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Synthesis of Globin Ribonucleic Acid from Duck-Reticulocyte Chromatin *In Vitro*

(DNA transcription/RNA–DNA hybridization)

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ABSTRACT **The proteins of chromatin serve to restrict the transcription of DNA. The relevance of these findings to the control of gene expression is contingent upon the demonstration that this restriction is specific and mirrors the patterns of RNA synthesis observed *in vivo*. In this study we demonstrate by RNA–DNA hybridization that the vast majority of the chromatin-directed RNA is synthesized from the unique regions of the reticulocyte genome. Furthermore, by use of the DNA complement of globin mRNA as a probe in annealing reactions, *de novo* synthesis of globin RNA was detected in RNA transcripts from duck reticulocyte chromatin. No globin sequences were detected in similar preparations of RNA *in vitro* either from liver chromatin or from DNA freed of protein. These results show that the proteins of chromatin serve to restrict transcription in a very specific manner and provide convincing evidence for the existence of transcriptional control factors in eukaryotes.**

Any attempt to elucidate the mechanism of gene expression in eukaryotes will inevitably require an understanding of the possible regulatory role of chromatin. Previous studies of transcription *in vitro* have demonstrated that chromatin, as compared to native DNA, is a poor template for RNA polymerase (1, 2). We have recently shown that this restriction is largely due to a striking decrease in the number of available initiation sites for RNA polymerase on chromatin (2). Studies of this kind, however, provide no information about the specificity of the RNA product, and this restriction in template activity cannot be interpreted as a regulatory phenomenon. For this reason, several investigators have attempted to characterize the products of *in vitro* transcription of chromatin by the use of RNA–DNA annealing techniques (3, 4). Most of these studies have, however, been done under conditions that permit the detection only of those RNA sequences that are present in multiple copies within the transcript population and provide little information as to the identity of the RNA transcript.

In this study we ask: Can one detect in RNA transcripts of chromatin any evidence for synthesis of an identifiable tissue-specific gene product that is not synthesized when comparable chromatin extracts from other tissues are used? Globin RNA synthesis in avian erythrocytes is most amenable to this sort of analysis. Globin messenger RNA has been isolated from this system, and its DNA complement has been synthesized by use of RNA-dependent DNA polymerase (5–7). Using a highly radioactive preparation of globin DNA as a probe for the presence of globin RNA in transcription experiments *in vitro*, we find that duck reticulocyte chromatin is a template for production of globin RNA. In contrast, no globin RNA can be detected in transcripts when either liver chromatin or protein-free reticulocyte DNA is used as template.

Abbreviation: Hb, hemoglobin.

METHODS

Preparation of Chromatin and DNA. Duck reticulocytes were prepared by injection of phenylhydrazine hydrochloride (5 mg/kg) for 5 days and obtained by cardiac puncture on day 8 as described (8). Duck-reticulocyte chromatin was prepared by a modification of the procedure of Simpson and Sober (9). Reticulocytes were homogenized in a Dounce homogenizer in five volumes of 10 mM Tris·HCl (pH 7.9)–1 mM $MgCl_2$–0.25 M sucrose. The nuclei were then washed extensively in the presence of 1% Triton X-100. After removal of the Triton, the nuclei were suspended and washed in 0.25 M NaCl–0.025 M Na EDTA (pH 6.0). Chromatin was then obtained from the nuclei by a stepwise reduction in ionic strength. The final preparation was then sheared in a Virtis homogenizer (1 min at 80 V) to an average DNA molecular weight of 3 to 5 $\times 10^6$. It had a protein to DNA ratio of 1.3 g/g. Duck-liver chromatin was prepared in a similar fashion except that the tissue was disrupted in 10 mM Tris·HCl (pH 7.9)–0.25 M sucrose–3 mM $CaCl_2$ in a Potter homogenizer, and the 0.25 M NaCl wash was omitted because of the increased fragility of liver nuclei in these preparations.

