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Fractionation of Mouse Myeloma Chromatin†

Edwin C. Murphy, Jr.,*‡ Stanton H. Hall,§ Judith H. Shepherd,¶ and Russell S. Weiser

ABSTRACT: Chromatin was isolated from several mouse myelomas and fractionated after shearing on both sucrose and glycerol gradients. The fractionation procedure resulted in the separation of two classes of chromatin as defined by their sedimentation properties and capacity for RNA synthesis *in vivo* and *in vitro*. The first class, slowly sedimenting chromatin, was composed of chromatin particles with widely varying template capacities as estimated with the RNA polymerase obtained from *Escherichia coli* and both the form II (nucleoplasmic) and form I (nucleolar) DNA-dependent RNA polymerases purified from a myeloma tumor. The second class, rapidly sedimenting chromatin, was heterogeneous with respect to size and uniformly unable to support RNA synthesis *in vitro*. Slowly sedimenting chromatin contained 10–20% of the total chromatin DNA and possessed >90% of the *in vitro* template activity. The validity of the *in vitro* assays for RNA synthesis as a method of estimating the fractionation of chromatin into functionally distinct classes was confirmed by the observation that newly synthesized RNA molecules were found selectively associated with slowly sedimenting chromatin *in vivo*. Analysis on polyacrylamide–sodium dodecyl sulfate gels revealed that rapidly sedimenting chromatin possessed two prominent high molecular weight nonhistone proteins which are absent in slowly sedimenting (template-active) chromatin. Conversely, four or more nonhistone proteins were selectively associated with the template-active chromatin. The capacity for specific interaction of DNA and chromosomal proteins was examined by reconstitution of dissociated chromatin. It was found that reconstituted chromatin, although not identical with native chromatin, retained a portion of its structural and functional heterogeneity.

Chromatin, the interphase form of chromosomes, is a complex of DNA, proteins, and RNA. In most cells, the major part of the genome is in the repressed state. Studies using RNA–DNA hybridization have suggested that only a minor yet tissue-specific fraction of the DNA sequences in chromatin (5–15%) is active in RNA synthesis and that the necessary elements for the control of tissue-specific RNA synthesis are retained in the isolated complex (Paul and Gilmour, 1968; Smith *et al.*, 1969; Huang and Huang, 1969; Hahn and Laird, 1971; Grouse *et al.*, 1972). Consequently, most studies on chromatin deal with material which is predominantly inactive.

One approach to the study of the relationship between the components of chromatin and its function as a template for RNA synthesis is to attempt the fractionation of chromatin into segments which are transcribed *in vivo* and those which are repressed. Electron microscopy has revealed that interphase chromatin is a mixture of electron-dense and diffuse regions. Radioautographic studies have established that the diffuse regions are template-active, whereas the condensed regions are not (Littau *et al.*, 1964, 1965). There have been several studies designed to separate the extended and condensed regions of chromatin. Frenster *et al.* (1963) reported the separation of sonicated thymocyte chromatin by centrifugation into a supernatant fraction with considerable

† From the Departments of Microbiology and Pathology, The University of Washington, Seattle, Washington 98105. *Received January 31, 1973.* Supported in part by U. S. Public Health Service Grants AM 08686 and CA 13600, American Cancer Society Grant NP-99C, and National Institutes of Health Training Grant 2-T01-CA05040.

‡ Present address: Department of Biology, The Johns Hopkins University, Baltimore, Md. 21218.

§ Postdoctoral Trainee in Experimental Pathology; supported by U. S. Public Health Service Grant GM-0100.

¶ Predoctoral Fellow supported by National Institutes of Health Grant 5-F01-GM-45030.

template activity and a highly condensed, inactive pellet. Chalkley and Jensen (1968) employed sucrose gradients to fractionate thymocyte chromatin into a template-active fraction (30 S) and aggregated material (130 S) with reduced template activity. Similar fractionations have been reported by Duercksen and McCarthy (1971) and Nishiura (1972). Other properties of chromatin components, such as differing solubilities at physiological salt concentrations (Marushige and Bonner, 1971), differing T_m values (McConaughy and McCarthy, 1972), and differing affinity for ion exchangers (Reeck *et al.*, 1972) have also been exploited in efforts to separate template-active and inactive DNA sequences.

The variety of mouse myelomas available for study provides a potentially useful system for the exploration of the regulation of RNA and protein synthesis in highly differentiated mammalian cells. Each of these tumors is thought to originate from a single clone of plasma cells and secretes large amounts of a single class of immunoglobulin. Moreover, variants have been obtained which are partially or totally defective in immunoglobulin production. In this study we report the fractionation of chromatin from mouse myeloma cells into template-active and inactive fractions, as estimated by their capacity to support RNA synthesis *in vivo* and *in vitro* using the *Escherichia coli* RNA polymerase and the endogenous myeloma RNA polymerases. We have also observed certain potentially interesting differences among the nonhistone chromosomal proteins of active and inactive chromatin.

Materials and Methods

Animals and Tumors. Balb/c-j mice were purchased from the Jackson Laboratories, Bar Harbor, Maine, and maintained as an inbred colony in our laboratory. Myelomas MOPC104E (μ, λ), MOPC173D (γ_{2a}, κ), ADJPC5 (γ_{2a}, κ), and MOPC21 (γ_1, κ) were provided by Dr. Robert Kreuger (The Mayo Clinic, Rochester, Minn.). Dr. H. Eisen (Department of Microbiology, Washington University, St. Louis, Mo.) kindly provided us with MOPC315 (α, λ).

Reagents. All chemicals were analytical reagent grade and were used without further purification. Tris(hydroxymethyl)-aminomethane (Tris), Trizma reagent grade, was supplied by the Sigma Chemical Co. The acrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylenediamine were of the "prep-grade" and were obtained from Canal Industries Corporation, Rockville, Md. L-[*methyl*-^{3}H]Methionine (4 Ci/mmol) and L-[^{35}S]methionine (203 Ci/mmol) were obtained from New England Nuclear Corporation. [5-^{3}H]Uridine 5'-triphosphate (15.9 and 1 Ci/mmol) was purchased from Amersham Searle Corporation. [8-^{3}H]Adenosine 5'-triphosphate (20.7 Ci/mmol) was obtained from Schwarz BioResearch. Nonidet P-40 was purchased from the Shell Oil Co.

Cell Labeling. Primary cultures of the myeloma cells (1×10^6 viable cells/ml) were labeled with [^{35}S]methionine (0.5 μCi/ml to 4 Ci/mmol) for 4 hr in Waymouth's medium (1959) containing no methionine and one-tenth the normal amount of the remaining amino acids, as described elsewhere (E. C. Murphy, J. H. Shepherd, and R. S. Weiser, manuscript in preparation).

Preparation of Chromatin. Nuclei were isolated by two different procedures, with no detectable difference in the chromatin obtained. In the first method, the excised tumors were minced and then homogenized in 5–10 volumes of 0.25 M sucrose–0.05 M Tris (pH 7.6) containing 0.003 M $MgCl_2$ (0.25 M STM[1]). Nonidet P-40 was added to a concentration of 0.1% and the cells were disrupted in a Dounce-type tissue homogenizer by six strokes of the loose-fitting pestle and six strokes of the tight-fitting pestle. Two volumes of 2.3 M sucrose–0.05 M Tris (pH 7.6) containing 0.003 M $MgCl_2$ were added and the nuclei were sedimented at 30,000 rpm for 60 min (no. 30 rotor, Spinco Model L ultracentrifuge). Alternately, nuclei were prepared by low-speed centrifugation (1000*g*, 7 min) of myeloma cell suspensions treated with 0.5% Nonidet P-40 for 5–10 min at 4°.

