A Class of Chromatin Particles Associated with Nonhistone Proteins†

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ABSTRACT: Unfixed nucleoproteins may be banded isopycnically in metrizamide (2-(3-acetamido-5-*N*-methylacetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose) according to the protein/nucleic acid ratio. Unsheared or lightly sheared chromatin bands sharply (ρ = 1.2 g/ml); it has a protein/DNA ratio of 1.4. Chromatin sheared by sonication to approximately 350 base pairs of DNA contains two components with protein/nucleic acid ratios of approximately 1.3 (ρ = 1.185 g/ml) and 2 (ρ = 1.245 g/ml). When chromatin is digested exhaustively with staphylococcal nuclease, two density components are found, one with a protein/DNA ratio of 1.5 (ρ = 1.21 g/ml), the other with a protein/DNA ratio of 2 (ρ = 1.24 g/ml). In both instances the denser particle (ρ = 1.24 g/ml) contains nearly all the nonhistone proteins, while both dense and light fractions contain histones in similar amounts. The base sequence complexity of DNA from the fractions is not distinguishable from that of total DNA and there is no evidence of any concentration of sequences complementary to polysomal polyadenylated RNA molecules.

Since Hewish and Burgoyne (1973) demonstrated that autodigestion of rat liver nuclei yields DNA fragments that are multiples of 200 base pairs, evidence has accumulated for the existence of nucleoprotein subunits in chromatin, referred to as nucleosomes (Rill and Van Holde, 1973; Noll, 1974; Kornberg and Thomas, 1974; Oudet et al., 1975). Kornberg (1974) has postulated that the basic unit consists of an octomer, containing stoichiometric amounts of histones H2A, H2B, H3, and H4, associated with about 200 base pairs of DNA. According to Noll (1974) 80–90% of the DNA in chromatin is associated with these structures. In all the studies so far reported, it has been assumed that nucleosomes are homogeneous, but Rickwood et al. (1973) found that when chromatin is sheared to about 350 base pairs, it can be separated into two buoyant density classes occurring in the ratio of approximately 2.6:1. The protein to DNA ratio in the less dense (major) fraction is about 1.3 and in the denser fraction at least 1.8 (Birnie et al., 1973). The denser peak also sediments with nascent ribonucleoprotein and has rather higher template activity for *E. coli* RNA polymerase. However, there is no difference in the distribution of specific DNA sequences in the two fractions (Rickwood et al., 1974).

In the work described here, the characteristics of these components were further investigated; it will be shown that the nonhistone proteins are associated with a dense particle which may belong to a subclass of nucleosomes.

Materials and Methods

Preparation of Cells. Cultured mouse Friend cells (Friend et al., 1971) (clone M2) were used as a source of chromatin. Proteins were labeled by adding [5-^{3}H]tryptophan (5 Ci/mmol, 1.2 mCi/l.) and [^{14}C]lysine (300 mCi/mmol; 25 μCi/l) (Radiochemical Centre, Amersham, England) to a 4-l. spinner culture of Friend cells at a cell density of 0.5–0.6 × 10^6 cells/ml and were harvested one cell cycle later (after 18 h). DNA was labeled with [2-^{14}C]thymidine (59 mCi/mmol, 0.05 mCi/l.) in the culture medium during 4 days.

Preparation of Chromatin. Nuclei were prepared as described previously (Gilmour et al., 1974). Cells were lysed in ice-cold 1 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.5 mM DTT;[1] 1 M sucrose was added to give a final sucrose concentration of 0.25 M and the nuclei were sedimented at 2000*g* for 10 min. The nuclei were dispersed by homogenization in 2.2 M sucrose, 1 mM $MgCl_2$, 0.1 mM DTT, and centrifuged at 18 000*g* for 60 min at 4 °C. The pellet was suspended in lysis buffer containing 1% Triton X-100 (Lennig Chemicals, Croydon, England), sucrose was added, and the nuclei were pelleted. Chromatin was prepared from nuclei by suspending in ice-cold saline (0.14 M NaCl, 50 mM Tris-HCl, pH 7.6, 5 mM EDTA) and stirring, on ice, for 20 min. The chromatin was pelleted by centrifuging at 2000*g* for 10 min at 4 °C. The extraction was repeated twice and the chromatin finally washed with 50 mM Tris-HCl, 5 mM EDTA, pH 7.6. The chromatin was suspended in water and a clear viscous gel was obtained; it was treated with 0.5 mM phenylmethanesulfonyl fluoride (Sigma Chemical Co. Ltd., St. Louis, Mo.) to inhibit proteolysis.

