Chromatin Fractionation Related to Cell Type and Chromosome Condensation but Perhaps Not to Transcriptional Activity*

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A chromatin fractionation procedure has been developed that involves the shearing of swollen chromatin and the separation of chromatin fragments by sucrose gradient centrifugation. When chromatin from rapidly growing HeLa cells was fractionated, four partially resolved peaks were obtained. Partial characterization of the chromatin fractions indicated that each contained similar lengths of DNA formed in a complex with histones in nucleosomes. The most likely difference between the fractions is the degree of intra- or interstrand association of the fibers of nucleosomes. Chromatins from chicken erythrocytes and mitotic chromatin were analyzed by the procedure. These chromatins yielded gradient profiles that were distinctly different from each other and from interphase chromatin. These results suggested that the fractionation procedure reflected at least some of the differences in the structure of chromatin known to exist in vivo.

The association of pulse-labeled RNA with a particular chromatin fraction is frequently used to support claims of successful separation of transcriptionally active chromatin from inactive chromatin. Since our data show that the slowly sedimenting fractions preferentially bind RNA whose synthesis was clearly not in progress at the start of the fractionation, this criterion is suspect. The presence of equal amounts of satellite DNA in all fractions of mouse L-cell chromatin indicated that the method did not fractionate on the basis of the in vivo transcriptional activity.

The organization and structure of eukaryotic chromosomes has been the subject of many studies (1). DNA is formed in a complex with chromosomal proteins in such a manner that a large amount of DNA is confined in a small volume. Chromosomal structure varies from one cell type to another and from one stage of the cell cycle to another. This variation in packaging is accompanied by changes in activities and functions of DNA metabolism such as transcription and replication. In interphase cells, chromatin is organized such that some DNA sequences are transcribed and all sequences can be replicated.

The chromatin in nucleated erythrocytes is more condensed and supports a very low level of transcription and no replication. Mitotic chromosomes are even more highly condensed and no transcription or replication occurs.

Thin section electron microscope studies show interphase chromatin to be a network of nucleoprotein fibers. These fibers are now known to consist of DNA packaged in λ bodies or nucleosomes (2, 3). Both transcribed and nontranscribed DNA sequences are found in nucleosomes (4). It has been accepted for a long time that the fibers containing DNA that is transcribed in vivo are loosely organized (often referred to as extended chromatin, or euchromatin), while nontranscribed DNA is present in fibers which are tightly packed (often called condensed chromatin, or heterochromatin). In this paper we shall refer to chromatin that was transcribed in vivo as active chromatin and that which was not, as inactive chromatin. Since one of the fundamental current questions in biology concerns the mechanism of gene regulation in eukaryotes, the separation and characterization of active and inactive chromatin is of interest.

Chromatin fractionation procedures have been described by Feuister et al. (5), Yasumichi and Yunis (6), Rechv et al. (7), Bonner et al. (8), and McCarthy et al. (9) among others. Although there is some variation in procedure, each method involves the fragmentation of chromatin and the separation of the fragments on the basis of physicochemical differences expected of active and inactive chromatin. The major goal of these studies was to separate chromatin components that were transcriptionally active in vivo from those that were inactive.

Criteria such as satellite DNA enrichment (10), in vitro template activity, and localization of pulse-labeled RNA (9, 11) were used to support claims for successful fractionation.

A procedure developed by McCarthy et al. (9) involved mechanical shearing of purified chromatin. The components in the sheared chromatin were separated by centrifugation on sucrose gradients. When chromatin from cultured Drosophila cells was fractionated this way two peaks of chromatin appeared in the gradient. The chromatin that sedimented more slowly was a much better template for Escherichia coli RNA polymerase than the faster sedimenting fraction. Similar results were obtained by Murphy et al. (11) with fractionated chromatin from mouse myeloma cells. Both groups reported that nascent RNA was localized in the slower sedimenting fraction. These and other data seemed to identify the slower sedimenting chromatin fraction as active chromatin. Since

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then Doenecke and McCarthy have presented further evidence to support this assignment (12).

All chromatin fractionation procedures employ a starting hypothesis that the structural distinctions between active and inactive chromatin will survive the solubilization and fragmentation of the chromatin, but a study by Howk et al. (13) suggests that this hypothesis may be invalid. They reported that their use of the procedure used by Murphy et al. (11) as well as the method described by Recce et al. (7) had failed to fractionate chromatin on the basis of authentic in vivo transcrip-
tional activity. They found that all chromatin fractions contained equal amounts of DNA sequences that were transcribed in vivo and also contained sequences that were not transcribed in vivo. Similar results have been reported by Krieg and Wells (14).

In this paper we describe a chromatin fractionation procedure based on the method developed by McCarthy et al. (9) and employed by Murphy et al. (11). This procedure appears successful in reflecting some aspects of endogenous chromatin structure and organization, but like the results of Howk et al. (13), and Krieg and Wells (14), ours suggest that the fractionation is not to be correlated with in vivo transcriptional activity.

**MATERIALS AND METHODS**

**Cells**

HeLa S1 and mouse L929 cells were grown in suspension culture in Joklik modified Eagle's medium supplemented with penicillin, streptomycin, and 5% calf serum (Gibco).