Duck-reticulocyte DNA was prepared from isolated nuclei by a modification of the procedure of Marmur (10). Reticulocyte nuclei were disrupted by suspension in 10 mM Tris·HCl (pH 7.9)–0.1 M NaCl–5 mM Na EDTA–1% sodium dodecyl sulfate–0.5 M $NaClO_4$, and the mixture was deproteinized by two extractions with an equal volume of chloroform–3% isoamyl alcohol (v/v). An equal volume of ethanol was added to the resulting aqueous phase. The DNA was spooled out of solution and dissolved in 10 mM Tris·HCl (pH 7.9)–5 mM Na EDTA and treated with pancreatic RNase (25 μg/ml) at 37° for 40 min. The solution was then brought to 0.1 M NaCl and incubated with Pronase (80 units/ml) for 4 hr at 37°. The reaction was then extracted with chloroform–3% isoamyl alcohol, and the aqueous phase was extracted with phenol. The DNA was again precipitated with ethanol, dissolved in 5 mM Na EDTA–0.01 M NaOH, and sonicated for 1 min at top speed in a Bronson sonicator. This solution was then neutralized and dialyzed overnight against 5 mM Na EDTA (pH 7.0) and stored at a concentration of 20 mg/ml.

In Vitro Synthesis and Isolation of RNA. RNA was synthesized in 5-ml reaction mixtures containing: 10 mM Tris·HCl (pH 7.9), 1 mM $MnCl_2$, 0.08 mM each of ATP, GTP,

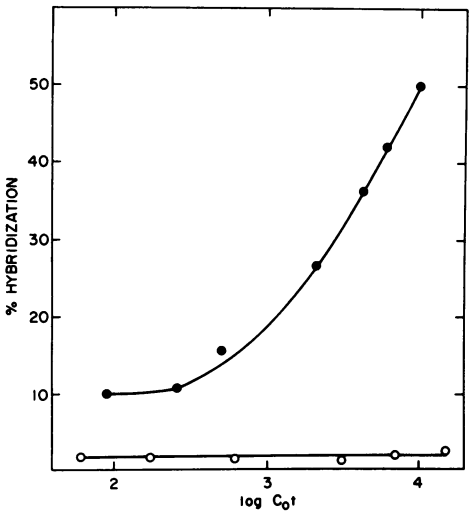

FIG. 1. Kinetics of annealing of duck-reticulocyte RNA to DNA: complementary [^{3}H]RNA was synthesized *in vitro* with duck-reticulocyte chromatin as template and was annealed to reticulocyte DNA (●——●) and *Micrococcus luteus* DNA (○——○) under conditions of vast DNA excess. Hybrid formation at various time intervals was assayed by ribonuclease resistance. The inherent ribonuclease resistance of the complementary [^{3}H]RNA was determined (4.8%) and was subtracted from all points. C_0t = mol of deoxynucleotides × sec/liter (16).

UTP, and [α-^{32}P]CTP (specific activity 3–5 cpm/pmol), 300 units of *Escherichia coli* RNA polymerase [Fraction IV of Berg *et al.* (11)], and 0.75 mg of either DNA or chromatin. Each preparation of chromatin was titrated with RNA polymerase to determine conditions that yield maximum synthesis of RNA at minimal chromatin concentrations in order to reduce the possibility of contamination of the transcripts by cellular RNA present within the chromatin. Where necessary, highly radioactive [^{3}H]RNA was synthesized by addition of 0.01 mM [^{3}H]UTP at a specific activity of 20,000 cpm/pmol. It is important to note that under these conditions chromatin is soluble and less than 3% of the histones undergo exchange (2). The reaction was incubated at 37° for 45 min, and was then brought to 1% Na dodecyl sulfate–0.4 M NaCl–5 mM Na EDTA. The reaction mixture was deproteinized by extraction with chloroform–isoamyl alcohol and subsequently with phenol saturated with 0.01 M Tris·HCl (pH 7.9)–0.1 M NaCl–5 mM Na EDTA. The aqueous phase was then precipated with ethanol. The resulting precipitate was suspended in 10 mM Tris·HCl (pH 7.4)–0.1 M NaCl–5 mM $MgCl_2$ and freed of template DNA by treatment with 40 μg/ml of DNase (Worthington) for 1 hr at 37°. This mixture was again extracted with phenol and freed of residual triphosphate by Sephadex G-50 chromatography. A typical 5-ml reaction mixture with chromatin as template yields from 40–60 μg of newly synthesized complementary RNA after purification.

