Chromatin: Its Isolation from Cultured Mammalian Cells with Particular Reference to Contamination by Nuclear Ribonucleoprotein Particles†

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ABSTRACT: A new approach to isolating chromatin from cultured mammalian cells is described. Clean nuclei are disrupted by sonication and the nucleoli are removed by brief centrifugation in 30% sucrose. The postnucleolar supernatant is then recentrifuged to separate chromatin from smaller ribonucleoprotein particles, especially those containing heterogeneous nuclear RNA. The final chromatin preparation is enriched fourfold in DNA relative to RNA, as compared to nuclei, and contains less than 2.0% of the nuclear phospholipid (as choline). The chromatin yield is approximately 70%. Chromatin isolated from HeLa cells by this method had only half as much RNA as conventional preparations and contained correspondingly less HnRNA-associated proteins. Fractionation of mechanically disrupted nuclei by differential centrifugation may be a useful complement to chromatin isolation methods involving the extraction of *intact* nuclei, particularly in cases where contamination by heterogeneous nuclear RNA associated proteins is to be minimized.

Chromosomes of eukaryotic cells are fibrillar complexes of DNA, histone and nonhistone proteins, and some RNA. Because chromosomes exist as cytologically defined structures during only a brief portion of the cell cycle (mitosis), it has been necessary to isolate presumably equivalent material ("chromatin") from the interphase nucleus. In the procedures developed so far, *intact* nuclei are extracted in media of low ionic strength (Bonner *et al.*, 1968a). While these methods have high yields (as DNA), the question of whether other nuclear constituents are also extracted has been ignored. Methods have not been developed to isolate chromatin from mechanically disrupted nuclei on the basis of intensive properties such as its size or density. We here describe a method of chromatin isolation based upon the fractionation, by size, of the major constituents of disrupted HeLa cell nuclei. We have been particularly concerned with minimizing contamination by ribonucleoprotein particles containing heterogeneous nuclear RNA.

Materials and Methods

Cells. HeLa cells (S_3 strain) and mouse L cells were grown between 2 and 4 $\times$ 10^5/ml in suspension culture at 37° in Joklik-modified Eagle's minimum essential medium (Eagle, 1959), supplemented with 3.5% each of calf and fetal calf serum. All cultures used in these experiments were free of mycoplasma (assayed microbiologically by HEM Research Inc., Rockville, Md). Cells were harvested by low-speed centrifugation (600 *g*, 3 min), and were washed twice in cold Earle's balanced salt solution (Earle, 1943).

Isolation of Nuclei. All procedures were carried out at 4°, unless noted otherwise. The washed cell pellet was suspended in 10 vol of reticulocyte standard buffer, hereafter termed RSB[1] (0.01 M NaCl–1.5 mM $MgCl_2$–0.01 M Tris-HCl, pH 7.0). The suspension was allowed to stand for 10 min and then disrupted with a tight-fitting Dounce homogenizer. Usually ten strokes were sufficient to give over 95% breakage as monitored by phase-contrast microscopy. The nuclei were pelleted by centrifugation at 1000*g* for 3 min and washed three times in 10 vol of RSB. At this stage, the nuclei were relatively free of microscopically visible cytoplasmic debris and had an average protein:DNA mass ratio of 3.0, and a RNA:DNA ratio of 0.20.

Isolation of Chromatin. The clean nuclei were suspended at 4 $\times$ 10^7/ml in RSB and disrupted by brief sonication (total of 1 min in 15-sec pulses at 40 W; Model W185, Heat Systems Ultrasonics, Plainview, N. Y.). Breakage was routinely 99% or more, as determined by phase-contrast microscopy. The sonicate was then layered over 30% sucrose in NaCl-Tris[2] (0.01 M NaCl–0.0025 M Tris-HCl, pH 7.2) and centrifuged at 4500*g* for 15 min (5000 rpm, Beckman Spinco SW-27 rotor); this pelleted most of the nucleoli, but only 2–8% of the chromatin (DNA). We wish to emphasize the importance of sonicating the nuclear suspension at 4 $\times$ 10^7 nuclei/ml or less, which results in free, intact nucleoli and finely diffuse chromatin. When more concentrated nuclear suspensions were sonicated, the nucleoli tended to aggregate and trap significant amounts of chromatin; in these instances, 15–25% of the chromatin (DNA) was lost as a result of cosedimentation with the nucleoli in the 4500*g* spin. The material that remained on top of 30% sucrose was then layered in 10.0-ml aliquots over 27.0 ml of 60% sucrose in NaCl–EDTA–Tris buffer (0.01 M NaCl–0.024 M EDTA–0.0025 M Tris-HCl, pH 7.2) in 1 $\times$ 3.5 in. nitrocellulose tubes. The upper two-thirds in each was then gently stirred and the tubes were centrifuged at 131,000*g* for 90 min in the SW-27 rotor. The resulting pellet was resuspended in 0.5–1.0 ml of NaCl-Tris buffer[2] and dialyzed overnight against NaCl-Tris[2] to remove sucrose.

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[1] RSB, 0.01 M NaCl–1.5 mM $MgCl_2$–0.01 M Tris-HCl (pH 7.0) (reticulocyte standard buffer); HnRNP, heterogeneous nuclear ribonucleoprotein.

[2] We have subsequently replaced this buffer with RSB, with equivalent results.