Erythrocytes were obtained from fresh rooster blood. Clotting was prevented by the addition of a few milliliters of 5 mM Na2EDTA, pH 7.5, and the cells were collected by centrifugation at 1500 × g. The cells were washed with Dulbecco's phosphate-buffered saline (15) and nuclei isolated as described for HeLa and L929 cells.

**Isolation of Nuclei**

Nuclei were isolated using a modification of a method described by Wray and Stubblefield (16). All cells were washed with Buffer A (1.0 mM calcium chloride, 0.5 mM Pipes (1.4-piperazineethanesulfonic acid) (Calbiochem), pH 6.8, 0.1 M hexylene glycol) (18). The cells were suspended at a concentration of 107/ml in Buffer A, pH 7.5, and the cells were collected by centrifugation at 1500 × g. The cells were washed with Dulbecco's phosphate-buffered saline (15) and nuclei isolated as described for HeLa and L929 cells.

**Isolation of Chromatin**

Chromatin was isolated from all cells by suspension of the nuclei in 10 mM Tris/HCl, 1 mM Na2EDTA, pH 8.0, followed by centrifugation at 10,000 × g for 10 min. The chromatin was washed by gentle teasing of the chromatin gel in the appropriate buffer (see "Results").

**Mitotic Chromosomes**

Mouse L929 cells were treated with vinblastine sulfate (Rl Lilly) at a concentration of 0.5 µg/ml. The drug was added directly to growing suspension cultures at a cell concentration of 2 × 106/ml. The percent of mitotic figures was determined by Giemsa staining of fixed cultures (17). The yield of cells arrested in metaphase was highly variable. On some occasions 30 to 40% mitotic arrest was observed and these preparations were used in the experiments described here. The chromosomes were purified by the method of Wray and Stubblefield (16) except that the 37°C incubation step was omitted. The purity of the chromosomes was monitored by phase contrast microscopy. There were no nuclei visible in the final chromosome preparations.

**Shearing of Chromatin**

Washed chromatin was suspended in the appropriate buffer and loosely homogenized in a Dounce glass homogenizer with a B pestle. The chromatin was adjusted to the desired concentration and then sheared by a Sorval Omni-Mixer microtubilization homogenizer in the mini cup. Usually 3 to 6 ml of chromatin suspension was sheared. A few drops of heparin were added just before shearing to reduce foaming. The voltage was controlled by a Variac powerstat.

**Separation of Chromatin Fractions**

Sheared chromatin was applied to 12.5 ml of 0.35 to 1.7 M sucrose, 10 mM Tris/HCl, pH 8.5, gradients. The gradients were centrifuged in an SW 41 rotor (Beckman) at 41,000 rpm for 190 min. The temperature was set at 4°C. The gradient was collected from the top by pumping 2 ml sucrose through the bottom of the cellulose nitrate tube. The gradients were pumped through a turbulence free flow cell (Molecular Instruments Co.) mounted in a Gilford 2000 continuously recording spectrophotometer. The absorbance was measured at 260 nm.

**DNA Molecular Weight Determination**

Chromatin (150 µl), isolated from cells which had been labeled with [3H]thymidine was mixed with 10 µl of 10 mM Na2EDTA, pH 7.5, 10 µl of 50% sodium dodecyl sulfate and 15 µl of nucleoside-free promise (Calbiochem) 10 mg/ml (18). The mixture was incubated at 37°C for 1 h and applied to linear to 5% sucrose gradients (in 10 mM Tris/HCl, 250 mM NaCl, 5 mM Na2EDTA). The gradients were centrifuged in an SW 41 rotor at 41,000 rpm for 240 min at 23°C. 

**DNA Labeling**

DNA Labeling—[methyl-3H]Thymidine (Amersham, 1 µCi/ml, 50 Ci/mmole) was added to exponentially growing cells to a final concentration of 0.5 µCi/ml. The cells were grown in the presence of the label for 48 h to insure extensive labeling.

**RNA Labeling**

RNA Labeling—[3H]Uridine (New England Nuclear), 1 mCi/ml, 40 µCi/mmol was added to exponentially growing cells to a final concentration of 5 µCi/ml. The cells were stirred for the indicated times, and then the incorporation was stopped by the addition of an equal volume of ice-cold 10 mM sodium phosphate in phosphate-buffered saline solution. The cells were collected and washed once with cold phosphate-buffered saline/sodium azide.

**DNA Labeling—[methyl-3H]Thymidine (Amersham, 1 µCi/ml, 50 Ci/mmole) was added to exponentially growing cells to a final concentration of 0.5 µCi/ml. The cells were grown in the presence of the label for 48 h to insure extensive labeling.

**RNA Extraction**

Chromatin was prepared from [3H]thymidine-labeled cells. The chromatin suspension was adjusted to 10 mM Tris/HCl, 1% sodium Sarkosyl, and 1 mM Na2EDTA, pH 8.0. The mixture was phenol-extracted and the aqueous phase was treated with alcohol. The precipitate was collected by centrifugation and dissolved in a small volume of 10 mM Tris/HCl, 1 mM Na2EDTA, pH 8.0.

