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Studies on the Role and Mode of Operation of the Very-Lysine-Rich Histones in Eukaryote Chromatin

Nuclear-Magnetic-Resonance Studies on Nucleoprotein and Histone φ1 · DNA Complexes from Marine Invertebrate Sperm

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Proton magnetic resonance and other measurements have been carried out in order to study the behaviour of the lysine-rich histones φ1 in the sperm chromatin of certain marine invertebrates. Well defined particles (12 S) have been obtained from this chromatin by nuclease treatment. Chromatin solubility as a function of ionic strength shows a relaxation at salt concentrations higher than in the case of calf thymus nucleoprotein. Nuclear magnetic resonance (NMR) studies show that the release of histone from DNA occurs both in chromatin and in the reconstituted complexes at practically the same ionic strength as solubility relaxation. The higher the arginine content of a given φ1, the higher the ionic strength at which both effects take place. The NMR results demonstrate that arginine residues are bound more strongly than lysine residues. The data overall show that the φ1 histones play a role in the contraction mechanism of sperm chromatin similar to that of H1 histone in calf thymus chromatin. The highly contracted state of sperm chromatin is directly related to the increased arginine content of the φ1 histone.

Histone H1 appears to play an important role in the control of the structure of chromatin. In this respect, a recently proposed model for chromatin structure [1] considers that this histone is not involved in the core of its globular subunits [2, 3]. On the other hand there is evidence supporting the view that the presence of histone H1 is a basic requirement for the packing of DNA within the nucleoprotein [4]. In this context, modification '*in vivo*' of a few residues of this histone seems to be related to the changes in chromatin contraction before mitosis [5]. Studies on calf thymus chromatin including high-resolution NMR [4, 6] have shown that the release of histone H1 is accompanied by a relaxation effect of contracted chromatin. In addition H1-depleted chromatin does not show the typical contraction behaviour of chromatin with an increase of salt concentration [4].

Sperm chromatin is a highly condensed material, packed within the spermatozoa head. The X-ray diffraction patterns of sperm nucleohistone show only slight differences among different species [7]. It is also well known that the basic proteins bound to sperm DNA are very different in different species, and in echinoderms they are closely related to somatic histones; in sea urchin sperm, for instance, the five typical fractions are found, with some modifications in amino acid composition when compared with calf thymus histones [8].

The H1-like histones extracted from the sperm of some echinoderms and molluscs, called histones φ1, show a number of differences in their amino acid composition with respect to calf thymus H1. Notable is a greater arginine content in most φ1 histones. The φ1 histones used in the present work were extracted from the sea urchins *Arbacia lixula* and *Paracentrotus lividus*, the sea cucumber *Holothuria tubulosa*, and the sea mussel *Mytilus edulis*. NMR studies [9] have suggested homologous features in the tertiary structure of certain echinoderm φ1 histones and calf thymus H1. It is thus possible that the function of φ1 histones is very similar in echinoderm sperm to that of H1 in somatic tissues. In that sense sea urchin chromatin may be taken as an extreme example of chromatin condensation and a suitable material for studying the

Abbreviation. NMR, nuclear magnetic resonance.

role in contraction of the very-lysine-rich histones. This paper considers the relationship between the φ1 histones and the better studied calf thymus H1.

EXPERIMENTAL PROCEDURE

Preparation of Chromatin

The extraction of chromatin was carried out following essentially the method of Zubay and Doty [10] and using some modifications for sperm, as described by Subirana [11]. Male gonads from sea urchins were suspended in sea water. The sperm was recovered by filtration through a double gauze. Coarse material was sedimented by centrifugation at $30 \times g$ for 10 min. The sperm was recovered as a loosely packed material by centrifugation at $2500 \times g$ for 10 min and then washed four times with ultrafiltered sea water.