The sedimented nuclei were washed twice with 0.25 M STM and twice with 0.01 M Tris (pH 8.0) (1000*g*, 7 min). Disruption of the washed nuclei (in 30 ml of 0.01 M Tris (pH 8.0)) was accomplished by five strokes of the tight-fitting pestle in a Dounce-type homogenizer. Ten milliliters of this preparation was layered over each of three sucrose step gradients consisting of 10 ml of 1.6 M sucrose and 10 ml of 1.3 M sucrose (both in 0.01 M Tris (pH 8.0)) and sedimented at 25,000 rpm for 180 min at 4° (SW 25.1 rotor). The chromatin pellet was resuspended and the last step repeated. For smaller preparations, it was found that sedimentation through sucrose could be performed quite rapidly by layering 5 ml of disrupted nuclei over 4 ml of 1.3 M sucrose and 4 ml of 1.6 M sucrose and centrifuging for 45–60 min at 4° at 39,000 rpm (SB-283 rotor, using an International B-60 ultracentrifuge). The chromatin pellet was resuspended in 5–15 ml of 0.01 M Tris (pH 8.0), dialyzed for 10–15 hr against the same buffer, and sheared in a Virtis "45" homogenizer (40 V, 3 min) or in a French pressure cell at 3000 psi. Any insoluble debris was removed by centrifugation at 10,000*g* for 30 min. The purity of the chromatin preparations was assessed optically (260/240 nm > 1.45, 260/280 nm > 1.65). The chromatin preparations were stored packed in ice in 1 mM NaN_3 or at −20° in 25% glycerol. In our experience, chromatin can be stored frozen in glycerol (10–50%) and thawed without any noticeable effect on its solubility, spectral properties, T_m (J. Chamberlain, personal communication), template activity, or gradient profile (E. C. Murphy, unpublished experiments).

Chromatin Fractionation. To isolate chromatin components of differing sedimentation coefficients, unfractionated chromatin (usually 3 ml, containing ~0.5–1.0 mg of chromatin DNA/ml) was layered on 30 ml of 0.17–1.7 M linear sucrose density gradients, buffered with 0.01 M Tris (pH 8.0), and centrifuged for 15 hr at 4° at 22,500 rpm in a Spinco SW 25.1 rotor. For smaller preparations, 12-ml gradients were centrifuged at 25,000 rpm for 12–14 hr at 4° in the International SB-283 rotor. Because of the high viscosity of 1.7 M sucrose all gradients were formed at room temperature and cooled to 4° before centrifugation. The gradients were monitored at 254 nm using an Isco Model D density gradient analyzer attached to a Gilford Model 2000 recorder, and collected into 0.5- or 1-ml fractions. As an alternate fractionation procedure, chromatin was centrifuged in 7.6–76% (v/v) linear glycerol gradients in 0.01 M Tris (pH 8.0) using the same conditions employed for the sucrose gradients.

Template Activity of Chromatin Fractions. (i) BACTERIAL RNA POLYMERASE. RNA polymerase purified from *E. coli* through the DEAE-Sephadex step (Burgess, 1969) was provided by J. Nishiura, Department of Genetics, University of Washington. The enzyme was precipitated by ammonium sulfate and stored at −20° at a concentration of 4 mg of

[1] Abbreviations used are: STM buffer, 0.25 M sucrose–0.05 M Tris (pH 7.6)–0.003 M $MgCl_2$; TGMED buffer, 0.05 M Tris (pH 7.9)–25% glycerol–5 mM $MgCl_2$–0.1 mM EDTA–0.5 mM dithiothreitol.

protein/ml in 0.01 M Tris (pH 7.9) containing 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.1 M KCl, and 50% glycerol (Burgess, 1969). Incorporation of radiolabeled precursors into RNA was linear at concentrations of 1–40 μg of DNA/ml and 1–36 μg of chromatin DNA/ml (Nishiura, 1972). The reaction mixtures (0.75 ml) contained 5 or 10 μl of polymerase, 0.5 ml of 0.1 M Tris (pH 7.9), containing 0.02 M $MgCl_2$, 0.001 M EDTA, and 0.1 M KCl, and 0.05 ml of 0.8 mM CTP, GTP, ATP, and UTP, containing 0.45 μCi of [^{3}H]UTP (15 Ci/mmol) or 0.05 ml of 0.8 mM CTP, GTP, and UTP containing 0.45 μCi of [^{3}H]ATP (20 Ci/mmol). Since 76% glycerol and 1.7 M sucrose had no effect on the activity of RNA polymerase, fractions from the chromatin gradients were assayed directly (3–5 μg of chromatin DNA in 0.1–0.20 ml of 0.01 M Tris (pH 8.0) containing sucrose or glycerol). After incubation at 37° for 10–15 min, the reaction mixtures were chilled on ice, 100 μg of bovine serum albumin (1 mg/ml) was added, and the reactions were stopped by adding an equal volume of 10% Cl_3CCOOH. After 30 min, the precipitates were collected on Whatman GF/c filters, washed with 35 ml of 5% Cl_3CCOOH, dried, and counted in 10 ml of a toluene–Spectrafluor mixture (958:42) in a Packard Tri-Carb Model 3320 liquid scintillation spectrometer.

(ii) MYELOMA RNA POLYMERASE. Forms I and II of RNA polymerase were isolated from myeloma MOPC21 (carried as solid tumors in Balb/c-j mice), by the method of Roeder and Rutter (1970), with the following modifications. Following ammonium sulfate precipitation and centrifugation, the pellet was resuspended in 0.05 M Tris (pH 7.9)–25% glycerol–5 mM $MgCl_2$–0.1 mM EDTA–0.5 mM dithiothreitol (TGMED buffer) containing 0.025 M $(NH_4)_2SO_4$ and was passed through a Bio-Gel A-5 M column (100 × 2.5 cm) equilibrated in the same buffer. The pooled polymerase activity was adsorbed to DEAE-Sephadex equilibrated in TGMED–0.025 M $(NH_4)_2SO_4$ and eluted with a linear $(NH_4)_2SO_4$ gradient (0.025–0.50 M) in TGMED. Fractions of RNA polymerases I and II with highest activity were used as the source of enzyme. A detailed description of the purification and properties of RNA polymerase from mouse myeloma cells will be presented elsewhere (S. Hall and E. Smuckler, manuscript in preparation). Incorporation of radiolabeled precursors was linear for 10 min with 30 μl of enzyme I or II and 1–250 μg/ml of chromatin DNA. The assay contained in 0.4 ml: 72.5 mM Tris-HCl (pH 7.9 at 20°); 0.7 mM ATP, GTP, and CTP; 6.25 μM [^{3}H]UTP (1 Ci/mmol); 1.25 mM $MgCl_2$; 3.1 mM $MnCl_2$; 0.025 mM EDTA; 0.125 mM dithiothreitol; 25 mM 2-mercaptoethanol; 50 mM KCl; 12.5–40% (v/v) glycerol; and chromatin DNA or myeloma DNA as indicated in the legend to the figures. The concentration of $(NH_4)_2SO_4$ was 40 mM for form I polymerase and 60 mM for form II polymerase. Incubations were stopped by placing the tubes in ice and adding 100 μg of bovine serum albumin and 1 volume of cold 10% Cl_3CCOOH. After 10 min samples were collected on Whatman GF/C filters, washed with 35 ml 5% Cl_3CCOOH, and dried. Dried filters were placed in 10 ml of toluene containing 0.1% 2,5-diphenyloxazole and 0.05% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene and counted in a Model 3375 Packard Tri-Carb liquid scintillation spectrometer.