**DNA Polynucleotide Assay**

The DNA polynucleotide activity of chromatin and chromatin fractions was assayed according to the method of Roeder and Rutter (20). The assays were incubated at 37°C for 20 min. The reactions were terminated by spotting 100 µl of the assay mixture onto DE81 paper circles (Whatman) which were processed for counting as described by Blatti et al. (21).

**Satellite DNA Analysis**

DNA was extracted from mouse L929 chromatin fractions by the method of Coch et al. (22). Analytical CsCl centrifugation was performed as described by these authors except centrifugation was at 44,000 rpm for 24 h in the Spinco model E ultracentrifuge. Optical grade CsCl (Harshaw) was used. Buoyant densities were measured relative to Micrococcus luteus chromosomal DNA with a density of 1.73 g/ml (a gift of Dr. J. E. Hearst of the University of California, Berkeley). Density differences between bands were calculated according to the method of Schmid and Hearst (23).
Chromatin Fractionation

Thermal Denaturation Analyses

Chromatin fractions and unfractionated chromatin were dialyzed versus 5 mM Tris, pH 8.0. Thermal denaturation analyses were performed in a Gilford model 2000 recording spectrophotometer. The range of the analysis was from 30-100°C with a temperature increase of 15°C/h. Absorbance was read every 2 min. The data were corrected for the expansion of the buffer.

Nuclease Digestion

Micrococcal nuclease digests of chromatin and chromatin fractions were performed as described by Axel et al. (24).

Polyacrylamide Gels

Electrophoresis of chromatin proteins on polyacrylamide gels containing sodium dodecyl sulfate was as described by Ferro-Luzzi and Huang (28). The method of Raynaud and Ohlenbusch (26) was used to determine DNA content (27). Histone:nonhistone ratios were determined by densitometer analysis of Coomassie blue-stained, sodium dodecyl sulfate containing polyacrylamide gels of chromatin proteins.

RESULTS

Application of Modified Procedure to HeLa Cell Interphase Chromatin—One of our initial goals was to study structures in active chromatin, such as transcriptional and replicative complexes, that might be more fragile than the bulk of the chromatin structure. In the procedure used by McCarthy et al. (9) chromatin was purified by centrifugation through concentrated sucrose (1.7 M), but because the structures of interest might not survive this step we dispensed with it. In adapting the fractionation procedure of McCarthy et al. (9) to chromatin from HeLa cells we used the following criterion for chromatin solubility; sheared chromatin that was not pelleted from 10 mM Tris/HCl, pH 8.0, when centrifuged at 10,000 × g for 30 min was considered soluble. When such chromatin was centrifuged on a sucrose gradient, a well resolved profile of absorbance at 260 nm was obtained and all chromatin was contained in the gradient. This behavior is characteristic of chromatin that had been purified by sucrose washing (9). However, when chromatin was prepared by lysis of nuclei in 10 mM Tris/HCl and sheared directly, more than 50% of the chromatin was found in a 10,000 × g pellet. When the sheared chromatin was centrifuged on a 0.35 to 1.7 M sucrose gradient, a well resolved profile of absorbance at 260 nm was obtained and all chromatin was contained in the gradient. This behavior is characteristic of chromatin that had been purified by sucrose washing (9). However, when chromatin was prepared by lysis of nuclei in 10 mM Tris/HCl and sheared directly, more than 50% of the chromatin was found in a 10,000 × g pellet. When the sheared chromatin was centrifuged on a 0.35 to 1.7 M sucrose gradient again more than 50% was found in the pellet. Consequently attention was given to preparing chromatin that would be solubilized by shearing.

Preparation of Soluble Chromatin—After considerable experimentation a solubilization method, based on that of Shaw and Huang (28), was devised. Chromatin was prepared from nuclei and then washed by pelleting at 10,000 × g from the following buffer series: 1 × 5 mM Tris/HCl, 0.5 mM Na2EDTA; 1 × 2.5 mM Tris/HCl, 0.25 mM Na2EDTA; 2 × 1 mM Tris/HCl; 1 × 0.5 mM Tris/HCl. All buffers were at pH 8.0.

The chromatin was visibly swollen after the wash procedure. The washed chromatin was suspended in 0.5 mM Tris/HCl at a concentration of 15 A260 units/ml and sheared in the Omni-Mixer at 40 V for 5 min. The sheared chromatin was completely soluble.

When the sheared chromatin was centrifuged on a 0.35 to 1.7 M sucrose gradient (in 10 mM Tris, pH 8.0) at 205,000 × g for 195 min the absorbance profile shown in Fig. 1d was obtained. The profile might be described as a series of overlapping peaks, A, B, C, and D as indicated in the figure. The D region peak is much broader than the others, suggesting a greater constituent heterogeneity.

Shearing to Limit—Fig. 1 also illustrates an experiment in which the shearing time was varied while the voltage was held constant. When unsheared chromatin was centrifuged most of the material pelleted. The soluble portion forms a low broad peak in the central part of the gradient. After 90 s of shearing the basic elements of the profile were apparent (Fig. 1b), however 20 to 30% of the chromatin pelleted. The A260 pattern was essentially constant across the range of 3 to 6 min of shearing and there was no pellet. These results indicate that shearing is necessary for the generation of the chromatin profile. That is, endogenous nucleolytic activity is not responsible for the general appearance of the pattern. In any case a "limit shear" condition is reached after 3 min of shearing.

