Chinault, A. C., Tan, K. H., Hassur, S. M., & Hecht, S. M. (1977) *Biochemistry 16*, 766–771.
Dudock, B., Katz, G., Taylor, E., & Holley, R. (1969) *Proc. Natl. Acad. Sci. U.S.A. 62*, 941–945.
Fersht, A. R., & Kaethner, M. M. (1976) *Biochemistry 15*, 3342–3346.
Fraser, T. H., & Rich, A. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2671–2675.
Fraser, T. H., & Rich, A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3044–3048.
Gordon, J. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 179–183.
Griffin, B. E., Jarman, M., Reese, C. D., Sulston, J. E., & Trentham, D. R. (1966) *Biochemistry 15*, 3638–3649.
Hecht, S. M., & Chinault, A. C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 405–409.
Hecht, S. M., Hawrelak, S. D., Kozarich, J. W., Schmidt, F. J., & Bock, R. M. (1973) *Biochem. Biophys. Res. Commun. 52*, 1341–1347.
Hecht, S. M., Tan, K. H., Chinault, A. C., & Acari, P. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 437–441.
Igloi, G. L., von der Haar, F., & Cramer, F. (1977) *Biochemistry 16*, 1696–1702.
Jekowksy, E. (1976) Ph.D. Thesis, Department of Biology, M.I.T.
Lucas-Lenard, J., & Lipmann, F. (1971) *Annu. Rev. Biochem. 40*, 409–448.
Miller, D. L., & Weissbach, H. (1970) *Arch. Biochem. Biophys. 141*, 26–34.
Sprinzl, M., & Cramer, F. (1973) *Nature (London), New Biol. 245*, 3–5.
Sprinzl, M., & Cramer, F. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3049–3053.
Sprinzl, M., Kucharzewski, M., Hobbs, J., & Cramer, F. (1977) *Eur. J. Biochem. 78*, 55–61.
von der Haar, F., & Cramer F. (1976) *Biochemistry 15*, 4131–4138.

# Simple Isolation of DNA Hydrophobically Complexed with Presumed Gene Regulatory Proteins ($M_3$)†

Isaac Bekhor* and Carol J. Mirell

ABSTRACT: Chromatin from chicken reticulocytes and mouse Ehrlich ascites tumor cells has been extracted with 2 M NaCl, leaving a portion of the DNA still complexed with a fraction of nonhistones (designated $M_3$, since it can be dissociated from DNA in solutions of 3 M NaCl containing 5 M urea). The DNA complexed with $M_3$, separated from the bulk DNA by centrifugation, was found to contain sequences poorly represented in bulk DNA. Specifically we found that DNA-$M_3$ complexes isolated from chicken reticulocyte chromatin were enriched in globin gene sequences by 20-fold relative to unfractionated DNA and by over 1000-fold relative to DNA rendered free of protein following the extraction of chromatin with 2 M NaCl. We have therefore isolated DNA fractions complexed with $M_3$ which are enriched in specific sequences as may be determined by $M_3$.

In this laboratory we have performed numerous experiments in order to classify the nonhistone chromosomal proteins into groups possessing distinguishable properties (see review by Bekhor, 1978). In the following experiments we have continued to study the characteristics of those nonhistones which are tightly bound to DNA. Those tightly bound proteins, designated $M_3$, require 3 M NaCl–5 M urea for dissociation and constitute about 5% of the total nonhistones.

The discovery of nucleosomes in chromatin (Olins & Olins, 1974; Kornberg, 1974; Sahasrabuddhe & Van Holde, 1974) has advanced our understanding of how the DNA is packaged with histones (Felsenfeld, 1978). The finding (Lacey & Axel, 1975; Kuo et al., 1976; Garel & Axel, 1976) that active genes may be clustered in nucleosomes suggested that both active and inactive chromatin may contain the general subunit structures with a repeating DNA chain length of 140 to 200 base pairs (Woodcock, 1973; Olins & Olins, 1973; Finch et al., 1975; Oudet et al., 1975; Johnson et al., 1976). Additional findings by Weintraub & Groudine (1976) and Levy & Dixon (1977) suggested that active genes may be more susceptible to digestion with DNase I than inactive genes. These findings supported the hypothesis that nucleosomes containing active genes are less rigid than DNA–histone complexes (Pyhtila et al., 1976). Such data led to the conclusion that nucleosomes are essential to the function of the genome, leaving unresolved the role of nonhistones in chromatin.

We have reported (Bekhor & Samal, 1977) that the tightly bound nonhistones showed dramatic effects on the transcription of DNA complexed with histones. Gadski & Chae (1978) reported that specific genetic activity might be determined by the DNA binding nonhistone fraction which reassociates prior to histones during reconstitution. Paulson & Laemmli (1977) and Adolph et al. (1977) have shown that HeLa cell metaphase chromosomes possessed a highly organized structure retaining the familiar metaphase morphology even after removal of the histones and most of the nonhistones. This structure was stabilized by a small number of nonhistones, which the authors (Adolph et al., 1977) called scaffolding proteins. The "scaffolding proteins", the tightly bound nonhistones of Gadski & Chae (1978), and our $M_3$ (Bekhor & Samal, 1977) probably are of the same class of proteins. Therefore, it is apparent that $M_3$ may contain proteins responsible for particular effects on the genome in general.

For these various reasons we have postulated that the removal of histones from chromatin into 2 M NaCl results in a fraction of DNA that is complexed with $M_3$ and enriched in specific sequences as determined by $M_3$. In previous communications (Samal & Bekhor, 1977; Bekhor & Samal,

† From the Department of Basic Sciences, School of Dentistry, and the Laboratory for Developmental Biology, Andrus Gerontology Center, University of Southern California, Los Angeles, California 90007. *Received September 28, 1978.* This work was supported in part by Grants DE-04255-03 and DE-04031-04 from the National Institute of Dental Research, National Institutes of Health, and in part by private funds.

0006-2960/79/0418-0609$01.00/0 

1977), we suggested the hypothesis that most of the proteins dissociated from DNA prior to $M_3$ may not be related to the activation of tissue-specific gene sequences in differentiated cells. Therefore, $M_3$ may include those regulatory proteins involved in determining specialized genetic expressions in various cells. In the following experiments we have isolated $M_3$ complexed with DNA enriched in specific sequences, thus supporting the conclusion that $M_3$ may contain proteins which are ultimately responsible for cell specialization.

## Experimental Procedures

*Cells, Preparation, and Labeling.* Mouse Ehrlich ascites tumor cells, propagated in the peritoneal cavity of adult Swiss–Webster mice with a culture from the Cancer Center at the University of Southern California, were harvested on the seventh day. Reticulocytes were prepared from adult white Leghorn chickens by five daily subcutaneous injections of 1.0 mL of phenylhydrazine (10 mg/mL). On the seventh day the chickens were decapitated and the blood was collected into 5 volumes of ice-cold deionized water. For labeling of DNA, the mice were injected with 100 $\mu$Ci/mouse of [$^3$H]thymidine (55 Ci/mmol, Schwarz/Mann) on the fourth day for harvesting on the seventh day. For labeling of proteins, the mice were injected with 50 $\mu$Ci/mouse of [$^3$H]leucine (59 Ci/mmol, Schwarz/Mann) on the fourth day for harvesting on the seventh day. Labeling experiments were not done with chicken reticulocytes.

