Volume 2 number 10 October 1975 Nucleic Acids Research

Evidence of altered histone interactions, as investigated by removal of histones, in chromatin isolated from rat liver nuclei by a conventional method

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Received 10 September 1975

ABSTRACT

It is shown that the release of the slightly lysine-rich histones f2a2 and f2b by 0.4 M ammonium sulfate from conventionally isolated chromatin is diminished in comparison to the lysed nuclei. The change in extractability is further demonstrated by the application of ethidium bromide. At a molar input ratio of 0.09 (moles ethidium bromide/moles nucleotide) and 0.4 M ammonium sulfate the slightly lysine-rich histones are released from the chromatin to 70 - 80 % if the lysed nuclei are used. At 0.1 M ammonium sulfate ethidium bromide effected also a release of 50 % of histone f1. Comparable effects could not be observed with chromatin prepared in a conventional way but instead a tendency towards loss of histone f3 in the presence of ethidium bromide was observed.

INTRODUCTION

The question in how far chromatin preserves essential "native" features after isolation from the nuclei has received increasing attention (1-5). In a previous paper we have shown that chromatin changes its transcription properties in the course of the isolation procedure (1). From this result we suggested that alterations of the DNA-protein interactions may arise during the isolation procedure of the chromatin from the nuclei (1). Recently, we could show that the ability of histone f1 to be extracted by ammonium sulfate in the region between 0.1 and 0.4 M depends on the way by which chromatin was obtained from the nuclei (5). From this result we concluded that deterioration of histone f1-chromatin interaction occurs during the conventionally applied preparation methods. In the present paper we have investigated whether also the extractability of the other histone types is changed after isolation

of the chromatin from the nuclei. It was further checked whether ethidium bromide, as a potential inducer of overall conformational changes in chromatin, influences the histone-DNA-interactions in such a way that their extractability is altered.

MATERIALS AND METHODS

Nuclei were isolated from the livers of 50-day-old Wistar rats immediately after killing the animals as described before (5).
Chromatin I: The nuclei were lysed in the presence of 0.1 or 0.4 M ammonium sulfate in a medium which was normally used for in vitro RNA synthesis. For the histone removal experiments, the whole nuclei lysates were used. In experiments, however, in which the solubility behaviour of the chromatin was to be investigated, the nuclei membranes with attached chromatin material were pelleted by centrifugation at 5 000 x g for 10 min. The clear supernatant obtained with 0.1 M ammonium sulfate contained approx. 40-50 % of the chromatin material of the nuclei in a soluble form.
Chromatin II was isolated from the freshly prepared nuclei according to the first steps described by Butterworth et al. (6). The nuclei were suspended in 0.025 M NaCl containing 8 mM EDTA, pH 8, and were sedimentated at 900 x g for 3 min. This step was repeated once. The pellet was suspended with 10 mM Tris-HCl-buffer, pH 7.9, gently stirred with a glass rod and kept at 0°C for 60 min before being centrifuged at 12 000 x g for 15 min. Finally the pellet was suspended in the ammonium sulfate solution for the histone removal experiments.
Removal of the histones by ammonium sulfate was performed by incubation of the different chromatin samples in the presence of 0.1 M or 0.4 M ammonium sulfate at 0°C for 30 min. The histones which were not removed by ammonium sulfate were extracted from the rest-chromatin pellets according to Johns (7) with 0.25 N HCl. Details of the extraction procedures as well as the conditions of the polyacrylamide gel electrophoresis (8) are described in (5). The relative amount of each histone type in the densitometric traces was determined by an analog computer program using the analog computer MEDA 81 P. The single curves were imitated as exactly as possible according to their shapes experimentally determined with the micro densitometer Vitatron.

For the calculation program of the analog computer the shapes of the typical experimental curves were used. Because we never found any histone f2a1 in the ammonium sulfate extract it is obvious that this histone type was not removed from the chromatin under our salt conditions. Therefore it was chosen as reference for calculation of the percentual removal of the other histone types.

