Complex of DNA with chromatin proteins investigated by isopycnic centrifugation in metrizamide

Joanna Rzeszowska-Wolny, J.Filipski, S.Gröbner and M.Chorazy

Department of Tumor Biology, Institute of Oncology, 44-100 Gliwice, Poland

Received 14 August 1978

ABSTRACT

Complexes of mouse main band DNA with a fraction of non-histone proteins (NHP), having a high affinity for DNA, in the absence or presence of histones have been investigated by gradient centrifugation in metrizamide. Two types of complexes were formed at an input ratio of NHP to DNA between 1 and 2.5. In metrizamide gradients a majority of SNA was found in the light complex (at the density of 1.14 - 1.16 g/cm³) even at the very high NHP to DNA ratio. When histones were present in the reaction mixture, most of the DNA was found in the heavy complex (1.19 - 1.21 g/cm³). The electrophoretic profiles of the proteins recovered from the heavy and light complexes were different; some fractions of nonhistone proteins were present only in the heavy component.

INTRODUCTION

Nonhistone proteins (NHP) - DNA interactions play an important role in the structure and function of chromatin (1,2). Nonhistone proteins take part in regulation of DNA transcription, DNA replication and in various enzymatic processes in the chromatin, and maintain the chromatin and chromosome structure (3-8).

We have studied the complexes of DNA with a fraction of nonhistone proteins having a high affinity for DNA by metrizamide gradient centrifugation. The isopycnic centrifugation in metrizamide enables separation of complexed and uncomplexed components on the basis of the differences in buoyant densities between DNA-protein complex, free DNA, and free proteins (9).
Isolation of DNA

Mouse liver DNA was isolated following the method of Kay et al. (10). Labelled DNA was isolated from mouse kidney cells cultured in Eagle's medium supplemented with 10% calf serum and containing 12 μCi/ml of [3H]-6-thymidine, specific activity 20 Ci/mM, (UVVVR, CSBB), and 10 μg/ml of chlorocyline (Polfa, Warsaw, Poland). Main band and satellite DNA were separated by Cs2SO4-Ag+ density gradient centrifugation following the procedure of Corneo et al. (11). In order to obtain appropriate specific activity, labelled DNA was diluted with nonlabelled DNA. Final activities, measured in the presence of metrizamide, were 2 600 cpm/μg for satellite DNA, and 4 500 cpm/μg for main band DNA. The molecular weight of the DNA was about 9 x 10^6 daltons.

Isolation of histones and nonhistone proteins

Histones and nonhistone proteins were obtained by chromatography of reassociated mouse liver chromatin on hydroxyapatite (12). Mouse liver nuclei prepared according to Widnell and Tata (13) were extracted three times with 0.14 M NaCl, 0.05 M Tris-HCl (pH 7.5). The chromatin was dissociated in 2 M NaCl, 5 M urea, 0.01 M Tris-HCl (pH 8.0) by homogenization in a knife homogenizer and stirred overnight. Undissolved fragments were removed by centrifugation for 1 hour at 27 000 rpm (Spinco, rotor 30). A soluble chromatin fraction was dialyzed overnight against 15 volumes of distilled water to lower the concentration of NaCl to 0.14 M. The precipitated chromatin was obtained by centrifugation for 30 min at 2 500 x g. Nonhistone proteins from reconstituted chromatin (about 20% of all mouse liver nonhistone proteins) were obtained by chromatography on hydroxyapatite. The reconstituted nucleoprotein redissolved in 0.001 M sodium phosphate (NaP) buffer (pH 8.0), 2 M NaCl, 5 M urea was sonicated 4 x 10 sec and layered on the top of the 2 x 40 cm hydroxyapatite column. Histones were eluted with 0.001 M and NHP with 0.05 M sodium phosphate buffer (pH 8.0), 2 M NaCl,
The affinity and the specificity of this nonhistone protein fraction for DNA was determined following the method of Thomas and Patel (14), and the affinity of binding to mouse main band DNA and E. coli DNA was compared.

