allowed to reaassociate with erythrocyte DNA by reconstituting the mixture in 2 M NaCl–5 M urea and dialysis against gradually decreasing concentrations of NaCl in the presence of 5 M urea. The final mixture in 5 M urea was centrifuged to recover the DNA–protein complex. The DNA–protein complexes formed were taken up in 5 M urea and erythrocyte histones were added to the DNA–nonhistone complex, and reticulocyte nonhistone proteins were added to the DNA–histone complex. The mixtures in 5 M urea were dialyzed against 10 mM Tris (pH 7.5). RNA was then transcribed from both reconstituted chromatin bridized to globin [$^3$H]cDNA as shown in Figure 2. If the nonhistone protein fraction is allowed to reassociate with DNA before the histones are added, the reconstituted chromatin complex formed is capable of transcribing the globin genes. On the other hand, if the histone fraction is first allowed to reassociate with DNA, before the nonhistone proteins are added, the reconstituted chromatin complex is not capable of transcribing the globin gene. Thus it appears that nonhistone proteins have to bind to DNA before histones for transcription of globin gene. However, in this experiment, the maximum level of hybridization of [$^3$H]cDNA to RNA was 35–40%. We found that, in our earlier study in the absence of histones, only 50% of the reticulocyte nonhistone proteins bind to DNA in the presence of 5 M urea (Gadski & Chae, 1976). Thus, it is expected that, in the experiment shown in Figure 2, only 50% of the nonhistone proteins bound to DNA before histones. It is possible that the rest of the nonhistone proteins are also required for the 60% of transcription of globin genes seen in the case of native chromatin.

*Reassociation to DNA of Elements Controlling Globin Gene Transcription.* Chromatin reconstitution is routinely carried out by dissociating chromatin in 2–3 M NaCl–5 M urea. This is usually followed by a gradient dialysis against decreasing salt concentrations in the presence of 5 M urea (Bekhor et al., 1969). In our studies, we have investigated the reassociation of histones and nonhistone proteins during the stepwise reduction of salt concentration down to 0.1 M in the presence of 5 M urea. This is followed by the removal of any remaining salt and urea. This procedure was adapted because it is convenient for our studies to follow the reassociation of nonhistone proteins and histones to the DNA during the chromatin reconstitution. Histone H5 reassociates with DNA first in 0.5 M NaCl–5 M urea, followed by H1 in 0.4 M NaCl–5 M urea, H2A in 0.3 M NaCl–5 M urea, and H3, H4, and H2A in 0.2 M NaCl–5 M urea. The binding of histones to DNA is complete in 0.1 M NaCl–5 M urea. The binding of nonhistone proteins to DNA

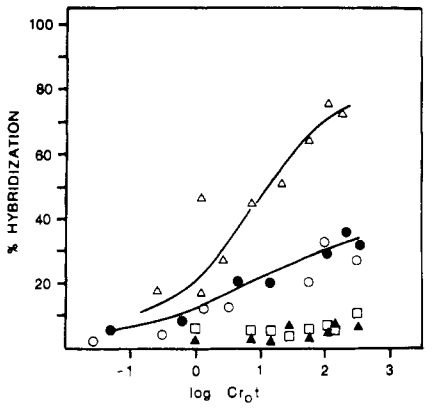


FIGURE 3: Kinetics of hybridization of RNA transcribed from reconstituted chromatin to globin [$^3$H]cDNA. Erythrocyte DNA and reticulocyte nonhistone proteins in 2 M NaCl–5 M urea–10 mM Tris-HCl (pH 7.5) were reconstituted at a ratio of 1:1 (w/w) by direct dialysis overnight against buffers containing 0.15 M NaCl (○), 0.3 M NaCl (●), 0.5 M NaCl (△), 1.0 M NaCl (▲), or 2.0 M NaCl (□) in 5 M urea–10 mM Tris-HCl (pH 7.5). The fractions were then centrifuged at 200 000$g$ for 4 h. The pellet was taken up in 5 M urea–10 mM Tris-HCl (pH 7.5) and erythrocyte histones in 5 M urea–10 mM Tris-HCl (pH 7.5) were slowly added to give what would be an initial DNA:histone ratio (w/w) of 1:1.2. The mixture was then dialyzed against 10 mM Tris-HCl (pH 7.5).