*Preparation of Chromatin.* All operations were done at 0–4 °C and all biochemicals were purchased from Calbiochem-Behring unless otherwise indicated. Chromatin was prepared from cells essentially according to Bhorjee & Pederson (1973) with modifications. The cells were first suspended in 5 volumes of deionized water and homogenized gently in a Dounce homogenizer with a B pestle. The cells were collected by centrifugation in a refrigerated Sorvall at 3000 rpm for 5 min. The packed cells were suspended into cell wash buffer (10 mM Tris-HCl, 1 mM EDTA, 0.14 M NaCl, pH 7.9) and repelleted at 3000 rpm for 5 min. Cells were lysed in lysing buffer (10 mM Tris-HCl, 0.1 M NaCl, 1.5 mM $MgCl_2$, 0.1 mM $PhCH_2SO_2F$,[1] pH 7.9) containing 0.2% Triton X-100. The released nuclei were pelleted at 3000 rpm for 5 min and washed twice with the lysing buffer. The nuclei were broken by homogenization in TPD (10 mM Tris-HCl, 0.2 mM DTT, 0.1 mM $PhCH_2SO_2F$, pH 8.0) at a concentration of about 50 $A_{260}$/mL. This suspension was layered over 3 volumes of 1.1 M sucrose in TPD and centrifuged at 2500 rpm for 15 min in a Beckman SW 27 rotor to pellet nucleoli. The crude chromatin supernatant was layered over 2 volumes of 1.7 M sucrose and centrifuged for 90 min at 25000 rpm in a Beckman SW 27 rotor to obtain purified chromatin.

*Fractionation of Chromatin–DNA into Soluble DNA (DNA-S) and Protein-Bound DNA (DNA-P).* The method used is summarized in Figure 1. Purified chromatin was immediately suspended into 2 M NaCl in TPD by homogenization to a uniform solution at concentrations of about 50 $A_{260}$/mL. This solution was placed over one-third volume of 1.0 M sucrose–2 M NaCl in TPD and centrifuged in a

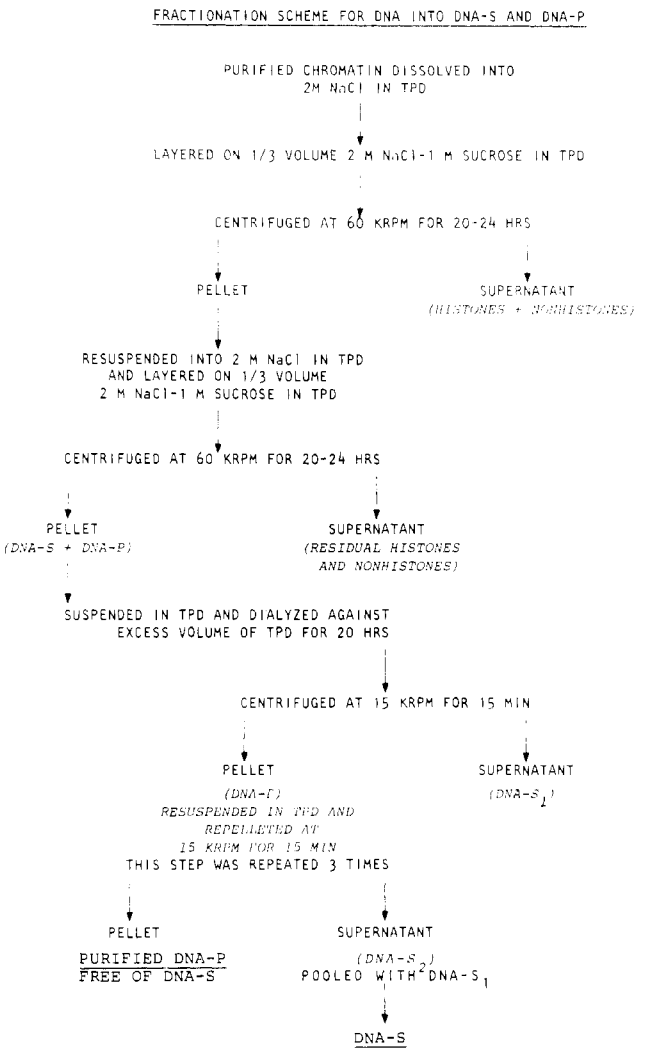


FIGURE 1: Fractionation scheme for chromatin–DNA into soluble DNA (DNA-S) and nonhistone chromosomal protein-bound DNA (DNA-P).

Beckman 60 Ti rotor at 60000 rpm for a minimum period of 20 h. This step was repeated, and the final pellet, consisting of a clear protein-free DNA (DNA-S) underlaid with a DNA–protein aggregate (DNA-P), was suspended into TPD and dialyzed against 1000 volumes of TPD. The DNA solution (DNA-S and DNA-P) was centrifuged in the Sorvall at 15000 rpm for 10 min to pellet DNA-P. The pelleted DNA-P was resuspended in TPD, homogenized to separate residual DNA-S from DNA-P, and centrifuged in the Sorvall at 15000 rpm for 10 min. This step was repeated until the resulting pellet was freed of DNA-S.

*Digestion with Micrococcal Nuclease.* Chromatin, DNA-P, DNA-S, and DNA were digested at 37 °C for 20 min with varying concentrations of micrococcal nuclease, as indicated in the appropriate experiments, in 1 mL of digestion buffer (10 mM Tris-HCl, 0.2 mM DTT, 0.1 mM $PhCH_2SO_2F$, 0.2 mM $CaCl_2$, pH 7.9). Where the DNA was labeled with [$^3$H]thymidine, the reaction was stopped by the addition of 5 mL of 6% $Cl_3CCOOH$ containing 100 $\mu$g of bovine serum albumin as a carrier. Resistant counts to micrococcal nuclease were collected on Millipore nitrocellulose filters, washed with 5% $Cl_3CCOOH$, and counted in a toluene/Triton X-100 (2:1) cocktail. For electrophoresis, the reaction was stopped by the addition of 100 $\mu$L of 0.1 M EDTA containing 10% sodium dodecyl sulfate, and the DNA was deproteinized and sized by electrophoresis as described below.

[1] Abbreviations used: TPD, 10 mM Tris-HCl, 0.2 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, pH 8.0; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; NHCP, nonhistone chromosomal proteins; EDTA, (ethylenedinitrilo)tetraacetic acid; $M_3$, 5 M urea–3 M NaCl–TPD soluble chromosomal proteins from 2 M NaCl–TPD extracted chromatin; DNA-S, soluble DNA obtained from 2 M NaCl–TPD extracted chromatin; DNA-P, chromosomal protein-bound DNA obtained from 2 M NaCl–TPD extracted chromatin.