Synthesis of Nascent RNA. The 66-2 myeloma tissue culture cells (kindly supplied by Dr. U. Storb, The University of Washington, Seattle, Wash.) were maintained in Dulbecco's modified Eagle's medium (Dulbecco and Freeman, 1959) supplemented with 10% horse serum. To label nascent RNA species, two cultures of 2×10^7 cells at a density of 2×10^6 cells/ml were exposed to 10 μCi/ml of [^{3}H]uridine (10 Ci/mmol) for 10 min at 37°. The cells were then collected by centrifugation and chromatin was immediately isolated from one culture as described below. The other culture was washed once in warm medium containing a 500-fold excess of unlabeled uridine, resuspended in medium containing excess uridine, and incubated at 37° for an additional 60 min. Both cultures were washed twice in cold 0.01 M Tris (pH 7.5) containing 0.145 M NaCl and suspended in 1 ml of 0.25 M STM, and the nuclei were released by treatment of the washed cells with 0.9% Triton X-100. Chromatin was isolated from these nuclei and fractionated on glycerol gradients as described earlier, except that the centrifugation through 1.3 and 1.6 M sucrose was omitted. The acid-precipitable radioactivity was determined in an aliquot of each gradient fraction. Because of the very small amounts of chromatin obtained (<1 A_{260} unit), unlabeled chromatin from 1×10^8 66-2 cells was isolated and fractionated in parallel with the experimental cultures in order to provide a reliable A_{260} profile.

Dissociation and Reassociation of Chromatin. Myeloma chromatin was dissociated and reassociated according to the method of Huang and Huang (1969) with minor modifications. As recommended by Hill *et al.* (1971), chromatin was dissociated at a concentration of DNA + chromosomal proteins no greater than 1.5 mg/ml (assuming a DNA:protein ratio of 1:2), in 2 M NaCl–5 M urea buffered with 0.01 M Tris (pH 8.0). As an additional precaution against aggregation of the chromosomal proteins, 6 mM 2-mercaptoethanol was included in all buffers. After the above treatment, chromatin components were dialyzed against 100 volumes of 5 M urea–0.006 M 2-mercaptoethanol–0.01 M Tris (pH 8.0)–0.6 M NaCl overnight, followed by successive dialysis for 3 hr each against the same buffer containing 0.4 and 0.2 M NaCl. In all of these steps 5 M urea and 0.01 M Tris (pH 8.0) were included in the buffer (Huang and Huang, 1969). In the final step, the urea and reducing agent were removed by overnight dialysis against 0.01 M Tris (pH 8.0). At this point, 1 mM NaN_3 was added and the reconstituted chromatin stored packed in ice.

Gel Electrophoresis of Chromosomal Proteins. Electrophoresis in 8.75% polyacrylamide gels containing 0.1% sodium dodecyl sulfate was performed according to Laemmli (1970), using a 9-cm lower gel. For the optimum resolution of chromosomal proteins directly from chromatin, prior hydrolysis of the chromatin DNA was essential. More than 20 μg of chromatin DNA per gel caused pronounced streaking and distortion of the polypeptide bands. To hydrolyze chromatin DNA, chromatin (80–100 μg of DNA) in a volume of 0.2 ml of 0.01 M Tris (pH 8.0) containing 2.5×10^{-5} M $CaCl_2$ was treated with staphylococcal deoxyribonuclease (DNase) at a concentration of 5 μg/ml. After 90 min at 37°, the chromosomal proteins, which precipitate as the DNA is digested, were dissolved in 0.5 volume of 3× concentrated sample buffer (1× sample buffer contains 0.0625 M Tris (pH 6.8), 1.0% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 5% glycerol, and 0.01% Bromophenol Blue). To prepare chromatin from the various gradient fractions for electrophoresis, pooled material (still in sucrose or glycerol) was treated with 5 μg/ml of DNase as described above, dialyzed overnight against two changes of 4000 ml of 0.001 M Tris (pH 8.0), lyophilized, and resuspended in an appropriate volume of electrophoresis sample buffer (about 500 μg of chromatin DNA/ml). To completely dissolve the proteins, the chromatin samples were heated at 100° for 1 min and cooled.

The dissolved chromosomal proteins were applied to the gels under the reservoir buffer (0.025 M Tris–0.15 M glycine (pH 8.3)). Stacking was carried out at a constant current of 1

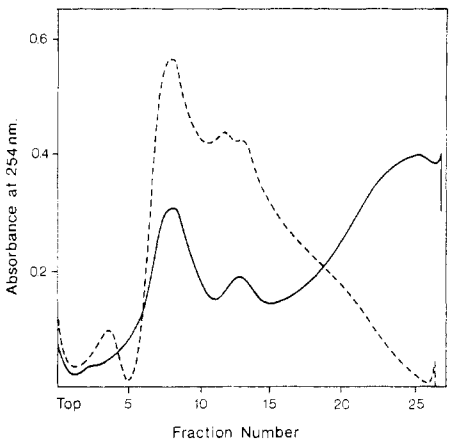

FIGURE 1: Sucrose density gradient centrifugation of ADJPC5 chromatin sheared in a Virtis homogenizer at 40 V for 5 min (—) or at 3000 psi in a French pressure cell (- - - -) and centrifuged at 10,000*g* for 30 min. The chromatin supernatants (300 μg of chromatin DNA) were layered on 12 ml of 0.17–1.7 M linear sucrose gradients and centrifuged at 25,000 rpm for 12 hr at 4° (SB-283 rotor, International B-60 ultracentrifuge).

mA/gel until the tracking dye reached the lower gel, after which time a constant voltage (60 V) was applied until the tracking dye was 5 mm from the bottom of the gel (9–10 hr). The gels were stained for 12–15 hr in a solution containing 0.05% Coomassie Brilliant Blue, 10% acetic acid, and 25% isopropyl alcohol, and diffusion destained in 10% acetic acid containing successively lower concentrations of dye and isopropyl alcohol (Fairbanks *et al.*, 1971). The destained gels were photographed using Polaroid type 55 P/N film with a yellow filter and scanned using a Gilford densitometer at 586 nm (King *et al.*, 1971). Gels containing radioactive proteins were frozen on Dry Ice and sliced transversely into 1.7-mm sections by a series of razor blades spaced by fiber washers. The slices were placed in scintillation vials, dissolved in 0.5 ml of 30% H_2O_2 overnight at 60°, and counted in 10 ml of Kinards solution (1957) using a Packard Tri-Carb Model 3302 liquid scintillation spectrometer.

Purification of DNA. DNA was prepared from myeloma cells by standard methods (Church and McCarthy, 1967) and sheared at 12,000 psi in a French pressure cell. Estimates of both purified and chromatin DNA concentrations were made by assuming an extinction coefficient of 22 at 260 nm (Tuan, 1967).

Results

Chromatin Fractionation. Myeloma chromatin was fractionated after shearing by centrifugation in linear sucrose density gradients (0.17–1.7 M sucrose in 0.01 M Tris (pH 8.0)) a procedure recently introduced for fractionation of *Drosophilia melanogaster* chromatin (J. Nishiura, 1972). This technique permits the separation of chromatin into slowly and rapidly sedimenting fractions.

Myeloma chromatin was routinely sheared in a Virtis homogenizer (40 V, 3 min). Other investigators have sheared chromatin in a French pressure cell at 3000 psi (Nishiura, 1972). To examine which of these procedures, if either, might permit a more effective separation of active and inactive chromatin, a preparation of myeloma chromatin was sheared by both methods and fractionated. As shown in Figure 1, shearing by Virtis homogenization yielded three major components with good separation of the rapidly and slowly sedimenting fractions. In contrast, shearing in a French pressure cell resulted in incomplete resolution of the chromatin components. The distribution of Virtis-sheared chromatin DNA in the gradient (as estimated by the absorbance at 260 nm) was as follows: the slowly sedimenting component contained 12–20% of the chromatin DNA, ~25% was found in the pellet, and the remainder appeared in the intermediate and rapidly sedimenting components.

TABLE I: Template Activity of Myeloma Chromatin and DNA Using *E. coli* RNA Polymerase.[a]

Template	cpm of [^{3}H]UTP Incorpd	Template Act. Relative to DNA	Template Act. Relative to Total Chromatin	Template Act. Relative to Rapidly Sedimenting Chromatin
DNA	31,800	1.0	—	—
Total chromatin	3,720	0.12	1.0	2.3
Slowly sedimenting chromatin	14,700	0.46	3.9	9.0
Rapidly sedimenting chromatin	1,610	0.06	0.43	1.0
Pelleted chromatin	1,930	0.07	0.51	1.2

[a] Chromatin from MOPC173D was fractionated as described in the legend for Figure 3. Assays were carried out using 5 μg of *E. coli* RNA polymerase and 5 μg of purified DNA or chromatin DNA from pooled preparations of slowly sedimenting material (fractions 7–12 of Figure 1), rapidly sedimenting material (fractions 26–31 of Figure 1), and the pellet fraction.