Chromatin was sheared by sonication using a Dawe-Soniprobe, 3.5 A at power setting 4. Staphylococcal nuclease (Worthington Biochemical Corp., N.J.) was used in the digestion experiments. A standard reaction mixture contained 5 mM Tris-HCl, pH 8.5, 1 mM $CaCl_2$, a chromatin-DNA concentration of 300 μg/ml, and staphylococcal nuclease 0.5 μg/ml. After a suitable time the reaction was quenched by the addition of EDTA to a final concentration of 10 mM.

Buoyant Density Centrifugation in Gradients of Metrizamide (2-(3-acetamido-5-N-methylacetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose). Stock solutions of metrizamide (87%, w/w) (Nyegaard and Co. A/S, Oslo) were prepared by slowly adding 50 g of metrizamide to 35 ml of water with stirring at room temperature (Rickwood et al., 1973). The pH of the solution was adjusted, where necessary, to 7.5 with 0.1 M NaOH. For centrifugation, the chromatin

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[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; cDNA, complementary DNA; DTT, dithiolthreitol; BSA, bovine serum albumin.

solution, or any other solution required, was mixed with stock metrizamide solution, buffered, and initial density was adjusted as required. Portions of 5 ml, overlaid with paraffin, were centrifuged in 10-ml polyallomer tubes in an aluminium 10 × 10 fixed angle rotor. The rotor was allowed to come to rest without braking, the gradients were unloaded by upward displacement with fluorochemical (3M Chemical Division, 3M House, London) and 0.25-ml fractions were collected. The refractive index of each fraction was measured and the density calculated using the relationship $3.350\eta - 3.462$. Standard conditions for the banding of chromatin in metrizamide gradients were, up to 1 mg of chromatin in 1 mM Hepes, 1 mM EDTA, pH 7.0, at a starting concentration of 41% metrizamide, centrifuged for 42 h, 30 000 rpm at 2 °C.

Fractions were analyzed for DNA content by adding 50 μl of BSA solution (10 mg/ml), 0.5 ml of 0.2 M sodium pyrophosphate, pH 7.4, and 5 ml of ice-cold 0.6 M Cl_3CCOOH, centrifuging at 1000g for 10 min at 4 °C and washing twice in 0.6 M Cl_3CCOOH. The pellets were digested in 0.5 ml of 0.3 M NaOH for 1.5 h at 37 °C. 0.4 ml of supernatant was incubated with 0.4 ml of orcinol reagent (1% orcinol, 0.5% $FeCl_3$ in concentrated HCl) at 100 °C for 20 min and the E_{660} read. The remaining solution was reacidified and washed with ice-cold 0.6 M Cl_3CCOOH. The pellets were digested at 90 °C for 20 min in 0.5 ml of 0.6 M Cl_3CCOOH. 0.4 ml of sample was incubated with 0.8 ml of diphenylamine reagent (1 g of diphenylamine dissolved in 98% acetic acid, 2% sulfuric acid) at 100 °C for 10 min and the E_{600} was read immediately after cooling.

Analysis of Proteins by Acrylamide Gel Electrophoresis. Total chromatin proteins were analyzed on one-dimensional polyacrylamide gels containing sodium dodecyl sulfate (Laemmli, 1970). The running gels were made up with Tris-HCl, pH 8.9, 4 M urea, containing 15% acrylamide, while the stacking gels were made up with Tris-HCl, pH 6.7, 4 M urea, containing 2.5% acrylamide. The samples were prepared in 8 M urea, 0.1% sodium dodecyl sulfate, 0.5% β-mercaptoethanol, 0.01 M phosphate, and mixed with 5 μl of 0.05% bromophenol blue. For radioactive samples, a Gilson automatic gel slicer (Villiers le Bel, France) was used to cut 1-mm slices directly into scintillation vials; 0.03 ml of 100 volumes of hydrogen peroxide was added to each vial and they were incubated overnight at 60 °C. Histones were analyzed by a modification of the method of Weintraub et al. (1974). Samples were prepared in 8 M urea containing 62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 5% mercaptoethanol, and 0.001% bromophenol blue. Two-dimensional analysis of proteins was carried out exactly according to the method of MacGillivray and Rickwood (1974).

Preparation and Analysis of Complementary DNA Hybrids. Reverse transcriptase was prepared from avian myeloblastosis virus and cDNA to total polysomal poly(A)-containing RNA, synthesised as described by Getz et al. (1975). DNA was isolated using CsCl gradients (Flamm et al., 1972).

