Isopycnic centrifugation of sheared L-cell chromatin in metrizamide gradients

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ABSTRACT

Isopycnic gradient centrifugation of L-929 cell chromatin in a 38% (W/V) metrizamide solution yields two distinct fractions. The fraction banding at a density of 1.24 gm/cm² (H chromatin) contains about 10% of the DNA present in the fraction banding at a density of 1.18 gm/cm² (L chromatin). Both fractions contain the same proportions of satellite to main band DNA's. Some differences can be seen in the DNA: protein ratios and types of proteins present in the H and L chromatin fractions.

INTRODUCTION

Metrizamide [2-(3-acetamido-5-N-methylacetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose] dissolves in water to give solutions of high density, low ionic strength and low viscosity.

It is possible to isopycnically band by centrifugation, unfixed ribonucleoproteins, DNA, RNA, proteins, polysaccharides and chromatin in concentrated metrizamide solutions^{1,2}. Because the buoyant densities of these compounds are quite different from each other in metrizamide solutions², isopycnic gradient centrifugation in metrizamide may be a powerful chromatin purification tool. Sheared chromatin (in the few cases that have been examined) appears to separate into two fractions of different buoyant densities after isopycnic gradient centrifugation in metrizamide solutions^{1,2}. Do

these two different fractions represent two different functional components of chromatin such as euchromatin and heterochromatin?

Distinct differences are seen in the protein components of sheared chromatin fractionated by other methods such as chromatography on EDTHAM-cellulose^{4,5} or after sedimentation through sucrose and glycerol gradients⁶. One aim of these chromatin fractionation techniques is to isolate distinct functional components such as euchromatin and heterochromatin. Because clear differences in the content of total RNA and protein are seen in "heavy" and "light" chromatin bands obtained from metrizamide gradients² we felt it would be of interest to analyze the two metrizamide chromatin fractions further to see if these fractions represent two different functional forms of chromatin.

However, purified proteins such as α -casein, urease or catalase also display a bimodal distribution after isopycnic gradient centrifugation in metrizamide solutions^{2,7}. The reason for this is not entirely clear. Rickwood *et al.*⁷ suggest that a reversible interaction of protein with metrizamide gives rise to the second denser protein band. It is important therefore to see if the bimodal distribution of chromatin in such metrizamide gradients actually represents two different chromatin functional fractions. The different content of RNA and the protein in the two mouse liver LS cell chromatin fractions² as well as the work described here would suggest that this is the case.

METHODS

Mouse L-929 cells were originally obtained from Microbiological Associates, Bethesda, Maryland. The line was grown in monolayer culture in roller bottles (700 cms²) at 37°C using a Forma Cel-Rol apparatus at 1/4 R.P.M. in MEM medium (GIBCO). Cells were labeled with ³²P-H₃PO₄ and ³H-thymidine, or ³H-leucine for 8 hours and collected as described previously⁸. Two methods were used to isolate L-cell nuclei.

1. Citric acid procedure: The low pH of the buffers used in this procedure insures an almost quantitative recovery of nuclear DNA with minimal nuclease and protease activity during nuclei isolation^{9,10}. However, the acidity of the buffers used lead to loss of nuclear proteins, particularly F1 histones¹⁰; chromatin isolated from such nuclei is probably not in its native state⁹.

Nuclei were isolated from monolayer culture of 5×10^7 cells with 0.05 M citric acid¹⁰.

2. Detergent procedure: The use of the nonionic detergent Triton X-100 has been shown to greatly facilitate the release of intact nuclei from some cells¹¹ without any major apparent effect on the yield of chromosomal proteins¹². Chromatin isolated from nuclei extracted with this procedure appeared to contain its full complement of histones^{8,12}.

For a typical nuclei preparation with this method, 5×10^7 L-cells were washed in MEM and allowed to swell in 10 mls of RSB buffer (0.01 M NaCl, 0.01 M ACES-NaOH, 1.5 mM MgCl₂, pH 7.0) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 2 units/ml rat liver RNase inhibitor¹³. With rapid mix-

ing 0.5 mls of 10% Triton X-100 was then added and the cell suspension was homogenized in a Dounce glass homogenizer (Kontes, type B) until all cells were broken. The solution was centrifuged at 700 xg for 10 min. and the above homogenization was repeated two further times with 5 mls of buffer. Nuclei prepared by either of the above procedures were washed twice in 0.14 M NaCl, 0.01 M Tris-HCL, 1 mM MgCl₂, 0.1 mM PMSF, 2 units/ml RNase inhibitor, pH 7.6. The washed nuclei were then sonicated in 5 mM EDTA, 1 mM HEPES, 0.01 mM PMSF, 2 units/ml RNase inhibitor, pH 8.3 in a volume of 4 mls. for 2 x 5 seconds at a setting of 2 on a Branson model sl25 sonifier. The supernatant after a low speed centrifugation (600 xg for 10 mins.) was used as our source of chromatin in the experiments to be desired. This material represents at least 95% of the cellular DNA²⁵.