The relationship between the limit shear chromatin profiles and the molecular weight of the DNA isolated from the chromatin at various shear times was determined in the experiment illustrated in Fig. 2. DNA was isolated from unfractionated chromatin after the indicated shearing times. The sedimentation coefficient of the DNA was determined by centri-
Chromatin Fractionation

FIG. 2. Sedimentation analysis of DNA extracted from sheared chromatin at different shearing times. Chromatin was prepared from HeLa cells which had been incubated with [3H]thymidine, and sheared for the times indicated. Aliquots were removed after each shearing time and the sedimentation properties of the DNA analyzed as described under "Materials and Methods."

FIG. 3. High voltage shearing profile of HeLa cell chromatin. Chromatin was prepared from HeLa cells and adjusted to the standard concentration of 15 A_260/ml. It was sheared in the Omni-Mixer at 80 V and applied to a gradient under the standard conditions. The positions of the chromatin fractions from the 40-V shearing are shown.

The stability of the gradient profile and relative constancy of DNA molecular weight over the range of shearing times suggest that the peaks in the chromatin gradient profile represent discrete structures rather than intermediates in the shearing process, some of which would disappear when shearing was complete.

Variation in Shearing Conditions—Shearing the chromatin at higher speed resulted in a loss of resolution in the gradient profile. When the chromatin was sheared at 80 V for the usual 5 min only a broad peak in the A region appeared. This is shown in Fig. 3. A similar result was obtained when chromatin concentration was reduced by a factor of 3 prior to shearing at the standard speed and time (40 V for 5 min). This last observation suggests that there is a self-protecting effect of the chromatin which may be similar to the results obtained when DNA solutions of varied concentrations are sheared at the same speed (29). The data from the experiments with high speed shear and low concentration indicate that the chromatin profiles of Fig. 1 are sensitive to factors which affect the extent of fragmentation of the chromatin.

Variability of Profile—In the course of this work chromatin was fractionated many times. A range of variability in the amount of material in each region of the profile is given in Table II. An interesting feature of these data is that while the amounts of Fractions A and D did vary from preparation to preparation, it is as if each did so at the expense of the other, for the relative amounts of Fractions B and C were always quite similar.

Not only was the chromatin fractionation profile reproducible under the standard conditions but a number of factors could be varied without significantly affecting the pattern. These include the use of either rapidly growing or stationary phase cells, the omission of the NaCl: Na_2EDTA and Triton washes of the nuclei, the isolation of the nuclei at pH 10.5 (to inhibit nucleases) (30) and centrifugation of the gradients at room temperature instead of 4°C.

Molecular Weight of DNA from Each Fraction—Chromatin was prepared and fractionated and the molecular weights of the DNA from the A, B, and D regions of the gradient were determined. The DNA from all fractions appeared heterogeneous in size with a sedimentation profile similar to Fig. 2c. The mean molecular weight of the DNA from Fraction A was 2.7 x 10^8, that from Fraction B, 3.0 x 10^8 and that from Fraction D, 3.3 x 10^8. While there was some fractionation on the basis of DNA length, the size of the DNA was plainly a very minor factor in the fractionation process.

Table 1

<table>
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<tr>
<th>Time (min)</th>
<th>Sedimentation coefficient</th>
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<td>Low Peak</td>
<td>High</td>
<td>Low Peak</td>
</tr>
<tr>
<td>2</td>
<td>10.2 15.2 23.5</td>
<td>0.92</td>
</tr>
<tr>
<td>3</td>
<td>10.6 15.2 23.5</td>
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</tr>
<tr>
<td>4</td>
<td>10.6 15.2 23.5</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>9.6 14.8 23.5</td>
<td>0.8</td>
</tr>
</tbody>
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* Low and high refer to measurements taken at 20% of maximum peak height on either side of the peak in the 5 to 20% linear sucrose gradient.
Proteins and Protein Content of Fractions The protein:DNA ratios (w/w) of the fractions were: Fraction A, 1.5, Fraction B, 1.7; Fraction D, 1.4. Histones were extracted from each of the chromatin fractions and analyzed by electrophoresis on sodium dodecyl sulfate containing polyacrylamide slab gels. There were no differences between the histone patterns of the fractions and little difference in the amount of total histone (relative to DNA). All five histones were present in all fractions. These results are similar to those reported by McCarthy et al. (9). Total proteins in each fraction were analyzed in other experiments. Each fraction gave a number of nonhistone bands on the gels. However, there did not appear to be any significant variation in the spectrum of proteins from one fraction to the next. There were differences in the nonhistoneprotein ratios of the fractions as reflected in the protein:DNA ratios cited above. The nonhistonehistone ratio of Fraction A was 1.0; that of B, 1.3; that of D, 0.6. The possibility of a significant degree of proteolytic degradation was ruled out by the appearance of well stained lysine-rich histone bands in electrophoretic gels along with the absence of the characteristic products of their degradation.