Preparation of Histones and Nonhistone Chromosomal Proteins. Histones were extracted from the dialyzed chromatin by adding H_2SO_4 to a concentration of 0.4 N and allowing the sample to stand for 30 min (4°). After centrifugation (37,000*g*, 15 min), the supernatant was aspirated and the pellet resuspended in 0.4 N H_2SO_4 and extracted again as above. The supernatants were pooled and made 1.0% in sodium dodecyl sulfate and 1% in 2-mercaptoethanol. The acid-extracted chromatin was then resuspended in NaCl-Tris, made 1% in sodium dodecyl sulfate and 1% in mercaptoethanol, and dissociated by incubation at 37° for 60 min with frequent stirring. The histone and nonhistone fractions were then dialyzed overnight against 0.1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, and 0.01 M sodium phosphate buffer, pH 7.0 (20°), for subsequent chemical analysis and gel electrophoresis. The histone and nonhistone protein fractions prepared in this fashion were free of insoluble material (no visible pellet after centrifugation at 100,000*g* for 2 hr).

Polyacrylamide Gel Electrophoresis. Initially, polyacrylamide gel electrophoresis of the nonhistone chromosomal proteins revealed significant "trapping" of material at the gel origin, resulting in poor resolution. The addition of 5 mM EDTA to both the gel polymerizing solution and electrode buffer, along with the use of a spacer gel, eliminated this problem.

Sodium dodecyl sulfate gel electrophoresis was carried out as detailed by Maizel (1971). Cylindrical gels (6 × 75 mm) were cast with the following final composition: 7.5% acrylamide, 0.28% *N,N'*-methylenebisacrylamide, 0.1% sodium dodecyl sulfate, 0.1 M sodium phosphate buffer (pH 7.0), 0.5 M urea, 5 mM EDTA, 0.05% *N,N,N',N'*-tetramethylenediamine, 0.1% ammonium persulfate. The gels were then overlaid with 0.5 ml of a solution containing 0.1% sodium dodecyl sulfate, 0.005% tetramethylenediamine, and 0.1% ammonium persulfate, until polymerized (approximately 45 min at 20°). The overlay was then decanted and a 20-mm long spacer gel was cast of the same composition, but with 2.5% acrylamide and 0.01 M sodium phosphate buffer at pH 6.0. The spacer gel was overlaid with water until polymerized. The stock solution of acrylamide and *N,N'*-methylenebisacrylamide (30%:1.12% w/v, respectively) was deionized before use with a mixed-bed ion-exchange resin (Bio-Rad Laboratories) until the solution had a conductivity of 10 μmhos/cm or less. Protein samples (generally 15 μg of histones, 30 μg of nonhistones, and 45 μg of total chromatin) in 15–50-μl volumes were made 0.25 M in sucrose and 0.1% in Bromophenol Blue just before loading. The electrode buffer was 0.1% sodium dodecyl sulfate, 0.1 M sodium phosphate, pH 7.0, and 5 mM EDTA. Electrophoresis was conducted at 8.0 mA/gel until the dye reached the last 50 mm of the lower gel (6.5–7 hr at 20°). Molecular weights were estimated by the procedure of Shapiro *et al.* (1967), with the marker proteins used previously (Bhorjee and Pederson, 1972).

Gels were stained with 0.05% Coomassie Brilliant Blue by the method of Fairbanks *et al.* (1971). Densitometry of stained gels was done at 550 mμ with a Beckman Model DU spectrophotometer equipped with a Gilford linear gel transport and strip-chart recorder.

Chemical Analyses. DNA was assayed by the diphenylamine method (Dische, 1955) using calf thymus DNA as a standard. Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard. RNA was separated from DNA by alkaline hydrolysis as described by Fleck and Munro (1962), and determined by the orcinol reaction (Dische, 1955), using yeast RNA as standard.

TABLE I: Chemical Analysis of Fractions.[a]

Fraction	% of Nuclear DNA	RNA: DNA	Protein: DNA
Nuclei	100	0.19	2.9
30% sucrose top	84	0.18	2.7
30% sucrose pellet (nucleoli)	5	0.70	6.7
60% sucrose pellet (chromatin)	73	0.05	1.8

[a] HeLa cells (2000 ml) at 3 × 10^5/ml were harvested and homogenized as described under Materials and Methods. Aliquots of the washed nuclei in RSB were taken for chemical analysis. After centrifugation of the nuclear sonicate on 30% sucrose, a portion of the nonsedimentable material was dialyzed against RSB for 4 hr to remove sucrose. The pellet was resuspended in RSB and also dialyzed. The remainder of the material above 30% sucrose was recentrifuged on 60% sucrose as detailed under Materials and Methods; the resulting pellet was resuspended in RSB and dialyzed. Aliquots of each dialysate were then analyzed chemically for DNA, RNA, and protein (see Materials and Methods). The experiment was then repeated on another day; the data given are the means of these two experiments.

Isotopes and Chemicals. L-[^{3}H] Fucose (4.8 Ci/mmol) and [^{14}C]choline (6.2 mCi/mmol) were obtained from New England Nuclear, Boston, Mass. 5-[^{3}H]Uridine (30.8 Ci/mmol), L-[^{3}H]tryptophan (1.8 Ci/mmol), L-[^{14}C]lysine (0.3 Ci/mmol), ultrapure sucrose (ribonuclease free), ultrapure urea, and Coomassie Brilliant Blue were purchased from Schwarz-Mann, Orangeburg, N. Y. Acrylamide and *N,N'*-methylenebisacrylamide were obtained from Eastman Organic Chemicals, Rochester, N. Y.