Template Activity of Fractionated Myeloma Chromatin. As estimated using the *E. coli* RNA polymerase, the slowly sedimenting chromatin component was nine times more active (Table I) as a template for *in vitro* RNA synthesis than rapidly sedimenting chromatin and approximately half as active as purified myeloma DNA. Both the rapidly sedimenting and pelleted chromatin components were less than half as active as unfractionated chromatin, suggesting that both had been separated from template-active DNA sequences by the fractionation procedure. The template activity of the rapidly sedimenting component of chromatin was not zero, suggesting either incomplete fractionation or that the *E. coli* RNA polymerase can transcribe inactive DNA sequences in mammalian cells. However, since the template activity of the rapidly sedimenting component is low relative to that of the slowly sedimenting component, they will hereafter be referred to as inactive and active chromatin, respectively.

Optimal Conditions for the Separation of Active and Inactive Chromatin. The results of the previous experiment suggested that although chromatin can be resolved into active and inactive components, such separation may not be complete. A longer shearing time might allow more efficient separation by reducing the size of the chromatin fragments generated which, ideally, should be shorter than the average

TABLE II: Template Capacity of Chromatin Fractions Sheared for Various Times.[a]

Shearing time (min)	Slowly Sedimenting Chromatin (cpm/μg of DNA)	Rapidly Sedimenting Chromatin (cpm/μg of DNA)
1	1780	128
2	2040	65
3	1850	
4	2130	82
5	1600	

[a] MOPC104E chromatin was sheared and fractionated as described under Figure 2. Aliquots were taken from the peak of the active fraction (tube 6) and from the leading edge of the inactive fraction (tube 18) in each gradient and their template activities assayed with *E. coli* RNA polymerase (5 μl) as described under Materials and Methods.

length of an active sequence. To test this possibility, chromatin was sheared at 40 V for 1, 2, 3, 4, and 5 min. The amount, distribution in glycerol gradients, and template activity of the solubilized chromatin DNA were examined. Chromatin DNA is rapidly solubilized by shearing, since only 15% more chromatin DNA was released by 5 min of shearing than by 1 min of shearing. On glycerol gradients, as illustrated by Figure 2, the effect of prolonged shearing was a progressive shift of chromatin DNA from the template-inactive to the active region, and a decrease in the amount of pelleted chromatin DNA. After a 1-min shear, only 22% of the chromatin DNA was found in the active fraction and 19% was pelleted. If shearing was continued for 5 min, the pellet contained only 10% of the chromatin DNA, while the amount of DNA in the active component increased to 38%. At 2, 3, and 4 min of shearing, 25, 30, and 32%, respectively, of the chromatin DNA were found in the active fraction. It was obvious from these data that lengthening the shearing time altered the sedimentation behavior of chromatin. Similar observations have been reported by Chalkley and Jensen (1968) and Duercksen and McCarthy (1971).

If additional active chromatin DNA sequences were shifted to the slowly sedimenting component between 1 and 5 min of shearing, the template activity of this fraction should either rise, due to a more efficient separation of active and inactive sequences, or at least remain constant. A decrease in template activity should result only if inactive sequences were caused to cosediment with active sequences as a result of prolonged shearing. As illustrated in Table II, the template activity of slowly sedimenting chromatin (using the peak fraction on the trailing side of the active peak) was approximately constant over 5 min of shearing.

It is likely that regions of chromatin DNA which are active in RNA synthesis *in vivo* are interspersed with regions which are not transcribed. Random breakage of chromatin by shear forces should generate a series of chromatin segments which vary from highly template-active to completely repressed. This possibility is rendered even more likely by the results of the previous sections which showed that some of the rapidly sedimenting chromatin can be converted to slowly sedimenting chromatin by increasing the shearing time or by varying the method of shear (Figure 1). To test this supposition and to determine whether any component of the fractionated chromatin is uniformly composed of active DNA sequences, the template activity of each fraction in the slowly sedimenting chromatin component and alternate fractions in the rapidly sedimenting component of MOPC-173D chromatin was assayed. The results, presented in Figure 3, clearly reveal a gradient of decreasing template activity in the direction of rapidly sedimenting chromatin. Only the first four fractions (fractions 7–10 in Figure 3) in the slowly sedimenting chro-

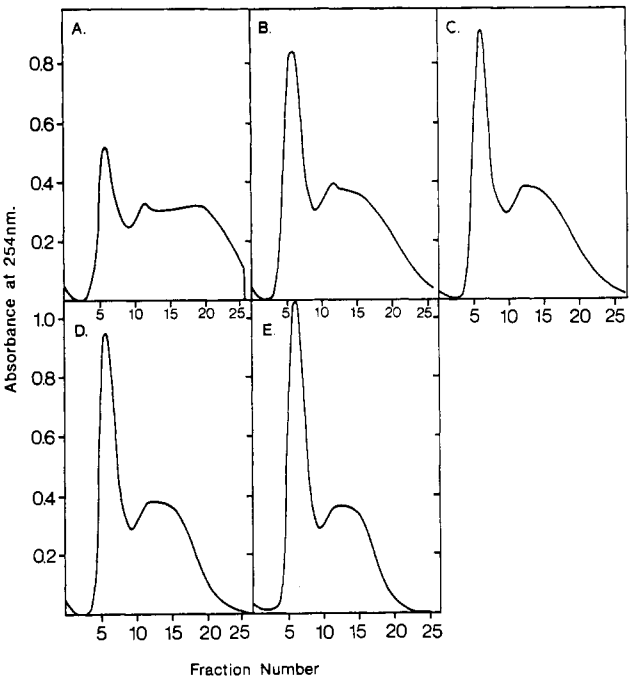

FIGURE 2: The distribution in glycerol gradients of myeloma chromatin subjected to shearing for varying lengths of time. Chromatin was obtained from MOPC104E and sheared in a Virtis homogenizer at 40 V for: (A) 1 min; (B) 2 min; (C) 3 min; (D) 4 min; (E) 5 min; centrifuged at 10,000*g* for 30 min. The supernatants (200 μg of chromatin DNA) were layered over 12 ml of 7.6–76% linear glycerol gradients and centrifuged for 12 hr at 4° at 25,000 rpm (SB-283 rotor, B-60 ultracentrifuge).

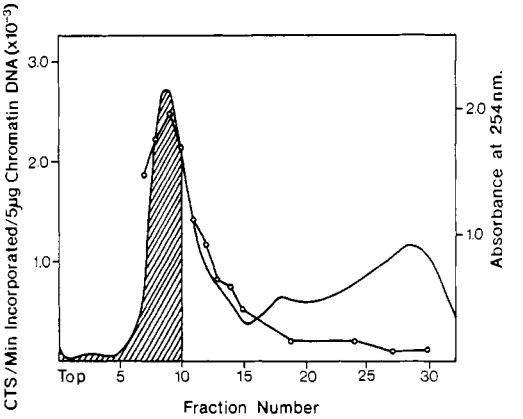

FIGURE 3: The template activity of individual fractions distributed across a sucrose gradient of myeloma chromatin. MOPC173D chromatin was sheared (40 V, 3 min) and 1.6 mg of chromatin DNA was fractionated on 30 ml of 0.17–1.7 M sucrose gradients (22,500 rpm for 15 hr, SW 25.1 rotor, Spinco Model L ultracentrifuge). The template activity of 5 μg of chromatin DNA from the area of the gradient indicated was assayed with the *E. coli* RNA polymerase (5 μl) as described under Materials and Methods. The results were expressed as radioactivity (O) superimposed on the A_{260} profile (—). The shaded area represents the fractions referred to as the trailing two-thirds of the active component.