Thermal Denaturation Analysis—When chromatin is melted and the hyperchromicity plotted versus temperature, a profile is obtained that has been interpreted as demonstrating the multiplicity of DNA:protein structures in chromatin. Since procedures, like the one described in this paper, are designed to fractionate such structures, melting analyses are frequently used to characterize chromatin fractions (9, 10, 14). Active chromatin is thought of as less constrained and thus its DNA is expected to be less thermally stable than that of inactive chromatin. This view of chromatin, when related to the chromatin fractionation procedure, predicts that the slowly sedimenting chromatin will have a lower melting temperature than that of the rapidly sedimenting chromatin.

The A, B, and D chromatin fractions were melted simultaneously to give the data shown in Fig. 4. The A and B fractions are quite similar, but differ from the D chromatin in two important respects. The first is the greater amount of the DNA in the A and B fractions which melts in the 40–65° range. The second is the melting temperature of the most thermally stable component of the chromatin fractions. The DNA in chromatin which melts below 65° (in this buffer) is thought to be either free of proteins or formed in a complex with nonhistone proteins (31). The hyperchromicity of the A and B chromatin in the 40–65° range is 25 and 23%, respectively, that of D, 6%. At the other end of the profiles the A and B chromatins have a melting temperature of 79–80° while that of the D chromatin is 83–84°. Although the interpretation of the higher temperature melting transitions has been discussed, a precise interpretation is still unavailable (31). It is clear, however, that the D region chromatin contains a larger proportion of DNA with a melting temperature greater than 65°. There also appears to be some structure(s) characterized by the 83-84° melting temperature which is not found in the A and B chromatin fractions.

In another experiment, A and D chromatin fractions and sheared, unfractinated chromatin were melted simultaneously. The temperatures at which the highest transitions occurred were: A, 80°, unfractinated chromatin, 82°, and D, 83.5°. This indicates that the fractionation procedure segregated components of differing thermal stability. The characteristics of the fractions are summarized in Table III.

Nuclease Digestion of Chromatin Fractions—The protein:DNA ratios and the histone content of the chromatin fractions seemed to rule out the possibility that the fractionation was based simply on protein content. However, the slowly sedimenting chromatin contained a significant amount of DNA that melted in the 40–65° range. This raised the possibility that the arrangement of proteins, particularly histones, might be different in the A fraction compared to the D chromatin. To test this possibility, micrococcal nuclease digests of the chromatin fractions were performed as described by Axel et al. (24). Digests of nuclei or unsheared chromatin yield nuclease-resistant DNA fragments of about 200 base pairs (32). When sheared chromatin is digested a more complicated pattern is obtained (33). The 200 base pair fragments are observed, but

**Table II**

| Fraction | Preparation
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**Table III**

| Characterization of chromatin fractions
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<th>Fraction D</th>
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<tr>
<td>DNA</td>
<td>9.7 x 10^6</td>
<td>3.0 x 10^6</td>
<td>3.3 x 10^6</td>
</tr>
<tr>
<td>Protein:DNA</td>
<td>1.5</td>
<td>1.7</td>
<td>1.4</td>
</tr>
<tr>
<td>Nonhistone:histone</td>
<td>1.0</td>
<td>1.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Hyperchromicity</td>
<td>25%</td>
<td>23%</td>
<td>6%</td>
</tr>
<tr>
<td>Highest T_m</td>
<td>79–80°</td>
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<td>83–84°</td>
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also fragments of approximately 145, 130, 90, and 80 base pairs (and smaller as well). The differences in digestion pattern have been attributed to the disruptive action of shearing on the nucleosomes that give rise to the 200 base pair fragments (34). A possible consequence of washing and shearing of chromatin during the fractionation procedure is the generation of a mixed population of DNA-protein complexes. There might be DNA loosely formed in a complex with histones in non-nucleosomal configurations, as well as intact nucleosomes. Differences in structure at this level could be the basis for the distribution of chromatin in the gradient.

The results of the nuclease digestion of the chromatin fractions ruled out this explanation. There were no significant differences in the patterns of nuclease-resistant DNA fragments of the fractions. All fractions contained 200, 145, 130, 90, 80, and smaller base pair fragments. The patterns were alike quantitatively and they were essentially identical with those of other investigators (24, 33).

These results indicated that the chromatin fractions did not differ at the level of the histone-DNA complexes, as assayed by the nuclease digestion, and that nucleosomes were present in all fractions. Interestingly, we observed identical patterns when swollen but unsheared chromatin was digested, indicating that the washing procedure itself greatly affects the native structure of the n-body.

The results of these experiments suggest that the fractionation is related to differences in the conformation of fibers of nucleosomes at a level above that of the individual nucleosome.

Extension of Method to Other Chromatins — One of the fundamental working assumptions in the fractionation of chromatin is that at least some elements of the in vivo structure and organization of chromatin fibers survive the process of solubilization, scission, and separation. One way of testing this assumption is to ask if chromatins known to differ from interphase chromatin in their in vivo structure would yield different profiles when analyzed by the fractionation procedure. Accordingly, the chromatins from chicken erythrocytes and mitotic chromosomes were chosen for study. Clearly, if these chromatins yielded profiles similar to that from interphase chromatin it would be difficult to argue that significant structural elements characteristic of the particular chromatin had survived the manipulation during the fractionation protocol.