Synthesis of Hemoglobin (Hb)-Specific [^{3}H]DNA. Duck reticulocytes were disrupted by Dounce homogenization in 5% sucrose in 10 mM Tris·HCl (pH 7.9)–0.1 M NaCl–5 mM $MgCl_2$. Polysomal RNA was prepared as described (12). A postmitochondrial supernatant from reticulocytes was layered on a 12-ml cushion of 25% sucrose in 10 mM Tris·HCl (pH 7.9)–0.1 M NaCl–5 mM $MgCl_2$ and centrifuged for 3 hr at 42,000 rpm at 4° in a Spinco 50.1 rotor. The resulting polysomal pellet was suspended in 10 mM Tris·HCl (pH 8.6)–0.1 M NaCl–5 mM Na EDTA–1% Na dodecyl sulfate and extracted twice with buffer-saturated phenol (pH 8.6). Two volumes of ethanol were added to the aqueous phase, and the nucleic acids were collected by centrifugation at 10,000 × *g* for 20 min. The resulting pellet of polysomal RNA was suspended in 10 mM Tris·HCl (pH 7.4)–0.5 M KCl–0.2 mM $MgCl_2$. Poly(A)-containing RNA was purified from this preparation by cellulose (Sigma) column chromatography (13). 300 A_{260} units of polysomal RNA in 10 mM Tris·HCl (pH 7.4)–0.5 M KCl–0.2 mM $MgCl_2$ was passed over 3 ml (packed volume) of cellulose. The column was eluted until the absorbance of the effluent dropped below 0.01. The column was then washed with water (pH 7.5) to remove the bound poly(A)-containing RNA (about 2% of total polysomal RNA).

RNA prepared in this way was 30-times as effective as an equivalent quantity of polysomal RNA in stimulating protein synthesis when added to a Krebs-ascites cell-free translating system. The protein product of this reaction consists largely of globin components (13). This preparation of RNA was then used as template for synthesis of globin-specific [^{3}H]DNA (Hb[^{3}H]DNA). [^{3}H]DNA complementary to Hb mRNA was synthesized in a 1-ml reaction mixture containing: 50 mM Tris·HCl (pH 8.3), 6 mM $MgCl_2$, 0.2 mM of dTTP, dCTP, and dATP, 0.02 mM [^{3}H]dGTP (15,000 cpm/pmol), 0.05 M KCl, 25 μg of actinomycin D, 3 μg of $(dT)_{10}$, 30 μg of mRNA, and 100 units of RNA-dependent DNA polymerase purified from avian myeloblastosis virus (a gift of Dr. T. Papas of the National Cancer Institute) (14). The reaction was incubated at 37° for 90 min, and the newly synthesized [^{3}H]DNA was freed of protein and template RNA as described (5). In these experiments we labeled the