**Isolation of phosphoproteins**

Phosphoproteins were isolated by the method of Kostraba et al. (15). Mouse liver nuclei were extracted three times with 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.4). The pellet was extracted three times with 0.2 N HCl, followed by extraction with a mixture of chloroform:methanol (1:1, 2:1, v/v) and extraction with ether. The final pellet was suspended in 0.01 M Tris-HCl (pH 7.4), 0.1 M CH₃COOH, 0.14 M β-mercaptoethanol and mixed with phenol (1:1, v/v) saturated with the same buffer. After centrifugation the phenol phase was dialysed for 12 hours against 0.01 M Tris-HCl (pH 7.4), 8.6 M urea and 0.14 M β-mercaptoethanol, then for 24 hours against 2 M NaCl, 5 M urea, 0.05 M sodium acetate buffer (pH 6.0).

**Polyacrylamide gel electrophoresis**

Fractions containing the protein-DNA complex were dialysed against 0.1 % SDS, 0.01 M NaP (pH 7.4), 5.6 mM β-mercaptoethanol and subjected to electrophoresis in 10 % SDS-polyacrylamide gel as described by Weber and Osborn (16). The gels were stained with 0.02 % Coomassie Blue R. After destaining, the absorption along the gels was measured with Joyce-Loebel densitometer. The distribution of [125I] labelled proteins was determined by counting 2 mm gel slices digested in 1 ml of Soluene 350 (Packard Instr. Co., Inc., USA) in a 3380 Packard Spectrometer.

**Labelling of proteins**

1 mC of Na [125I] (Institute for Nuclear Research, Świerk, Poland) in 0.1 ml of 0.05 M NaP buffer was added to 4 ml of protein solution in 0.05 M NaP (pH 8.0), 5 M urea and 2 M NaCl. Subsequently monochloramine B in 6 ml of 0.05 M NaP, 5 M urea was mixed with the protein solution. The mixture was allowed to stand for 2 minutes at room temperature, then
the solution of sodium pyrosulphite was added in 5 ml 0.05 M NaP (pH 8.0), 5 M urea. For each mg of protein 4 mg of monochloramine and 48 mg of pyrosulphite was used. Labelled protein solutions were dialysed exhaustively against 2 M NaCl, 5 M urea, 0.05 M sodium acetate (pH 6.0). The specific activity of histones, measured in the presence of metrizamide was 3 000 cpm/μg. NHP fractions with high affinity for DNA had a specific activity of 2 400 cpm/μg, and phosphoprotein fraction 2 100 cpm/μg.

Preparation of a DNA-protein complex

200 μg DNA and appropriate quantities of proteins in 2 M NaCl, 5 M urea, 0.05 M sodium acetate (pH 6.0) were mixed and adjusted with the same buffer to 1.5 ml, then dialysed against buffers containing decreasing concentration of NaCl: 1, 0.6, 0.4, and 0.15 M NaCl, 5 M urea and 0.05 M sodium acetate (pH 6.0). The last dialysis was against 0.15 M NaCl, 0.05 M sodium acetate (pH 6.0). The recoveries after the final dialysis were 95 to 99 % for DNA and 80 to 85 % for proteins.

Ultracentrifugation in metrizamide

After final dialysis solutions of DNA-protein complex were adjusted to 3.75 ml with 0.15 M NaCl, 0.05 M sodium acetate (pH 6.0), then 1.75 g of metrizamide (Nyeaard and Co. A/S., Oslo, Norway) was added and the samples were centrifuged for 42 hours at 30 000 rpm, 4°C (Spinco, rotor 40). Samples were collected by sucking from the bottom, using a peristaltic pump. The density of each fraction was determined by measuring the refractive index using the relation given by Bickwood and Birnie (17) and correcting the data for the refraction of the buffer. The recoveries from the gradient were 80-85 % for DNA and 80-95 % for proteins.

Determination of radioactivity

20 μl samples from each fraction of the gradient were digested with 0.3 ml of Soluene 350 and radioactivity was counted in a 3380 Packard Spectrometer (A-B channel 50-250 units, A = 4.48 and EF channel 800 - 1 000 units, A = 4.48).
RESULTS AND DISCUSSION

1. Complexes of DNA with nonhistone proteins

In this work we have studied the complexes of nonhistone proteins with main band DNA, formed under conditions similar to those used by Rickwood and McGillvray (18), with the exception that we have reconstituted the complexes at pH 6.0 and that we have worked with a different nonhistone proteins fraction. Our fraction of nonhistone proteins was isolated by the method of Patel and Thomas (12). This fraction had a high affinity for DNA; 70% of its proteins complexed with mouse main band DNA when reconstituted in pH 8.0 following the method of Thomas and Patel (14). However, its complexing specificity was low as it complexed to the same extent with E. coli DNA.