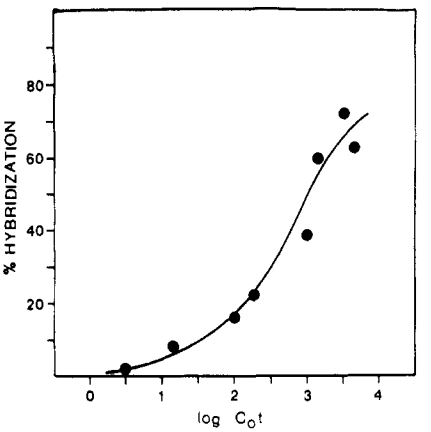


FIGURE 4: Kinetics of hybridization of DNA isolated from chromatin monomer to globin [$^3$H]cDNA. Hybridization was carried out following the same procedure as described for RNA-DNA hybridization except that the DNA was incubated in a boiling water bath for 5 min followed by rapid cooling before hybridization.

is gradual, and there are nonhistone proteins which reassociate with DNA before, at the same time, and after the binding of histones (Gadski & Chae, 1976). To determine at what salt concentration elements responsible for globin gene transcription reassociate with DNA, the total nonhistone protein fraction was allowed to reassociate with the DNA at various NaCl concentrations in 5 M urea. The DNA–nonhistone protein complex formed was then separated from the nonhistone proteins that did not reassociate to DNA by high-speed centrifugation. The histones were then added to the DNA–nonhistone protein complex. RNA transcribed from the various reconstituted chromatin complexes was hybridized to globin [$^3$H]cDNA, as shown in Figure 3. From the hybridization patterns obtained, it is apparent that elements in the nonhistone protein fraction responsible for globin gene transcription reassociate to the DNA at a salt concentration between 1.0 and 0.5 M NaCl in 5 M urea. (More recent evidence shows that this range is between 1.0 and 0.6 M NaCl.) Furthermore, the RNA transcribed from chromatin reconstituted with this DNA–nonhistone complex hybridized to approximately 70% of the globin [$^3$H]cDNA, a percentage which is higher than the value obtained from native chromatin (Figure 1). On the other hand, RNA transcribed from chromatin reconstituted with DNA–nonhistone protein complexes formed at 0.3 M NaCl–5 M urea or 0.15 M NaCl–5 M urea (Figure 3) can only hybridize to approximately 40% of the globin [$^3$H]cDNA. This value is very close to the value obtained with RNA transcribed from chromatin reconstituted with DNA–nonhistone protein complex formed at 5 M urea as shown in Figure 2. At the present time, we do not know the reason for the reduced transcription of the amount of RNA which hybridizes to globin [$^3$H]cDNA as more nonhistone proteins bind to DNA (as the concentration of salt is reduced from 0.5 M NaCl to 0 M). It is possible that there are nonhistone proteins which also inhibit the transcription of segments of globin gene or different globin gene subunits.

*Globin Gene Sequences Associated with Reconstituted Chromatin Monomers.* To determine whether histones had reassociated with DNA sequences coding for the globin genes during reconstitution, chromatin subunits were isolated from reconstituted chromatin. DNA was then purified from the fractions containing chromatin monomer and hybridized to globin [$^3$H]cDNA (Figure 4); the DNA hybridized to approximately 70% of globin [$^3$H]cDNA, suggesting that in reconstituted chromatin, histones are associated with at least 70% of the globin genes (in length). This experiment, however, does not distinguish between the globin gene sequences that are being transcribed and those that are not. Our data with reconstituted chromatin are consistent with that of Lacy & Axel (1975), who have shown that histones are associated with actively transcribed DNA sequences (globin genes) in native chromatin. Although the yield of chromatin monomer could have been lower the DNA isolated from the monomer obtained from the reconstituted chromatin hybridizes to the globin [$^3$H]cDNA at a rate similar to the rate obtained with total chicken erythrocyte DNA. Therefore, we can conclude that the binding of histones to DNA is essentially random during chromatin reconstitution.