*Digestion with Restriction Nucleases.* DNA fractions were purified as described below and digested for 1 h at 37 °C with 5 units of enzyme per 1 μg of DNA (where 1 unit is defined as that amount of enzyme required to digest 1 μg of λ DNA per h). Digestions were done with *EcoRI*, *EcoRII*, and *HaeIII* in the appropriate buffers: (*EcoRI* buffer) 100 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM mercaptoethanol, 50 mM NaCl, pH 7.2; (*EcoRII* buffer) 100 mM Tris-HCl, 5 mM $MgCl_2$, pH 8.0; (*HaeIII* buffer) 50 mM Tris-HCl, 5 mM $MgCl_2$, 0.5 mM DTT, pH 7.5. DNA from each assay reaction is electrophoresed on horizontal agarose slab gels, as described below, developed with 0.5 μg/mL of ethidium bromide for visualization over a short UV light source, and photographed with a Polaroid MP-4 camera using a Kodak 23A phase filter.

*Preparation of DNA.* All DNA samples were purified by the same method. DNA was first RNased with 10 units/mL of RNase $T_1$ and 20 μg/mL of pancreatic RNase A by incubation for 1 h at room temperature in TPD and then digested with 50 μg of Pronase/mL in a solution of 1% sodium dodecyl sulfate–1 N NaCl for 30 min at 37 °C. Residual proteins were removed by several chloroform/isopentyl alcohol (24:1) extractions. The purified DNA was precipitated with 2 volumes of ethanol at −20 °C overnight. The resultant DNAs were dissolved in a minimum volume of distilled water and dialyzed against distilled water. The DNAs were spectrally pure ($A_{260}/A_{230}$ = 2.4) and were used immediately for the various assays.

*Preparation of RNA Polymerase from Escherichia coli and Assay for Transcription. E. coli* RNA polymerase was prepared by the method of Burgess (1969). The enzyme eluted from DEAE-cellulose was precipitated with ammonium sulfate (0.32 g/mL), collected by centrifugation, dissolved, and dialyzed overnight against 50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM $PhCH_2SO_2F$, 0.2 mM DTT, 0.3 M NaCl, and 50% glycerol, pH 7.9. The dialyzed enzyme was stored in aliquots at −80 °C. For transcription the method of Roeder (1974) was followed at the ammonium sulfate concentration of 0.075 M. The reaction mixture contained, in addition to DNA and *E. coli* RNA polymerase, ATP, CTP, GTP, [$^3$H]UTP (15 Ci/mmol, Amersham), $MnCl_2$, and Tris-HCl to give final concentrations as indicated by Roeder (1974). The final volume of the mixture was 50 μL. The incubation was for 20 min at 37 °C, and amounts of [$^3$H]UTP incorporated into RNA were assayed for as described by Roeder (1974).

*Preparation of cDNA from Chicken Globin mRNA and Hybridization to Various DNA Fractions.* Chicken reticulocyte globin mRNA was a gift from Dr. Chi-Bom Chae of the University of North Carolina. Complementary DNA (cDNA) was synthesized from the template globin mRNA essentially as described by Friedman & Rosbash (1977). The reaction mixture contained the following: 44.4 μL of [$^3$H]-dCTP (24 Ci/mmol, 0.5 Ci/mL; Amersham) which had previously been evaporated to dryness under nitrogen at 0 °C in sterile Falcon tubes, 50 mM Tris-HCl, pH 8.3, 6 mM $MgCl_2$, 60 mM NaCl, 20 mM DTT, 100 μg/mL of actinomycin D, 8 μg/mL of oligo(dT), 1 mM each of dATP, dGTP, dTTP, 20 μg/mL of globin mRNA, and 400 units/mL of avian myeloblastosis virus reverse transcriptase (a gift from Dr. J. Beard of Life Sciences, Inc.) in a final volume of 25 μL. After incubating for 20 min at 37 °C, the reaction was terminated by the addition of EDTA and sodium dodecyl sulfate at final concentrations of 10 mM and 1%, respectively. This solution was made 0.25 N in NaOH and incubated at 70 °C from 15 min to hydrolyze the RNA template. After the solution was cooled to 4 °C, it was neutralized with 1 N HCl and loaded with 50 μg of *E. coli* tRNA as carrier on a sterile Sephadex G-50 column (Bio-Rad Econo-column, 50 × 0.7 cm), which had been previously equilibrated with the running buffer (10 mM EDTA, 65 mM NaCl, 20 mM Tris-HCl, pH 7.5). Fractions of 0.5 mL were collected in sterile plastic tubes; 15 μL of each fraction was counted in a Triton X-100/toluene (1:2) cocktail. The fractions containing [$^3$H]cDNA were pooled, ammonium acetate was added at a final concentration of 0.24 M, and the cDNA was precipitated at −20 °C with 2.5 volumes of ethanol. The final cDNA pellet was dissolved in sterile distilled water ($10^6$ cpm/mL) and frozen in aliquots at −80 °C.

Complementary DNA (500 cpm/10 μL reaction mixture volume) was hybridized at 68 °C with sheared DNA-S, DNA-P, and total DNA up to a $C_0t$ of 10000 (mol s)/L as described by Weintraub & Groudine (1976). The cDNA probe had a specific activity of $2.5 \times 10^7$ cpm/μg and was 550–600 nucleotides long as measured by fluorography (Bonner & Laskey, 1974). The concentrations of DNA and the time of incubation were varied to obtain specific $C_0t$ values. Hybridizations were done with excess heat-denatured DNA in 0.3 M NaCl, 50 mM Tris-HCl, 0.1% sodium dodecyl sulfate, pH 7.4. Polypropylene tubes overlaid with paraffin oil were used for the hybridizations. The tubes were first heated at 90 °C for 10 min and then transferred to a 68 °C water bath for initiation of hybridizations. The reactions were stopped by adding 0.4 mL of a mixture of 30 mM sodium acetate (pH 4.5), 0.15 M NaCl, and 1 mM $ZnSO_4$. The samples were later incubated with 1000 units of $S_1$ nuclease at 45 °C for 40 min. After digestion, 100 μg of carrier *E. coli* tRNA was added to each sample, and the hybrids were precipitated by the addition of equal volume of cold 16% trichloroacetic acid. The hybrids were collected on nitrocellulose filters, washed with 5% trichloroacetic acid, and counted in Triton X-100/toluene (1:2) cocktail. The data are plotted as the percentage of cDNA hybridized as a function of the $C_0t$ (Britten & Kohne, 1968).

*Electrophoresis.* Electrophoresis of DNA was performed on 1–3% horizontal-slab agarose gels using the Tris/borate system of Peacock & Dingman (1967). The DNA samples were dissolved in 20 μL of 50 mM Tris-HCl, pH 8.3, containing 1% sucrose. Bromophenol blue was added as the tracking dye. We used φχ174 RF DNA cleaved with *Hae*III, and λ DNA cleaved with *Eco*RI as standards. The standards varied in base lengths from 234 to 21400 base pairs. The gels were run in Tris/borate buffer at 50 V for 5 to 7 h and later developed with ethidium bromide. Electrophoresis of the proteins was done on polyacrylamide disc gels as described by Laemmli (1970).