A sperm suspension in 0.1 M sodium citrate (10 ml/g wet residue) was prepared by a gentle homogenization with the aid of a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle (0.2 mm clearance). The suspension was kept at rest for 1 h, and then it was spun at $2500 \times g$ for 10 min. Homogenization and centrifugation under the same conditions were repeated once more. The pellet was resuspended in 0.15 M NaCl (15 ml/g wet residue) and the suspension was centrifuged at $11\,000 \times g$ for 10 min. The pellet obtained was homogenized with 0.15 M NaCl (2 ml/g wet residue) and centrifuged at $11\,000 \times g$ for 10 min. The sediment was poured into a solution of 1 mM EDTA, 1 mM sodium cacodylate, pH 8. The final ratio solvent to wet sediment was 25 ml/g. The suspension was centrifuged at $7500 \times g$ for 10 min. The sediment was resuspended in the same buffer (2 ml/g wet residue) and left overnight. This material was centrifuged at $7500 \times g$ for 10 min and the pellet obtained was homogenized in a high-speed blender for 1 min with EDTA-cacodylate buffer (15 ml/g of wet sediment). The suspension was centrifuged at $7500 \times g$ for 10 min. The pellet was discarded and the supernatant was centrifuged at $25\,000 \times g$ for 30 min. The sediment obtained in this centrifugation was the nucleoprotein used for our study.

Between 1 ml and 2 ml of the material obtained as in the preceding paragraph was placed in 0.25 -in (6.3 mm) dialysing tubing which had been previously boiled twice with 10 mM EDTA and washed extensively with distilled water. The samples were dialysed against three changes (about ten times the sample volume each time) of the corresponding salt solution in ^2H_2O (99.8% deuterated, Ryvan Chemical Co.). The gel was then placed in the NMR tube and spun in a small hand centrifuge.

Aliquots of the same sample were diluted 100 times with variable proportions of distilled water and a solution of 1 M NaCl, to be used for sedimentation studies.

Preparation of Histone · DNA Complexes

Histones were prepared as is described in preceding papers [8,9]. The extraction followed essentially the method 1 of Johns [12], with the exception of that for preparing histone φ1 from *M. edulis*, in which case a solution of 5% of acetic acid was used instead of perchloric acid [13]. Calf thymus DNA was purchased from Sigma Chemical Co. (type I) or prepared in the laboratory by the method of Kay et al. [14]. The A_{260}/A_{230} ratio for DNA was always higher than 2.3 and its hyperchromic effect between 20 °C and 80 °C in 10 mM NaCl solutions was about 40%.

The procedure for preparing histone · DNA complexes was essentially the same as in [6]. For NMR studies solutions in 1 M NaCl, 10 mM EDTA, pH 7, of protein and DNA were mixed and aliquots of the resulting solution (1 – 2 ml) were placed in 6-mm dialysis tubing and successively dialysed against solutions of decreasing salt concentration and lastly against pure water. Finally they were dialysed against different solutions of salt in ^2H_2O. The dialysis against each solvent was repeated three times, the outside volume being at least ten times that of the sample.

The same procedure was followed for preparing samples for sedimentation studies. In this case the volume of the sample was 4 ml and a 15-mm dialysis tubing was used.

Nuclease Digestion of Chromatin

50 ml of a chromatin solution (0.65 M NaCl, 20 mM Tris, pH 7) corresponding to 1 mg/ml of DNA was left overnight stirring with 15 ml of wet volume of BioRad AG 50W-X2 ion-exchange resin. The resin was washed twice with 1 M HCl, twice with 1 M NaOH and twice with the saline buffer, followed each time by intermediate washings with distilled water. The final material was dialysed against a solution of 1 mM Tris, pH 7. Bovine pancreatic DNase I (Sigma Chemical Co.), 2000 units, was dissolved in 15 ml of a solvent containing 20 mM Tris, 5 mM Mg^{2+}, pH 7.7. The digestion was carried out at 23 °C at a ratio of 1000 enzyme units/42 mg DNA. The reaction was stopped with 8 mM EDTA, 0.1 M Tris. Analytical ultracentrifugation was carried out by using an MSE Centriscan 75, at 20 °C and with intervals of 3 min for observation.

Nuclear Magnetic Resonance (NMR)

Spectra at 270 MHz were run on a Bruker WH-270 Fourier transform spectrometer. The preparation of the samples is described in preceding paragraphs.