DNA:polysomal cDNA of hybridizations were set up with a DNA:cDNA excess of 10^4:1. 50 μg of DNA was mixed with 4 ng of polysomal cDNA (20 000 cpm) and dissolved in 10 μl of hybridization buffer (0.5 M NaCl, 0.025 M Hepes, 0.01 M EDTA, pH 7.0, 50% formamide (Fluka AG, Fluorochem Ltd., Glossop, Derbyshire, England). Samples (1 μl) were sealed in capillaries and denatured at 70 °C for 5 min. Reannealing was carried out at 43 °C for varying periods of time up to a C_0t of 15 000 moles l^{-1} s. The proportion of cDNA in the hybrid and the extent of DNA reannealing was determined using S1 nuclease (prepared by the method of Sutton, 1971) by measuring the proportion of radioactivity rendered acid soluble after incubation with S1 nuclease for 2 h at 37 °C (Harrison et al., 1974).

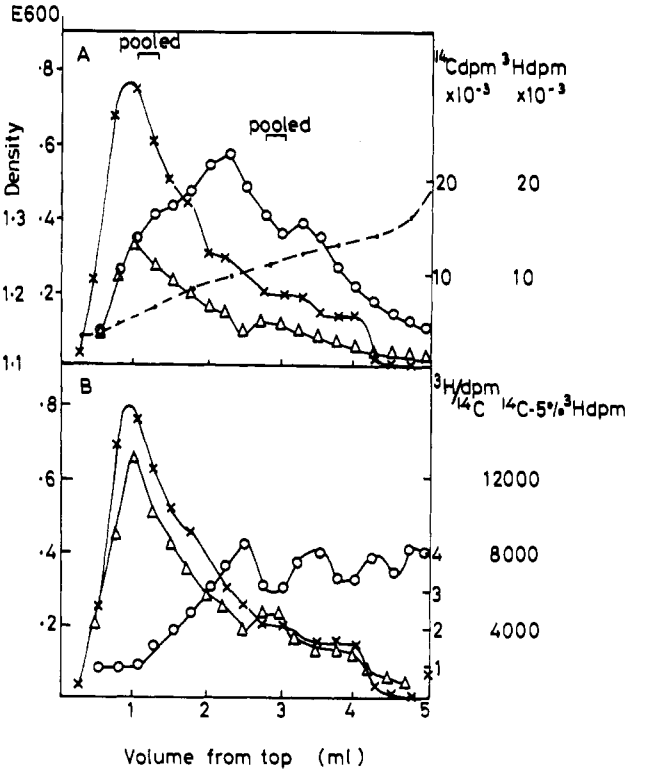


FIGURE 1: Isopycnic banding of Friend cell chromatin labeled with [^{3}H]tryptophan and [^{14}C]lysine. The cells were labeled for 18 h before harvesting and preparing chromatin from them. The chromatin was sonicated for 120 S before fractionation in metrizamide gradients. (A) Distribution of proteins. (×—×), DNA; (○—○), [^{3}H]tryptophan; (△—△), [^{14}C]lysine; (- - -), density. (B) (○—○), ratio of [^{3}H]tryptophan dpm to [^{14}C]lysine dpm; (△—△), estimate of histones ([^{14}C]lysine dpm minus 5% [^{3}H]tryptophan dpm).

Determination of Polylysine Binding to Chromatin Fractions. A constant volume of each chromatin sample (50 μM DNA-phosphate) was mixed with varying volumes of polylysine solution. The polylysine concentration varied from 50–200 μM. Distilled water was added to each sample to give a final volume of 1 ml. The mixtures were shaken for 30 min, centrifuged at 8000g for 15 min, and 0.8 ml of the supernatants was counted. Samples without metrizamide were measured directly by absorbance.

Results

Protein Distribution in Fractions from Mechanically Sheared Chromatin. To obtain an estimate of the distribution of histones and nonhistone proteins in these fractions, a 4 l. spinner culture of Friend cells was labeled with [^{14}C]lysine and [^{3}H]tryptophan for 18 h. Chromatin was isolated from these, sonicated for 120 s, and then centrifuged to equilibrium in a 20–63% (w/v) metrizamide gradient. Two density components are apparent, a major lighter component (1.185 g/ml) and a minor, more diffuse denser component. The results are plotted in Figure 1. In general, the histone distribution (estimated by [^{14}C]lysine dpm, with a suitable correction made for lysine incorporated into nonhistone proteins) follows that of DNA, but there is a very marked enrichment of the higher density fraction in [^{3}H]tryptophan. As there are no tryptophan residues in any of the histones, this implies that the denser particles