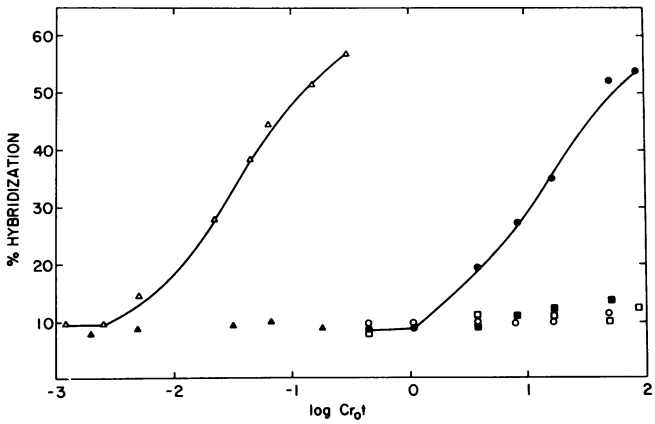

FIG. 2. Kinetics of annealing of Hb DNA to *in vivo* and *in vitro* RNA: Hb-specific [^{3}H]DNA (10,000 cpm) was annealed to 0.3 μg of duck-reticulocyte polysomal RNA (△——△), 0.3 μg of calf-thymus polysomal RNA (▲——▲), 10 μg of RNA made with reticulocyte chromatin template (●——●), 10 μg of RNA made with reticulocyte DNA template (□——□), 10 μg of RNA made with liver chromatin template (■——■), and 10 μg of RNA made with *E. coli* DNA template added to control preparations of reticulocyte chromatin (○——○). Hybrid formation was assayed by resistance to staphylococcal nuclease under conditions where this enzyme specifically degrades only single-stranded DNA. No background values have been subtracted. Cr_0t = mol of ribonucleotides × sec/liter.

Hb DNA with [^{3}H]GTP and assayed for hybrid structures using single-strand nuclease (see below). Any hybrid structures resulting from the annealing of the poly(dT) region of this newly synthesized DNA to the poly(A) region of mRNA will not be detected under our assay conditions.

Annealing Reactions. RNA–DNA annealing reactions between complementary [^{3}H]RNA and cellular DNAs were performed in vast DNA excess (15). Sonicated cellular DNA was denatured by boiling for 10 min immediately before addition to the annealing reaction. Annealing was done at 69° in 0.1 ml containing: 0.4 M NaCl, 1 mM Tris·HCl (pH 7.5), 0.5 mM Na EDTA, 1 mg of denatured DNA, and 10,000 cpm of complementary [^{3}H]RNA (specific activity, 2×10^7 cpm/μg). 10-μl Aliquots were then sealed in siliconized capillaries and incubated for various times. At each time point the contents of one capillary were diluted with 0.5 ml of 10 mM Tris·HCl (pH 7.4)–0.4 M NaCl–5 mM Na EDTA (pH 7). 0.25 ml was precipitated immediately with trichloroacetic acid, and the remaining aliquot was incubated for 30 min at 37° in the presence of 40 μg/ml of pancreatic RNase.

Hb [^{3}H]DNA and unlabeled complementary RNA were similarly hybridized at 69° in 50 μl containing: 1 mM Tris·HCl (pH 7.9), 0.4 M NaCl, 0.5 mM Na EDTA, 10 μg of complementary RNA, and 10,000 cpm of Hb [^{3}H]DNA (specific activity, 1.2×10^7 cpm/μg). 5-μl Aliquots were sealed in siliconized capillaries and assayed for hybrid formation at various time points with staphylococcocal nuclease under conditions where the enzyme specifically degrades only single-stranded DNA (Kacian, D. & Spiegleman, S., manuscript in preparation). Each 5-μl aliquot was diluted in 0.5 ml of 10 mM Tris·HCl (pH 7.9)–0.4 M NaCl–10 mM $MgCl_2$–0.1 mM $CaCl_2$. 0.25 ml was immediately precipitated with trichloroacetic acid and the other half was incubated at 37° for 1 hr in the presence of staphylococcal nuclease at a concentration of 80 μg/ml. The amount of radioactive DNA resistant to digestion was determined by trichloroacetic acid precipitation.

RESULTS

Transcription of unique sequences in chromatin

In vivo, the bulk of the large nuclear RNA is transcribed from the slowly renaturing or unique sequences of the eukaryotic genome (15). It was of obvious interest to determine whether RNA transcript *in vitro* mirrors the situation observed *in vivo*. We therefore hybridized the *in vitro* RNA transcript from duck-reticulocyte chromatin to duck DNA under conditions of vast DNA excess, a technique that reveals the relative abundance of RNA transcripts as a function of the repetitiveness of the DNA from which they are copied.

