Nonhistone Proteins Control Gene Expression in Reconstituted Chromatin

(nonhistones/histones/chicken globin mRNA/complementary DNA/RNA polymerase)

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ABSTRACT Chromatin was reconstituted from the purified DNA and histones of chicken erythrocytes and the nonhistone proteins of either chicken reticulocytes or chicken liver. Reconstituted chromatins, native chicken reticulocyte chromatin, and free DNA were transcribed with Escherichia coli RNA polymerase and the concentrations of globin-specific sequences in the RNA products were measured by hybridization with [3H]DNA complementary to chicken globin messenger RNA. Reticulocyte, but not liver, nonhistone proteins were shown to activate the globin genes in reconstituted erythrocyte chromatin. The transcripts of native and reconstituted chromatins were indistinguishable in respect of both the total yield of the RNA and the fractional yield of globin-specific sequences.

Pioneering work by Paul and Gilmour (1–3) implicated the nonhistone proteins of chromatin in determining the specificity of RNA synthesis in eukaryotic systems. The relevant experiments performed by these authors entailed the following steps: (1) fractionation of three chromatin constituents, DNA, histones, and nonhistone proteins; (2) reconstitution of homologous and heterologous chromatins by dialysis of the required components from 6 M urea and 2 M NaCl into 0.2 M NaCl; (3) transcription of native and reconstituted chromatins with bacterial RNA polymerase; and (4) characterization of the nucleotide sequences in the synthesized RNA by the hybridization tests then available. These tests enabled Paul and Gilmour to analyze only a small fraction of the transcribed RNA, that transcribed from the highly reiterated sequences in the DNA. This “redundant” DNA does not appear to include the genes for cytoplasmic messenger RNAs, e.g., globin messenger RNA (4). The RNA transcribed from the highly reiterated DNA sequences is mainly confined to the nucleus and its function is unknown. Nevertheless, the experiments were interesting because they showed for the first time that a substantial proportion of this type of RNA synthesized in vitro is “organ specific” (1), that chromatin could be reconstituted from its constituents, apparently without changing the specificity of transcription (2), and that the nonhistone proteins control transcription of at least one fraction of the DNA (3). In other words, it seemed possible that the reconstitution procedure originally devised by Dahmus and Bonner (5) may suffice to restore the biological function of chromatin.

That the nonhistone protein fraction might be responsible for differential gene expression is suggested by a number of less direct observations. The nonhistone protein fraction exhibits both greater diversity and more obvious tissue specificity than the histones (6). The activity of chromatin in vitro bears a proportional relation to the nonhistone protein content (7). The more active parts of chromatin contain a higher proportion of these proteins than the less active parts (8).

The importance of translational control of protein synthesis in eukaryotes is currently at issue. However, the synthesis of any specific protein requires the presence of the corresponding messenger RNA, and the range of specific messenger RNAs available for translation is very restricted in highly differentiated tissues such as reticulocytes (9). The control mechanism operating on the messenger population to a large extent determines the spectrum of proteins and thus the phenotypic characteristics of the differentiated cell. Elucidation of these control mechanisms is fundamental to the understanding of differentiation.

The discovery of RNA-dependent DNA polymerase (reverse transcriptase) in RNA viruses immediately suggested to several groups of workers a possible way of measuring the concentrations of specific messenger RNAs. Conditions were reported simultaneously from three laboratories (10–12) that stimulate the enzyme to copy globin messenger RNA. The complementary DNA product synthesized in vitro from radioactive precursors is an extremely sensitive “probe” for globin messenger RNA. Axel et al. (13) and Gilmour and Paul (14) were the first to take advantage of this “probe” to measure the concentrations of globin-specific sequences in chromatin transcripts. Both groups recently showed that chromatin isolated from erythropoietic tissues, but not from other tissue types examined, serves as a template for the synthesis of globin-specific sequences in vitro. Like Paul and Gilmour’s original experiments on the transcription of the highly reiterated DNA (1), the more recent results indicate that the control mechanisms operating on the genetic material in vivo are conserved in isolated chromatin.