*Other Methods.* Protein was determined according to Hartree (1972) with bovine serum albumin as standard. DNA was determined by the diphenylamine method of Burton (1955). Chromatin was dehistonized in 0.2 N $H_2SO_4$ prior to measurements of the nonhistone/DNA ratios in the various DNA/chromosomal protein fractions. Thermal denaturation of chromatin, DNA-P, DNA-S, and unfractionated DNA was done in $2.5 \times 10^{-4}$ M EDTA (pH 8.0) in an automated Pye-Unicam spectrophotometer Model SP 1700, equipped with a Temperature Programme Controller Model SP 876.

## Results

*Properties of DNA-S and DNA-P from Mouse Ehrlich Cells.* DNA-S and DNA-P were isolated from mouse Ehrlich cells as described in Figure 1. The DNA/nonhistone protein ratios observed in the various fractions investigated are shown

Table I: Ratios of Nonhistone Chromosomal Proteins to DNA in Various DNA Fractions

| tissue | component | NHCP/DNA | yield in DNA (%) |
|---|---|---|---|
| mouse Ehrlich cells | chromatin | 1.20 | 100 |
| | DNA-S | 0.12 | 96.8 |
| | DNA-P | 0.64 | 3.2 |
| chicken reticulocytes | chromatin | 0.93 | 100 |
| | DNA-S | 0.02 | 98.8 |
| | DNA-P | 0.72 | 1.2 |

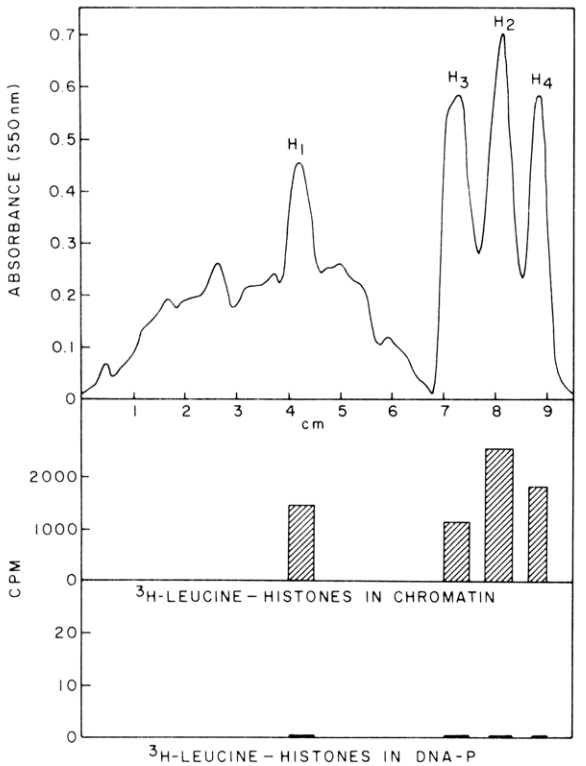


FIGURE 2: Analysis of [$^3$H]leucine–histones found in chromatin and in DNA-P from mouse Ehrlich cells. Chromatin and DNA-P were dehistonized with 0.2 N $H_2SO_4$ as described in Experimental Procedures, and the sulfuric acid supernatants were dialyzed against deionized water, lyophilized, and dissolved into a minimum volume of 4 M urea/0.1% sodium dodecyl sulfate. We loaded about 50 $\mu$g of protein (100 $\mu$L) per column for polyacrylamide disc gel electrophoresis as described in Experimental Procedures. The gels were developed with Coomassie blue R and scanned at 550 nm in a Pye-Unicam spectrophotometer; the histone bands from the labeled chromatin were cut, dissolved into 0.5 mL of 5% $H_2O_2$ at 60 °C for ~12 h, and counted in Triton X-100/toluene (1:2) cocktail. The proteins obtained from DNA-P were fortified with 10 $\mu$g/mL of unlabeled histones as markers. The histone bands in the developed gels were again cut and analyzed for radioactivity as above.

in Table I. The DNA-P fraction represents 1–3% of the total DNA. The ratio of proteins to DNA in DNA-P was greater than 0.6 in all fractions, while the protein/DNA ratio in DNA-S was 0.12 in mouse Ehrlich cells and 0.02 in chicken reticulocytes. To determine whether any histones still remained associated with DNA-P, mouse Ehrlich cells were labeled in vivo with [$^3$H]leucine. The data in Figure 2 suggest that 2 M NaCl extraction of chromatin yields a DNA-P fraction that is free of histones. The specific activity of the histones in these experiments was 450 cpm/$\mu$g. Since DNA-P was apparently devoid of histones, we investigated whether this fraction contained fragments which were resistant to digestion with micrococcal nuclease. For this experiment we have labeled the DNA in mouse Ehrlich cells in vivo with

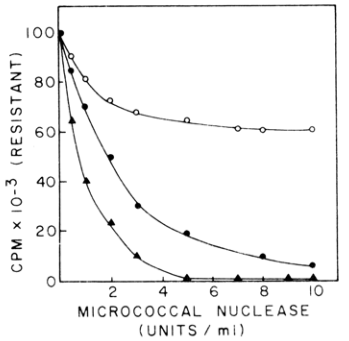


FIGURE 3: Micrococcal nuclease digestion kinetics of [$^3$H]thymidine labeled chromatin (○), DNA-P (●), DNA-S or unfractionated DNA (▲) isolated from mouse Ehrlich cells.

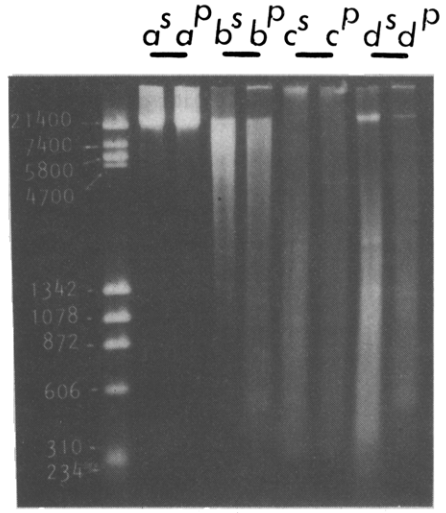


FIGURE 4: Restriction endonuclease digestion patterns of mouse Ehrlich cell DNA-S (s) and DNA-P (p) with *Eco*RI (b), *Eco*RII (c), and *Hae*III (d), as seen by electrophoresis at 50 V for 7 h on 1.5% horizontal agarose slab gels. "a" indicates the position of the undigested DNA-S and DNA-P. Number of base-pair markers were obtained by digestion of λ DNA with *Eco*RI, and $\phi\chi$174 DNA with *Hae*III. Positions of the various bands are analyzed in Table II. Concentrations of DNA per well varied between 1.5 and 2 $\mu$g.

