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Localization of testis-variant histones in rat testis chromatin

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Nucleosome core particles and oligonucleosomes were isolated by digesting rat testis nuclei with micrococcal nuclease to 20% acid-solubility, followed by fractionation of the digest on a Bio-Gel A-5m column. The core particles thus isolated were characterized on the basis of their DNA length of 151 ± 5 base-pairs and sedimentation coefficient of 11.4S. Analysis of the acid-soluble proteins of the core particles indicated that histones TH2B and X2 are constituents of the core particles, in addition to the somatic histones H2A, H2B, H3 and H4. The acid-soluble proteins of the oligonucleosomes comprised all the histones, including both the somatic (H1, H2A, H2B, H3, H4 and X2) and the testis-specific ones (TH1 and TH2B). It was also observed that histones TH1 and H1 are absent from the core particles and were readily extracted from the chromatin by 0.6M-NaCl, which indicated that both of them are bound to the linker DNA.

Spermatogenesis in mammalian testis is accompanied by a sequential transition of the basic proteins of the chromatin (Kumaroo *et al.*, 1975; Platz *et al.*, 1975; Meistrich *et al.*, 1978). Several elegant studies have shown that such replacement of the histones by more basic proteins eventually brings about the total condensation of the chromatin, with complete cessation of transcriptional activity (Dooher & Bennet, 1973; Kierzenbaum & Tres, 1975; Marushige & Marushige, 1975; Meistrich *et al.*, 1976).

Branson *et al.* (1975) had shown the presence of three testis-specific histones (X1, X2 and X3) together with the somatic histones in rat testis. Subsequent evidence, based on the amino acid composition of these testis-specific histones and on their property of co-purification with some specific somatic histones, had indicated that histones X1 (TH1) and X3 (TH2B) are actually testis variants of the somatic histones H1 and H2B respectively, and histone X2, which is also present in normal rat liver, though in a much smaller amount, behaves like histone H3 during fractionation (Branson *et al.*, 1975; Shires *et al.*, 1976). It has also been demonstrated that histones TH1 and X2 appear first in the spermatogonial cells of rat testis, whereas histone TH2B appears at a later stage, probably in the primary spermatocytes (Grimes *et al.*, 1975).

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Observations on the structural organization of the eukaryotic chromatin have led to the general concept of the nucleosome model as the basic structural repeat of the chromatin (Kornberg, 1977). According to this model the nucleosomes are made up of core particles containing an octamer of histones $(H2A, H2B, H3, H4)_2$, over which 146 base-pairs of DNA are wound, and two such core particles are connected by linear DNA of varying length to which histone H1 is bound (Kornberg, 1977; Lutter, 1978; Thoma *et al.*, 1979). The nucleosome type of chromatin organization has been shown to persist in the mammalian germ cells until the early round-spermatid stage, after which the organization of the chromatin changes markedly, resulting in the condensed chromatin containing smooth fibres (Kierzenbaum & Tres, 1975, 1978).

The possible significance of such a transition in the nuclear basic proteins resulting from the gradual and partial replacement of the somatic histones by the testis-variant histones appears to be obscure, although Branson *et al.* (1975) suggested that variant histones may play a role in the meiotic events. With a view to understanding the significance of such a programmed and well-regulated transition of the chromatin proteins, in the present work we attempted to study the structural localization of the testis-variant histones in the chromatin. The results reported here have indicated that, although histones TH2B and X2 are found in the

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core particles, histone TH1 is present in the linker regions of rat testis chromatin.

Materials and methods

Materials

Micrococcal nuclease, phenylmethanesulphonyl fluoride, acrylamide, *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethylethylenediamine ('TEMED') were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Bio-Gel A-5m was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. The sources of all other materials used here have been described previously (Rao *et al.*, 1979, 1980).

Treatment of animals

Male albino rats of this Institute strain, weighing 150 ± 10g and about 2–3 months old, were used throughout this work. All rats were killed by cervical dislocation and the testes were quickly removed, decapsulated and processed for the isolation of nuclei.

Isolation of nuclei and chromatin from rat testes

The nuclei were isolated from rat testes as described by Rao *et al.* (1980), except that 0.04M-$NaHSO_3$ was included in the homogenization buffer. Chromatin was prepared from the purified nuclei by the method described by Rao *et al.* (1979) for hen oviduct chromatin.