Preparative CsCl isopycnic gradient centrifugation was carried out as described by Flamm *et al.*¹⁴. The histone and nonhistone proteins (NHP's) were analyzed on 7.5% acrylamide gels as described by Bhorjee and Pederson¹⁵. Cylindrical gels 18 cms. long x 0.75 cms. were used. Protein samples (10 - 30 µg) were dissolved in 50 µl of electrode buffer containing a few crystals of urea, 1 µl of 0.05% bromophenol blue, 0.05% trypan red and carefully layered on top of the 2.5% spacer gel. Electrophoresis was conducted at 8.0 mA/gel for 12 hrs. at room temperature. Gels were sliced into 2 mm slices and counted in xylene scintillation fluid¹⁶.

Only the purest available chemicals were used. Metrizamide was obtained from Nyegaard and Co., Oslo, Norway. (4, 5-³H)-leucine 40,000 mCi/mmol, (CH₃-³H) thymidine 50,000

mCi/mmole and $^{32}\text{P-H}_3\text{PO}_4$ (carrier free) were obtained from New England Nuclear, Montreal.

The density of the metrizamide gradient fractions was measured with a 100 μl pipet calibrated with water or from the refractive index².

RESULTS

About 8-10% of mouse L-cell DNA occurs as a satellite band with a buoyant density lighter than that of the main band^{17,23}. It has been shown that the amount of satellite is markedly increased in DNA isolated from heterochromatin¹⁹. If, therefore, there is any fractionation of chromatin in metrizamide into eu- and heterochromatin we might expect to see a difference in the content of satellite DNA in the H and L chromatin fractions.

L-cells (5×10^7 cells) were labeled with $^{32}\text{P-H}_3\text{PO}_4$ (100 mCi/ml) and ^3H thymidine (10 mCi/ml) for 8 hours. Chromatin was isolated as described in methods using the detergent procedure to isolate the nuclei. Figure 1 shows the ^{32}P and ^3H C.P.M. distribution in a 6 ml 38% (w/v) metrizamide gradient after 40 hours centrifugation at 25,000 R.P.M. in an IEC A321 rotor at 5°C . Six drop fractions were collected from the bottom of the tube.

Figure 1: Fractionation of L-cell chromatin labeled with $^{32}\text{P}\text{-H}_3\text{PO}_4$ (●—●) and ^3H -thymidine (Δ — Δ) on a 38% (w/v) Metrizamide isopycnic gradient.

The ^{32}P broad shoulder with a buoyant density of about 1.28 g/cm^2 does not contain any ^3H -thymidine labeled material and appears to consist mainly of ribonucleoprotein particles (RNP's) not removed from the nuclei. [unpublished observations and (1)]. Chromatin bands as two peaks of buoyant densities 1.24 g/cm^2 (H chromatin) and 1.18 g/cm^2 (L chromatin). Approximately 90% of the ^3H -thymidine labeled material is present in the L chromatin fraction.

The H and L chromatin peaks were collected. To each fraction was added 0.2 vols. of 2M sodium acetate (pH 4.5) and 3 vols. of 95% EtOH. The precipitate of chromatin was left overnight at -20°C . After centrifugation, the pellets were deproteinized as described by Marmur¹⁸. Aliquots of the purified DNA were resuspended in 6 mls. of 0.01 M Tris-HCl, 0.002 M EDTA, 0.01% sodium Sarkosyl, pH 7.4. The density was adjusted to 1.7100 with CsCl. Figure 2 shows the

distribution of ^3H -thymidine labeled DNA obtained from the H and L chromatin peaks in the CsCl gradient after centrifugation at 35,000 R.P.M. in an IEC A321 rotor for 64 hours at

Figure 2: Distribution of L-cell mainband and satellite DNA from H (●—●) and L (▲---▲) chromatin fractions in a preparative CsCl density gradient.

15°C . It is clear that the ratio of satellite DNA ($\rho = 1.690 \text{ gm/ml}$) to mainband DNA ($\rho = 1.700 \text{ gm/ml}$) is the same for both H and L chromatin fractions.

It would appear, therefore, that the H and L chromatin fractions obtained from metrizamide do not give rise to a significant enrichment of heterochromatin over euchromatin. We should qualify this statement, however, by saying there is no enrichment of heterochromatin that contains satellite DNA such as pericentromeric heterochromatin²⁴ and perinuclear heterochromatin²⁰. An enrichment of heterochromatin not containing a large proportion of satellite DNA, or the nucleolus itself²⁰ would not be detected in these experiments.