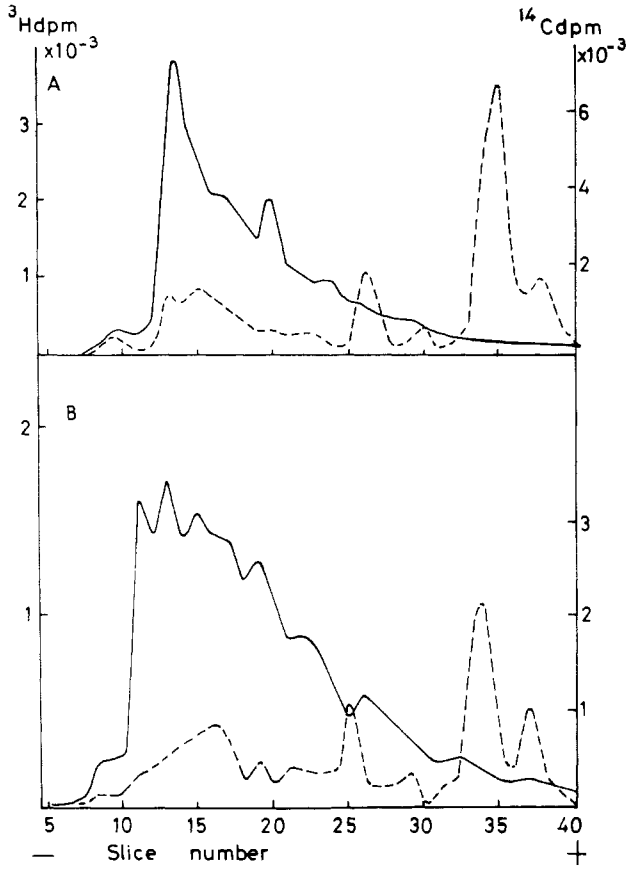


FIGURE 2: Sodium dodecyl sulfate–Polyacrylamide gel electrophoresis of the proteins from the dense and light fractions isolated from isopycnically banded, sonicated chromatin. Friend cells were labeled with [^{3}H]tryptophan and [^{14}C]lysine. (A) Proteins from the lighter peak (ρ = 1.185 g/ml). (B) Proteins from the denser peak (ρ = 1.245 g/ml). (—) [^{3}H]Tryptophan; (- - -) [^{14}C]lysine.

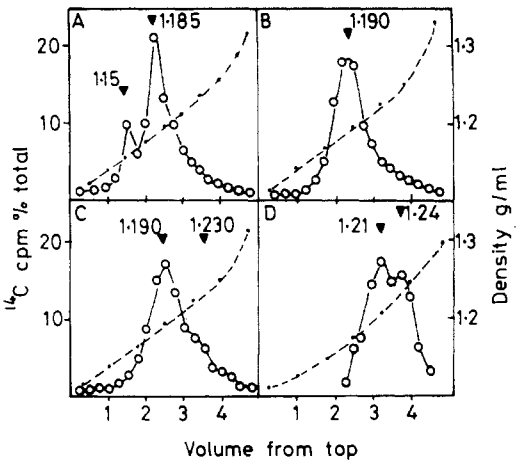


FIGURE 3: Isopycnic banding of chromatin in metrizamide gradients after staphylococcal nuclease digestion. (O—O), [^{14}C]DNA; (· - · -), density; (A) 30 S, (B) 10 min, (C) 30 min, (D) 70 min, nuclease digestion.

are enriched in nonhistone proteins. This conclusion was substantiated by extracting the proteins and subjecting them to electrophoresis in sodium dodecyl sulfate polyacrylamide gels (Figure 2). Lysine is incorporated extensively into histones, represented mainly by the peaks in the low-molecular-weight region of the gel (Slices 25–40), although some lysine is also incorporated into high-molecular-weight (nonhistone) proteins. Tryptophan, on the other hand, appears to be incorporated almost entirely into nonhistone proteins in both fractions.

The histones were isolated from the two fractions and analyzed by gel electrophoresis according to Weintraub et al. (1974). These analyses reveal that histones H2A, H2B, H3, and H4 were present in approximately equal amounts in both fractions; histone H1 was slightly enriched in the light fraction. It is already apparent from Figure 2 that there is a qualitative difference in the non-histone proteins from these fractions but a more detailed analysis was carried out using the two-dimensional system of MacGillivray and Rickwood (1974), which revealed that, not only is the dense fraction quantitatively richer in nonhistone proteins, but there is a more complex mixture of peptides in this fraction which is particularly evident in a group with molecular weights between 30 000 and 80 000, prominent in the dense fraction but absent from the light one. Since all these components could be identified in preparations of nonhistone proteins from total chromatin, they are unlikely to be breakdown products formed in the course of processing. In the light fraction, about 3% of the nucleic acids was estimated to be RNA and in the dense fraction, this was estimated at 12%.