Standard 5-mm tubes were used, and the spectra were obtained by transforming the sum of 8000 free induction decays accumulated over periods of about 70 min. No irradiation of residual water (H^2HO) signals was used.

Sedimentation Studies

Two different methods for studying the influence of the ionic strength on the sedimentation of chromatin and of $\varphi 1$ · DNA complexes were used. One procedure consists of monitoring the turbidity of a collection of solutions at different ionic strengths by measuring the absorbance at 600 nm. In the other method the samples were centrifuged at $25000 \times g$ for 30 min in a Beckman L2-65B ultracentrifuge. The absorbance of the supernatant at 260 nm was measured and plotted against salt concentration. In both methods the dilution due to dialysis at different salt concentrations was corrected by measuring the weight of the dialysis bags before and after dialysis.

Ultraviolet measurements were carried out on a Zeiss DMR-21 double-beam spectrophotometer using 1-cm path-length cells.

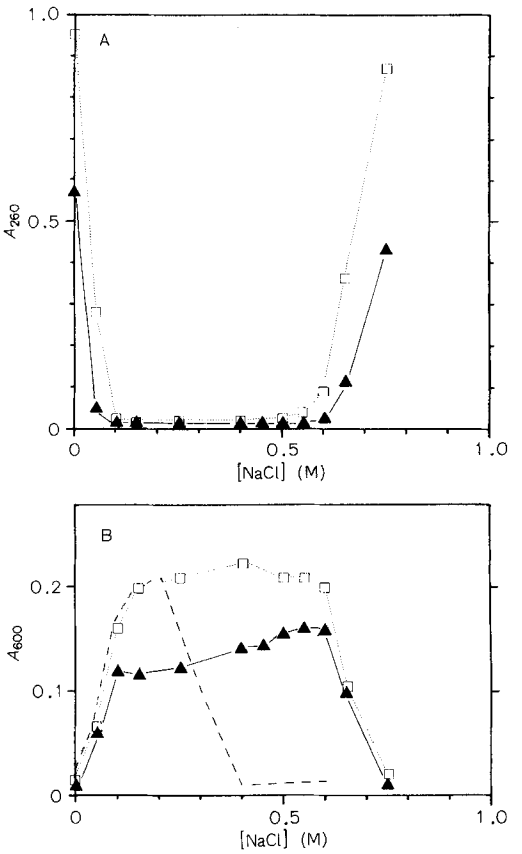

Fig. 1. *Sedimentation (A) and turbidity (B) curves for chromatin at different ionic strengths.* 50 μg/ml in DNA concentration. *A. lixula* sperm (▲), *P. lividus* sperm (□), calf thymus (----)

RESULTS AND DISCUSSION

Sea Urchin Chromatin

The state of chromatin can be studied by investigating the products of nuclease digestion. The chromatin used in the present study was submitted to bovine nuclease digestion and well-defined particles of 15 S were obtained after 10 s digestion. Particles of 12 S were also obtained after 30 s digestion. These values for the sedimentation coefficient correspond to those observed for other tissues [15]. Recently the existence of a subunit structure of sea urchin sperm chromatin has also been shown by analyzing the DNA length of the products of nuclease digestion [16].

In calf thymus chromatin the release of histone H1 occurs at the same ionic strength ($\approx$ 0.5 M NaCl) at which the relaxation of the contracted state takes place. The solubility of sea urchin chromatin with increasing ionic strength was therefore studied, and the lower part of Fig. 1 shows the variation of turbidity for chromatin from two related sea urchins *A. lixula* and *P. lividus*. Also shown for comparison are the data for calf thymus chromatin previously reported [4]. The turbidity results at 600 nm were checked by noting the absorbance at 260 nm of the supernatant of an identical chromatin after centrifugation at $25000 \times g$ for 3 min. and these data are presented in the upper part of Fig. 1. In both experiments the chromatin solubility at high salt concentration appears very similar to that in pure water.