Birnie et al.^{1,2} have shown that there was a large difference in the protein to DNA ratios between H and L chromatin fractions. We have made a similar observation for L-929 cell chromatin. We have analyzed the total protein content (NHP's + histones) of H and L chromatin fractions by polyacrylamide gel electrophoresis. The overall "banding patterns" of the H and L chromosomal proteins are similar but do display some differences, suggesting that they do represent two different chromatin fractions.

About 5×10^7 L-929 cells were labeled with ^3H -leucine (50 mCi/ml) for 8 hours. Half of the nuclei were isolated with the citric acid procedure, the other half with the detergent procedure as described in methods. Figure 3 shows the distribution of ^3H -leucine on the 38% (w/v) metrizamide gradient. There appears to be a difference in the distribution of ^3H labeled protein between the two chromatin fractions

Figure 3: Fractionation of L-cell chromatin labeled with ^3H -leucine on a 38% (w/v) metrizamide isopycnic gradient. (o---o) nuclei isolated with citric acid procedure. (●—●) nuclei isolated with detergent procedure.

for citric acid isolated nuclei compared with chromatin isolated from detergent extracted nuclei. Chromatin from nuclei isolated by the citric acid method has an almost equal amount of ^3H labeled protein in both the H and L chromatin bands. Chromatin from detergent extracted nuclei has more ^3H labeled protein in the H chromatin band compared to the L chromatin band. Since almost 90% of the DNA is in the L chromatin fraction it is clear that the DNA: protein ratios are quite different for the H and L chromatin fractions.

The chromatin from each of these fractions was precipitated with EtOH as described above. The pellets were resuspended in 0.01 M sodium phosphate pH 7.0. This solution was made 1% with mercaptoethanol and SDS and incubated at 60°C for 30 minutes. The ^3H -histone and NHP's were then dialyzed against 0.1% SDS, 0.1% mercaptoethanol, 0.01 M sodium phosphate pH 7.0 and analyzed by electrophoresis on 7.5% acrylamide gels¹⁵. Figures 4a and b show the electrophoretic distribution of ^3H -leucine labeled proteins obtained from chromatin isolated from detergent and citric acid extracted nuclei respectively. It can be seen that for

Figure 4: Polyacrylamide gel electrophoresis of total ^3H -leucine labeled protein from H chromatin (●—●) and L chromatin (●---●) fractions. A. nuclei isolated with citric acid procedure, B. nuclei isolated with detergent procedure.

chromatin from citric acid isolated nuclei the ^3H -leucine profiles are quite similar for the heavy and light chromatin fractions. The positions of ^{14}C labeled L-cell histone fractions isolated from chromatin²¹ with $0.4\text{N H}_2\text{SO}_4$ is shown on each graph. The loss of F_1 histone from chromatin isolated from citric acid isolated nuclei¹⁰ is apparent from a diminished peak in this region of the gel.

Some more distinct differences can be seen between the heavy and light chromatin fractions with chromatin from detergent isolated nuclei. There appears to be less F_1 histone in the light chromatin fraction. Also present in the heavy chromatin fraction is a distinct peak at slice 39. This peak appears to be considerably reduced in the light chromatin fraction. There appears to be some difference in the ratios of NHP's to Histones in the H and L chromatin fractions. The H fraction has a larger amount of NHP's while the L fractions has a larger amount of histones.

DISCUSSION

Metrizamide appears to have considerable potential as a compound for isopycnic gradient centrifugation of chromatin preparations. There appears to be little loss of histone or nonhistone protein components from chromatin during centrifugation. Striking differences in the histone or nonhistone protein components between the H and L chromatin fractions obtained by isopycnic banding of chromatin in 38% W/V metrizamide were not apparent at the present level of resolution. Some minor qualitative differences were seen.

Since only minor qualitative differences in the non-histone proteins between extranucleolar and nucleolar rat liver chromatin preparation have been found²², it may be that many functional forms of chromatin are constructed of the same major protein components and that our analytical techniques at present do not resolve key components present in smaller amounts.

Large differences were seen in the RNA associated with H and L chromatin fractions obtained from metrizamide gradients². This, together with some minor differences seen here in the nonhistone protein components (as well as the content of F₁ histone in chromatin extracted from nuclei isolated by the detergent method) would appear to suggest that the heavy and light chromatin fractions do represent two different chromatin fractions, and that one band is not a "satellite band" of the other as was observed for purified proteins².

ABBREVIATIONS

NHP's, nonhistone proteins; ACES, N-(2-Acetamido)-2-amino-ethanesulfonic acid; HEPES, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PMSF, Phenylmethylsulfonyl fluoride.

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