In collaboration with Dr. Itzhaki, polylysine titrations were undertaken of the nucleoprotein in the light and dense fractions and these were compared with total chromatin. In these experiments (not shown), it was found that whereas 44% of the DNA in total chromatin was available for polylysine binding, 40% was available in the light fraction, while 56% was available in the dense fraction. In their polylysine binding studies, Clark and Felsenfeld (1971) suggested that this might be due to alternating covered and uncovered stretches of DNA. In these gradients, there was, in fact, no evidence of free DNA, which would have sedimented at a density of about 1.12 g/ml. On the other hand, it is possible that polylysine binds to very short DNA stretches associated with the protein-containing particles. An alternative possibility is that much of the DNA is free for binding of polylysine. If, as has been postulated, it lies on the outside of nucleosomes, it may well be extensively accessible. Even if the N-terminal regions of the histones fit into one groove of DNA, polylysine might bind in the opposite groove. On any of these interpretations, it has to be assumed that the presence of nonhistone proteins in some way is associated with greater accessibility of DNA. On the other hand, it cannot be excluded that some of the extra binding seen in the dense fraction is simply due to binding of polylysine to the RNA in that fraction or even to nonhistone protein, although work by Itzhaki (Itzhaki and Cooper, 1973) makes this latter interpretation unlikely.

Nuclease-Sheared Chromatin. In these experiments, soluble chromatin was prepared in the standard way from Friend cells except that EDTA was omitted from all solutions. It was subjected to digestion by staphylococcal nuclease for different times and the reaction quenched with EDTA. When DNA was isolated from these digests and electrophoresed in polyacrylamide gels, it formed the typical series of bands described by Hewish and Burgoyne (1973) and Noll (1974). The logarithm of the band number was plotted against the square root of the mobility and a straight line was obtained. This has been taken to imply that the bands are multiples of a common subunit that we estimated, by comparison with cytoplasmic DNA markers (which had previously been sized; Williamson, 1970), to be 135 000.

When chromatin at different stages of digestion is banded in metrizamide, the pattern seen in Figure 3 is obtained. After a very short digestion time (Figure 3A), most of the chromatin has a buoyant density of 1.185 g/ml but a small proportion

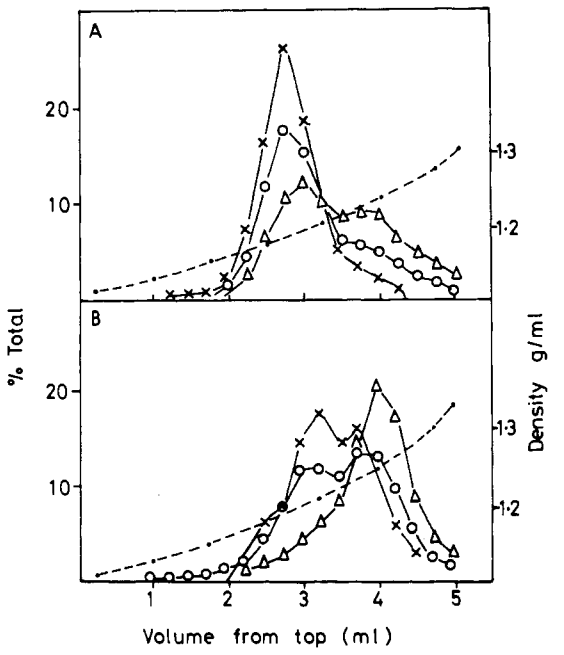


FIGURE 4: Isopycnic banding in metrizamide gradients of chromatin, labeled with [^{3}H]tryptophan and [^{14}C]lysine, after staphylococcal nuclease digestion. (A) No added nuclease. Chromatin sonicated for 5 S. (B) Chromatin digested with nuclease for 70 min. (×—×), DNA; (○—○), [^{14}C]lysine; (△—△), [^{3}H]tryptophan; (· - · -), density.