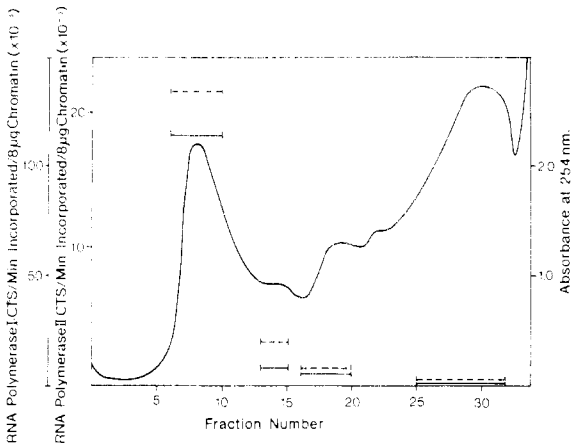

FIGURE 4: A comparison of the template activity of fractionated myeloma chromatin with the MOPC21 forms I and II RNA polymerases. Chromtain from MOPC173D was sheared (40 V, 3 min) and 3 mg of chromatin DNA was fractionated on sucrose gradients as described under Figure 3. Fractions in various portions of the gradient were pooled and 8 μg of chromatin DNA (in 200 μl of 0.01 M Tris (pH 8.0)) were incubated with 100 μl of either the MOPC21 form I or form II RNA polymerases for 10 min as described under Materials and Methods. The results were expressed as the cpm of [^{3}H]UTP incorporated/8 μg of DNA, for the form I (|- - -|) and form II (|—|) RNA polymerase. The assay conditions are the same as described under Materials and Methods.

matin (approximately one-half to two-thirds of the entire component, containing 11% of the chromatin DNA applied to the gradient) possessed a high and nearly constant template activity for *in vitro* RNA synthesis. The fraction of maximal activity was 17 times as efficient a template as the rapidly sedimenting chromatin and possessed 80% of the template activity of purified MOPC173D DNA. A close correspondence was observed between the fractional amount of DNA in the highly active chromatin (11%) and the template activity of the unfractionated chromatin preparation relative to purified DNA (12%). From these data, it seems clear that the fractionation procedure is capable of resolving the expected series of molecular species, and that the most highly template-active chromatin DNA sequences are concentrated in the trailing edge of the slowly sedimenting chromatin component.

In Vitro Template Activity of Myeloma Chromatin Fractions Using RNA Polymerase from Myeloma Cells. The biological relevance of the results in the preceding sections can be challenged on the grounds that use of a bacterial polymerase and a eukaryote template may generate misleading results. Moreover, there is evidence which suggests that some transcriptional control may reside at the level of RNA polymerase. For example, Roeder and Rutter (1969) have isolated RNA polymerases having different template and salt optima from eukaryote nuclei.

In order to examine the validity of the results obtained previously with the bacterial polymerase, the template capacity of MOPC173D chromatin components was tested with both the nucleolar (form I) and nucleoplasmic (form II) MOPC21 RNA polymerases. As shown in Figure 4, the fractions of chromatin which were found to be template active and inactive with the homologous polymerase correspond exactly to the chromatin fractions designated as active and inactive with the bacterial enzyme. The template capacity of slowly sedimenting chromatin using the form II RNA polymerase was 15 times that of rapidly sedimenting chromatin and possessed 50% of the template activity of purified MOPC173D DNA. The form I MOPC21 RNA polymerase exhibited even more preference for the slowly sedimenting component of MOPC173D chromatin than did RNA polymerase II (Figure 4, Table III). With the form I polymerase, slowly sedimenting chromatin was 62 times as efficient a template as rapidly sedimenting chromatin. Similar results were obtained using fractionated MOPC21 chromatin in conjunction with the MOPC21 RNA polymerases (S. H. Hall and E. C. Murphy, unpublished data).

TABLE III: Template Activity of Myeloma Chromatin Fractions Using MOPC21 RNA Polymerases I and II.[a]

Template	cpm/μg	Template Act. Relative to DNA	Template Act. Relative to Total Chromatin	Template Act. Relative to Slowly Sedimenting Chromatin
	RNA Polymerase I			
DNA	26,600	1.0		
Total chromatin	2,180	0.08	1.0	7.9
Slowly sedimenting chromatin	17,100	0.65	7.9	62.0
Intermediate chromatin (A)	2,550	0.10	1.2	9.3
Intermediate chromatin (B)	945	0.04	0.43	3.4
Rapidly-sedimenting chromatin	275	0.01	0.13	1.0
	RNA Polymerase II			
DNA	4,580	1.0		
Total chromatin	434	0.10	1.0	2.9
Slowly sedimenting chromatin	2,180	0.50	5.2	15.0
Intermediate chromatin (A)	640	0.15	1.6	4.5
Intermediate chromatin (B)	580	0.13	1.3	3.8
Rapidly sedimenting chromatin	152	0.03	0.35	1.0

[a] Assay conditions are described in Figure 5 but with 8 μg of chromatin DNA. $(NH_4)_2SO_4$ was 40 mM for form I polymerase incubations.

Association of Newly Synthesized RNA with Fractionated Chromatin. The results of the previous sections have shown that the slowly sedimenting component of chromatin contained most of the *in vitro* template capacity for RNA synthesis. If this fraction represents, in fact, the portion of the genome which is active in RNA synthesis *in vivo*, newly synthesized RNA molecules might be found associated with slowly sedimenting chromatin after a brief exposure to a radiolabeled RNA precursor and absent from the other fractions of chromatin. To test this prediction, myeloma cells in tissue culture (myeloma 66-2) were exposed to [^{3}H]uridine for 10 min, after which one-half of the culture was incubated with a large excess of unlabeled uridine for an additional 60 min. Chromatin from both the "pulsed" and "chased" cultures was isolated, fractionated, and examined for the presence of nascent RNA. The results, presented in Figure 5, show a peak of specific activity over the slowly sedimenting chromatin frac-

TABLE IV: Association of Newly Synthesized RNA with Myeloma Nuclei and Chromatin.

Culture	Nuclei cpm	Proportion of Nuclear Radioact. in: Unfractionated Chromatin	Proportion of Nuclear Radioact. in: Slowly Sedimenting Chromatin[a]
Pulse	754,000	0.47	0.21
Chase	965,000	0.27	0.08

[a] Fractions 4–9 in the gradient shown in Figure 5.

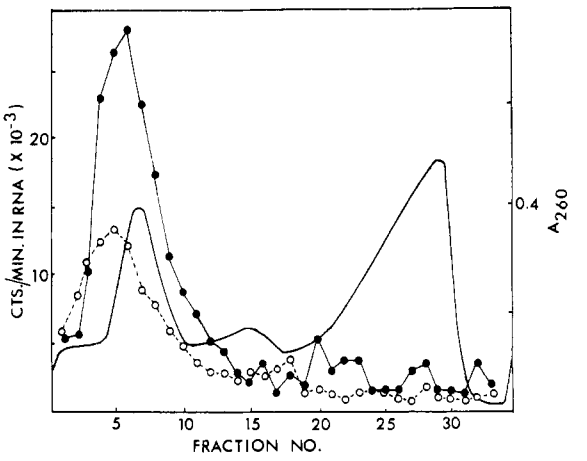

FIGURE 5: The association of newly synthesized RNA with fractionated chromatin. As described under Materials and Methods, two cultures of 66-2 myeloma cells were exposed to 10 μCi/ml of [^{3}H]uridine for 10 min, and one of these was incubated with a large excess of unlabeled uridine for an additional 60 min. Chromatin was isolated from both cultures, sheared, and fractionated on glycerol gradients. The radioactivity was determined throughout the gradient in the "pulsed" culture (●—●) and in the "chased" culture (O- - -O) and superimpsoed on the A_{260} profile of a gradient of chromatin from the 66-2 tissue culture cells containing ten times the A_{260} input as the experimental cultures (—).

tion which can be diminished by exposure to unlabeled uridine, as one might expect if the radioactivity represents partially synthesized RNA molecules.