The kinetics of annealing of chromatin [^{3}H]RNA with reticulocyte DNA are shown in Fig. 1. More than 85% of the *in vitro* complementary RNA anneals to the slowly renaturing portion of the DNA with less than 10% annealed at a C_0t of 250. Virtually no hybridization is detected when this complementary RNA is annealed with identical amounts of DNA obtained from the bacterium *Micrococcus luteus*. These data demonstrate that at least 85% of the RNA synthesized *in vitro* with chromatin templates is transcribed from the nonreiterated portions of the genome, a situation analogous to that described for nuclear RNA isolated *in vivo* (15). It should be noted that these annealing reactions are done in vast DNA excess and, therefore, provide no information as to what proportion of the genome is represented in the chromatin-directed transcripts.

Transcription of globin-specific sequences

Definitive proof of a regulatory role for the proteins of chromatin requires that we demonstrate restriction of transcription for a specific gene or set of genes, similar to that which occurs *in vivo* in the specific tissue from which the chromatin was initially isolated. These experiments were made feasible by the demonstration (5–7) that hemoglobin messenger RNA can serve as a template for RNA-dependent DNA polymerase, resulting in the synthesis of DNA complementary to Hb RNA. We find that the DNA product synthesized in this way anneals well to polysomal RNA from duck reticulocytes but shows no evidence of annealing either to polysomal RNA from calf thymus (Fig. 2) or poly(A) (data not shown).

Since the [^{3}H]DNA has been shown to be specific for populations of RNA containing globin sequences, this [^{3}H]DNA can now be used to detect Hb-specific RNA in *in vitro* transcripts synthesized by various chromatin and DNA templates. Fig. 2 shows the results of annealing reactions between Hb [^{3}H]DNA and 10 μg of RNA synthesized *in vitro* with reticulocyte chromatin as template. The annealing data clearly demonstrate the presence of Hb-specific sequences within RNA transcribed from reticulocyte chromatin, with more than 50% of the Hb [^{3}H]DNA annealed at 24 hr (corresponding to a Cr_0t of 48). To rule out the possibility that the Hb sequences detected in this reaction were not synthesized *de novo* but resulted from contamination by cellular Hb-specific RNA present within the chromatin, we performed the following control experiment: Reticulocyte chromatin was incubated in the standard reaction mixture in the absence of RNA polymerase. After 45 min of incubation, 50 μg of RNA transcribed from *E. coli* DNA was added and the RNA was then freed of proteins and DNA. Any endogenous RNA contaminating the chromatin preparation would be expected to be present in this control population of *E. coli* transcripts. When 10 μg of this control RNA was reacted with Hb DNA, less than 3% annealing could be detected at time points where virtually all of the hybridizable Hb DNA was annealed to RNA transcribed from reticulocyte chromatin (Fig. 2). The absence of hybridizable RNA in these control preparations clearly demonstrates that the Hb-specific sequences detected within RNA transcripts synthesized with chromatin template are not the result of cellular RNA contaminating the reticulocyte chromatin.

Having demonstrated the *in vitro* synthesis of Hb-specific RNA by reticulocyte chromatin, we now asked whether similar RNA was present in transcripts from purified DNA. 10 μg of RNA transcribed from purified duck-reticulocyte DNA was reacted with Hb [^{3}H]DNA; there was virtually no detectable annealing at any time (Fig. 2). The inability to detect Hb-specific sequences in transcripts from reticulocyte DNA suggests that these sequences are present at much higher dilution and that the restriction of transcription conferred upon DNA by the proteins of chromatin is specific in nature. More revealing information would be obtained, however, if one could demonstrate the absence of Hb sequences in transcripts of chromatin derived from tissues not actively engaged in hemoglobin biosyntheis. To this end, RNA was synthesized *in vitro* with normal duck-liver chromatin as

template. At times where more than 55% of the Hb [^{3}H]DNA is annealed to reticulocyte chromatin transcripts, less than 5% annealing is observed with comparable RNA transcribed from liver chromatin (Fig. 2).