Results

The chromatin isolation procedure used here consists of two steps: (1) removal of nucleoli from sonicated nuclei as described previously (Maggio *et al.*, 1963; Muramatsu *et al.*, 1963; Schildkraut and Maio, 1968), and (2) recovery of chromatin from the postnucleolar fraction by high-speed centrifugation, leaving ribonucleoprotein particles and nuclear membrane fragments in the supernatant. To assess this, chemical measurements of DNA, RNA, and protein were performed at each stage of the procedure (Table I). As isolated here, HeLa nuclei have a RNA:DNA mass ratio of 0.19 and a protein:DNA ratio of 2.9. Centrifugation of the nuclear sonicate on 30% sucrose (4500*g*, 15 min) resulted in a pellet of microscopically definable nucleoli. Compared to intact nuclei, this nucleolar pellet was enriched threefold in RNA, relative to DNA, and over twofold in protein. Conversely, material remaining on top of the sucrose retained the RNA:DNA and protein:DNA ratios characteristic of intact nuclei, and contained approximately 85% of the initial nuclear DNA. The amount of DNA in the nucleolar pellet (about 5%) is similar to values obtained in other cell types and to that found in an earlier study with HeLa nucleoli (McConkey and Hopkins, 1964); it probably consists of the ribosomal RNA cistrons and nucleolar associated constitutive hetero-

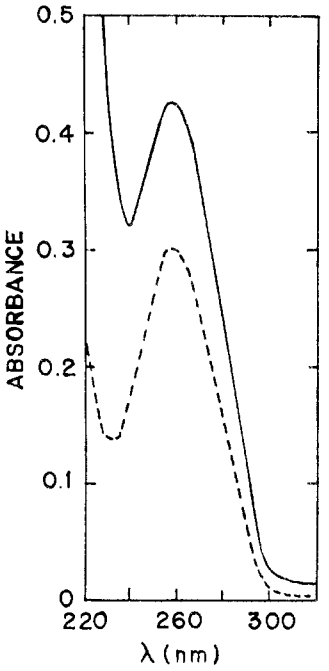

FIGURE 1: Absorption spectra of HeLa chromatin and DNA. The DNA was prepared from chromatin by the sodium dodecyl sulfate–Pronase method (Schildkraut and Maio, 1968). The solvent was 0.01 M NaCl–0.025 M Tris-HCl, pH 7.0: (—) chromatin; (- - -) DNA.

chromatin. Clearly, very little bulk chromatin is lost with the nucleoli.

When the material remaining above 30% sucrose was centrifuged through 60% sucrose (131,000*g*, 90 min), an ivory-colored pellet was obtained which had approximately 73% of the initial nuclear DNA (87% of that layered on 60% sucrose) and which was enriched nearly fourfold in DNA relative to RNA (Table I). The protein:DNA ratio of the pellet (1.8) was similar to that of conventional chromatin preparations. As defined by solubility in 0.4 N H_2SO_4, approximately two-thirds of this protein is histone (Table II). The relative proportions of histones, nonhistone protein, and RNA, per unit DNA, are in general agreement with values for chromatin prepared by other techniques from several tissues (Dingman and Sporn, 1964; Bonner *et al.*, 1968b). However, the RNA:DNA ratio of 0.05 is almost 50% less than that obtained for HeLa chromatin prepared by extraction of intact nuclei in media of low ionic strength, pH 8.0 (*e.g.*, Sadgopal and Bonner, 1970). We shall return to this point later.

TABLE II: Chemical Composition of HeLa Chromatin.[a]

μg/μg of DNA			
Total Protein	Histone	Nonhistone	RNA
1.80 ± 0.05	1.08 ± 0.02	0.70 ± 0.05	0.05 ± 0.005

[a] The 60% sucrose pellet (see Materials and Methods) was dialyzed against RSB to remove sucrose. Three aliquots were then taken for determination of DNA, RNA, and protein. The remaining chromatin was then extracted in 0.4 N H_2SO_4 as detailed in the text; an aliquot of the acid-soluble fraction (histones) was then analyzed for protein. The acid-extracted chromatin (DNA and nonhistone protein) was then analyzed for DNA and protein. This analysis was repeated on two other occasions with freshly prepared chromatin. The values given in the table are the means and standard errors for these three separate groups of determinations.

TABLE III: Assessment of Membrane Contamination.[a]

Fraction	Cl_3CCOOH Insoluble Radioactivity in Total Fraction	
	L-[^{3}H]-Fucose (cpm)	[^{14}C]-Choline[b] (cpm)
Cytoplasm	4,080,000	1,730,000
Nuclei	952,000	550,000
30% sucrose top	681,000	261,000
30% sucrose pellet (nucleoli)	15,300	15,800
60% sucrose pellet (chromatin)	20,400	7,600
% in chromatin rel to nuclei[c]	2.8	1.7
% in chromatin rel to whole cell[c]	0.5	0.4

[a] HeLa cells (2500 ml) at 2 × 10^5/ml were incubated for 18 hr with L-[^{3}H]fucose, 0.5 μCi/ml, and [^{14}C]choline, 0.025 μCi/ml. The cells were harvested, washed, and homogenized as detailed under Materials and Methods. At each point in the chromatin isolation procedure, replicate aliquots were taken for the determination of ^{3}H and ^{14}C radioactivity by liquid scintillation counting. These were made 10% in Cl_3CCOOH, collected on nitrocellulose filters, and counted in a toluene-based scintillation cocktail (Pederson and Kumar, 1971). The values given are counts per minute at a counting efficiency of 12.8% for ^{3}H and 64% for ^{14}C. [b] 98% of the Cl_3CCOOH-insoluble choline is extractable in acetone (10 min, 20°) or in chloroform–methanol (2:1, 60°, 30 min) (Robbins and Pederson, 1970). [c] Normalized to a chromatin yield of 100%.