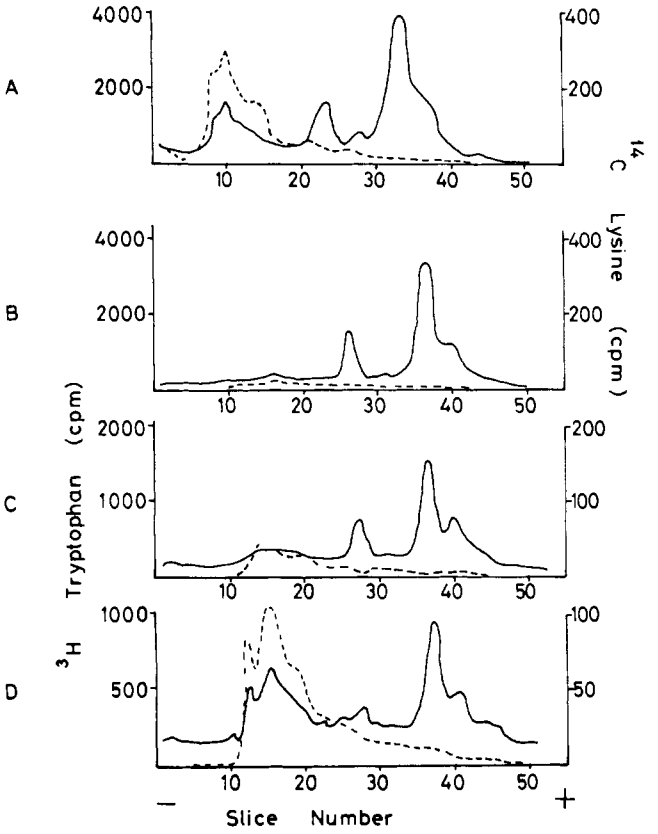


FIGURE 5: Polyacrylamide gel electrophoresis of chromatin proteins after staphylococcal nuclease digestion; (A) Total chromatin proteins. (B) Proteins extracted with 0.4 N HCl. (C) Proteins from nuclease resistant chromatin ρ = 1.21 g/ml. (D) Proteins from nuclease resistant chromatin ρ = 1.245 g/ml. (—) [^{14}C]Lysine; (- - - -) [^{3}H]tryptophan.

bands at a lower density of 1.15 g/ml. This material appears only transiently and is digested away in 10 min; it seems quite likely that it corresponds to the nuclease-sensitive protein-poor component recognized by Gottesfeld et al. (1974). After digestion for 10 min, the main peak has a mean buoyant density of 1.19 g/cm^3 and shows a distinct broadening on the heavy side. After 30 min, this broadening forms a distinct shoulder with a density of approximately 1.23 g/ml. When digestion is continued for 70 min, two distinct subunits emerge, one at 1.21 g/ml and one at 1.24 g/ml. By this stage, approximately 50% of the chromatin has been digested into soluble products that do not appear in the metrizamide fractionation.

Clearly, it was of interest to determine whether these components corresponded to fractions revealed by mechanical shearing of chromatin and, in particular, whether the 1.24 g/ml component revealed by nuclease digestion had properties similar to the denser component revealed by mechanical shearing. Accordingly, chromatin was prepared from Friend cells that had been labeled with [^{14}C]lysine and [^{3}H]tryptophan for 18 h before harvesting. Samples of chromatin were digested for varying times with staphylococcal nuclease and centrifuged to equilibrium in metrizamide gradients as before. A sample which was not treated with nuclease (but was sonicated for a minimum time to obtain dispersed material) and a sample which was digested for 70 min are compared in Figure 4. The undigested material (Figure 4A) in general shows banding of [^{14}C]lysine, [^{3}H]tryptophan, and DNA at a buoyant density of about 1.2 g/ml. Even with this small amount of mechanical shearing, the appearance of a protein-rich shoulder can be discerned. This occurred reproducibly with minimal shearing and may represent another minor component, but it was not investigated further in this study. After exhaustive digestion, by which time approximately half of the DNA and associated proteins had been removed, the two peaks at buoyant densities of about 1.21 and 1.245 g/cm^3 emerged as previously described; it is very apparent that the tryptophan-containing protein is confined to the more dense of these. (It may be noted that this is also relatively richer in lysine-containing proteins than the lighter component.) The nature of the proteins in these components was further studied by isolating samples of the two fractions and analyzing them by electrophoresing in polyacrylamide gels (Figure 5). The fraction with buoyant density of 1.21 g/cm^3 contained almost entirely lysine-containing proteins with only a trace of high-molecular-weight tryptophan-containing material. Moreover, the lysine-containing peaks exhibited the mobilities typical of histones. By contrast, the 1.245 g/cm^3 fraction was much richer in nonhistone proteins, although typical histone peaks were also evident. Further analysis was performed on DNA from the two fractions. First the size of the DNA fragments was determined by isolating DNA from each of the peaks and sedimenting on neutral sucrose gradients. The DNA from the lighter peak (1.21 g/ml) had a peak sedimentation coefficient of 5 S, corresponding to a molecular weight of about 120 000, while the DNA from the denser fraction was of smaller size, with a rather diffuse peak at about 4 S.

Further experiments were undertaken to determine whether the DNA sequences in the nuclease-resistant particles represented subsets or a random representation of the DNA in the total genome. To investigate this, an experiment was performed that combined a measurement of the annealing kinetics of the total DNA of each fraction and of the component of the DNA that is expressed in Friend cells.