## Discussion

Recently great confusion has been generated with regard to the chromatin transcription in vitro due to the significant level of gene-specific mRNA sequences attached to chromatin and due to the reports on the possible RNA transcription from mRNA template by *E. coli* RNA polymerase. In our case, however, the critical problem could have been the possible synthesis of RNA from undetectable amounts of globin mRNA, in the reticulocyte nonhistone protein fractions we used, by *E. coli* RNA polymerase. We found, however, that very little RNA was synthesized from globin mRNA by *E. coli* RNA polymerase under our reaction conditions which contain 5 mM $MgCl_2$. Tsai et al. also found that ribosomal RNA cannot be transcribed by *E. coli* RNA polymerase in the presence of $Mg^{2+}$ (Tsai, Tsai, Chang, and O'Malley, submitted for publication).

Most of the claim that endogenous mRNA can serve as template for *E. coli* RNA polymerase was derived from the use of HgUTP for the synthesis of HgRNA which can be separated from nonmercurated RNA by sulfhydryl (SH) affinity chromatography. However, HgRNA has a great tendency to aggregate with nonmercurated RNA and the aggregated RNA binds to an SH affinity column (Tsai, Tsai, Chang, and O'Malley, submitted for publication). In fact, the evidence for the transcription of endogenous RNA was based on the fact that the globin mRNA added to the DNA–histone complex binds to an SH column together with the in vitro

transcribed HgRNA (Zasloff & Felsenfeld, 1977) and that the in vivo labeled chromatin-bound RNA and in vitro transcribed HgRNA bind to a SH column together (Shih et al., 1977). These results were assumed to be due to the formation of hybrid between the endogenous RNA and in vitro transcribed HgRNA or the elongation of the in vivo initiated RNA. It now appears that these results were due to the nonspecific aggregation of HgRNA and nonmercurated RNA (Tsai, Tsai, Chang, and O'Malley, submitted for publication).

Also, in order to generate the level of globin mRNA sequences we see from the undetectable amount of endogenous globin mRNA sequences eventually an equal amount of the opposite strand of globin mRNA sequences has to be generated by *E. coli* RNA polymerase. However, at the ratio of the polymerase to chromatin used we found that asymmetric transcription occurs in the case of the transcription of simian virus 40 genes from chromatin isolated from a cell line transformed by the virus (Wong et al., manuscript in preparation). Others also reported assymmetric transcription of specific genes (Towle et al., 1977).

Therefore, this evidence, plus the fact that the chromatin reconstituted with *E. coli* DNA does not mediate transcription of globin mRNA by *E. coli* RNA polymerase, makes it highly unlikely that our results are due to the transcription of endogenous RNA. We also rule out the possible elongation of the in vivo initiated globin mRNA since we used RNase-treated chicken erythrocyte DNA in our studies. Chicken erythrocytes do not synthesize globin mRNA and the chromatin isolated from chicken erythrocyte does not mediate transcription of globin mRNA (Barrett et al., 1974; our unpublished results).

There also has been criticism for using *E. coli* RNA polymerase for chromatin transcription studies. The main problem is that a large amount of RNA is needed to detect single-gene transcripts by RNA-DNA hybridization, and this requires the use of several hundred nanomole units of RNA polymerase for each transcription experiment. The maximum reported nanomole units for RNA polymerase II from 1 kg of wheat germ is 70 (Jendrisak & Burgess, 1975). Unless we can study the reconstitution of chromatin using an isolated gene system it is difficult to use RNA polymerase II. Another difficulty is the uncertainty in the true initiation sites for most single copy genes. A precursor of cytoplasmic globin mRNA as large as 27 S (~5000 nucleotides) has been reported (Bastos & Aviv, 1977). Therefore, there is the possibility that the true globin gene could be several-fold larger than the coding region which is about 600 base pairs. The transcription of ribosomal RNA genes (Ballal et al., 1977) and 5S RNA gene (Parker & Roeder, 1977) by *E. coli* RNA polymerase is nonspecific and the strand selection for gene transcription is also poor compared to the respective eucaryotic RNA polymerase. Therefore, there is the good possibility that the results we have obtained could have been due to the nonspecific transcription of the globin gene by *E. coli* RNA polymerase as far as the initiation is concerned, but *E. coli* RNA polymerase most likely recognizes the structural feature of transcriptionally active chromatin. We are presently studying this possibility with chromatin reconstituted with double-stranded globin gene produced from globin mRNA which contains only the coding region of the gene. As long as *E. coli* RNA polymerase transcribes tissue-specific genes from isolated chromatin, which appears to be the case, the enzyme can be useful as a probe for studies on the structure of transcriptionally active chromatin.