Salt extraction of the linker-bound proteins

The procedure used for extraction of the linker-bound histones of the chromatin was similar to that described by Oudet *et al.* (1975). The chromatin pellet was suspended in 10ml of 0.01M-Tris/HCl buffer, pH 7.4, containing 0.6M-NaCl and 0.002M-phenylmethanesulphonyl fluoride to give a final concentration of 20A_{260}units/ml, after which the suspension was slowly stirred overnight at 4°C. Then 2ml of this suspension was layered on a discontinuous gradient of 30% and 60% (w/v) sucrose in the same buffer (1.5ml each) and centrifuged at 84000***g*** for 2h in a Beckman ultracentrifuge (SW 50.1 rotor). The uppermost layer, containing the dissociated linker proteins of the chromatin, was removed along with 0.5ml of 30% sucrose, and the pellet at the bottom of the 60%-sucrose layer represented the chromatin fraction depleted of the linker proteins.

Isolation of the nucleosome core particles

This was done by the procedure of Martinson *et al.* (1979), except that 0.04M-$NaHSO_3$ was included in the digestion buffer and the amount of the micrococcal nuclease used was 5 units (1μmol of acid-soluble polynucleotides released from DNA/min) of the enzyme/100A_{260} units of the nuclei. Digestion was performed at 37°C until 20% of the DNA became acid-soluble, which was ascertained by the measurement of the A_{260} of a sample in 1M-$HClO_4$/1M-NaCl. The digestion was arrested by immediate chilling, which was followed by centrifugation at 27000***g*** for 15min.

At this stage the procedure described by Whittaker *et al.* (1979) for selective solubilization of the mononucleosomes and of the core particles was used as follows. The pellet obtained after the centrifugation was resuspended in 0.01M-Tris/HCl buffer, pH 7.5, containing 0.01M-EDTA, 0.04M-$NaHSO_3$ and 0.002M-phenylmethanesulphonyl fluoride. The suspension was centrifuged at 27000***g*** for 10min, and to the supernatant 0.5M-NaH_2PO_4 was added to give a final concentration of 0.1M. The pellet obtained after centrifugation at 27000***g*** for 10min contained oligonucleosomes, and the supernatant was enriched with the nucleosome core particles. The supernatant was dialysed against 0.01M-Tris/HCl buffer, pH 7.5, containing 0.01M-EDTA and 0.002M-phenylmethanesulphonyl fluoride, after which it was concentrated to 1–2ml by using polyvinylpyrrolidone-360. The concentrate was loaded on a column of Bio-Gel A-5m (100cm × 2.5cm), which had previously been equilibrated with the same Tris/HCl/EDTA/phenylmethanesulphonyl fluoride buffer, pH 7.5. The column was washed with the same buffer, and 3ml fractions were monitored at 260nm, after which the fractions under the symmetrical peak were pooled and concentrated against polyvinylpyrrolidone-360 to give a final concentration of 20–30A_{260}units/ml.

Sedimentation analysis of the core preparation

Sedimentation-velocity measurements were performed at 45000***g*** at 20°C with a single-sector 12mm cell in a Beckman model E ultracentrifuge equipped with Schlieren optics. Samples containing 0.9ml of freshly prepared core preparations (20A_{260}units/ml) were used in the study, and the sedimentation coefficient was calculated by the procedure of Schachman (1957).

Extraction and determination of the size of the DNA

DNA was extracted from the core preparation by using phenol/chloroform, as described by Rill *et al.* (1978), after which the length of the DNA was determined by electrophoresis of the sample in non-denaturing 3.5%-polyacrylamide slab gels (Maniatis *et al.*, 1975) with a sample of endonuclease-*Hae*III digest of phage-M13 DNA as the standard. Xylene cyanol and Bromophenol Blue were used as the tracking dyes, and the bands were detected under u.v. light after staining with 1μg of ethidium bromide/ml in water for 30min.

Extraction and analysis of the acid-soluble proteins

The acid-soluble proteins of the different chromatin fractions were extracted by the methods of Platz *et al.* (1977) and Rao *et al.* (1980) and were analysed by electrophoresis on 15%-polyacrylamide/acid/urea gels as described by Panyim & Chalkley (1969). At the end of the electrophoresis the gels were stained with 0.2% Amido Black in methanol/acetic acid/water (43:7:50, by vol.) for 30 min and destained with the same solvent. More satisfactory resolution of the core histones could be effected by previous electrophoresis of the proteins in 15% polyacrylamide/acid/urea disc gels for separation in the first dimension, which was followed by electrophoresis in the second dimension on 12% polyacrylamide/0.4% Triton X-100/acid/urea slab gels, as described by Candido *et al.* (1974). The second-dimension slab gels were stained and destained as described above.