Friend cells were first labeled by incubation with [^{14}C]-thymidine. Chromatin from these cells was fractionated and DNA was prepared from: (1) total Friend cell chromatin, (2) the light fraction (1.21 g/ml) from chromatin digested with

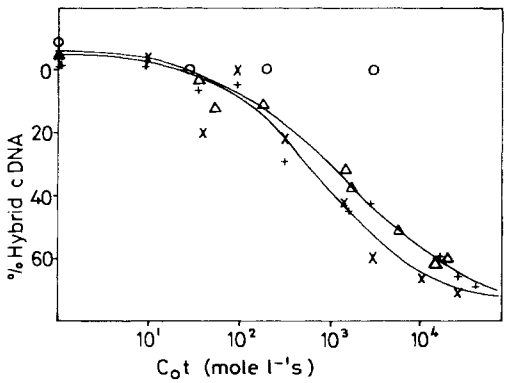


FIGURE 6: Annealing of polysomal cDNA to a vast excess of *E. coli* DNA or DNA prepared from chromatin digested with staphylococcal nuclease. (○) *E. coli* DNA; (+–+) total chromatin DNA; (△–△) chromatin of density 1.21 g/ml (60-min digestion); (×–×) chromatin of density 1.245 g/ml (60-ml digestion).

nuclease for 60 min, and (3) the dense fraction (1.245 g/ml) from chromatin digested with nuclease for 60 min. As described under Materials and Methods, [^{3}H]cDNA was prepared by copying polyadenylated polysomal RNA, from growing Friend cells, with reverse transcriptase. Characterization of Friend cell polysomal cDNA has been published previously (Birnie et al., 1974). Each [^{14}C]DNA fraction was mixed with [^{3}H]cDNA in the ratio 10^4:1. The DNA mixture was then denatured and permitted to reanneal; samples were taken for analysis at intervals up to C_0t 15 000. Both the reannealing of the carrier DNA and annealing of the cDNA probe to the DNA were measured. No significant differences could be detected in the rates of annealing of the [^{14}C]DNA from different fractions. This implies that, within the limits of error of the method, no specific subset of DNA sequences is present in any fraction but that they all correspond to total Friend cell DNA. The annealing of polysomal cDNA to all the fractions behaved very similarly (Figure 6). Since polysomal cDNA provides a probe for those DNA sequences which are being expressed in the cell (presumably mainly in euchromatin), this is strong evidence that these sequences are not enriched in either fraction.

This experiment shows clearly that none of the fractions studied contains a subset of DNA sequences in terms of complexity. It does not rule out the possibility that the nuclease-sensitive sequences represent a subset.

Discussion

The main conclusion that can be drawn from this work is that fragments of nucleoprotein of the size of "nucleosomes" obtained by shearing by sonication or by micrococcal nuclease are heterogeneous. In particular, we have obtained evidence that the bulk of nonhistone proteins are confined to a protein-rich class. The evidence, in fact, suggests there are two distinct protein-rich classes. This is evident in the nuclease-resistant fragments (Figure 3). In the fragments obtained by sonication, the transition is not quite so marked, but from Figure 1 it seems very likely that the same is true. From this figure there is clearly a light fraction that incorporates very little tryptophan and a heavy fraction that incorporates substantial amounts of tryptophan. The transitional region is likely to represent the effect of random shearing rather than a series of transition fragments, although this is also a possible explanation. In the conditions used in these experiments, nonhistone proteins do not band (D. Rickwood, personal communication). On more extended centrifugation, they band at a density of at least 1.28. The bands observed are, therefore, due to nucleoprotein particles and the observed densities agree well with the chemical analyses.

The evidence we present here does not prove that the fragments obtained by the two shearing methods are identical and it must be emphasized that the protein-rich components obtained by sonic shearing contain about 350 base pairs, whereas those obtained by nuclease-shearing contain about half as much DNA. In all other respects, however, their properties are strikingly similar and the possibility has to be entertained that the fragments produced by sonication represent dimers or trimers, while the fragments obtained by nuclease are monomers. Since the components produced by sonication do not appear until shearing has been carried down to a level equivalent to about 2 nucleosomes, the particles which are rich in non-histone proteins must therefore be randomly distributed throughout the chromatin and cannot form large clusters. A problem with all experiments of this kind is the possibility of nonhistone protein exchange. In unpublished results, we have obtained no evidence for aggregation of non-histone proteins per se into particles with a buoyant density as low as 1.24. It cannot be excluded that some displaced nonhistone proteins become associated with the dense fraction, but it seems rather unlikely that all non-histone proteins would behave like this.