The up-field NMR spectra of chromatin from *A. lixula* sperm at different concentrations of NaCl are presented in Fig. 2 (the low-field part of the spectrum not being visible under the conditions used). At intermediate ionic strengths (0.25 – 0.50 M NaCl), when the chromatin is in its contracted state, the only peaks clearly visible are at 0.93 ppm and 1.35 ppm and can be assigned to the CH_3 groups of valine, leucine and isoleucine and of alanine respectively. There is also a weak resonance at about 2.0 ppm due to acidic residues but no evidence of peaks due to lysine or arginine. At very low salt and in pure water the same peaks are also observed, albeit somewhat stronger, indicating that although the chromatin is highly expanded at low ionic strength the basic residues are still strongly bound to DNA, whereas the apolar regions are freer than in the highly contracted state. At high ionic strength (0.7 – 0.8 M NaCl) the spectrum is close to that expected for $\varphi 1$ largely free from DNA [9] with the possible addition of some resonance from the H2A and H2B histones, which are released to a small extent at this ionic strength. Only at 0.8 M NaCl is the resonance from lysine and arginine fully developed: for example the δ-CH_2 peak

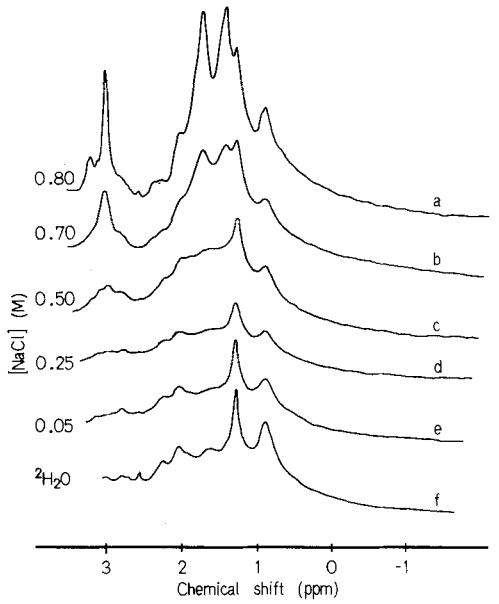

Fig. 2. *270 MHz NMR spectra of* A. lixula *sperm chromatin at different ionic strengths.* (a) 0.80 M NaCl; (b) 0.70 M NaCl; (c) 0.50 M NaCl; (d) 0.25 M NaCl; (e) 0.05 M NaCl; (f) ^2H_2O

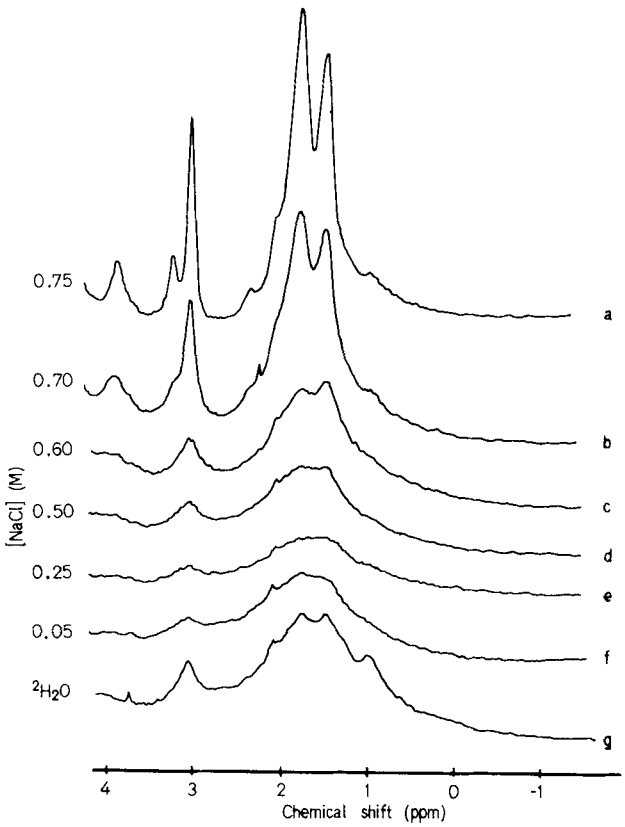

Fig. 3. *270 MHz NMR spectra of the complex of DNA with histone φ1 from* A. lixula *sperm.* Histone/DNA ratio, 0.2; protein concentration 2 mg/ml. (a) 0.75 M NaCl; (b) 0.70 M NaCl; (c) 0.60 M NaCl; (d) 0.50 M NaCl; (e) 0.25 M NaCl; (f) 0.05 M NaCl; (g) ^2H_2O

of arginine at 3.2 ppm is not observed at all at 0.7 M NaCl.