The ultraviolet absorption spectra of HeLa chromatin and DNA are illustrated in Figure 1. The chromatin 260/240 and 260/280 ratios of 1.35 and 1.70, respectively, are similar to values for chromatin prepared by other methods (Bonner *et al.*, 1968a; Shaw and Huang, 1970). Since neither nucleic acids nor proteins absorb significantly at 320 mμ, this region provides an internal assessment of light scattering due to aggregation. The 320/260 ratio of 0.03 is lower than that obtained by other isolation methods (usually 0.07–0.10).

We next explored the degree of contamination of this chromatin preparation by nuclear membrane and plasma membrane fragments. Cells were incubated for one generation with L-[^{3}H]fucose and [^{14}C]choline. L-Fucose is incorporated predominantly into plasma membrane glycoproteins (Atkinson and Summers, 1971) while choline is a general lipid label in HeLa cells (Penman, 1965; Robbins and Pederson, 1970). Chromatin was prepared as usual and the amounts of trichloroacetic acid insoluble fucose and choline were monitored throughout the procedure (Table III). After centrifugation at 4500*g*, most of the nuclear fucose-containing material remained on top of the 30% sucrose and only about 1.6% was found in the nucleolar pellet. In the case of choline, 50% remained above the sucrose and only 2.9% was found in the nucleolar pellet. When the material above 30% sucrose (681,000 cpm fucose and 261,000 cpm choline) was centrifuged

TABLE IV: Lysine–Tryptophan Labeling of Chromosomal Proteins.[a]

Electrophoretic Band	cpm of [^{3}H]-Tryptophan:cpm [^{14}C]Lysine[b]
Nonhistone (14,000 daltons)	4.5
Histone III (14,000 daltons)	1.0

[a] HeLa cells (900 ml) (4×10^5 cells/ml) were concentrated 20-fold in Joklik-modified Eagle's MEM minus lysine and tryptophan containing 2% dialyzed calf serum and labeled for 30 min with [^{3}H]tryptophan (5 μCi/ml) and [^{14}C]lysine (0.5 μCi/ml). Chromatin was prepared and the histone and nonhistone proteins were electrophoresed in 12-cm 7.5% gels. After mechanical fractionation (Maizel, 1966), the amount of [^{3}H]tryptophan and [^{14}C]lysine in each gel fraction was determined by double channel liquid scintillation counting. To facilitate selection of the fractions of interest, parallel gels were stained. [b] It is to be noted that these cpm ratios are determined by the amounts and specific activities of the two precursors used and the intracellular amino acid pools. They do not relate in a direct way to the molar abundance of the two amino acids in a given protein.

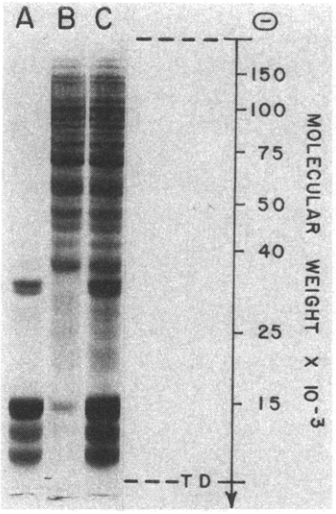

FIGURE 2: Sodium dodecyl sulfate gel electrophoresis of HeLa chromosomal proteins: (A) histones, 15 μg loaded; (B) nonhistone proteins, 30 μg; (C) total chromatin proteins, 50 μg. Direction of electrophoresis was from top (cathode) to bottom (anode). T.D. indicates the tracking dye front. It is to be noted that the apparent molecular weights of histones in sodium dodecyl sulfate gel electrophoresis differ significantly from the values determined by conventional methods.

on 60% sucrose, the chromatin pellet contained only 2% of the nuclear fucose and a similar fraction of the choline. When normalized to a chromatin yield of 100%, this corresponds to 2.8% of the initial nuclear fucose and 1.7% of the choline.

Figure 2 illustrates the sodium dodecyl sulfate–polyacrylamide gel electrophoretic patterns obtained for H_2SO_4-soluble chromatin proteins (histones, gel A), acid-insoluble proteins (B), and total chromatin (C). All of the electrophoretic bands present in total chromatin are found in either the histone or nonhistone fractions; comparative densitometry and Lowry determinations (not shown) revealed that approximately 95% of the chromatin proteins were recovered as either histone or nonhistone bands. Figure 2 also illustrates the absence of Coomassie Blue positive material trapped at the gel face; the first detectable band appears at about 3.5 mm down the gel. We previously found that no DNA, or DNA–protein complexes, entered the gel under these conditions by electrophoresing proteins from chromatin labeled with [^{14}C]thymidine (Bhorjee and Pederson, 1972). We have now also explored the possibility of RNA entering the gel by electrophoresis of chromatin proteins from 5-[^{3}H]uridine-labeled cells. In a typical experiment in which 45–50 μg of protein from total chromatin was loaded, approximately 0.8 μg of RNA was found in the gel, but as a polydisperse zone confined to the first 5 mm. The [^{3}H]uridine radioactivity was not coincident with either of the first two Coomassie Blue bands illustrated in Figures 2B and 2C. We conclude that some RNA may be heterogeneously distributed at the gel starting zone, but that the Coomassie Blue positive bands represent polypeptides which are free of nucleic acid.