The procedure of chromatin reconstitution used in this report does not necessarily produce a good yield of chromatin subunit structures compared to other variations in the dialysis of the chromatin in 2 M NaCl-5 M urea (Woodcock, 1977). However, the sequence of binding of histones and nonhistone proteins to DNA can be followed more systematically in the procedure we used in this report. Also, during the fractionation of chromosomal proteins and reconstitution, chromosomal proteins are exposed to the denaturing agent, 5 M urea, and it is quite conceivable that a significant amount of proteins will not renature during the removal of urea. It could be for this reason that we had to add a twofold excess of nonhistone proteins to the reconstitution mixture in order to obtain the amount of globin mRNA synthesized by native chromatin.

There are at least three possibilities for chromatin reassembly that would allow for selected gene transcription in the reconstituted chromatin. The first possibility was that, during chromatin reconstitution, no proteins bind to the regions of DNA that are being actively transcribed. This would be unlikely, since this does not take into account any factors necessary for restricted gene transcription, nor the fact that, when histones alone reassociate with DNA, they bind randomly along the DNA (Polisky & McCarthy, 1975). Also, there are reports that suggest that histones are associated with regions of DNA that are being actively transcribed in native chromatin (Lacy & Axel, 1975; Weintraub & Groudine, 1976). The second possibility was that specific nonhistone proteins reassociate with an already formed DNA-histone complex. Elements in the nonhistone protein fraction would then bind to the DNA-histone complex, altering it in such a way that selected sequences of DNA would become accessible for RNA transcription. A third possibility was that nonhistone proteins first specifically associate with the DNA, before the histones, and are then capable of directing the reassociation of the histones in regions of DNA being transcribed. This direction could be in the form of the inhibition of histones binding to selected regions of DNA, or an alteration in the manner in which the histones are associated to DNA.

The data presented in this report suggest that there is a specific order for reassociation of the chromosomal proteins to DNA for correct globin gene transcription. Under the conditions used for reticulocyte chromatin reconstitution in this work, it was found that, for the globin gene to be transcribed, specific elements in the nonhistone protein fraction must reassociate with the DNA before reassociation of the histones. During chromatin reconstitution, elements controlling globin gene transcription appear to reassociate with the DNA before histones when the salt concentration is reduced from 1.0 M NaCl to 0.6 M NaCl in 5 M urea. In a previous report, we have shown that histone H5 first reassociates with DNA in 0.5 M NaCl-5 M urea, and no other histones are bound to DNA at 0.5 M NaCl-5 M urea (Gadski & Chae, 1976). Also, the majority of the nonhistone proteins do not bind to DNA until the NaCl is removed in 5 M urea (Gadski & Chae, 1976). From this we conclude that the fidelity of chromatin reconstitution is probably a result of the high affinity that the elements responsible for specific sequence transcription have for DNA. The early reassociation of these elements to specific DNA sequences could foreordain the manner of reassociation of other proteins to those selected regions of DNA resulting in a product capable of specific sequence transcription. It is quite likely that the actively transcribed globin gene is bound by histones since the amount of the globin gene present in the chromatin subunit isolated from reconstituted chromatin is roughly the same as the amount present in the total chick DNA.

Although the reconstitution carried out under the denaturing conditions does not resemble the in vivo conditions, the fact that certain nonhistone elements (most likely proteins)

must reassociate before histones for transcription of the globin gene suggests the possibility that the transcriptionally active structure of chromatin probably is assembled during DNA replication as also suggested by Weintraub & Groudine (1976).

It appears from our data that the extent of globin gene transcription varies depending on the nonhistone protein fractions that bind to DNA at various salt concentrations in 5 M urea. This observation may be the result of various nonhistone protein fractions contributing to control mechanism for globin gene transcription. Also, there is the possibility that this variation is a result of the reconstitution technique used in this work. These possibilities are being investigated.