RESULTS

A summary of the experimental data, which represent the non-removed portion of the 5 histone types after treatment of the two different chromatin preparations with ammonium sulfate and ethidium bromide, respectively, is given in Table 1. No significant amounts of histones are removed from the chromatin at 0.1 M ammonium sulfate if the extraction step was performed with lysed nuclei (Chromatin I) (5). Quite simular results can be demonstrated at this salt concentration with chromatin which was isolated from the nuclei by the technique of Butterworth et al. (6). A comparison of the two different chromatin preparations, however, yielded significant differences as to the extractability of the slightly lysine-rich histones f2a2 and f2b at 0.4 M ammonium sulfate. As can be seen from Table 1, at 0.4 M ammonium sulfate about 40 % of f2b and 50 % of f2a2 were removed from Chromatin I. But only a slight release of this two histone types was found with Chromatin II under the same conditions. As to the degree of extractability of histone f1 from both kinds of chromatin preparations we could reproduce our previous results (5), which showed that in contrast to Chromatin I an appreciable amount of histone f1 remained at the rest-Chromatin II after treatment with 0.4 M ammonium sulfate (Table 1).

In order to see whether alterations of the secondary structure of the chromosomal DNA which are induced by ethidium bromide (9) result also in changed histone-DNA interactions we have investigated the extractability of different histone types from chromatin at two ammonium sulfate concentrations. The results obtained with the two kinds of chromatin preparations are demonstrated by the electrophoresis profiles of the histones extracted from the rest-chromatin samples after treatment with

$(NH_4)_2SO_4$:	0.1 M			0.4 M		
EB/ Nucleot.	0	0.03	0.09	0	0.03	0.09
CHROMATIN I						
F2a1	100	100	100	100	100	100
F3	100	86	82	72	75	83
F2b	95	94	94	61	30	24
F2a2	98	76	79	46	28	18
F1	92	52	49	7	0	0
CHROMATIN II						
F2a1	100	100	100	100	100	100
F3	88	75	56	97	81	56
F2b	100	86	83	83	100	78
F2a2	100	90	90	91	82	79
F1	86	84	78	32	30	0

TABLE 1

Extractability of the different histone types from two chromatin preparations: CHROMATIN I and CHROMATIN II in dependence on the ammonium sulfate and ethidium bromide (EB) concentration.

The data represent rest-histone (%) after treatment with the indicated concentration of ammonium sulfate and ethidium bromide as mean values of 3 different experiments and each of 2 electrophoretic runs.

ethidium bromide (Fig. 1 and 2).

As can be seen from Fig. 1, addition of ethidium bromide to Chromatin I at 0.1 M ammonium sulfate leads to release especially of histone f1. A quantitative evaluation of the electrophoresis profiles has shown that, as a consequence of the treatment of Chromatin I with ethidium bromide in a molar input ratio of 0.03 (moles ethidium bromide/moles nucleotide), approx. 50 % of histone f1 and 20 % of histone f2a2 were released. Raising the ethidium bromide concentration up to a molar input ratio of 0.09 (moles ethidium bromide/moles nucleotide) failed to bring about further enhancement of the removal of histone f1 and histone f2a2 (Fig. 1 and Table 1). If the chromatin, however, was prepared according to the method of Butterworth et al. (6), on the average, no significant effect on the extractability of the lysine-rich histones could be observed. From the experiments performed with Chromatin II at 0.1 M ammonium sulfate it seemed, however, that there is