Figure 2 also illustrates the high efficiency of histone extraction in 0.4 N H_2SO_4 (pH ~0.1). We chose this instead of 0.25 N HCl since Murray (1969) found that complete histone extraction occurs only at pH 0.6 or lower. However, we consistently noted a faint histone-like band (approximately 14,000 daltons) in gels of the acid-insoluble proteins (Figure 2B). Electrophoresis of these proteins from cells which had been pulse labeled with [^{3}H]tryptophan and [^{14}C]lysine revealed that this 14,000-dalton "nonhistone" protein had a tryptophan:lysine isotope ratio of 4.5, while in a parallel gel from the same cells, the acid-soluble protein of similar mobility (histone III) had a tryptophan:lysine ratio of 1.0 (Table IV). This analysis indicates that little, if any, of the 14,000-dalton protein in the acid-insoluble fraction is histone contamination, despite the similarity of molecular weights. An acid-insoluble protein of similar size has been noted in chromatin from rat liver and kidney (Teng *et al.*, 1971) and sea urchin embryos (Hill *et al.*, 1971).

The data in Table I indicate that the chromatin preparation is enriched at least fourfold in DNA, relative to RNA. The majority of HeLa nuclear RNA is ribosomal (Warner *et al.*, 1966), and it is associated with a specific class of proteins having molecular weights between 10,000 and 55,000 (Vaughan *et al.*, 1967; Warner and Soeiro, 1967; Kumar and Warner, 1972). Figure 3B illustrates that the acid-soluble fraction of intact HeLa nuclei contains, in addition to histones, three proteins in the 10,000–55,000 mol wt range (approximate mol wt 50,000, 36,000, and 22,000). These coelectrophorese with three of the bands seen in authentic ribosomal structural proteins, prepared from 50S cytoplasmic ribosomal subunits (Figure 3A). The relative absence of these bands from the basic proteins of the purified chromatin (Figure 2A) indicates little contamination by either cytoplasmic ribosomes or nuclear ribosomal precursor particles.

To compare the present method of chromatin isolation with existing techniques, we prepared chromatin by the extraction of intact nuclei in distilled water at pH 8.0 (Paoletti and Huang, 1969; Shaw and Huang, 1970). Chromatin prepared by this latter method had a protein:DNA ratio of 2.1 and a RNA:DNA ratio of 0.12. Electrophoresis of the proteins from this preparation (Figure 4B) revealed several differences as compared to the presently described technique (Figure 4A), hereafter termed "sonication method." Relative to the histones, many of the bands above 40,000 daltons were significantly enriched in distilled water-extracted preparation. This is especially apparent in the densitometer tracings (Figure 5).

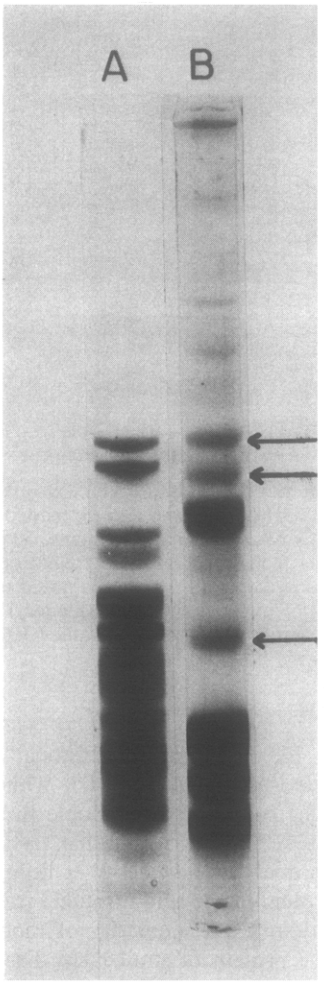

FIGURE 3: Comparison of basic nuclear proteins and ribosomal proteins: (A) proteins from 50S cytoplasmic ribosomal subunits; sample load, 35 μg; (B) proteins extracted from intact HeLa nuclei in 0.4 N H_2SO_4; sample load, 20 μg. Preparation of ribosomes was as follows. Cytoplasmic extracts prepared in RSB were centrifuged on 7–47% linear sucrose gradients containing 0.5 M NaCl and 50 mM $MgCl_2$ (SW-27 rotor, 27,000 rpm, 2.5 hr). The polyribosome region was collected and dialyzed to 0.01 M NaCl; EDTA was then added to a final concentration of 10 mM and the material was centrifuged in 15–30% linear sucrose gradients containing 0.01 M NaCl and 10 mM EDTA (SW-27 rotor, 24,000 rpm, 8 hr). The region containing the 50S A_{260} peak was collected and centrifuged at 30,000 rpm for 18 hr (Spinco type 60 Ti rotor); the pellets were resuspended in 1% sodium dodecyl sulfate–1% 2-mercaptoethanol–0.01 M phosphate buffer and processed for gel electrophoresis as detailed under Materials and Methods. Arrows indicate basic nuclear proteins that comigrate with ribosomal proteins.