[$^3$H]thymidine. The labeled chromatin was isolated, and the DNA again fractionated into DNA-S and DNA-P (Figure 1). The specific activity of DNA was 1100 cpm/$\mu$g when harvested. Chromatin, DNA-S, DNA-P, and unfractionated DNA, all labeled with [$^3$H]thymidine, were treated with varying concentrations of micrococcal nuclease as shown in Figure 3. The results indicate that, while chromatin was 65% resistant to digestion, DNA-P was totally digested, but at a slower rate than DNA.

Restriction endonucleases were also used to assay for possible differences in sequences between DNA-P and DNA-S fractions of Ehrlich cell chromatin. Pfeiffer et al. (1975) and Horz et al. (1976) reported that treatment of nuclei and DNA from mouse and rat livers with *Eco*RII and *Hae*III results in the appearance of DNA fragments with a repeat pattern of 225–245 base pairs. Figure 4 shows the results of such an experiment. We find that the repeat pattern is present in our preparations as well. *EcoRII* cleavage of DNA-S and DNA-P results in repeat patterns of multiples of 250 base pairs (Table II). *Hae*III digestion shows a repeat pattern of about 650 base pairs (Table II), with minor differences between the two fractions, if any. However, digestion with EcoRI certainly suggests a major difference in band patterns between DNA-S and DNA-P (Figure 4 and Table II). Therefore the data from digestion with *Eco*RI and *Hae*III imply that the DNA-P and

Table II: Number of Base Pairs in the Various DNA-S and DNA-P Bands as Seen in Figure 4

| *Eco*RI | | *Eco*RII | | *Hae*III | |
|---|---|---|---|---|---|
| $b^s$ | $b^p$ | $c^s$ | $c^p$ | $d^s$ | $d^p$ |
| | | | | 4300 | |
| | | 2450 | 2450 | 2600 | 2600 |
| 1475 | 1475 | | | | |
| | 1350 | | | 1300 | 1300 |
| | | 750 | 750 | | |
| | 500 | 500 | 500 | | 600 |
| | | 250 | 250 | | |

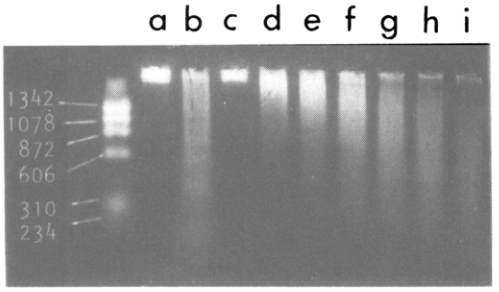


FIGURE 5: Micrococcal nuclease digestion pattern of chicken reticulocyte chromatin (a, undigested; b, digested with 10 units/100 μg of DNA) or DNA-P (c, undigested; d–i, digested with increasing amounts of micrococcal nuclease, 1 unit, 2, 3, 5, 7.5, and 10 units/100 μg of DNA), all incubated for 20 min at 37 °C, and the DNA freed of protein as described in Experimental Procedures. Concentrations of DNA per well varied between 0.5 and 1 μg. Electrophoresis was at 50 V for 5 h on 3% agarose slab gels.

DNA-S fractions from Ehrlich cells show differences in specific repeating sequences.

*Properties of DNA-S and DNA-P from Chicken Reticulocytes.* Unfractionated chromatin and DNA-P (Figure 1) from chicken reticulocytes were digested with micrococcal nuclease as shown in Figure 5. The chromatin subunits are evident with the monomer occurring at 190 base pairs. The data suggest that (1) there are no repeating units in DNA-P; (2) histone octamers are not found in DNA-P; and (3) all of the DNA in DNA-P is degradable with micrococcal nuclease. These findings support our previous conclusions gained from our studies on Ehrlich cells (Figures 2 and 3). Digestion of chicken reticulocyte DNA-P and DNA-S with *Eco*RI, *Eco*RII, and *Hae*III (Figure 6) did not reveal any differences between DNA-S and DNA-P. In both cases *Eco*RI digestion resulted in a minor band occurring at 1225 base pairs. Digestion with *Eco*RII and *Hae*III resulted in smears, indicating that these cleavage sites were randomly distributed throughout the DNA. This correlates with the data reported by Doel et al. (1977) that chicken DNA digestion with *Hae*II and *Hind*III revealed the complete absence of detectable banding in the digests.

Thermal denaturation (Figure 7) shows that the $T_m$ of DNA-S was 41 °C, while DNA-P exhibited a $T_{m1}$ at 46 °C and a $T_{m2}$ at 58 °C, with a $T_m$ at 50% hyperchromicity of 49 °C. The transitions observed in DNA-S and DNA-P suggest negligible cross-contamination. Chromatin exhibited two transitions, one occurring at $T_m$ = °C and a second at $T_m$ = 83 °C. The template activity of DNA-S and DNA-P was also examined with *E. coli* RNA polymerase as shown in Figure 8. These experiments indicated that the template activity of DNA-P was substantially the same as that observed for DNA-S, and equivalent to the activity obtained with protein-free DNA.

Assessments of differences in specific DNA sequences between chicken reticulocyte DNA-P and DNA-S came from studies on the distribution of globin-gene sequences in these

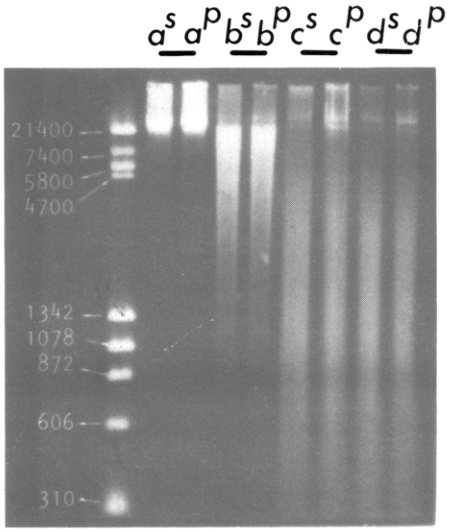


FIGURE 6: Restriction endonuclease digestion of chicken reticulocyte DNA-S (s) and DNA-P (p) with *Eco*RI (b), *Eco*RII (c), and *Hae*III (d). Electrophoresis was done as described under Figure 4. "a" designates the positions of the undigested DNA-S and DNA-P on the gel.

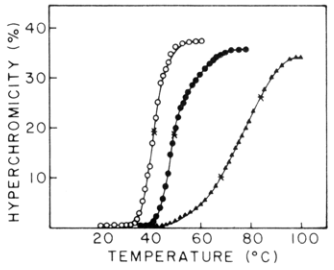


FIGURE 7: Thermal denaturation profiles of DNA-S or unfractionated protein-free DNA (○), DNA-P (●), and chromatin (▲) isolated from chicken reticulocytes. Asterisks indicate $T_m$ for the various transitions.