It should be noted that the DNA probe used in these experiments was synthesized with globin message purified by cellulose chromatography (13). When analogous experiments are performed with Hb [^{3}H]DNA (supplied by Dr. P. Leder) templated by a globin message isolated by poly-(dT)–cellulose chromatography followed by sucrose gradient sedimentation (6), identical results are obtained.

DISCUSSION

The mechanism by which chromatin proteins might specifically restrict DNA template activity is a problem central to the understanding of cellular differentiation in higher organisms. In our laboratory an attempt is being made to relate the structure of the nucleoprotein complex to its function. This is a meaningful subject of study only if it can be shown that isolated chromatin contains the information necessary for regulation of gene expression. For this reason, we have chosen to study the *in vitro* synthesis of a well-defined species of RNA, the globin-specific RNA of duck reticulocytes.

Virtually all past attempts (3, 4) to characterize the products of transcription of chromatin by annealing experiments have been performed under conditions that permit detection only of RNA sequences either synthesized by the reiterated regions of DNA or present in multiple copies in the RNA transcript. Those studies that have used conditions of RNA excess sufficient to permit the analysis of unique RNA sequences have demonstrated that more than half of the template activity for total *in vivo* RNA is contained in 3% of the chromatin (17). Using highly radioactive RNA transcribed from chromatin, we have demonstrated by RNA–DNA annealing that the vast majority of the *in vitro* RNA is transcribed from the unique regions of the reticulocyte genome, a situation analogous to that observed when similar annealings are performed with *in vivo* nuclear RNA (15).

Having demonstrated that a large proportion of the complementary RNA is transcribed from the nonrepetitive portion of the genome, we next asked whether specific RNA or gene products could be detected in this population of *in vitro* RNA. Using purified Hb [^{3}H]DNA as a probe in annealing reactions, we detected Hb-specific RNA in transcripts from duck-reticulocyte chromatin but not in similar preparations of *in vitro* RNA from duck liver. The possibility that these sequences result not from *de novo* synthesis but from either contamination by cellular RNA or from the mere elongation of previously initiated Hb RNA sequences in chromatin is unlikely, since Hb-specific RNA cannot be detected when chromatin is incubated in the absence of RNA polymerase. Furthermore, no detectable Hb RNA was found in RNA transcripts synthesized with purified reticulocyte DNA as template. The amount of Hb RNA present within the chromatin-directed transcripts can be estimated by comparing the kinetics of annealing of this chromatin RNA with RNA of known Hb RNA content. Duck-reticulocyte polysomal RNA contains 1–2% Hb RNA and anneals to Hb [^{3}H]DNA with a $Cr_0t_{1/2}$ of 3.3×10^{-2}. With RNA synthesized with chromatin template, the reaction is 1/300 as rapid, with a $Cr_0t_{1/2}$ of 11. Thus, if polysomal RNA consists of 2% mRNA, 0.007% of the chromatin transcripts are Hb specific. This is clearly a rough estimate, and no attempt has been made to correct for such varying factors as RNA size or fragmentation and degradation of the RNA during annealing.

Our results show that the proteins of chromatin serve to restrict DNA transcription in a very specific fashion, and that the structures that determine this restriction are not lost during chromatin isolation procedures. Furthermore, they show that a bacterial polymerase is at least qualitatively capable of responding faithfully to these restrictions.

Finally, our results appear to provide convincing evidence of transcriptional control factors for an identifiable gene product in eukaryotes. The observation that these factors survive the chromatin isolation procedures provides both the justification and the technology for studies relating chromatin structure to function.

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