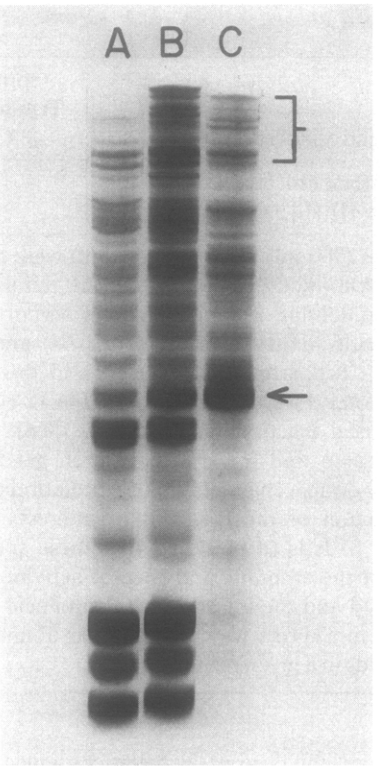

FIGURE 4: Sodium dodecyl sulfate gel electrophoresis of proteins from HeLa chromatin and heterogeneous nuclear ribonucleoprotein particles: (A) total proteins from chromatin prepared by the present method, sample load 45 μg; (B) total protein from chromatin extracted from intact nuclei in H_2O, pH 8.0, sample load 60 μg; (C) proteins from ribonucleoprotein particles containing heterogeneous nuclear RNA, sample load 30 μg. This latter material was prepared as follows. The fraction remaining above 30% sucrose was layered on a 34.0-ml 15–30% linear sucrose gradient (in RSB) and centrifuged at 15,000 rpm for 17 hr (SW-27 rotor). Under these conditions, all of the chromatin is pelleted, but most of the HnRNA containing material remains in the gradient as a polydisperse zone, from 40 to 250 S. These gradient fractions were pooled and diluted with 1 vol of RSB, and the particles were collected by centrifugation at 368,000g for 6 hr. The resulting pellet was resuspended in 1% sodium dodecyl sulfate–1% 2-mercaptoethanol,–0.01 M sodium phosphate, pH 7.0, and prepared for electrophoresis by dialysis against 0.1% sodium dodecyl sulfate–0.1% mercaptoethanol–0.01 M sodium phosphate, pH 7.0.

The most pronounced difference resides in a protein of approximately 40,000 daltons, indicated by the arrows in Figures 4 and 5. Relative to histone I, there is approximately twice as much of this protein in the distilled water preparation. Furthermore, this latter preparation had several additional bands near the gel origin which were absent from chromatin prepared by the sonication method (bracket in Figure 4). Interestingly, both of these differences can be explained on the basis of the proteins which are associated with HeLa heterogeneous nuclear RNA, illustrated in Figure 4C. This material, hereafter termed HnRNP, contains an electrophoretically complex population of proteins, in which a 40,000-dalton species predominates (see legend to Figure 4 for details of HnRNP preparation). The RNA content of the distilled water chromatin preparation was twice that of the other (RNA:DNA = 0.12 and 0.05, respectively); this is precisely the difference in the relative amounts of the 40,000-dalton protein, as shown in Figure 5. In addition, the high molecular weight bands seen in HnRNP (bracket in Figure 4C) are considerably more prominent in the H_2O preparation. There is thus a good correlation between the amount of RNA in these chromatin preparations and their content of proteins which comigrate with those associated with HnRNA.

It was noted above that HeLa nuclei contain three basic (ribosomal) proteins, which are almost completely absent from the chromatin preparation; in other words, there are clearly basic nuclear proteins other than histones. To explore whether a similar situation might exist with respect to acidic proteins, we electrophoresed the acid-insoluble proteins of *intact* HeLa nuclei. The results are illustrated in Figure 6A, along with a representative gel (6B) of the nonhistone proteins from chromatin prepared by sonication. The pattern of the *nuclear*

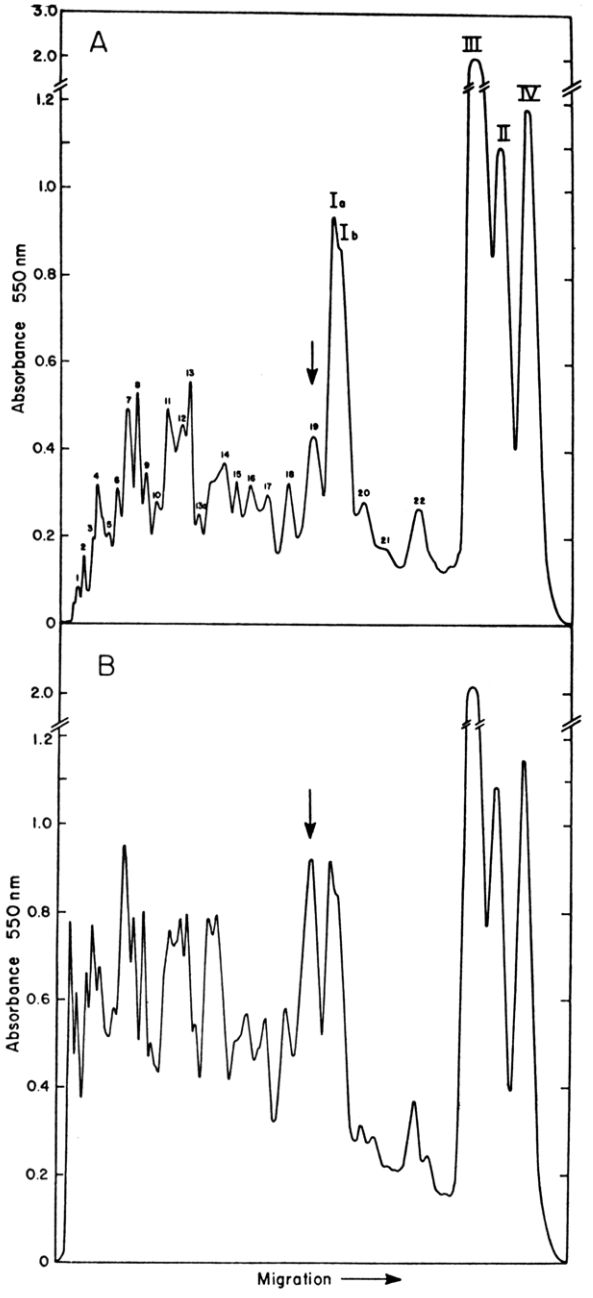

FIGURE 5: Densitometer scans of stained sodium dodecyl sulfate gels: (A) the gel illustrated in Figure 4A; (B) the gel illustrated in Figure 4B (*i.e.*, chromatin extracted from nuclei in H_2O, pH 8.0). Arrows indicate the polypeptide (approximately 40,000) which co-electrophoreses with the major HnRNP protein (Figure 4C). The direction of migration is left to right.