After the stepwise dialysis from 2 M NaCl, 5 M urea, 0.05 M sodium acetate (pH 6.0) to 0.15 M NaCl, 0.05 M sodium acetate (pH 6.0) our NHP fraction formed two kinds of complexes with the mouse main band DNA. Densities of these complexes were similar to those obtained by Rickwood and McGillvray (18). Fig. 1 shows the metrizamide gradient centrifugation profiles of complexes obtained at DNA to protein input ratios of 1:1, 1:1.5, 1:2 and 1:2.5. At all input ratios two types of complexes (light with density of 1.13-1.14 g/cm$^3$ and heavy of 1.20 - 1.22 g/cm$^3$) were seen. With increased NHP to DNA ratio, more proteins were found in the heavy complex (from 19% for DNA:NHP input ratio of 1:1 to 39% for DNA:NHP ratio of 1:2.5). However, in all the cases a majority of DNA was found in the light component. For example at the DNA:NHP input ratio of 1:1 as much as 61% of DNA was found in the light complex, while at DNA:NHP ratio equal to 1:2.5 the amount of DNA decreases to 48%.

It seems that during reconstitution in the first row protein-protein complexing takes place, and subsequently this complex interacts with DNA forming heavy DNA-protein complex in metrizamide gradient. Single protein molecules bound with DNA form the light complex.
Fig. 1. Metrizamide gradient centrifugation profiles of mouse main-band DNA-NHP complexes at (A) 1:1, (B) 1:1.5, (C) 1:2 and (D) 1:2.5 input ratios. (o) [125I] labelled nonhistone proteins, (x) [3H] DNA. MB, main-band DNA. NHP, nonhistone proteins. Asterisks on Fig. 1 and Fig. 2 denote compound labelled.
Blokwood and McGillvray (18) have observed considerable amounts of uncomplexed proteins and DNA after the reconstitution in 0.14 M NaCl. In our conditions the amount of uncomplexed material is negligible. It seems that this results from the difference in binding affinity between their HAP2 and our high affinity NHP fraction. Our high affinity NHP fraction forms complexes with DNA at higher ionic strength than HAP2 fraction. For example, in 0.3 M NaCl HAP2 fraction did not bind to DNA at all, whereas with our NHP fraction no uncomplexed material in 0.25 M NaCl was observed (data not shown). Thus, our proteins have the binding characteristics more similar to the histones than to HAP2 fraction.

Fig. 2 shows the metrizamide gradient centrifugation profile of nonhistone phosphoprotein fraction to mouse main band DNA at the input ratio of 1:1; the conditions of reconstitution were like those in the case of high affinity NHP fraction.

Phosphoproteins and DNA form a heavy complex with a density in metrizamide of 1.224 g/cm$^3$. A small amount of the proteins bound to the bulk of DNA forms a second light complex with a density of 1.132 g/cm$^3$. From the gradient of metrizamide we recovered 89% of DNA, and only 60% of phosphoproteins. Since phosphoproteins are known to aggregate very easily, we suggest that this feature is responsible for the formation of a complex having a density greater than that found for heavy complex of DNA with high affinity nonhistone proteins. In some cases (see e.g. Fig. 1C) a heavy fraction containing a small amount of DNA associated with a significant amount of proteins could be observed. It can be caused by a contamination of a metrizamide gradient with some material from the bottom of the centrifuge tube during the collection of fractions.

2. Complexes of DNA with histones and nonhistone proteins

During the reconstitution of nucleohistone some DNA molecules bind more histone than others (21). This effect can be explained chiefly by the cooperativity of complex formation,
but preferential binding of some histones to regions enriched in AT or GC could also be of importance (19,20).

When the concentration of histones in the reaction mixture is sufficiently low, they form fibrillar structures with DNA. By increasing the concentration of histones, more heavy complexes with globular structure are obtained (21).