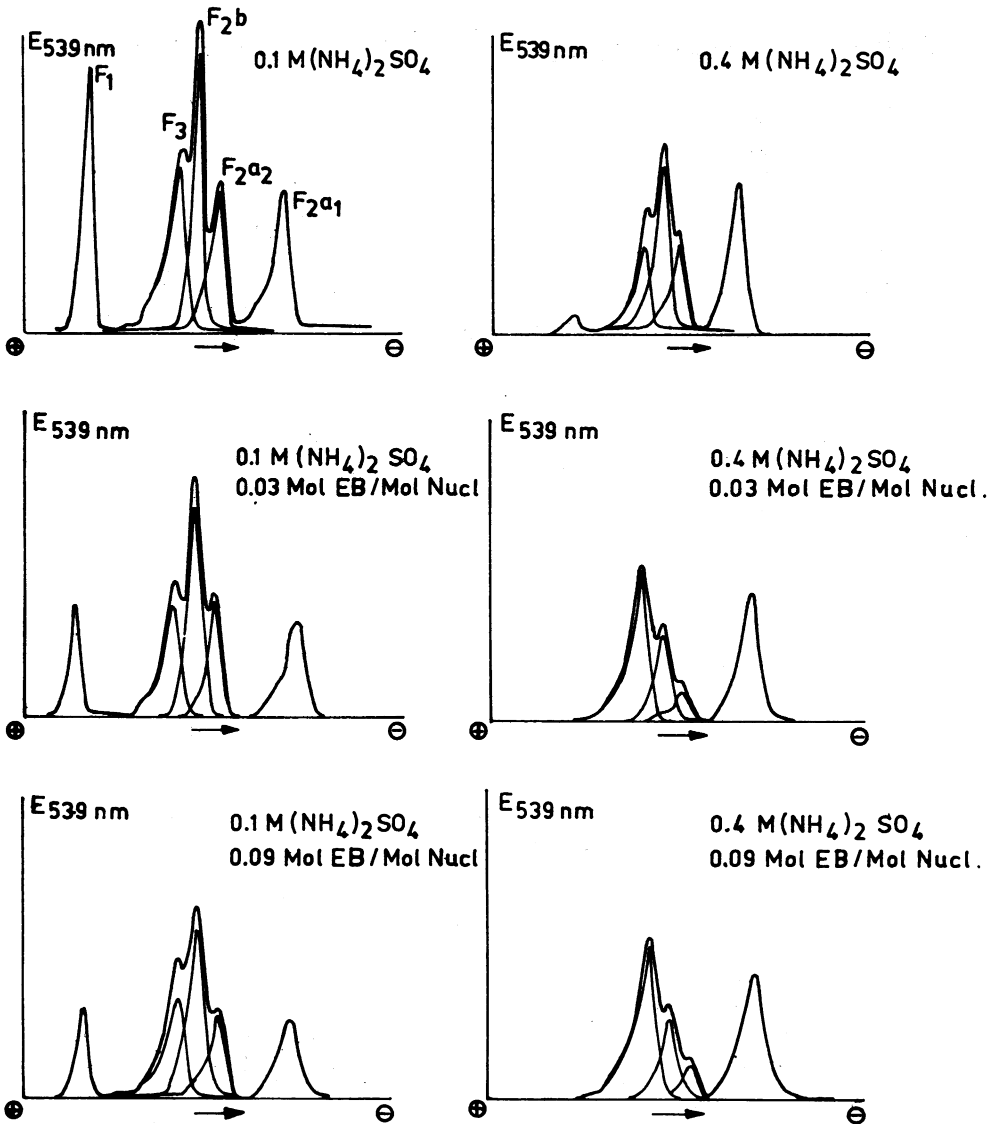

Fig. 1: Extractability of the different histone types from Chromatin I.

Typical densitometer tracings of polyacrylamide gels containing histones from the rest-chromatin pellet obtained from Chromatin I pre-treated as indicated in the Figures.