preparation (gel A) is dominated by two major bands at approximately 50,000 and 40,000 daltons. The latter is likely to represent the major HnRNP polypeptide (Figure 4C) since (1) HnRNA is completely retained by acid-extracted HeLa nuclei (Table V), and (2) most of the HnRNP proteins (Figure 4C) have isoelectric points below 7.0 and are insoluble in 0.4 N H_2SO_4 (T. Pederson, manuscript submitted for publication). The mol wt 50,000 protein (Figure 6A) probably represents a component of either the nucleolus or nuclear membrane, since it is found in neither chromatin nor HnRNP. As is the case with basic proteins, the HeLa nucleus obviously contains some acidic proteins which are not found in purified chromatin.

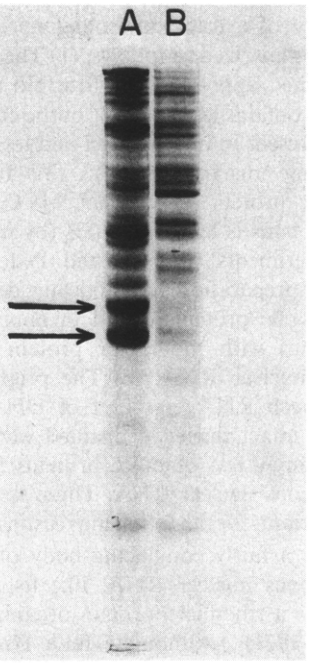

FIGURE 6: Comparison of nuclear and chromosomal acidic proteins: (A) proteins of intact HeLa nuclei insoluble in 0.4 N H_2SO_4; sample load, 50 μg; (B) proteins of purified chromatin insoluble in 0.4 N H_2SO_4; sample load, 30 μg. Arrows indicate approximately 50,000 and 40,000 mol wt.

Discussion

Our purpose in undertaking this work was not to discredit existing techniques for isolating chromatin, but rather to develop an alternative method, based upon fractionation of nuclear constituents by size. With the exception of the possible contamination of conventional chromatin preparations by heterogeneous nuclear RNA-associated proteins (Figures 4 and 5), we do not wish to suggest that the presently described method yields a chromatin preparation that is inherently superior to that obtained by other techniques. In fact, one might be inclined to question the degree to which any of these isolated materials resembles the interphase chromosomes of living cells. Nevertheless, in this study we have used several criteria of purity which have not been applied heretofore, and

TABLE V: Retention of HnRNA by Acid-Extracted HeLa Nuclei.[a]

Fraction	cpm
Nuclei	68,500
Acid soluble	3,200
Acid insoluble	66,800

[a] HeLa cells (200 ml) (2.5×10^5/ml) were incubated for 30 min with a low dose of actinomycin D (0.04 μg/ml), to selectively suppress ribosomal RNA synthesis. [^{3}H]Uridine, 2 μCi/ml, was then added for an additional 30 min. Nuclei were isolated and extracted in 0.4 N H_2SO_4. Aliquots of nuclei, acid-soluble, and acid-insoluble fractions were precipitated with 10% trichloroacetic acid, collected on glass fiber filters, and counted.

we conclude that chromatin prepared by the present method is relatively free of extrachromosomal nuclear constituents. This may be summarized as follows. (1) The chromatin preparation is enriched approximately fourfold in DNA relative to RNA. (2) It contains less than 2% of the choline-containing phospholipid present in intact nuclei and less than 3% of the fucose-containing macromolecules. (3) It contains less ribosomal basic protein than nuclei. (4) Contamination by cytoplasmic proteins is less than 2.0% (by mass), as assessed by mixing experiments (Bhorjee and Pederson, 1972). (5) The chromatin preparation is practically devoid of the two predominant acidic proteins found in *intact* nuclei, one of which comigrates with the major protein associated with heterogeneous nuclear RNA. (6) The preparation contains only half as much RNA, per unit of DNA, as chromatin extracted from intact nuclei in distilled water, pH 8.0, and has correspondingly less of those proteins found associated with heterogeneous nuclear RNA. These last two points are especially important, for the following reasons.

There is now a fairly convincing body of data indicating that heterogeneous nuclear RNA, like its ribosomal RNA counterpart, has a ribonucleoprotein organization (Georgiev and Samarina, 1971). Although HeLa HnRNA-associated proteins are electrophoretically complex (Figure 4C), they are nevertheless readily distinguishable from chromatin proteins in sodium dodecyl sulfate–polyacrylamide gels (Figure 4A–C). To a degree, it is therefore possible to assess contamination of a given chromatin preparation by HnRNA proteins merely by inspection of its sodium dodecyl sulfate–gel electropherogram. This is especially so for the predominant, 40,000-dalton HnRNP protein and some of the very high molecular weight bands near the gel origin (see Figure 4C). Such contamination, when indicated, could arise in one of two ways. First, in methods based upon the extraction of intact nuclei, RNP particles containing fully synthesized HnRNA molecules could be coextracted with chromatin. However, since these HnRNP particles are only 40–250 S (Samarina *et al.*, 1968; Niessing and Sekeris, 1970; Armelin and Marques, 1972), in practice they should be separable from chromatin by a subsequent centrifugation step, which is in fact sometimes done. We refer to this as type I contamination. Second, in some cases HnRNP particles may bind tightly to chromatin and not be removed by subsequent differential centrifugation (type II contamination); this could occur both in methods based upon extraction of intact nuclei as well as the one we have described here. To complicate matters further, electron microscopic studies have suggested that the HnRNA specific proteins combine with nascent RNA transcripts that are still attached to chromatin (Miller and Bakken, 1973). We may thus begin to grasp the subtlety of the distinction between proteins that are truly chromosomal and those that are not. The data presented in Figures 4 and 5 indicate that chromatin prepared by extraction of intact HeLa nuclei in distilled water, pH 8.0, contains considerably more HnRNA specific proteins than that prepared by the sonication method. However, the latter preparation does contain *some* 40,000-dalton protein (Figure 4A). Whether this represents a true chromosomal protein distinct from the one associated with HnRNA, type II contamination, or protein combining with nascent RNA transcripts will only be determined through further work. However, type I contamination of our chromatin preparation is unlikely, since free, extrachromosomal HnRNP particles are only 40–250 S and do not enter 60% sucrose under the conditions of centrifugation we have deliberately chosen.