The association of newly synthesized RNA molecules with the *in vitro* template-active fraction of chromatin represents the single most convincing piece of evidence that this fraction is indeed the *in vivo* template. It seems unlikely that the RNA associated with the slowly sedimenting chromatin could be nonspecifically bound. In the experiment cited above, as shown in Table IV, there was more radioactivity in the nuclei after the 60-min chase than after the 10-min pulse. However, a greater proportion of the counts was found bound both to the chromatin and its slowly sedimenting component in the pulsed cells than in the chased cells, a result which can best be explained by a selective association of RNA with the slowly sedimenting chromatin fraction during the period of the pulse.

Dissociation and Reassociation of Chromatin. It has been reported that under the appropriate conditions, chromatin can be dissociated and reassociated without untoward damage to its capacity to regulate the specificity of RNA synthesis (Paul and Gilmour, 1968; Gilmour and Paul, 1970; Huang, and Huang, 1969; Bekhor *et al.*, 1969; Spelsberg and Hnilica 1970; Spelsberg *et al.*, 1971) or to hormone receptors (O'Malley *et al.*, 1972). To test whether chromatin might be able to maintain its structural and functional heterogeneity under these conditions, MOPC315 chromatin was dissociated and reconstituted. The template capacity of the reconstituted material was 1.6 times that of native chromatin (Table V), suggesting that some originally template-inactive DNA sequences in native chromatin were accessible to RNA polymerase after reconstitution or that a minor fraction of the chromatin DNA remained uncombined with chromosomal proteins. The glycerol gradient profile of reconstituted chromatin was strikingly different from that of native chromatin. As illustrated in Figure 6, nearly all of the reconstituted chromatin remaining in the gradient cosedimented with the active component of native chromatin. The slowly sedimenting component in reconstituted chromatin, while significantly more template active than the unfractionated preparation (Table V), contained 40% more DNA and 50% less template activity (per microgram of DNA) than the corresponding component in native chromatin. These data also indicate a shift of some of the inactive chromatin to the active region of the gradient. The absence of a rapidly sedimenting chromatin

TABLE V: Template Activity of Native and Reconstituted Chromatin Using *E. coli* RNA Polymerase.

	Native Chromatin: Total Chromatin	Native Chromatin: Slowly Sedimenting Chromatin	Reconstituted Chromatin: Total Chromatin	Reconstituted Chromatin: Slowly Sedimenting Chromatin
Cpm incorpd	1390	7480	2200	5050
Rel template act.[a]	1.0	5.4	1.60	3.7

[a] As compared to the template activity of unfractionated native chromatin.

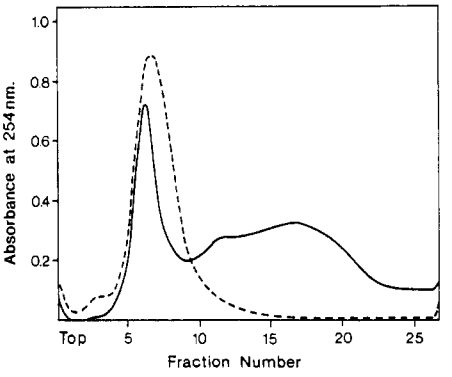

FIGURE 6: A comparison of the sedimentation properties of native and reconstituted chromatin. Chromatin (1.3 mg of chromatin DNA) obtained from MOPC315 was dissociated and reconstituted as described under Materials and Methods. A portion of this chromatin (150 μg of chromatin DNA) was centrifuged (along with an equal amount of native chromatin) in 12-ml 7.6–76% glycerol gradients at 25,000 rpm for 12 hr (SB-283 rotor, B-60 ultracentrifuge). The profiles of native (—) and reconstituted (- - - -) chromatin were analyzed as described under Materials and Methods.

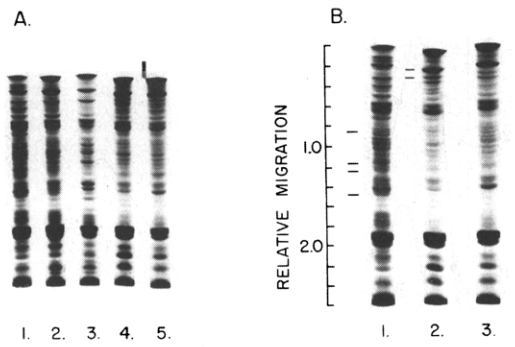

FIGURE 7: Sodium dodecyl sulfate–polyacrylamide electrophoresis of the proteins associated with the various components of fractionated chromatin. MOPC173D chromatin (3.33 mg of chromatin DNA) was fractionated on each of three sucrose density gradients as described under Figure 4. Fractions were pooled from five regions of the gradient: (1) slowly sedimenting chromatin-trailing edge; (2) slowly sedimenting chromatin-leading edge; (3) intermediate chromatin; (4) rapidly sedimenting chromatin; and (5) pelleted chromatin. These were dialyzed against 0.001 M Tris (pH 8.0) and concentrated to a small volume in an air stream. The chromatin (60 μg of chromatin DNA) was treated with DNase as described and subjected to electrophoresis as described under Materials and Methods: (A) the protein populations associated with each of the regions of the gradient; (B) a comparison of (1) slowly sedimenting, (2) rapidly sedimenting, and (3) unfractionated chromatin. Proteins unique to a given chromatin fraction are marked.

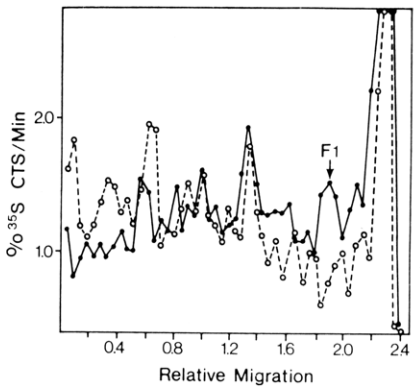

FIGURE 8: A comparison of radioactive chromosomal proteins from MOPC 173D active and inactive chromatin in sodium dodecyl sulfate–polyacrylamide gels. The cells were labeled with [^{35}S]-methionine as described under Materials and Methods and the chromatin fractionated as described under Figure 7. Chromatin from the active and inactive regions was treated with DNase and subjected to electrophoresis. The radioactivity in the chromosomal proteins from active (O—O) and inactive (O- - -O) chromatin was determined as described under Materials and Methods.

component in the reconstituted chromatin lent additional support to this assumption. However, not all of the rapidly sedimenting component was reduced in sedimentation rate in reconstituted chromatin. Most of it, in fact, appeared in the pellet, which contained 30% of the reconstituted chromatin DNA, as compared with 5% in native chromatin. From these data it was concluded that alterations of an unspecified magnitude occur when DNA and chromosomal proteins are reconstituted.

Proteins Associated with Active and Inactive Chromatin. Each differentiated cell type is programmed to synthesize a unique population of RNA species whose spectrum is apparently controlled by nonhistone chromosomal proteins (Paul and Gilmour, 1968; Gilmour and Paul, 1970; Spelsberg and Hnilica, 1970; Spelsberg *et al.*, 1971). If nonhistone chromosomal proteins control the functional state of the genome, it might be predicted that differences in this class of proteins would be apparent in a comparison of template-active and inactive chromatin.

To examine the distribution of chromosomal proteins in fractionated chromatin, a MOPC173D chromatin gradient was divided into four regions corresponding to those regions assayed previously with RNA polymerases I and II (refer to Figure 4). Chromatin from these regions and the pellet was treated with DNase and applied to sodium dodecyl sulfate gels, photographs of which appear in Figure 7A. Superficially, the protein patterns obtained from the various regions of the gradient were remarkably similar. Unfractionated chromatin contained more than 50 clearly resolved nonhistone proteins (Figure 7B, gel 3), all of which appear to a greater or lesser extent in the various chromatin fractions, a result which argues against the loss of chromosomal proteins by proteolysis during fractionation or during the preparation for electrophoresis. A close comparison of the proteins associated with active and inactive chromatin revealed that at least four nonhistones were preferentially localized on active chromatin, while inactive chromatin contained two nonhistone proteins absent or diminished in active chromatin.