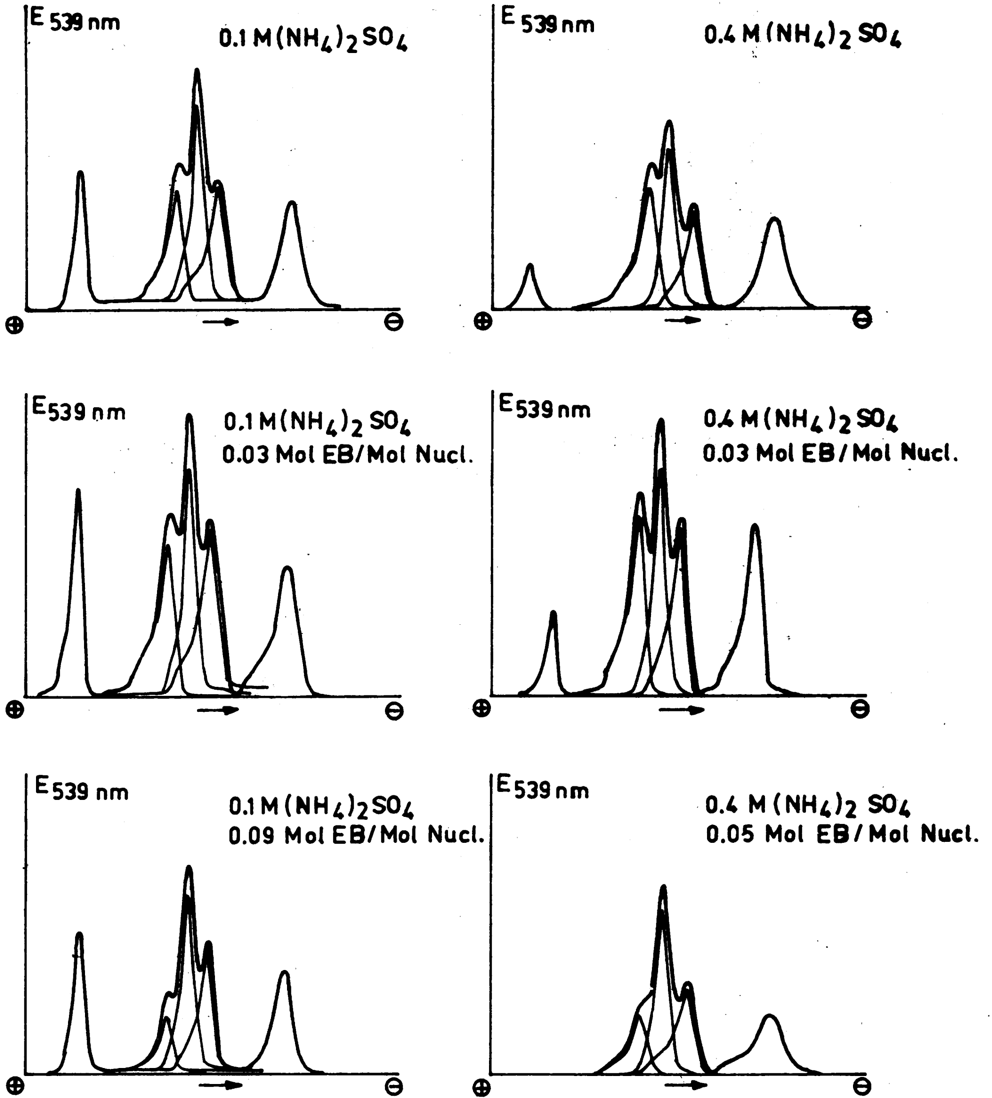

Fig. 2: Extractability of the different histone types from Chromatin II.

Typical densitometer tracings of polyacrylamide gels containing the histones from the rest-chromatin pellet obtained from Chromatin II pre-treated as indicated in the Figures.

a certain tendency towards loss of histone f3 as a consequence of the presence of ethidium bromide. The influence of ethidium bromide on the removal of histones from the chromatin preparations was also checked at 0.4 M ammonium sulfate. From the electrophoresis profiles shown in Fig. 1 it can be seen that the partial removal of the histones f2a2 and f2b from Chromatin I which was already obtained with 0.4 M ammonium sulfate was strengthened after the addition of ethidium bromide. As a result of this treatment only about 20 % of the slightly lysine-rich histones remained at the rest-chromatin. A comparable effect on the removal of the slightly lysine-rich histones with Chromatin II which was prepared in a conventional way using a low ionic strength and EDTA for the isolation procedure could not be ovserved. Instead, however, a partical release of histone f3 was obtained at the increased ethidium bromide concentration at 0.4 M ammonium sulfate.