We have now performed reconstitution experiments analogous to the earlier work of Paul and Gilmour (2, 3). The results presented below demonstrate that the capacity of chromatin to serve as a template for globin-specific RNA sequences is recovered during the reconstitution from DNA, histones, and nonhistone proteins. Moreover, the nonhistone protein fraction from erythropoietic tissue is specifically required for expression of the globin genes in the reconstituted chromatin.

METHODS

Preparation and Characterization of Chicken Globin Messenger RNA. Chicken globin messenger ribonucleoprotein was prepared from chicken reticulocytes according to the methods previously devised for rabbit globin messenger ribonucleoprotein (9). The RNA and protein were dissociated by treat-
ment with 1.0% sodium dodecyl sulfate. The RNA was further purified by centrifugation through isokinetic 5.09–42.9% (w/v) sucrose gradients containing 10 mM Tris·HCl, 1.0 mM EDTA, 0.1 M LiCl, and 1% sodium dodecyl sulfate, pH 7.5, using the Spinco SW27 rotor at 27,000 rpm for 17.5 hr at 15°. RNA sedimentating at 9S was precipitated with 2 volumes ethanol and chromatographed on an oligo(dT) cellulose column eluted stepwise successively with 0.5 M NaCl, 10 mM Tris, pH 7.5, and with 10 mM Tris, pH 7.5 (15). The second (adenylated) fraction was used as a template for reverse transcriptase. The molecular weight distribution of messenger RNA was determined by polyacrylamide gel electrophoresis in the presence of formamide (16).

Preparation and Characterization of DNA Complementary to Chicken Globin Messenger RNA. Complementary DNA (cDNA) was prepared using reverse transcriptase from avian myeloblastosis virus in standard conditions (9). The template activity of the chicken messenger was first assayed by titrating the enzyme with various amounts of the RNA. Purified rabbit globin messenger RNA was assayed in the same experiment at a typical concentration. Experimental details are given in the legend to Fig. 2. To prepare a larger quantity of cDNA for hybridization experiments, the reaction was scaled up 10 times by using 52 μg chicken globin messenger RNA, which gave 0.9 μg cDNA (specific activity 5 × 10^6 cpm/μg). DNA was extracted from the incubation mixture (10) and its size was determined from its sedimentation rate in alkaline sucrose gradients (17) (Fig. 3).

Preparation of Nuclei. Chicken liver nuclei were prepared by the method of Blobel and Potter (18) except that the nuclei were centrifuged through 2.1 instead of 2.4 M sucrose.

Chickens were made anemic [80–90% reticulocytosis (19)], by 6 daily injections of 0.8 ml 2.5% phenylhydrazine. The nuclei were prepared by a modification of the Zentgraf procedure (20) in which the saturated sucrose solution was replaced by 2.0 M sucrose, with 1 mM MgCl₂, 10 mM Tris·HCl, pH 7.5, 4 mM octanoil, and 0.5% Triton X-100.

Preparation of Chromatin. The nuclear pellets from liver or reticulocytes were extracted twice for 30 min with 200 ml of 0.35 M NaCl, 10 mM Tris·HCl, pH 7.5, and homogenized twice with 100 ml water, with recovery of the pellets between homogenization by sedimentation in the Sorvall HB-4 rotor for 10 min at 10,000 rpm.

Preparation of Chicken Erythrocyte DNA. The method of Bellard et al. (21) for extracting rat liver DNA of high molecular weight was applied to chicken erythrocytes. The resulting DNA was dissolved at 1–2 mg/ml in 5 M urea, 10 mM NaCl, 10 mM Tris·HCl, pH 8.3.

Preparation of Chicken Erythrocyte Histones. Histones were prepared from chicken erythrocyte chromatin by sulfuric acid extraction according to the method of Panyim et al. (22). The histones were dissolved at 1 mg/ml in buffer R 5 M urea, 2 M NaCl, 10 mM Tris·HCl, pH 8.3.

Preparation of Nonhistone Proteins. Chromatin containing not more than 100 mg DNA was homogenized in 200 ml of 6 M urea (deionized), 0.1 M sodium phosphate, pH 7.0, 0.35 M guanidinium chloride, and 0.1% 2-mercaptoethanol. The solution was centrifuged in the Sorvall HB-4 rotor at 5000 rpm for 5 min and the supernatant was centrifuged in the Spinco T60 rotor at 58,000 rpm for 15 hr. The nonhistone proteins were separated from histones on two Bio-Rex 70, 200–400 mesh, columns, 2.5 × 40 cm, by the method of Levy et al. (23). The proteins were concentrated to 1–2 mg/ml and dialyzed against buffer R in the Amicon apparatus.