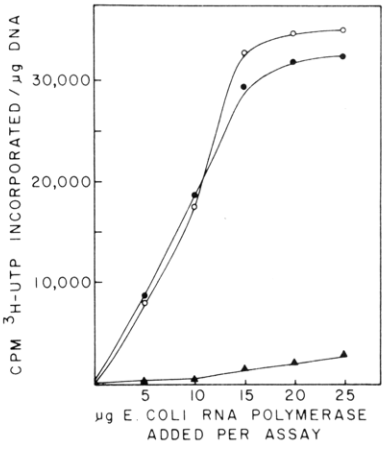


FIGURE 8: The template activity of chicken reticulocyte DNA-S or unfractionated DNA (○) and DNA-P (●), as measured by *E. coli* RNA polymerase. [$^3$H]UTP incorporated into RNA by *E. coli* RNA polymerase in the absence of added template is shown by ▲.

two fractions. The globin mRNA, obtained from Dr. Chae, had a $R_0t_{1/2}$ = 1000 (mol s)/L, indicating that it is composed of at least two mRNA species (Groudine & Weintraub, 1975; Jackson et al., 1976). In our experiments we used this mRNA for synthesis of cDNA by methods optimized by Friedman & Rosbash (1977), yielding a cDNA of an average length of 600 nucleotides as measured by fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975). This is essentially full length; the size of chicken globin mRNA was estimated to be 650 nucleotides (Crouse et al., 1976).

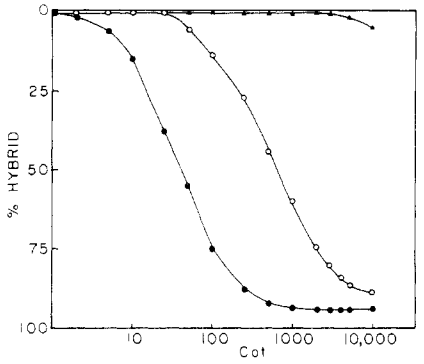


FIGURE 9: Hybridization kinetics of globin cDNA with chicken reticulocyte unfractionated DNA (○), DNA-P (●), and DNA-S (▲) measured as described in Experimental Procedures. $C_0t_{1/2}$ for unfractionated DNA = 650; $C_0t_{1/2}$ for DNA-P = 35; extrapolated $C_0t_{1/2}$ for DNA-S = 50 000.

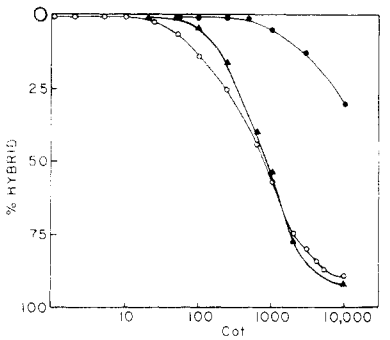


FIGURE 10: Hybridization kinetics of globin cDNA with chicken liver unfractionated DNA (○), DNA-P (●), and DNA-S (▲) measured as described in Experimental Procedures. $C_0t_{1/2}$ for unfractionated DNA = 650; $C_0t_{1/2}$ for DNA-S = 750; extrapolated $C_0t_{1/2}$ for DNA-P = 25 000.

The DNA used for hybridization was isolated from chromatin, DNA-S, and DNA-P, either from chicken reticulocytes or chicken liver, a nonerythroid tissue (Freeman, 1971). The DNA was sheared to an average length of about 900 base pairs by sonication with a Bronwill sonifier at maximum intensity in the cold for 5 min. The results from the hybridization experiments are shown in Figure 9 for chicken reticulocytes and Figure 10 for chicken liver. DNA-P from chicken reticulocytes showed a $C_0t_{1/2}$ = 35, while the total unfractionated DNA showed a $C_0t_{1/2}$ of 650. Similar $C_0t_{1/2}$ values for chicken DNA hybridizing with globin cDNA have been reported by Weintraub & Groudine (1976) and by Gadski & Chae (1978). DNA-S showed negligible hybridization with globin cDNA. If the data for DNA-S are extrapolated to give a rate of hybridization of a similar slope as found for total DNA, we would obtain a $C_0t_{1/2}$ for DNA-S of about 50 000, thereby suggesting that in chicken reticulocytes DNA-P may contain virtually all of the globin-gene sequences. Therefore, enrichment of the globin gene obtained in DNA-P relative to total DNA is about 20-fold, and relative to DNA-S it exceeds 1000-fold. The fact that the slopes of the hybridization curves of DNA-P and total DNA were similar implies that the fractionation of DNA as shown in Figure 1 results in actual enrichment of the globin-gene sequences. This conclusion was further confirmed with chicken liver (Figure 10). The hybridization kinetics shows that, in contrast to chicken reticulocytes, liver DNA-S did hybridize with globin cDNA to a $C_0t_{1/2}$ of 750 in comparison to a $C_0t_{1/2}$ of 650 for the unfractionated DNA, while DNA-P showed little hybridization, with an extrapolated $C_0t_{1/2}$ of 25 000. Therefore, the data of Figures 9 and 10 strongly suggest that the DNA sequences found in DNA-P may be determined by its associated proteins, the $M_3$ proteins. We regard it significant that we find a specific enrichment of gene sequences in DNA-P from a tissue actively transcribing a gene product (in our case globin mRNA), while an enrichment of this type is not seen in the DNA-P of a tissue not producing this product.

## Discussion

Traditionally, chromatin has been fractionated into transcriptionally active (euchromatin) and inactive (heterochromatin) components in order to identify and eventually characterize the transcriptional units. Recently, Hendrick et al. (1977), using methods of Gottesfeld et al. (1974) to fractionate chromatin, were able to enrich the globin coding gene of chicken reticulocyte genome by three- to fivefold. Although DNase II digestion of chromatin yields fragments which are transcriptionally active, the composition of these units cannot be ascertained because of their high impurity. It is well established that the transcriptional units contain in the least the histone octamers (Lacy & Axel, 1975; Axel et al., 1975; Kuo et al., 1976) in a conformation that is sensitive to digestion with DNase I (Weintraub & Groudine, 1976; Garel & Axel, 1976; Flint & Weintraub, 1977; Panet & Cedar, 1977). Recently, Neumann et al. (1978) studied the nucleosome-associated proteins and reported the presence of an array of nonhistones in mononucleosomes from uninduced Friend cells, a part of which become undetectable upon induction with dimethyl sulfoxide or *n*-butyrate. The presence of nonhistones in nucleosomes has been reported by others (Liew & Chan, 1976; Chan & Liew, 1977; Bohm et al., 1977). Thus the role of nonhistones in chromatin is a major problem in biology. The nonhistones have been implicated in gene regulation solely based on indirect information obtained from overextended methods of reconstitution. The discovery of nucleosomes has shifted experimentations from the role of nonhistones to the role of histones in the genome.