DISCUSSION

The two kinds of chromatin preparations used in our experiments show a strictly different solubility behaviour. Chromatin I which is obtained directly by lysis of the isolated nuclei with 0.1 M ammonium sulfate is clearly soluble at this salt concentration (10) in spite of the fact that it contains still all of the histones including histone f1 in an undegraded state. It has a remarkably high average sedimentation velocity, a low intrinsic viscosity (2, 10, 11) and the electron microscopic analysis has revealed the presence of well defined nucleosomes along the chromatin fiber which disappeared at 0.4 M ammonium sulfate (12). The sedimentation characteristics as well as the intrinsic viscosity of the soluble Chromatin I remains unchanged for at least 2 days if kept at $0^{o}C$. Dialysis, however, against a lower salt concentration (i.e. 0.05 M ammonium sulfate) induced marked turbidity which is greatly irreversible and not reversed if the samples are re-dialysed against 0.1 M ammonium sulfate. In contrast, Chromatin II which was obtained by a conventional isolation procedure (6) behaved in its solubility as described by Bradbury et al. (13) and also by other authors, that is, it is insoluble at a physiological salt concentration of 0.1 to 0.15 M NaCl and, as

we have checked,also at 0.1 M ammonium sulfate. Because Chromatin II prepared in a conventional way does not become soluble when subsequently brought into the same salt solution as used for the direct lysis of the nuclei, it is evident that the changes of the chromatin structure, once arisen, cannot be reversed.

Recently, Rees et al. (3) have also described a chromatin preparation soluble at 0.15 M NaCl which they obtained by autolysis of rabbit thymus nuclei. This chromatin, prepared avoiding shear, shows similarly high sedimentation velocity and low intrinsic viscosity as we found for Chromatin I at 0.1 M ammonium sulfate (2, 10, 11). Similarly to our experience their nucleohistone showed no tendency to aggregate if gently handled but a certain sensibility against homogenization and sedimentation and the change of salt concentration.

In connection with the organization of the chromatin structure in repeating units, the so-called nucleosomes, (14-18) the question was raised whether chromatin extracted from the nuclei by conventional methods retains these basic structure (4). Noll et al. (4) could show that chromatin prepared by conventional methods involving shear does not really preserve the native structure.

Evidence is presented in this paper that the two chromatin preparations which we used in our experiments and which differ in their solubility show also remarkable differences in the extractability of their histones with ammonium sulfate concentrations at 0.1 and 0.4 M. In this line we have found that only from Chromatin I the slightly lysine-rich histones f2a2 and f2b are removed under our extraction conditions with 0.4 M ammonium sulfate to about 50 %. The non-extractability of the two slightly lysine-rich histones from Chromatin II under the same conditions is of special interest, because it is now widely accepted that these histone types are localized within the nucleosomes (18). Assuming that the different extraction behaviour of the histones from the two chromatin preparations reflect various histone interactions in the chromatin preparations we conclude that the native histone interactions in the nucleosomes are rather sensitive against "unphysiological"

salt conditions as well as against shear. In spite of the fact that the conventional methods of chromatin preparation does not effect the disappearance of the nucleosome structure in the chromatin as checked by electron microscope analysis (18) it is clear from our results that they become altered in some structural details concerning the histone interactions. We have shown earlier (5) that also the interactions of the very lysine-rich histone f1 which is not included in the basic nucleosome structure (18) are changed in the course of the conventional isolation procedures. From the fact that after washing the nuclei with saline-EDTA and 0.01 M Tris-HCl, pH 8, the extractability by ammonium sulfate of the very lysine-rich histone f1 and also of the slightly lysine-rich histones f2a2 and f2b from chromatin is diminished we conclude that as a result of the washing procedure used in conventional isolation methods, new, artificial histone-DNA interactions arise. We cannot expect chromatin treated in a way causing changed histone-DNA interactions will react in in vitro transcription experiments in a native manner.