Chicken erythrocytes were obtained from fresh rooster blood. The cells were washed and the nuclei and chromatin prepared exactly as described for the HeLa cell interphase chromatin. The chromatin was visibly swollen after the wash series, as the HeLa chromatin had been. When the chromatin was sheared (40 V for 5 min) and centrifuged on a gradient, more than 80% of the material pelleted. There was a small peak before the A region of the gradient. This was a highly reproducible observation. When the erythrocyte chromatin was sheared for 5 min at 80 V, a procedure which rendered all of the material soluble, the profile shown in Fig. 5 was obtained. There are two well resolved peaks, one in front of the A region of the gradient and one in the D. It will be recalled that shearing interphase chromatin at 80 V did not produce any material in the D region although it did convert all the A300 to A region material (Fig. 3).

Mitotic chromosomes were obtained from vinblastine-treated mouse LA9-cells and purified as described by Wray and Stubblefield (16). The purified chromosomes were run through the standard wash procedure. The chromosomes did not swell to form a gel during the wash procedure as did the interphase and erythrocyte chromatins. Instead, they had a granular appearance and were readily dispersed in each wash buffer. The profile of the sheared chromatin is shown in Fig. 6. There was only a small shoulder in Region I where the A peak was observed with interphase chromatin. This was followed by a sharp Region II where the B peak runs, a broad Region III corresponding to C, and Regions IV and V where the D peak runs. The relative amounts of A300 in each region are as follows: I 3%, II 17%, III 24%, IV 25%, V 29%. The profile shown in Fig. 6 was from chromatin sheared for 6 min, but there were no significant differences in the profiles from 4 to 7 min of shearing indicating that this was the limit sheared profile.

The difference between the mouse mitotic chromatin profile and that from HeLa cell interphase chromatin was not due to the use of cells of different species. This was demonstrated by the limit sheared profile of chromatin from interphase LA9-cells shown in Fig. 7. There was a large A peak, a small B peak, and a broad D region; there was no C peak. The profile differed in both peak position and material distribution from the mitotic chromatin profile of the same cells. The LA9 interphase profile did show the same peaks seen in the HeLa chromatin profile; however, there were substantial and highly reproducible quantitative differences. The relative amounts of A300 in...
Each region of the L9 interphase profile were A, 29%; B, 18%; C, 11%; D, 41%.

The results of these experiments with erythrocyte and mitotic chromatin indicate that the fractionation procedure is sensitive to differences in endogenous chromatin structure and organization and these differences are reflected in the gradient profile. It is also apparent that the procedure reflects differences between interphase chromatin from different cell types.

**Nascent RNA**—The procedure developed by McCarthy et al. (9) and employed by Murphy et al. (11) was reported to separate transcriptionally active chromatin from the inactive form. It seemed appropriate to apply to the HeLa chromatin fractions some of the criteria used to support these claims. One method of identifying a chromatin fraction as having been transcriptionally active in vivo is the localization of nascent RNA in the chromatin fraction (11, 35, 36). The experimental design is straightforward. Cells are labeled for a short time (10 to 15 min) with radioactive uridine and then chromatin from these cells is isolated and fractionated. The radioactive RNA associated with the chromatin is considered nascent RNA, i.e. RNA that was being transcribed at the time of the chromatin isolation and is in a hybrid structure with the template DNA for at least part of its length. The presence of the labeled RNA in a particular chromatin fraction suggests that chromatin was transcriptionally active in vivo.

The HeLa cell chromatin fractions were examined for the presence of nascent RNA in the following experiment. Cells were labeled with [3H]uridine for 15 min at 37°. Chromatin was isolated, run through the usual washing procedure, sheared, and centrifuged on the sucrose gradients. Fractions were collected from each gradient and the radioactivity in each fraction determined. The results are shown in Fig. 8a. The peak of the radioactivity was coincident with the B fraction. There was a shoulder of label in the A region and no significant radioactivity in the D region. When radioactive uridine was mixed with unlabeled chromatin and run on the gradient, the label was found to sediment more slowly than the in vivo label as shown in Fig. 8a. This result might suggest that the chromatin in the B region and perhaps in A as well, was transcriptionally active in vivo. This result is similar to those reported by several authors (9, 11, 36).

The location of nascent RNA in the A-B region was further tested by labeling the cells for 1 h with [3H]uridine. In this time period a considerable amount of completed RNA chains would be synthesized (37). At the time of chromatin isolation from these cells, label will be found in mature RNA species as well as nascent chains. If the RNA associated with the chromatin is exclusively nascent RNA, then the label should appear in the same position in the gradient as in the short time label experiment. The results are shown in Fig. 8b. The label again appears in the A and B regions but there is more label in the A region than the B. One possible explanation for this is that the B region might really be the site of nascent RNA formation and that the label in the A region represents RNA that is not nascent but that is nonetheless chromatin-associated. The level of this RNA would be expected to rise as a function of labeling time. This explanation raises the possibility that the label in the B region also represents chromatin-bound RNA that is not nascent. A number of RNA species are known to be associated with chromatin (38), but are not present as RNA-DNA hybrids (39). The obvious control experiment is to mix labeled RNA with unlabeled chromatin and determine the position of the label in the gradient after centrifugation of the mixture.