Comparing Fig. 1 and 2 it is interesting to note that the release of histone φ1 takes place at the same ionic strength, 0.70–0.75 M NaCl, as the relaxation of the contracted state of the chromatin. A similar situation is found for calf thymus chromatin at an ionic strength of about 0.40–0.50 M NaCl. Our results suggest strong similarities in the action of histones φ1 and H1 in the contraction of sperm and somatic chromatin, despite the marked differences in the ionic strength dependence of the phenomenon.

Interactions between DNA and Sea Urchin φ1 Histone

The NMR spectra of reconstituted complexes of calf thymus DNA with histone φ1 from *A. lixula* and *P. lividus* are presented in Fig. 3 and 4. From these spectra it can be seen that the release of φ1 from both complexes occurs at about 0.70–0.75 M NaCl, which is the same range of ionic strengths where the release of φ1 takes place in *A. lixula* chromatin. Furthermore, the lysine ε-CH_2 peak becomes apparent in the spectrum at lower ionic strengths than does the arginine δ-CH_2 peak, as in the case of chromatin (*cf.* Fig. 2 and 3). The peak at 1.35 ppm, interpreted in the case of chromatin as corresponding to a shifted signal of alanine, is also present for complexes of *P. lividus* with DNA and its sharpness at very low ionic strength is remarkable. It has also been found for φ1 · DNA complexes of the species *H. tubulosa* and *M. edulis*, (see Fig. 6 and 7), whereas for *A. lixula* this peak is not visible at any ionic strength. It has been reported for calf thymus H1 interactions [6] that the appearance of such a signal might be dependent on the preparation of the sample. This peak has also been found for complexes of DNA with synthetic co-polypeptides, containing lysine, alanine and proline (unpublished results). This suggests that it may be due to some conformational behaviour of the C-terminal part of the very-lysine-rich histone molecule, inasmuch as calf thymus H1 is very rich in these 3 amino acids in the C-terminal half.

Fig. 5 presents the solubility of complexes of DNA with different φ1 histones, including *A. lixula* φ1. In the lower part of the figure the variation of turbidity for φ1 · DNA complexes is shown as a function of NaCl concentration. The solubilization of the complex begins, in the case of *A. lixula*, at about 0.5 M NaCl and continues up to about 0.7 M NaCl. This interval corresponds to that in which the release of histone φ1 from the complex occurs, as seen by NMR (Fig. 3). The results can also be compared with those for chromatin presented in Fig. 1 and 2 indicating that solubilization for both natural and artificial complexes occurs over the range of salt concentrations at which

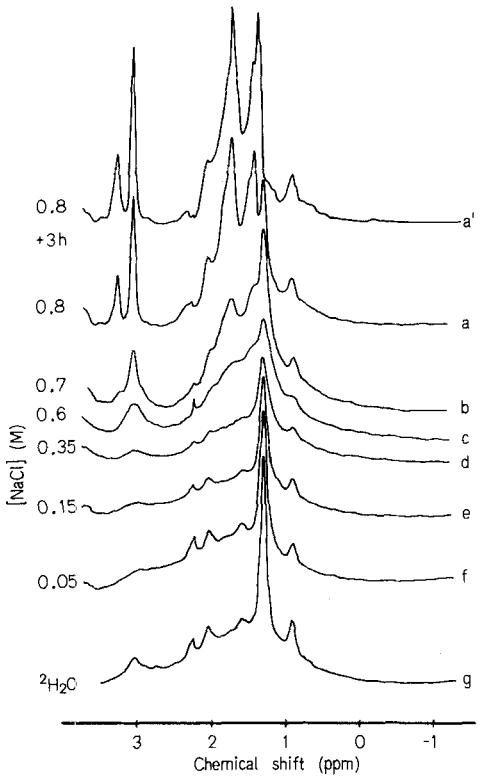

Fig. 4. *270 MHz NMR spectra of the complex of DNA with histone φ1 from* P. lividus *sperm.* Histone/DNA ratio 0.2; (a) 0.8 M NaCl (a') the same sample as (a) after 3 h at room temperature; (b) 0.7 M NaCl; (c) 0.6 M NaCl; (d) 0.35 M NaCl; (e) 0.15 M NaCl; (f) 0.05 M NaCl; (g) ^2H_2O