Characterization of Histone and Nonhistone Protein Fractions. Proteins were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (24).

Reconstitution of Chromatin. DNA, histones, and nonhistone proteins were recombined by gradient dialysis from buffer R according to Gilmour and Paul (3) except that the final dialysis buffer contained 10 mM Tris·HCl buffer, pH 7.9. The DNA:protein ratios were 1:1 (w/w) for histones and 1:0.8 (w/w) for nonhistone proteins. Each dialyzed sample contained 0.8 mg DNA, the required proteins, and buffer R in a volume of 4.0 ml.

Transcription. Samples containing 0.8 mg DNA, free or recombined with proteins (total contents of dialysis bag), 0.4 mM each of CTP, GTP, ATP, and [3H]UTP (specific activity about 3 Ci/mmol on day of incubation), 6 mM MgCl₂, 1.6 mM MnCl₂, 50 mM Tris·HCl, pH 7.9, 0.15 M NaCl, and 300 units of *Escherichia coli* RNA polymerase (Sigma Chemical Co.) in a total volume of 6 ml, were incubated in a shaking water bath for 1 hr. at 37°.

Extraction of RNA. Extraction of RNA from the incubation mixtures was carried out according to the method of Penman (25) with the following modifications: (1) Digestion with 100 μg of DNase (RNase-free from Worthington Biochemicals) was allowed to proceed for 60 min at 37°; (2) the reaction was stopped by the addition of 0.6 ml 10% sodium dodecyl sulfate, 0.15 ml 0.2 M EDTA, at room temperature; (3) 6 ml of molten phenol were added and a hot phenol extraction was performed at 55° followed by 3 chloroform extractions. RNA in the final aqueous phase was precipitated with 2 volumes of ethanol at −20°, pelleted by centrifuging in the HB-4 rotor for 15 min at 10,000 rpm, and dried in vacuo. RNA was separated from unpolimerized nucleotides on a Sephadex G-50 column, 1 × 50 cm. Fractions containing the RNA, usually eluting in 10–12 ml, were pooled and mixed with 0.1 volume 4 M LiCl and 900 μg carrier tRNA. The RNA was recovered by precipitation with 2 volumes of ethanol at −20°.

Hybridization Experiments. 10,000 cpm [H]cDNA (5 × 10^8 cpm/μg) and 9–50 μg RNA transcript were annealed at 69° in 40–200 μl containing 1 mM Tris·HCl, pH 8.0, 0.5 M NaCl, 0.5 mM EDTA, and 30 μg sonicated calf thymus DNA. The reaction mixtures were overlayed with a drop of paraffin oil to prevent evaporation. Aliquots, 0.1 of the total volume, were taken at selected intervals (15 min–72 hr) and diluted into 0.5 ml 40 mM NaCl, 30 mM sodium acetate, pH 4.5, 0.12 mM ZnSO₄. The RNA in 0.25 ml aliquots was immediately precipitated with 20% trichloroacetic acid and the remainder was incubated for 1 hr at 37° with S1 DNase before similar precipitation.

RESULTS

Characterization of Chicken Globin Messenger RNA. The electrophoretic pattern of the purified chicken globin messenger RNA in polyacrylamide gels containing formamide exhibits a single broad zone in the region between the two zones corresponding to the rabbit α- and β-globin messenger RNAs. (Fig. 1). The molecular weights of the chicken globin mes-
Electrophoretic patterns on proteins (5).