Accordingly chromatin was isolated from cells which had been labeled with [3H]uridine for 10 min and from cells labeled for 60 min. The chromatin was washed and sheared and then the RNA was extracted from each preparation with phenol. These RNA samples were mixed with unlabeled, sheared chromatin, and centrifuged on the gradients. For comparison an aliquot of each RNA sample was run on a gradient in the absence of unlabeled chromatin. The results are shown in Fig. 9. Both RNA samples when run alone sediment less rapidly...
the RNA extracted. The labeled RNA was mixed with unlabeled, loss of polymerase activity from the chromatin is a consequence of the extensive washing. Additionally, the polymerase activity is sensitive to shearing (41). Since a single wash of the chromatin (measured by absorbance at 260 nm) are shown in b and d, superimposed on the radioactive profile (---).

RNA Polymerase Activity—A more direct way to analyze the relationship of the chromatin fractions to the site of the in vivo transcriptional activity would be to assay the fractions for RNA polymerase activity. Such an experiment has been reported by Doenecke and McCarthy (40). Accordingly, chromatin was prepared as described except that 1 mM dithiothreitol was added to all buffers. Nuclei and chromatin were assayed for RNA polymerase activity. Although the nuclei displayed significant levels of α-amanitin-sensitive RNA polymerase and some α-amanitin-insensitive activity, the activity in the sheared unfractionated chromatin was approximately 1% that of the nuclear activity. The omission of Na,EDTA in the wash buffers did not enhance the activity significantly nor did the addition of exonuclease DNA. No attempt was made to distinguish RNA polymerase III activity (sensitive to high levels of α-amanitin) from RNA polymerase I activity (insensitive to α-amanitin). When the chromatin was fractionated and the fractions assayed, no activity could be detected in any of the chromatin regions in the gradient. Since a single wash of the nuclei significantly reduced activity (40%), it is likely that the lack of polymerase activity from the chromatin is a consequence of the extensive washing. Additionally, the polymerase activity is sensitive to shearing (41).

Localization of Satellite DNA-In order to test the relationship between the in vivo transcriptional activity and the chromatin fractions, the latter were analyzed for the presence of satellite DNA. Mouse satellite DNA is rarely if ever transcribed (49). If the method fractionated chromatin on the basis of the in vivo transcriptional activity, then the presence of this DNA species in a chromatin fraction(s) would strongly suggest that this chromatin had not been transcriptionally active. DNA was extracted from the chromatin fractions from LA9 interphase cells. For comparison DNA from unfractionated chromatin was prepared. The DNA samples were analyzed in CsCl gradients by equilibrium centrifugation. The results are shown in Fig. 10. In all samples the main band of DNA appears as a broad peak with a well defined shoulder. The shoulder peak is at a density of 1.681 g/ml, the known density of mouse satellite DNA (42). The lack of clear resolution between the main band and satellite DNA is probably due to the low molecular weight of the DNA. Nevertheless it is apparent that satellite DNA is found in all fractions and in comparable amount relative to the main band.

DISCUSSION

The chromatin fractionation procedure described in this paper yields four partially resolved fractions which appear as a series of overlapping peaks when the sheared chromatin is centrifuged on a sucrose gradient. The stability of the chromatin profile over a range of shearing times suggests that the chromatin fractions are discrete structures and do not arise as the result of incomplete shearing of the chromatin. The characteristics of the chromatin fractions indicate that the fractionation is most likely based on structural differences in nucleosome-containing fibers other than length of DNA. For example, interstrand or intrastrand associations could yield chromatin particles that would be different in sedimentation constant and perhaps melting temperature, but still be similar in DNA length, protein:DNA ratio, etc. The relationship of such structures to the structure of chromatin in vivo is unclear.

To test whether the chromatin fractions represent structures found in vivo, rather than artifacts of washing and shearing, the procedure was extended to erythrocyte and mitotic chromatin. They are well known to differ substantially from interphase chromatin in degree of condensation and compaction of structure. Like the interphase chromatin, they contain nucleosomes (43, 44), and therefore, the structural differences between the chromatin would appear to be in the higher order organization of the fibers of nucleosomes. It seemed possible that the manipulation, shearing, and solubilization of the chromatin, which constitute the fractionation procedure, would destroy the higher order chromatin organi-
The melting analysis in Fig. 4 (9). Such DNA could provide binding sites generated during the fractionation procedure and that the gradient profiles reflect endogenous differences in chromatin structure.

The major interest in the field of chromatin fractionation is to relate the fractionated chromatin structures to the chromatin templates that were transcriptionally active in vivo. Although the criterion of nascent RNA localization at first appeared to be a reliable index of in vivo transcriptional activity, the results shown in Figs. 8 and 9 suggest that such an interpretation of the labeling pattern is subject to challenge. The data from the mixing experiment (Fig. 9) suggest that the appearance of the radioactivity in the A and B fractions may represent a capacity of those chromatins to bind RNA, rather than representing the site of in vivo transcription. Since the interaction of RNA with chromatin in vivo is likely to be more intimate than can be the case in these mixing experiments, these are conservative control experiments. The extensive washing of the chromatin does not remove the associated RNA. When labeled RNA was added to unlabeled nuclei, 10% of the label survived the chromatin isolation and wash procedure, and sedimented with the A and B chromatin fractions. These results, together with the observations of Monahan and Hall (38), challenge the validity of the “nascent RNA” criterion as a means of identifying a chromatin fraction as having been transcriptionally active in vivo. There is no way to eliminate the possibility that transcription in vivo took place in chromatin templates that are found in one or all chromatin fractions and that the work-up and centrifugation of the chromatin results in the relocation of the once nascent RNA to the A and B regions. While it is not possible to rule out the presence of nascent RNA in the A-B regions, its presence cannot be convincingly demonstrated by this approach.