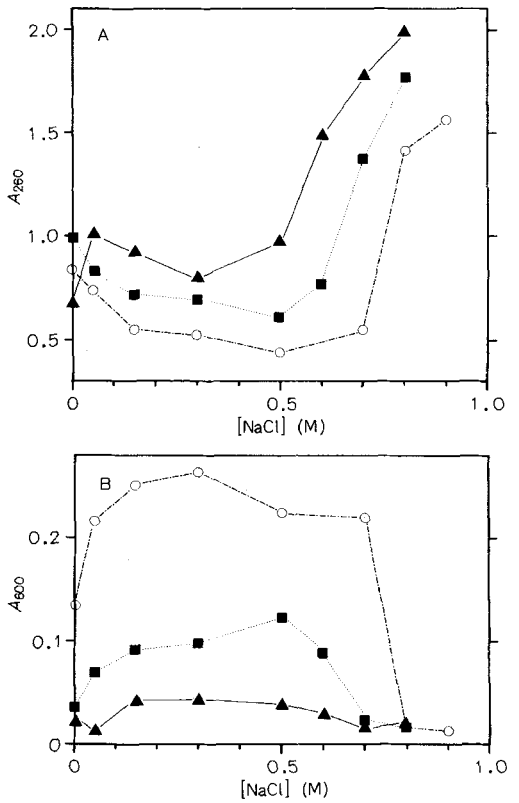

Fig. 5. *Sedimentation (A) and turbidity (B) curves of the artificial complexes of DNA with histone φ1 from sperm of* A. lixula (■), H. tubulosa (▲) *and* M. edulis (○)

release of φ1 takes place. To summarize the above results it can be said that the great similarity in solubility and NMR behaviour of chromatin and of artificial complexes demonstrates that the very-lysine-rich φ1 histone plays a definitive role in the mechanism of contraction and expansion of these chromatins. The results also indicate that the major conformational requirement for the interactions of DNA and very-lysine-rich histones in chromatin is met by simple duplex DNA and isolated protein, *i.e.*, is not dependent on the prior formation of chromatin subunits.

Interactions between DNA and Histone φ1 from Other Marine Invertebrates

The NMR spectra of complexes of DNA with *H. tubulosa* histone φ1 and with *M. edulis* histone φ1 at different ionic strengths are presented in Fig. 6 and 7. The general features of these spectra are very similar to those for *P. lividus* histone φ1 presented in Fig. 4. It is pertinent to note the relationship existing between the arginine content in the different φ1 histones and the release of these proteins from the DNA at different ionic strengths. Histone φ1 from

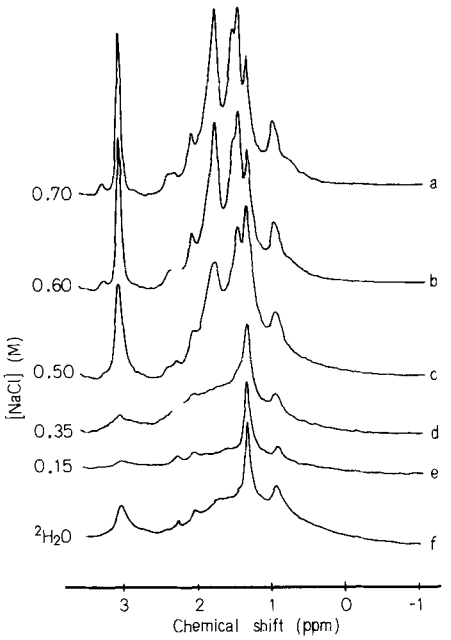

Fig. 6. *270 MHz NMR spectra of the complex of DNA with histone φ1 from* H. tubulosa *sperm.* Histone/DNA ratio 0.2; (a) 0.70 M NaCl; (b) 0.60 M NaCl; (c) 0.50 M NaCl; (d) 0.35 M NaCl; (e) 0.15 M NaCl; (f) ^2H_2O