The different features of the two chromatin preparations become even more clear from the experiments performed with ethidium bromide as a molecular probe. Only in the case of Chromatin I does addition of ethidium bromide at 0.1 M ammonium sulfate cause partial removal of histone f1 from the chromatin to about 50 % and, at 0.4 M ammonium sulfate, a further removal of the histones f2a2 and f2b to about 80 %. The results of these experiments show for the first time that due to the ethidium bromide binding to the chromosomal DNA the association of the lysine-rich histones is changed in such a way that they become easier to extract. In the case of Chromatin II at 0.1 M ammonium sulfate, however, the addition of ethidium bromide leads to the tendency of partial release of histone f3.

Since the effects are already found at the relatively low ratio of ethidium bromide to nucleotide of 0.03 on a molar input basis we suggest that conformational changes of the DNA caused by intercalation of the dye with the DNA bring about changes of the DNA-histone interactions in the chromatin.

This means that the conformational state of the DNA in the chromatin is of importance for the histone-DNA interactions. Partial unwinding of the DNA double helix by ethidium bromide seems to weaken the histone-DNA interactions leading to their release under appropiate ionic conditions. From this and unpublished results (11) obtained with hydrodynamic methods it seems that relatively small changes of the secondary structure of the DNA as induced by ethidium bromide can cause strong overall conformational changes in the chromatin with changed histone-DNA and histone-histone interactions, respectively. This may be a principle by which the regulation of condensation or decondensation in the chromatin in the regulation of the transcription process is governed.

REFERENCES

1 Lindigkeit, R., Fenske, H., Grade, K., Schmidt, G. and Eckel, R. (1972) Studia biophys. 31/32, 75-89

2 Fenske, H., Eichhorn, I., Böttger, M. and Lindigkeit, R. FEBS-Proceedings (1974) Budapest (in press)

3 Rees, A. W., Debuysere, M. S. and Lewis, E. A. (1974) Biochim. Biophys. Acta 361, 97-108

4 Noll, M., Thomas, J. O. and Kornberg, R. D. (1975) Science, 187, 1203-1206

5 Fenske, H., Eichhorn, I. and Lindigkeit, R. (1975) Acta biol. med. germ. 34, 303-307

6 Butterworth, P. H. W., Cox, R. F. and Chesterton, C. J. (1971) Eur. J. Biochem. 23, 229-241

7 Johns, W. E.: (1971) Histones and Nucleohistones. D. M. P. Phillips (Ed.) Plenum Press, London and New York, p. 10

8 Panyim, S. and Chalkley, R. (1969) Arch. Biochem. Biophys. 130, 337-346

9 Reinert, K. E. (1973) Biochim. Biophys. Acta 319, 135-139

10 Lindigkeit, R., Bellmann, K., Fenske, H., Böttger, M., Holtzhauer, M. and Eichhorn, I. (1974) FEBS-Letters, 44, 146-151

11 Böttger, M., Fenske, H. and Lindigkeit, R. (unpublished)

12 Ladhoff, A. M., Schmidt, G. and Lindigkeit, R. (unpublished)

13 Bradbury, E. M., Carpenter, B. G. and Rattle, H. W. E. (1973) Nature 241, 123-126

14 Sahasvabuddhe, G. G. and Van Holde, K. E. (1974) J. Biol. Chem. 249, 152-156

15 Olins, A. L. and Olins, D. E. (1974) Science 183, 330-332

16 Kornberg, R. D and Thomas, J. O. (1974) Science 184, 865-868

17 Kornberg, R. D. (1974) Science 184, 868-871

18 Oudet, P., Gross-Bellard, M. and Chambon, P. (1975) Cell 4, 281-300