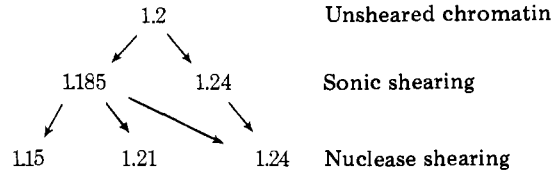
In chromatin from Friend cells, the ratio DNA:histone:non-histone proteins is 1:1:0.5. In the light fragments produced by sonication the ratio is 1:1:0.2 and in the dense fragments 1:1:1.2. From this it can be calculated that whereas 75% of the total histones occur in the light fraction, nearly 70% of the nonhistone proteins occur in the dense fraction. The nature of the nonhistone proteins associated with the dense fraction is itself of interest. As was shown in Figure 2 and in two-dimensional separations, a particular class of intermediate-molecular-weight proteins is found in the dense fraction. The nature of these proteins is a matter for speculation. Some may be associated with ribonucleoprotein particles but, knowing the RNA content of this fraction, it can be calculated that not more than 17% of the nonhistone proteins in the dense fraction can be in ribonucleoprotein. Since nonhistone proteins do not band in these conditions, the rest must presumably be associated with DNA. Another possibility is that some of these non-histone proteins may be membrane-associated proteins. However, the nuclei from which the chromatin was prepared were washed with 1% Triton-X which, according to Tata et al. (1972), strips off the nuclear membrane.

Several authors have proposed that chromatin may contain free DNA (Clark and Felsenfeld, 1971; Varshavsky et al. 1973, 1974) but in our experiments we see no evidence of free DNA and this would be clearly shown in the metrizamide gradients. The evidence that Varshavsky et al. (1973, 1974) obtained was based on buoyant density centrifugation of formaldehyde-fixed chromatin in cesium chloride. Doenecke and McCarthy (1975) have, however, provided evidence that only a minor fraction of nonhistone protein is fixed by formaldehyde treatment and, if stretches of chromatin were associated mainly with nonhistone proteins, these might well be stripped off in high ionic conditions.

Gottesfeld et al. (1975) have obtained evidence for stretches of nucleoproteins that are particularly nuclease sensitive and are associated with nonhistone proteins. In our own nuclease-digestion studies, we observed the initial appearance of a protein-poor component that was rapidly digested by micrococcal nuclease. A possible explanation of these phenomena that would reconcile the different observations is that there is a third component of chromatin consisting of protein-poor

stretches of DNA associated with nonhistone protein-containing protein-rich particles, the whole complex having a buoyant density of about 1.19. On nuclease digestion this might give rise to small particles with a buoyant density of 1.15 (Figure 4) and particles with a buoyant density of 1.24. If this were true, then the fragments obtained by shearing and those obtained by nuclease might be related according to Scheme I.

Scheme I



One of the original aims in undertaking this work was to attempt to fractionate hetero- and euchromatin, since the properties ascribed to these fractions lead to the suggestion that euchromatin might contain more protein than heterochromatin. A clear conclusion from our experiments is that the fractions we obtained by buoyant density centrifugation in metrizamide do not represent euchromatin and heterochromatin. On the contrary, no different classes of nucleoproteins could be distinguished until the nucleoprotein was sheared down to about 350 base pairs, which is considerably smaller than most genes. The two components that then appeared had properties that in some respects resembled those ascribed to hetero- and euchromatin. For example, the light fraction consisted mainly of DNA and histones, while the dense fraction not only contained nonhistone proteins but was associated with nascent RNA, and had a rather higher template activity and greater polylysine binding; moreover, it contained about 25% of the total DNA. It was shown previously (Rickwood et al., 1974) that, both in erythroid and nonerythroid tissue, globin genes are randomly distributed between the two fractions obtained by isopycnic separation of sonicated chromatin fragments. This criterion could be objected to on the grounds that not all globin genes may be active—or inactive—in erythroid and nonerythroid tissue. A more general but more exacting test has been used in the present studies. It has been shown that nuclease-resistant fragments of chromatin have similar properties to the fragments obtained by sonication but that in them too there is no evidence for any difference in DNA complexity or in the concentration of genes giving rise to polysomal messenger RNA in either fraction. Monahan and Hall (1974) carried out similar studies with sonically sheared chromatin and observed no fractionation of satellite DNA in the dense and light chromatin fractions.

Other workers have described particles that probably correspond to those we have characterized. After micrococcal nuclease digestion, Rill and Van Holde (1974) found nucleoprotein fragments with a protein:DNA ratio of 1.8:1 which comprised 25% of the total DNA. Arnold and Young (1974) found that 20–25% of the chromatin from sonicated rat liver remained soluble in 1.5 mM magnesium chloride; this fraction contained more protein and RNA than the total but had a similar histone content. They found that this fraction had a higher polylysine binding, that most of the newly labeled RNA was associated with it, and that the template activity of the soluble fraction was about double that of the insoluble fraction. These authors claimed that they had obtained fractionation into eu- and heterochromatin but the properties of their fractions correspond rather closely to properties of those we describe and clearly cannot represent eu- and heterochromatin in view of the random distribution of gene sequences.

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