A comparison of radiolabeled proteins from a separate preparation of MOPC173D active and inactive chromatin yielded the results shown in Figure 8. As observed in the stained gels, it was evident that two proteins of high molecular weight were concentrated on inactive chromatin and that several peaks of radioactivity corresponding to proteins designated as active unique in the stained gels appeared to be present in active chromatin and absent or depressed in inactive chromatin. The distribution of radioactivity in the gels of active and inactive chromatin confirmed another impression gained from examining the stained gels, namely that active chromatin was relatively rich in nonhistone proteins of lower molecular weight and deficient in high molecular weight nonhistone proteins, while the converse applied to proteins associated with inactive chromatin.

A comparision of the proteins associated with active and inactive chromatin in MOPC104E yielded similar results, as shown in Figure 9. Because of the difficulty in displaying histones and nonhistones on the same scale, the region of the gel which contains the histones was not shown. Again two nonhistone proteins of high molecular weight were present on inactive but not active chromatin, and in this case, the four or more nonhistone proteins unique to active chromatin were well resolved. As with MOPC173D, we were able to obtain confirmatory evidence relating to the proteins unique to MOPC104E active and inactive chromatin from gels containing radioactive proteins (E. C. Murphy and J. H. Shepherd, unpublished data).

Discussion

The results of several investigations have established that chromatin can be resolved into a slowly sedimenting, template-active component and a more rapidly sedimenting inactive component (Frenster *et al.*, 1963; Chalkley and Jensen, 1968; Duercksen and McCarthy, 1971). Likewise, we have found that chromatin from mouse myelomas can be fractionated into components which differ in their physical and functional characteristics. Equivalent results were obtained with both sucrose and glycerol gradients. However, the glycerol gradients were considered superior, since chromatin could be frozen and thawed in glycerol without detectable alteration of several of

its physical, chemical, or functional properties. The slowly sedimenting chromatin fraction, containing 10–20% of the total chromatin DNA, possessed >90% of the template activity for *in vitro* RNA synthesis with exogenous RNA polymerase and was selectively associated with nascent RNA molecules *in vivo*. The rapidly sedimenting chromatin fraction contained the bulk of the DNA and had little template activity *in vitro* or *in vivo*. Between these two extremes is found a series of chromatin subfractions possessing intermediate template capacities and sedimentation properties. Assuming that template-active DNA sequences are interspersed among repressed sequences along the length of the genome and that shearing produces breakage at random, the observed gradient in template activity of the chromatin segments generated is expected. Although shearing appears to produce the desired results, the conversion of rapidly sedimenting to slowly sedimenting chromatin as a result of increased shearing emphasizes that the native structure of chromatin is fragile and that excessive shearing may produce artifacts.

A close examination of the template efficiency of the various chromatin fractions reveals the presence of two distinct classes of chromatin particles. The first class, which includes the entire slowly sedimenting component (30–40% of the fraction of the genome that can be solubilized by shearing), is composed of a mixture of highly template-active, partially active, and nearly inactive chromatin particles. This class of chromatin particle, we propose, represents the randomly sheared portion of the genome which is transcribed *in vivo*. This proposal is supported by the association of nascent RNA with this class of chromatin particle *in vivo*. These chromatin particles probably contain structural genes continuous with "controller" loci, and whether a given chromatin particle contains more or less structural or "controller" sequences will determine its relative template efficiency *in vitro* and probably the number of nascent RNA chains associated with it *in vivo*. The second class of chromatin particle, which constitutes 60–70% of the chromatin DNA, appears to be nearly completely repressed throughout.

The factors which operate to influence the sedimentation properties of the classes of chromatin are not well understood, although it is likely that molecular weight, conformation, and aggregation all affect the sedimentation of template-active and inactive chromatin. The effect of molecular weight cannot be large, since the DNA isolated from both slowly and rapidly sedimenting chromatin has similar molecular weights (Chalkley and Jensen, 1968) and our results indicate that rapidly sedimenting chromatin contains only a moderately higher protein:DNA ratio than does slowly sedimenting chromatin (E. C. Murphy and J. H. Shepherd, unpublished data). From the observations of Littau *et al.* (1964, 1965) that the conformation of interphase chromosomes active in RNA synthesis *in vivo* was diffuse, while the inactive regions were condensed, it might be predicted that diffusely organized (transcribed) chromatin would sediment less rapidly than condensed (repressed) portions, and, in fact, this assumption has been confirmed. Chalkley and Jensen (1968), Nishiura (1972), and we in this study have all reported that slowly sedimenting chromatin is a more efficient template for RNA synthesis *in vitro* than is rapidly sedimenting chromatin, and it has been observed that the highly condensed satellite DNA of mammalian (Yunis and Yasmineh, 1970; Yasmineh and Yunis, 1969) and crab cells (Duercksen and McCarthy, 1971) sediments rapidly in sucrose gradients. Chalkley and Jensen (1968) have proposed that aggregation may provide an independent explanation for the rapid sedimentation behavior of

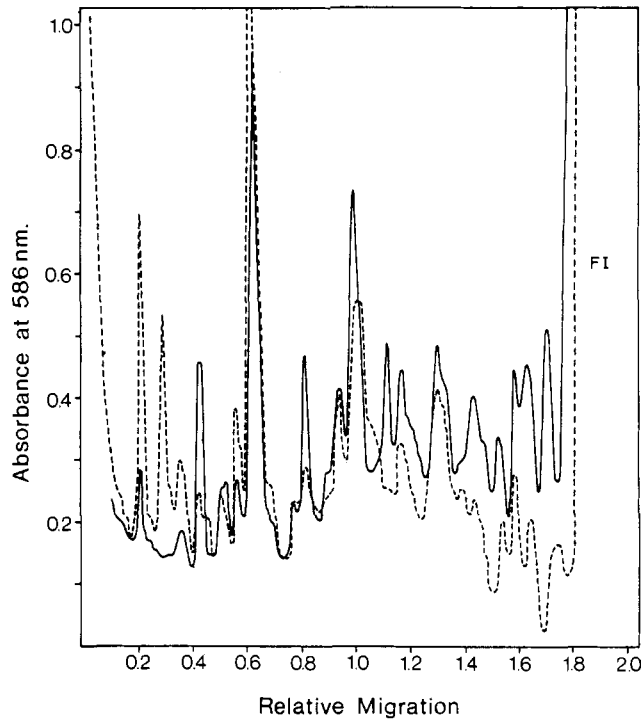

FIGURE 9: An optical scan of sodium dodecyl sulfate–polyacrylamide gels of proteins from the the active and inactive regions of MOPC104E chromatin. The chromatin (1.8 mg of chromatin) was fractionated by centrifugation on 7.6–76% glycerol gradients at 25,000 rpm for 14 hr (SW 25.1 rotor, Model L ultracentrifuge) and electrophoresis was carried out on the proteins of DNase-treated active and inactive chromatin as described under Materials and Methods. Scanning of the stained proteins from active (—) and inactive chromatin (- - -) was performed as described under Materials and Methods.

template-inactive chromatin particles, since exposure to 4 M urea (a procedure reported to remove no protein) influenced inactive 130S chromatin to cosediment with template-active 30S chromatin particles.