Goodwin & Brahms (1978), using physical methods for determining effects of chromosomal proteins on the conformation of DNA in chromatin and in isolated nucleosomes, have shown that the nonhistones interact with DNA. Goodwin & Brahms (1978) elegantly showed by Raman spectral analysis that the histones interact with the DNA in chromatin in the minor groove, while the nonhistones in chromatin from tissues with high template activities interact with DNA in the major groove. Therefore, according to Goodwin & Brahms, the sites of interaction of the nonhistones and histones are different. These investigators also proposed that the major groove may involve hydrogen bonding between the N-7 group of guanine and the side chains of the nonhistone amino acid residues. Other interacting sites, including hydrophobic interactions, must also be considered to confer specificity to bonding between DNA and the nonhistones. If such a specificity actually occurs, then the use of solutions of 2 M NaCl cannot destroy such an interaction. On the other hand, use of solvents that tend to weaken hydrogen and hydrophobic bonding can cause dissociation of the molecules from each other. Therefore, it is not surprising that a solution of 5 M urea has been used quite successfully in studies on chromosomal protein interactions with DNA.

The findings that modifications in the primary structures of histones through phosphorylation and acetylation (Balhorn et al., 1972; Gurley et al., 1974; Riuz-Carrillo et al., 1975; Jackson et al., 1975) result in increased nuclease sensitivity of the chromatin tentatively provide a mechanism by which RNA polymerase could migrate through loosened nucleosome regions (Seally & Chalkley, 1978). Such a mechanism,

however, still leaves the question of specificity totally unresolved. Studies on DNA complexed in vitro with nonhistones seem to form a reasonable approach to analyzing protein interactions with DNA in the absence of histones. Jagodzinski et al. (1978) have studied total rat liver nonhistones interacting with DNA and reported a fourfold enrichment in DNA/protein binding sites by such methods. The drawback of such experiments is that the nonhistones, which may total in the hundreds (although they may have resulted in enrichment of presumed specific DNA sequences), cannot be assigned a function or a specificity. More convincing are those experiments reported on the fractionation of chromatin itself, which have resulted in a three- to fivefold enrichment of the globin gene (Hendrick et al., 1977). It is unlikely that such an enrichment was obtained by Jagodzinski et al. (1978), since total DNA and unfractionated nonhistones were used in their studies.

The experiments reported in this communication were essentially a direct result of the methods of fractionation of nonhistones which we have introduced in 1974 (Bekhor et al., 1974). The data reported establish three fundamental principles: (1) the nonhistones are fractionated into strong hydrophobically bound proteins; (2) they can be used to separate the DNA into specific DNA sequences reflecting the activity of at least one specific structural gene and probably other gene-specific DNA sequences, and into DNA representing the balance of the total animal genome; and (3) the protein-complexed DNA is isolated in its "native" state. Native simply means that these nonhistone proteins are probably still bound to their original specific DNA sequences as found in native chromatin in spite of the removal of histones, since the binding sites for histones and nonhistones appear to be different (Goodwin & Brahms, 1978). In addition, such proteins, which we have designated $M_3$ and seem to form very strong hydrophobic, and possibly hydrogen bonding with specific DNA sequences, can be dissociated from DNA in solutions of 2–3 M NaCl containing 5 M urea, as reported in this communication and elsewhere (Bekhor, 1978; Gadski & Chae, 1978).

In the present work we have studied various properties of such a complex between $M_3$ and DNA, isolated by methods which do not result in the destruction of such complexes (Figure 1). The complex of $M_3$–DNA appears to be (1) free of histones (Figures 2 and 5; if residual histones are still present in $M_3$, DNA-P would be expected to exhibit a $C_0t_{1/2}$ value shifted toward that of total DNA; thus the actual $C_0t_{1/2}$ may be less than what we have estimated for globin cDNA/DNA-P reassociation); (2) its rate of digestion with micrococcal nuclease is slower than DNA free of protein (Figure 3), and shows no subunit formation (Figure 5); (3) it does not contain large regions of free DNA as analyzed by the method of thermal denaturation (Figure 7); (4) it shows some differences in its susceptibility to digestion with restriction nucleases (Figure 4 and Table II); (5) its template activity as measured with *E. coli* RNA polymerase is of the same magnitude as found for DNA free of protein (Figure 8); and finally (6) it shows a very significant enrichment in specific DNA sequences (globin-gene sequences) relative to unfractionated DNA (Figure 9). The experiments which suggest that $M_3$ may be in part regulatory originate from the data reported in Figures 9 and 10. DNA-P from chicken reticulocytes (Figure 9) shows almost total enrichment in globin sequences relative to DNA-S, while in the case for chicken liver no such enrichment is observed (Figure 10). DNA-P from chicken liver shows insignificant hybridization to globin cDNA.

We therefore conclude that we have isolated a DNA fraction in part complexed with proteins ($M_3$) which may be responsible for the expression of globin-gene sequences in chicken reticulocytes.

Acknowledgments

We thank Dr. Chi-Bom Chae for his generous supply of chicken reticulocyte globin mRNA. We are also indebted to our colleagues Gary Norman for the synthesis and sizing of globin cDNA and David Gates for his critical evaluations of our experiments.

References

Adolph, K. W., Chang, S. M., & Laemmli, U. K. (1977) *Cell 12*, 805–816.
Axel, R., Cedar, H., & Felsenfeld, G. (1975) *Biochemistry 14*, 2489–2495.
Balhorn, R., Oliver, D., Hohmann, P., Chalkley, R., & Granner, D. (1972) *Biochemistry 11*, 3921–3925.
Bekhor, I. (1978) in *The Cell Nucleus* (Bush, H., Ed.) Vol. 5, pp 137–166, Academic Press, New York.
Bekhor, I., & Samal, B. (1977) *Arch. Biochem. Biophys. 179*, 537–544.
Bekhor, I., Lapeyre, J. N., & Kim, J. (1974) *Arch. Biochem. Biophys. 161*, 1–10.
Bhorjee, J. S., & Pederson, T. (1973) *Biochemistry 12*, 2766–27773.
Bohm, J., Keil, G., & Knippers, R. (1977) *Eur. J. Biochem. 78*, 251–266.
Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.
Britten, R. J., & Kohne, D. (1968) *Science 161*, 529.
Burgess, R. (1969) *J. Biol. Chem. 244*, 6160–6167.
Burton, K. (1955) *Biochem. J. 62*, 315–323.
Chan, P. K., & Liew, C. C. (1977) *Can. J. Biochem. 55*, 847–855.
Crouse, G. F., Fodor, E. J. B., & Doty, P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1564–1567.
Doel, N. T., Houghton, M., Cook, E. A., & Carey, N. H. (1977) *Nucleic Acids Res. 4*, 3701–3713.
Felsenfeld, G. (1978) *Nature (London) 271*, 115–122.
Finch, J. T., Noll, M., & Kornberg, R. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3320–3322.
Flint, S. J., & Weintraub, H. (1977) *Cell 12*, 783–794.
Freeman, B. M. (1971) in *Physiology and Biochemistry of The Domestic Fowl* (Bell, D. J., & Freeman, B. M. Eds.) Vol. 2, 841–852, Academic Press, New York.
Friedman, E. Y., & Rosbash, M. (1977) *Nucleic Acids Res. 4*, 3455–3471.
Gadski, R. A., & Chae, C. B. (1978) *Biochemistry 17*, 869–874.
Garel, A., & Axel, R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3966–3970.
Goodwin, D. C., & Brahms, J. (1978) *Nucleic Acids Res. 5*, 835–850.
Gottesfeld, J., Garrard, W., Baqi, G., Wilson, R., & Bonner, J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2193–2197.
Groudine, M., & Weintraub, H. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4464–4468.
Gurley, L. R., Walter, R. A., & Tobey, R. A. (1974) *J. Cell Biol. 60*, 356–364.
Hartree, E. F. (1972) *Anal. Biochem. 48*, 422–427.
Hendrick, D., Tolstoshev, P., & Randlett, D. (1977) *Gene 2*, 147–158.
Horz, W., Igo-Kemenes, T., Pfeiffer, W., & Zachau, H. G. (1976) *Nucleic Acids Res. 3*, 3213–3226.