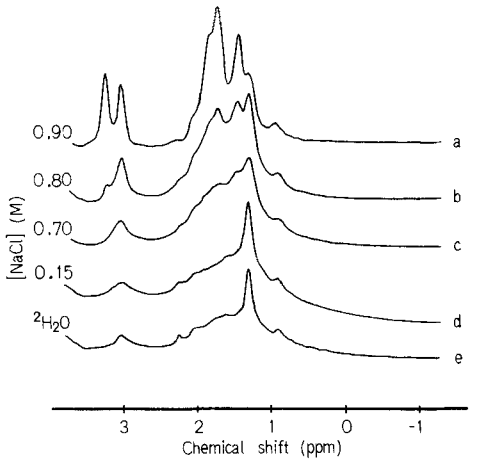

Fig. 7. *270 MHz NMR spectra of the complex of DNA with histone φ1 from* M. edulis *sperm.* Histone/DNA ratio 0.2; (a) 0.90 M NaCl; (b) 0.80 M NaCl; (c) 0.70 M NaCl; (d) 0.15 M NaCl; (e) ^2H_2O

H. tubulosa is released at 0.5–0.6 M NaCl, lower than in the case of sea urchin histone φ1 (0.7–0.75 M NaCl), but somewhat higher than in the case of calf thymus H1 (0.4–0.5 M NaCl). On the other hand *M. edulis* histone φ1, with the highest arginine content, is released from DNA at 0.8–0.9 M NaCl. Due to the particular amino acid composition of the histone φ1 from *M. edulis* sperm, (22.2% lysine and 29.4% arginine [9]), the NMR spectra of the complexes of DNA with this protein show very clearly that lysine is released from the complexes at a lower salt concentration than arginine.

Sedimentation experiments performed on complexes of DNA with histone φ1 from *H. tubulosa* and *M. edulis* are presented in Fig. 5. The differences with respect to *A. lixula* φ1 · DNA are clearly seen and are in the same sense as the release of histone φ1 from the complexes as seen by NMR and discussed in the preceding paragraph. The displacement of the various curves correlates with the arginine content of the φ1 used in the experiment. The φ1 from *M. edulis* has the greatest effect in terms of insolubility of the complex, and solubilization of all the complexes occurs at salt concentrations very close to that at which total release of histone φ1 occurs as observed by NMR. In particular full solubilisation coincides with complete release of the basic residues, in particular the arginines.

The correlation of arginine content with the ionic strength of histone release as well as with the solubility relaxation of the complexes are evident from our results. The sea water in which the species studied live, has a salt concentration of about 0.5 M NaCl. It seems, therefore, that the proportion of arginine content could be an efficient mechanism for the condensation and protection of chromatin in spermatozoa heads. In this sense histone φ1 plays an important role for species which posses histones in their sperm chromatin. The other type of sperm basic proteins, *i.e.* protamines, contain a large amount of arginine, and therefore this basic residue appears to play an important role in maintaining the structure of sperm nucleoprotein.

CONCLUSIONS

Well defined particles from sea urchin chromatin have been obtained by a nuclease treatment. This suggests that the chromatin contains a subunit arrangement, similar to other chromatins studied from somatic tissues.

Contraction of sperm chromatin from sea urchin occurs at the same salt concentration as for calf thymus chromatin (< 0.1 M NaCl) but relaxation of the contracted state occurs at higher salt concentration in the sperm chromatin.

The relaxation effect in sea urchin sperm chromatin is accompanied by the release of histone φ1. This suggests that this protein is responsible for chromatin contraction in the spermatozoa.

The arginine content of different φ1 histones correlates with the ionic strength at which the complex with DNA is relaxed and the protein is released. It also indicates a possible mechanism for condensing and protecting the sperm chromatin in some marine invertebrates.

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