Fig. 1. 4% Polyacrylamide-formamide gel electrophoresis of RNA. Left to right: 1, 17 μg chicken 9S RNA from an isokinetic gradient. 2, 20 μg chicken rRNA eluted from an oligo(dT)-cellulose column with 0.5 M NaCl, 10 mM Tris·HCl, pH 7.5. 3, 20 μg chicken globin mRNA eluted from an oligo(dT)-cellulose column with 10 mM Tris, pH 7.5 (adenylated fraction). 4, 10 μg rabbit globin mRNA prepared as described by Gould and Hamlyn (9). Electrophoresis was for 4 hours at 70 volts. The running buffer (0.02 M NaCl) was constantly circulated. Gels were stained with 0.1% pyronin Y in 0.5% glacial acetic acid, 1 mM citric acid.

 messenger RNAs must therefore fall in the same range, from 202,000 to 270,000 (14). The observed polydispersity of chicken globin messenger RNA is consistent with the presence of two major hemoglobins in chicken (cf. one in rabbit) made up of four (cf. two in rabbit) different polypeptide chains (26).

Chicken globin messenger RNA is equally efficient as rabbit globin messenger RNA as a template for reverse transcriptase from avian myeloblastosis virus (Fig. 2).

Characterization of Complementary DNA. The molecular weight of the cDNA was estimated to be 136,000 from its relative sedimentation rate in alkaline sucrose gradients (Fig. 3) using the Studier equation (17).

The specificity of the “probe” was tested by annealing to the chicken globin messenger RNA template, to rabbit globin messenger RNA and to calf thymus messenger RNA. The DNA hybridized only with the homologous RNA. The kinetics of the reaction between cDNA and its template RNA were analyzed. The Constant value derived from the curve shown in Fig. 4 is 2.5 × 10⁻³ mol·sec/liter, in close agreement with the values found for duck globin messenger RNA of 2.0 × 10⁻³ (G. Felsenfeld, personal communication) and rabbit globin messenger RNA of 3.0 × 10⁻³ (27).

Characterization of the Chromatin Protein Fractions. The electrophoretic patterns of the histones and the nonhistone proteins on sodium dodecyl sulfate-polyacrylamide gels (Fig. 5) exhibit negligible cross-contamination.

Hybridization of Chromatin Transcripts with cDNA. A description of the experiments performed together with the results derived from kinetic analysis (Figs. 6a–d) may be found in Table 1. We have found that native reticulocyte chromatin (Sample 9 in Table 1) and the chromatin reconstituted with DNA and histones from erythrocytes and the nonhistone protein fraction from reticulocytes (Sample 7) were transcribed by E. coli RNA polymerase with about equal efficiency.

Fig. 2. [³H]cDNA synthesis as a function of globin mRNA concentration. Assays were performed in 100 μl incubations at 37°C for 30 min. Assays contained 5 μg oligo(dT), 5 μg actinomycin D, 2 mM dithiothreitol, 0.2 mM each dATP, dCTP, and dTTP, 0.013 mM [³H]dGTP (6.5 Ci/mmol), 0.02 M NaCl, 6 mM Mg acetate, 0.1 M Tris·HCl, pH 7.8, and 0.04 M KCl. The reaction was stopped by addition of 20% trichloroacetic acid and the acid-precipitable radioactive material was determined. ●, Chicken globin mRNA; ▲, rabbit globin mRNA.

Fig. 3. Alkaline sucrose gradient of chicken globin [³H]-cDNA. The sample (30,000 cpm) was applied to a 5–20% linear sucrose gradient containing 0.9 M NaCl, 1 mM EDTA, adjusted to pH 12.5 with NaOH. Centrifugation was carried out at 40,500 rpm for 30 hr in a Beckman SW41 rotor at 4°C. 50 μg salmon sperm DNA sheared to a molecular weight of 3.74 × 10⁶ was added as a marker (arrow). The gradient was fractionated with an ISCO apparatus. Samples (5 μl) were taken from each fraction and the trichloroacetic-acid-precipitable radioactive material was determined. The molecular weight was calculated according to the equation of Studier (17).
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Fig. 4. Kinetics of annealing of chicken globin mRNA to complementary [H]cDNA. [H]cDNA (10,000 cpm) was annealed to 0.015 µg chicken globin mRNA in a 200 µl incubation. Hybridization was carried out as described in Methods.