Jackson, V., Shires, A., Chalkley, R., & Granner, D. K. (1975) *J. Biol. Chem. 250*, 4856–4863.
Jackson, J. F., Tolstoshev, P., Williamson, R., & Hendrick, D. (1976) *Nucleic Acids Res. 3*, 2019–2026.
Jagodzinski, L. L., Chilton, J. C., & Sevall, J. S. (1978) *Nucleic Acids Res. 5*, 1487–1499.
Johnson, E. M., Littau, V. C., Allfrey, V. G., & Bradbury, E. M. (1976) *Nucleic Acids Res. 3*, 3313–3329
Kornberg, R. D. (1974) *Science 184*, 868–871.
Kuo, M. T., Sahasrabuddhe, D. G., & Saunders, G. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1572–1575.
Lacy, E., & Axel, R. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3978–3982.
Laemmli, U. K. (1970) *Nature (London) 277*, 315–323.
Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335–341.
Levy, B. W., & Dixon, G. H. (1977) *Eur. J. Biochem. 74*, 61–67.
Liew, C. C., & Chan, P. K. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3458–3462.
Neumann, J., Whittaker, B., Blanchard, B., & Ingram, V. (1978) *Nucleic Acids Res. 5*, 1675–1678.
Olins, A. L., & Olins, D. E. (1973) *J. Cell Biol. 59*, 252a.
Olins, A. L., & Olins, D. E. (1974) *Science 183*, 330–332.
Oudet, P., Gross-Ballard, M., & Chambon, P. (1975) *Cell 4*, 281–300.
Panet, A., & Cedar, H. (1977) *Cell 11*, 933–940.
Paulson, J. R., & Laemmli, U. K. (1977) *Cell 12*, 817–828.
Peacock, A. C., & Dingman, C. W. (1967) *Biochemistry 6*, 1818–1827.
Pfeiffer, W., Hprz, W., Igo-Kemenes, T., & Zachau, H. G. (1975) *Nature (London) 258*, 450–452.
Pyhtila, M. J., Miettola, I., Kurkela, T., & Hoyhtya, A. (1976) *Acta. Chem. Scand. 30*, 797–798.
Roeder, R. G. (1974) *J. Biol. Chem. 244*, 6160–6167.
Ruiz-Carrillo, A., Wangh, L. J., & Allfrey, V. G. (1975) *Science 190*, 117–128.
Sahasrabuddhe, C. G., & Van Holde, K. E. (1974) *J. Biol. Chem. 249*, 152–156.
Samal, B., & Bekhor, I. (1977) *Arch. Biochem. Biophys. 179*, 527–536.
Sealy, L., & Chalkley, R. (1978) *Nucleic Acids Res. 5*, 1863–1876.
Weintraub, H., & Groudine, M. (1976) *Science 193*, 848–856.
Woodcock, C. L. F. (1973) *J. Cell Biol. 59*, 368a.

# Control of Cellular Content of Chicken Egg White Protein Specific RNA during Estrogen Administration and Withdrawal†

Nancy E. Hynes,‡ Bernd Groner,‡ Albrecht E. Sippel, Sigrid Jeep, Tilmann Wurtz, M. Chi Nguyen-Huu, Klaus Giesecke, and Günther Schütz*

ABSTRACT: The mRNAs for the egg white proteins ovalbumin, ovomucoid, and lysozyme have been isolated [Groner, B., Hynes, N. E., Sippel, A. E., Jeep, S., Nguyen-Huu, M. C., & Schütz, G. (1977) *J. Biol. Chem. 252*, 6666]. In this communication we describe the isolation of conalbumin-specific mRNA. DNA complementary to these mRNAs served as hybridization probes to determine the content of egg white protein mRNA sequences in total RNA of oviducts from chicks after estradiol administration and withdrawal. Chicks that had been hormone stimulated and subsequently rapidly withdrawn from the hormone show a rapid and preferential loss of egg white protein mRNAs. Within 1 day of hormone withdrawal ovalbumin mRNA content had decreased 50-fold and conalbumin mRNA content 17-fold, while the concentration of non-egg-white protein mRNAs did not change significantly. When chicks were restimulated with estrogens following acute hormone withdrawal, ovalbumin mRNA, ovomucoid mRNA, and lysozyme mRNA accumulate with a constant rate only after a lag of approximately 3 h while conalbumin mRNA accumulates with no lag. The method of hormone administration and withdrawal used in these studies does not lead to changes in the proportion of tubular gland cells in the oviduct. Therefore, the accumulation pattern observed cannot arise from complex effects due to cellular differentiation. Transferrin which is synthesized in the liver has a protein moiety identical with conalbumin. Using stringent hybridization and assay conditions we have found that conalbumin cDNA hybridizes to liver RNA. We have determined that 500–600 molecules per cell of conalbumin mRNA are present in livers isolated from immature chicks and from hens. This result shows that estrogen can affect regulation of the same gene product in two different target tissues in a different manner.

The chicken oviduct is an organ which is sensitive to the action of the steroid hormones estradiol, progesterone, and testosterone. In the tubular gland cells of the hen oviduct approximately 75% of the proteins synthesized are the egg white proteins ovalbumin, conalbumin, ovomucoid, and lysozyme (Palmiter, 1972), while in the immature or hormone-withdrawn organ their synthesis is not detectable (Oka & Schimke, 1969; Palmiter et al., 1970). It has been shown that the hormonal induction of egg white proteins is a consequence of accumulation of their mRNAs (Chan et al., 1973; Rhoads et al., 1973; Palmiter & Smith, 1973), and investigations are aimed at determining the mechanism by which steroid hormones exert control of mRNA content.

† From the Max-Planck-Institut für Molekulare Genetik, 1000 Berlin 33 Dahlem, West Germany. *Received February 2, 1978; revised manuscript received September 15, 1978.*

‡ Present address: Swiss Institute for Experimental Cancer Research, Ch. des Boveresses, 1066 Epalinges, S/Lausanne, Switzerland.

0006-2960/79/0418-0616$01.00/0 