Contamination of type II, as well as the adventitious binding to chromatin of soluble nuclear proteins in general, may be rather common in methods using low ionic strengths. One advantage of the technique described here is that it does not preclude the use of isotonic buffers. For example, we have recently performed the isolation procedure on both HeLa and mouse L cells using an isotonic buffer throughout (0.15 M NaCl–1.5 mM $MgCl_2$–0.01 M Tris-HCl, pH 7.0), including cell homogenization. All of the electrophoretic bands normally seen were still present, and in the same relative proportions to one another. Of course, working at still higher salt concentrations is not feasible, due to the lability of the chromatin itself.

It should be noted that while contamination of chromatin can be evaluated along the lines we have suggested, the question of whether some chromosomal constituents are *lost* during isolation is far more difficult. For example, in the present technique certain chromosomal components could be sheared off during nuclear sonication.

Nonhistone "chromosomal" proteins have been implicated in the positive control of transcription, particularly in biological transitions thought to involve the reading of new DNA sequences ("gene activation"). To demonstrate such a mechansim beyond reasonable doubt, it is necessary to physically separate the seats of transcription (chromatin) from the primary gene products themselves, which we now know to be RNP complexes, not naked RNA. In the absence of such a separation, an increase in "chromatin" proteins during gene activation is as likely to be a *result* of transcription as it is a causative factor. For this reason, all chromatin isolation methods, including that described here, should receive continued evaluation with respect to contamination by other nuclear materials in general, and by HnRNA-specific proteins in particular.

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Binding of Thyroxine and Thyroxine Analogs to Human Serum Prealbumin†

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ABSTRACT: The interaction of human serum prealbumin with thyroxine and thyroxine analogs was examined by equilibrium dialysis and ultraviolet absorption. Iodothyronines had a single binding site with the following association constants at pH 7.4, 0.1 M NaCl, 25°: L-thyroxine: 1.3×10^8 M^{-1}; 3,5,3′-triiodo-L-thyronine: 1.2×10^7 M^{-1}; 3,5-diiodo-3′,5′-dinitro-L-thyronine: $\geq 10^7$ M^{-1}. Iodotyrosine analogs were studied at pH 8.6, 0.1 M NaCl, 25°, and had the following values: 4-hydroxy-3,5-diiodobenzaldehyde: $n = 2$, $K = 5.5 \times 10^7$ M^{-1}; 3-(4-hydroxy-3,5-diiodophenyl)propionic acid: $n = 1$, $K = 2.5 \times 10^8$ M^{-1}; 4-hydroxy-3,5-diiodocinnamic acid: $n = 1$. The intensities of the circular dichroic bands due to bound ligands were consistent with one binding site for thyroxine and two for 4-hydroxy-3,5-diiodobenzaldehyde. The findings showed that thyroxine is bound more strongly to prealbumin than recently reported, that triiodothyronine is bound somewhat less strongly, and that iodotyrosine analogs are bound with similar affinities to either one or two sites. The spectra of thyroxine and the various analogs showed red shifts in 50% dioxane–water mixtures. When bound to human serum prealbumin they showed blue shifts except for 3,5-diiodo-3′,5′-dinitro-L-thyronine which showed a red shift.

Thyroxine-binding prealbumin is a serum protein with a molecular weight of 54,000 which is composed of four apparently identical subunits (Branch *et al.*, 1971). The conformation of the polypeptide chain has been determined recently by X-ray studies and the amino acid sequence and residue positions should be known soon (Blake *et al.*, 1971; Morgan *et al.*, 1971; Gonzales and Offord, 1971). Prealbumin is of considerable biological interest in that it transports both a hormone and a vitamin in serum. Thyroxine (T_4)[1] is bound directly to prealbumin whereas retinol (vitamin A) is bound by virtue of the binding of its protein carrier, retinol binding protein (Kanai *et al.*, 1968). The binding of hormone and protein is independent and therefore prealbumin must contain five binding sites for these two ligands since it binds 1 mol of thyroxine and 4 of protein (van Jaarsveld *et al.*, 1973a).

The interaction between T_4 and human prealbumin has been studied recently in two laboratories. Raz and Goodman (1969) measured the binding by equilibrium dialysis at pH 7.4 and reported a single site for T_4 with an association constant of 1.6×10^7 M^{-1}. Nilsson and Peterson (1971) using the rate of dialysis method of Colowick and Womack (1969) and the quenching of tryptophanyl fluorescence reported a single, strong binding site at pH 7.4 with $K = 1.1 \times 10^7$ M^{-1}, and three sites of much lower affinity.

In studies seeking to determine the functional groups of T_4 which are responsible for its high free energy of binding to

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[1] The following abbreviations are used: T_4, thyroxine, L form; T_3, triiodothyronine, L form.