0.137 µg RNA/mg DNA per unit enzyme for native and 0.115 µg RNA/mg DNA per unit enzyme for reconstituted chromatin. Moreover, the fractional yields of globin sequences for these two templates were very similar, 0.019% for native chromatin (Sample 2), and 0.007-0.025% for five samples of reconstituted chromatin (Samples 4-7, 11). The corresponding C_{50}, values (10-34 mol/sec/liter) are in close agreement with the values observed for native duck reticulocyte chromatin (11 mol/sec/liter) and for native rabbit bone marrow chromatin.

Fig. 5. Polyacrylamide–sodium dodecyl sulfate gel electrophoresis of nonhistone and histone proteins. Left to right: 1, 20 µg chicken erythrocyte histone; 2, 23 µg chicken liver histone; 3, 94 µg chicken reticulocyte nonhistone protein prepared on a Bio-Rex column; 4, 65 µg chicken liver nonhistone protein prepared on a hydroxylapatite column according to the method of MacGillivray et al. (31); 5, 68 µg chicken liver nonhistone protein prepared on a Bio-Rex column. Electrophoresis was at 50 volts for 6 hours. The gels were stained overnight in 0.1% Coomassie blue, 10% acetic acid, 50% methanol, and destained electrophoretically in 10% acetic acid, 10% methanol.

Fig. 6. Kinetics of annealing of chicken globin [H]cDNA to various RNA transcripts. [H]cDNA (10,000 cpm) was annealed to 9-50 µg of transcript (see Table 1) in 40 µl incubations. O, transcripts from reconstituted reticulocyte chromatin; ●, transcripts from (a) purified DNA (upper left); (b) DNA reconstituted with erythrocyte histone (upper right); (c) reconstituted liver chromatin (lower left); (d) no enzyme control (lower right).

(15 mol/sec/liter) reported by Axel et al. (13) and Steggles et al. (27), respectively. No globin-specific sequences were detectable in the transcript of erythrocyte chromatin reconstituted with the nonhistone fraction from liver (Sample 3), though this sample was an efficient template for enzyme. Neither free DNA, a good template (Sample 1), nor reconstituted erythrocyte nucleohistone, a poor template (Sample 2), yielded RNA with detectable concentrations of globin-specific sequences. The globin-specific sequences detected in the transcripts of the chromatin reconstituted with reticulocyte nonhistone proteins were synthesized de novo by E. coli RNA polymerase, as indicated by the enzyme requirement (see Samples 4-7 and 8, Table 1 and Fig. 6) and histone requirements (see Samples 11 and 12, Table 1). These "controls" exclude the possibility that the globin-specific sequences were already present in the nonhistone "protein" fraction.

**DISCUSSION**

Two conclusions may be justified by the results summarized in Table 1: (1) Chromatin with the original specificity for the synthesis of globin messenger RNA sequences may be reconstituted from the purified DNA, histone, and appropriate nonhistone protein fractions; (2) The nonhistone protein fraction controls the expression of the globin genes in the reconstituted chromatin.

The extent of the hybridization (up to 70% in Fig. 6) of cDNA with the transcribed RNA indicates that a high proportion of the sequences present in the cDNA are represented in the transcript. Since the cDNA itself is based on the basis of its size (Fig. 3) and specificity (Fig. 4) appears to be a reasonably complete and faithful copy of the original messenger RNA, this suggests that a substantial fraction of the messenger RNA sequences themselves are synthesized in vivo. The present data

* During the preparation of this manuscript similar results were briefly reported by Paul et al. (32).
TABLE 1. Hybridization of RNA transcripts with globin-specific cDNA*

<table>
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<tr>
<th>Sample number</th>
<th>Template</th>
<th>Product RNA/Unit enzyme per mg DNA</th>
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<tr>
<td></td>
<td>N or R'</td>
<td>DNA Histones Non-histones</td>
<td>Transcript hybridized (μg)</td>
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<tr>
<td>1</td>
<td>R'(a)</td>
<td>E — — — A</td>
<td>Not measured</td>
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<tr>
<td>2</td>
<td>R'(b)</td>
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<td>&quot;</td>
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<td>R'(f)</td>
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* Abbreviations used in the table: R' = reconstituted, N = native; (a)-(f) represent experiments performed at different times; E = erythrocyte, R = reticulocyte, L = liver; A, B, and C represent different batches of E. coli RNA polymerase.

† With corresponding no enzyme (Sample 10) background values subtracted.

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