However unclear the physical basis for chromatin fractionation, it is certain that chromatin classes differing in at least one functional property are produced. Our results with both the *E. coli* RNA polymerase and forms I and II of the MOPC-21 RNA polymerase clearly demonstrated that the trailing edge of the slowly sedimenting chromatin class was a far better template for *in vitro* RNA synthesis than its own leading edge and the rapidly sedimenting chromatin class. Both the bacterial polymerase and the form II RNA polymerase exhibited about the same preference for active chromatin over inactive chromatin (15–17-fold). With both enzymes, moreover, active chromatin possessed ~45% of the template activity of purified DNA, while the activity of unfractionated chromatin was only 9–12% that of DNA. The form I myeloma RNA polymerase exhibited an even greater preference for active over inactive chromatin (62-fold). This result in itself is curious since Butterworth *et al.* (1971) reported that in their hands the rat liver form I RNA polymerase was inactive with rat liver chromatin. No convincing explanation for the divergence of results is available, although it might be that the myeloma chromatin preparations contain a portion of the nucleolar ribosomal genes, whereas rat liver chromatin may not. Since all of these template assays were performed at limiting concentrations of DNA, using RNA polymerase preparations of undefined specific activity, no strict compari-

son of the bacterial and myeloma polymerases, or even of polymerases I and II, can be made.

It is quite probable that the fractionation procedure described here substantially conserves the *in vivo* structure of the genome. Aside from shearing, the method involves no harsh manipulations. Moreover, the sedimentation properties of template-active and inactive chromatin conform to what might be predicted based on cytological observations of RNA synthesis *in vivo* (Littau *et al.*, 1964, 1965), and the proportion of active to inactive DNA sequences generated corresponds well to that estimated from RNA–DNA hybridization experiments. Furthermore, McConaughy and McCarthy (1972) have reported that a low-melting minor component of chick erythrocyte chromatin DNA was complementary to 25–30% of the total erythrocyte RNA, while higher melting chromatin components did not hybridize. They proposed that a low T_m is typical of DNA sequences active in transcription. It has been found that template-active chromatin of *D. melanogaster* (Nishiura, 1972), calf thymus (Chalkley and Jensen, 1968), rabbit liver (Reeck *et al.*, 1972), and mouse myelomas (E. C. Murphy, unpublished data) has a T_m varying from 2 to 10° below that of template-inactive chromatin. Thus, by extension, chromatin which possesses a low T_m may well contain the DNA sequences which are transcribed *in vivo*. The most convincing evidence for the biological relevance of the chromatin fractionation procedure was provided by the observation that newly synthesized RNA was found selectively associated with the slowly sedimenting chromatin fraction. Whether or not the chromatin, after isolation, solubilization, and fractionation will prove to be a suitable template for the *in vitro* synthesis of functional RNA species remains an interesting and as yet unanswered question.

There are striking differences among the nonhistones associated with template-active and inactive chromatin. The repressed template is associated with a relatively large amount of high molecular weight (>100,000) nonhistones and the presence of two high molecular weight nonhistones in particular. An open template is characterized by the absence of these two large nonhistones, the presence of four or more nonhistones not associated with template-inactive chromatin, and a predominance of the smaller (<100,000 mol wt) species of nonhistones. It should be emphasized that the two nonhistones unique to inactive chromatin and the four or more associated preferentially with active chromatin must be regarded as the minimum number of variations between the two components. A number of distinct chromosomal proteins may occupy a single band in the sodium dodecyl sulfate gel (Elgin and Bonner, 1972) and it is clear that under certain conditions chromosomal proteins can undergo redistribution (Clark and Felsenfeld, 1971).

The function of the nonhistones unique to active and inactive chromatin is, of course, obscure. Possibly one or both of the two nonhistones restricted to template-inactive chromatin effect the specific aggregation of inactive chromatin by binding similar sequences on separate chromatin particles. It is entirely possible, however, that these two nonhistones have nothing to do with maintaining chromatin aggregates, but instead may function together with the histones to prevent RNA synthesis on repressed DNA sequences. In this connection, Salas and Green (1972) have reported the presence of a high molecular weight DNA-binding protein in cultured mouse fibroblasts resting at saturation density. They suggested that this protein may prevent DNA replication, since its synthesis was inversely related to the synthesis of another, smaller, DNA-binding protein, whose synthesis, in turn, was tightly coupled to the onset of DNA replication. In an analogous fashion, RNA synthesis might be inhibited by certain high molecular weight nonhistone chromosomal proteins.

The proteins associated preferentially with active chromatin might be no more than enzymes involved in the processing and transport of newly synthesized RNA. On the other hand, these proteins might well operate to maintain chromatin in an extended conformation and antagonize, at specific sequences, the generalized repression imposed by histones on the major portion of the genome. Lacking any additional information, no stronger statement can be made, except to recall that a number of studies have suggested that the nonhistone protein population harbors the regulators of tissue-specific RNA synthesis (Paul and Gilmour, 1968; Gilmour and Paul, 1970; Bekhor *et al.*, 1969; Spelsberg *et al.*, 1971) and receptors of hormone–cytoplasmic receptor complexes (O'Malley *et al.*, 1972). Moreover, certain nonhistone proteins respond to hormones by rapid and selective synthesis (Teng and Hamilton, 1970; Shelton and Allfrey, 1970). Thus the weight of evidence points to a direct connection between tissue-specific RNA synthesis, gene activation, and nonhistone chromosomal proteins. The association of unique nonhistone proteins with chromatin known to be highly active in RNA synthesis *in vivo* and *in vitro* strengthens this connection.

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A Comparison of the Proteins of Condensed and Extended Chromatin Fractions of Rabbit Liver and Calf Thymus†

Robert T. Simpson* and Gerald R. Reeck

ABSTRACT: Chromatography of sonicated chromatin on Ectham-cellulose allows the resolution of a spectrum of nucleoprotein species differing in melting properties (Reeck, G. R., Simpson, R. T., and Sober, H. A. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 2317). The early eluting, high melting fractions are condensed in structure, while the late eluting, low melting fractions are in an extended DNA-like conformation. In contrast to early eluting rabbit liver chromatin fractions, late eluted chromatin segments are depleted of the lysine-rich histone and contain a markedly increased amount of nonhistone protein, amounting to 2.5 times the mass of the DNA. While calf thymus chromatin can also be fractionated into low and high melting segments with altered histone contents, the total nonhistone protein content of all the fraction is low and constant. For both tissues the relative concentrations of the detected microheterogeneous variants of the histones are unaltered. Superimposed on a background of overall similarity, the nonhistone proteins of early and late eluted rabbit liver chromatin nevertheless show both qualitative and quantitative differences.

Chromatography of sonicated chromatin on Ectham-cellulose allows the partial resolution of a spectrum of nucleoprotein species which differ in their melting properties (Reeck *et al.*, 1972). Further studies have indicated that the early eluted, high melting chromatin fraction exist in a highly condensed or supercoiled conformation while the late eluted, low melting fractions have an extended DNA-like conformation in solution (Polacow and Simpson, 1973a,b). We now report a comparison of the protein contents and distributions of condensed and extended chromatin segments isolated by preparative level fractionation on Ectham-cellulose more detailed than previously reported (Reeck *et al.*, 1972). Further, we compare rabbit liver and calf thymus chromatins which differ markedly in their content of nonhistone proteins. Our data indicate that extended chromatin is characterized by a decreased content of histone, primarily due to a decrease in the lysine-rich histones. In rabbit liver, the nonhistone protein content of the extended chromatin fractions is nearly three times that of the condensed material. The types of nonhistone proteins present in the two chromatin fractions differ significantly but yet maintain a surprising overall similarity. In contrast to these differences, both condensed and extended chromatins appear to contain identical proportions of the various naturally occurring chemically modified histones which can be detected by high-resolution polyacrylamide gel electrophoresis.

Experimental Section

Chromatin was isolated from livers of mature male New Zealand White rabbits or from frozen calf thymus by previously detailed methods (Simpson, 1971). After isolation, shearing, and centrifugation to remove unsheared materials all chromatin samples were sonicated for 2 min at 0–2° and 70 W using a microtip and the Branson Model W185 sonifier (Reeck *et al.*, 1972). Chromatin was utilized for chromatog-

† From the Laboratory of Nutrition and Endocrinology, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received March 22, 1973.*