It has been postulated that this heavy complex results from the binding of excess of histone to other histone molecules. The histone-histone interactions are assumed to be essential for the formation of the globular type of complex. This kind of interaction may be similar to that existing in native chromatin where histones are assembled in the form of nucleosomes. Thus the study of the influence of histones on the DNA-high affinity NHP complex formation seems to be of interest. In this study the ratio of histone to DNA was constant, and the amount of nonhistone proteins was changed. The centrifugation profile of the DNA-histone-NHP mixed at the input ratio of 1:0.8:0.5 shows two (heavy and light) com-
ponents (Fig. 3). The density of the heavy fraction (1.211 g/cm$^3$) is very close to the 1.220 g/cm$^3$ density of the $^{[125]}$I labelled mouse liver chromatin, sedimented in the same conditions (data not shown). Increasing the input ratio of NHP to DNA the amount of both DNA and histones in the heavy component increases and the amount of DNA and proteins in the light complex diminishes considerably (Fig. 3 A,B,C). For example, at a DNA to NHP ratio of 1:1.5 in the presence of histones (the DNA to total proteins ratio was 1:2.3, Fig. 3C), as much as 77 % of DNA is found in the heavy component (1.208 g/cm$^3$) and the rest of the DNA (23 %) is found in the light component. The complex formed in the absence of histones at a DNA to NHP ratio of 1:2.5 contained only 20 % of total DNA in the heavy component (Fig. 1D), in spite of the fact that the total quantity of proteins was greater than in the system with histones in the mixture (Fig. 3C). Thus the presence of histones favours the formation of the heavy component, and it is possible that the histones which are known to form strong complexes with the DNA could play a role of "aggregation centres" for nonhistone proteins during the reconstitution process.

The profiles of polyacrylamide gel electrophoresis of the proteins recovered from the light and heavy components of DNA-histones-NHP mixed at the ratio of 1:0.8:0.5 show that the distribution patterns of total $^{[125]}$I labelled histones in these components are similar (Fig. 4). The distribution patterns of total proteins in the heavy and light components are different (Fig. 5); there are some NHP fractions present in the heavy component (marked by arrows on Fig. 5B) and absent in the light one.

The centrifugation profile of the complex formed in the mixture of satellite DNA-histones-NHP in the ratio 1:0.8:0.5 (Fig. 3D) differs from that formed with the main band DNA (Fig. 3A) under the same conditions. With satellite DNA the majority of histones and DNA are found in the heavy peak (Fig. 3D). This profile resembles the one obtained in the experiment illustrated in Fig. 3C, where main band DNA was
Fig. 4. Electrophoretic profiles of the $^{125I}$ labelled histones present in (A) light (fractions 8-10) and (B) heavy (fractions 12-15) component from the gradient shown on Fig. 3A. NBP were not labelled in this experiment.

complexed with histones and with a three-fold greater quantity of NHP.

CONCLUSIONS

A fraction of nonhistone proteins, having high affinity for DNA, forms two kinds of complexes with DNA. We suggest that the light complex arises by association of a single protein molecule with DNA. Binding of protein-protein complexes to DNA brings about the formation of the heavy complex. We suppose that binding of these protein aggregates is responsible for the comparatively low recovery of DNA from the heavy fraction even at the high NHP:DNA ratio.
Fig. 5. Electrophoretic profiles of the total proteins recovered from (A) light and (B) heavy components of main-band DNA-histone-NHP complexed at the input ratio 1:0.8:0.5 (the densitograms from the polyacrylamide gels shown in Fig. 4.

Histones present in the reaction mixture favour the formation of the heavy complex with DNA. Histones probably serve as an "aggregation centre" for nonhistone proteins in the reconstitution process. Some of the nonhistone proteins can bind to DNA only in the form of aggregates (protein--protein complexes). They are absent from the light complex.

ACKNOWLEDGEMENTS

The authors are indebted to Dr D. Bickwood for the helpful discussion. The technical assistance of Mrs K. Chorąży is acknowledged. Nyegaard and Co. A/S (Oslo, Norway)
kindly supplied us with a sample of metrizamide.

This work was partly carried out within the research project 09.71. of the Polish Academy of Sciences, and partly supported by National Cancer Program PB-6/1301.

REFERENCES