The results obtained so far certainly do not offer a complete picture of how transcriptionally active chromatin is reassembled during reconstitution. For example, we used [$^3$H]-cDNA, which is complementary to $\alpha$- and $\beta$-globin mRNA; only half of the [$^3$H]cDNA was full length. Also only 60% of the globin gene (in length) appears to be transcribed from reticulocyte chromatin, as others have also noted (Axel et al., 1973; Barrett et al., 1974). Therefore, uncertainty remains as to whether or not both $\alpha$ and $\beta$-globin genes are regulated in the same manner. Since the predominant form of [$^3$H]cDNA is copied from the 3′-terminal half of mRNA, it is not certain whether the incomplete hybridization of [$^3$H]cDNA to chromatin transcripts is due to partial transcription of the globin gene. We plan to answer these questions with full-length [$^3$H]cDNA of $\alpha$- and $\beta$-globin mRNA. The experimental approaches used in our study could be useful for investigating the regulation of different globin subunit genes during differentiation of avian red blood cells, as well as for characterizing the elements controlling globin genes and the structure of transcriptionally active chromatin.

## References


Aviv, H., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69,* 1408–1412.

Axel, R., Cedar, H., & Felsenfeld, G. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 2029–2032.

Axel, R., Meschion, W., Sollner-Webb, B., & Felsenfeld, G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 4104–4105.

Ballal, N. R., Choi, Y. C., Mouche, R., & Busch, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 2446–2450.

Barrett, T., Maryanka, D., Hamlyn, P. H. & Gould, H. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 5057–5061.

Bastos, R. N., & Aviv, H. (1977) *Cell 11,* 641–650.

Bekhor, I., Kung, G., & Bonner, J. (1969) *J. Mol. Biol. 39,* 351–364.

Burgess, R. R., & Jendrisak, J. J. (1976) *Biochemistry 15,* 4634–4638.

Gadski, R. A., & Chae, C.-B. (1976) *Biochemistry 15,* 3812–3817.

Gilmour, R. S., & Paul, J. (1975) *Chromosomal Proteins and Their Role in the Regulation of Gene Expression* (Stein, G. S., & Kleinsmith, L. J., Eds.) pp 19–33, Academic Press, New York, N.Y.

Graziano, S. L., & Huang, R. C. (1971) *Biochemistry 10,* 4770–4777.

Huang, R. C., & Huang, P. C. (1969) *J. Mol. Biol. 39,* 365–378.

Jendrisak, J. J., & Burgess, R. R. (1975) *Biochemistry 14,* 4639–4645.

Lacy, E., & Axel, R. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 3978–3982.

Noll, H. (1967), *Nature (London) 215,* 360–363.

Noll, M. (1974) *Nature (London) 251,* 249–251.

Oudet, P., Gross-Bellard, M., & Chambon, P. (1975) *Cell 4,* 281–300.

Parker, C. S., & Roeder, R. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 44–48.

Paul, J., & Gilmour, R. S. (1968) *J. Mol. Biol. 34,* 305–316.

Paul, J., Gilmour, R. S., Alfara, N., Birnie, G., Harrison, P., Hell, A., Humphres, S., Windass, J., & Young, B. (1973) *Cold Spring Harbor Symp. Quant. Biol. 38,* 885–898.

Pemberton, R. E., Housman, D., Lodish, H. F., & Baglioni, J. (1972) *Nature (London), New Biol. 235,* 99–102.

Perry, R. P., LaTorre, J., Kelly, D. E., & Greenberg, J. R. (1972) *Biochim. Biophys. Acta 262,* 220–226.

Polisky, B., & McCarthy, B. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 2895–2899.

Roti, J., Stein, G. S., & Cerutti, P. (1974) *Biochemistry 13,* 1900–1904.

Shih, T. Y., Young, H. A., Parks, W. P., & Scolnick, E. M. (1977) *Biochemistry 16,* 1795–1801.

Spelsberg, T. C., Hnilica, L. S., & Ansevin, A. T. (1971) *Biochim. Biophys. Acta 228,* 550–562.

Staynov, D. Z., Pinder, J. C., & Gratzer, W. B. (1972) *Nature (London), New Biol. 235,* 108–110.

Towle, H. C., Tsai, M-J., Tsai, S. Y., & O'Malley, B. W. (1977) *J. Biol. Chem. 252,* 2396–2404.

Weintraub, J., & Groudine, M. (1976) *Science 193,* 848–956.

Wilson, G., Steggles, A., Kantor, J., Nienhuis, A., & Anderson, W. (1975) *J. Biol. Chem. 250,* 8604.

Woodcock, C. L. F. (1977) *Science 195,* 1350.

Zasloff, M. Z. & Felsenfeld, G. (1977) *Biochem. Biophys. Res. Commun. 75,* 598–603.