Although the requirements for a stable hybrid of nascent RNA and DNA template have not been ascertained for eukaryotic chromatin, the situation has been analyzed in detail for the transcription of phage DNAs by Escherichia coli polymerase (45), and the transcription of superhelical viral DNA (46). The conclusion of these studies, based on the size of the RNA-DNA hybrid during transcription and the stability of RNA-DNA hybrids relative to DNA-RNA hybrids is that the presence of the RNA polymerase is required to retain the nascent RNA on the DNA. If a similar situation exists in the eukaryotic systems then the failure of our chromatin fractions to display measurable RNA polymerase activity is another demonstration of the difficulty of interpreting the data from the nascent RNA localization experiments. Doenecke and McCarthy have prepared chromatin with measurable levels of endogenous RNA polymerase activity (40). Chromatin with endogenous RNA polymerase was fractionated and a peak of activity was found in the more slowly sedimenting chromatin, although some activity was found throughout the gradient. However, as noted by these authors, the problem of artificial binding sites generated during the fractionation procedure renders these data inconclusive with respect to the in vivo-in vitro relationships of the fractions. The presence in the slowly sedimenting fraction of an appreciable amount of free DNA, or DNA loosely formed in a complex with protein, is indicated by the melting analysis in Fig. 4 (9). Such DNA could provide initiation sites for RNA polymerase in vitro.

A more reliable approach to the problem of the relevance of the chromatin fractions to transcription is that of the satellite DNA localization experiment. Our results show that the mouse satellite is present in comparable amounts in all fractions. Since the satellite DNA is not transcribed and since, quite obviously, some chromatin must be transcribed in vivo, the results indicate that the fractionation procedure does not fractionate primarily on the basis of the in vivo transcriptional activity. Howk et al. (13), and Krieg and Wells (14), have reached a similar conclusion in their study of both transcribed and nontranscribed DNA sequences in chromatin fractions prepared as described by Murphy et al. (11), Reec et al. (7), and Janowsky et al. (47).

Many of the physical characteristics of the “active chromatin” fractions prepared by several groups (7-9, 11, 35, 48) are consistent with the notion that this chromatin was transcriptionally active in vivo, but the properties of this chromatin are also consistent with those of damaged (nicked, degraded etc.) chromatin fibers. No direct, unambiguous evidence has been obtained to support the claims for successful fractionation. Moreover, our results (particularly with satellite DNA) and those of Howk et al. (13), Lewis et al. (49), Varshavsky et al. (60), and Krieg and Wells (14), strongly suggest that those fractionation techniques are inappropriate for most studies of questions about the regulation of transcription in vivo.

Irrespective of the distribution of any particular DNA sequence, higher orders of chromatin structure might be too delicate to withstand the manipulations that are a part of all published fractionation procedures, and a rearrangement in one of the higher orders of chromatin structure might produce various states of aggregation, differing in sedimentation properties. Lewis et al. (49) have demonstrated striking changes in the physical properties of chromatin exposed to even mild shearing. These included changes in the solubility and viscosity of the chromatin. They found that chromatin prepared so as to be soluble in 0.15 M buffer aggregated and precipitated when sheared. Their results suggest that the operations of the fractionation protocol may generate chromatin aggregates. Recently, Varshavsky et al. discussed the possible role of divalent cations in chromatin fractionation in light of the sedimentation of chromatin exposed to low concentrations of Mg++. They suggested that the cations might affect the association of chromatin fragments leading to aggregates with differing sedimentation properties; such aggregation might even explain differences in melting temperatures and variability of gradient profiles such as observed by us and by others. Whatever the mechanism of aggregation, it is plausible that the distribution of sheared chromatin that we have observed in sucrose gradients is an expression of different extents of aggregation. The A region chromatin, for example, might contain relatively nonaggregated chromatin fibers while the D region material would consist of chromatin aggregates of various size. The amount of DNA in a given region would reflect the probability of formation of an aggregate of that S value, but the actual DNA sequence would not influence the aggregation process and thus all DNA sequences would appear in all chromatin fractions. If aggregation is a major factor in determining the distribution of chromatin fragments in sucrose density centrifugation, it is interesting that it is affected by endogenous factor(s) specific to cell type, mitotic stage, etc. that are not directly related to transcription. The present procedures might be useful in the identification of such factors, and perhaps even particular structural features of chromatin that are not concerned directly with gene expression.
This prospect seems especially promising in the case of mitotic chromosomes and erythrocyte chromatin which may be protected from physical damage by their highly condensed state.

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