Results and discussion

Isolation and characterization of the nucleosome core particles

The kinetics of digestion of the rat testis nuclei with micrococcal nuclease showed that the rate of digestion was linear up to 4 min, after which it decreased markedly. Analysis of the DNA of the digest on 3.5%-polyacrylamide gels indicated that the maximum yield of the mononucleosomes could be obtained at 20% acid-solubility of the DNA. A typical profile of the fractionation of the nuclease digest, obtained at 20% acid-solubility, on a column of Bio-Gel A-5m, as given in Fig. 1 closely agreed with that reported for a similar digest of chicken erythrocyte nuclei by Ramsay-Shaw *et al.* (1975). The major peak (peak C) in the column profile represented the mononucleosome fraction, and peaks A and B represented oligonucleosomes and dinucleosomes respectively, as evidenced by analysis of DNA on polyacrylamide gels (results not shown). On the other hand, peak D probably represented linker DNA with attached histones, and peak E contained mostly oligodeoxyribonucleotides. Fig. 1 also shows that, after solubilization of the nuclease digest in 0.1 M-NaH_2PO_4 and fractionation of the soluble material on the same column of Bio-Gel A-5 m, the oligonucleosome peak disappeared, and only a hump could be seen near the dinucleosome region. However, most of the material was eluted at the mononucleosome region.

The fractions marked by the arrows in peak C of Fig. 1 were pooled and concentrated for obtaining the limit digest of the chromatin, which contained the nucleosome core particles. The core particles thus prepared were characterized on the basis of the following criteria.

(*a*) Sedimentation-velocity studies at 45000 ***g*** in a Beckman model E analytical ultracentrifuge of the preparation suspended in 0.01 M-Tris/HCl (pH 7.4)/0.01 M-EDTA revealed that the particles sedimented as a single peak. Computation of the sedimentation coefficient, $s_{20,w}$, by the procedure of Schachman (1957) gave a value of 11.4 S, which agreed well with that reported for the core particles isolated from chicken erythrocytes (Rill *et al.*, 1978).

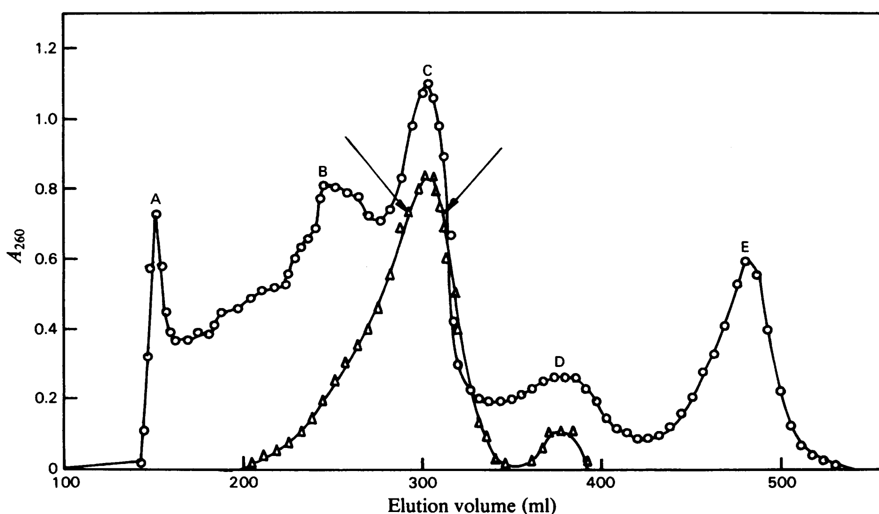

Fig. 1. *Gel-filtration profile of micrococcal-nuclease digest from rat testis nuclei*
The nuclease digest was chromatographed on a Bio-Gel A-5m column (100 cm × 2.5 cm) and 3 ml fractions were collected and monitored at 260 nm. ○, Total nuclease digest; △, digest soluble in 0.1 M-NaH_2PO_4 buffer, pH 7.5.

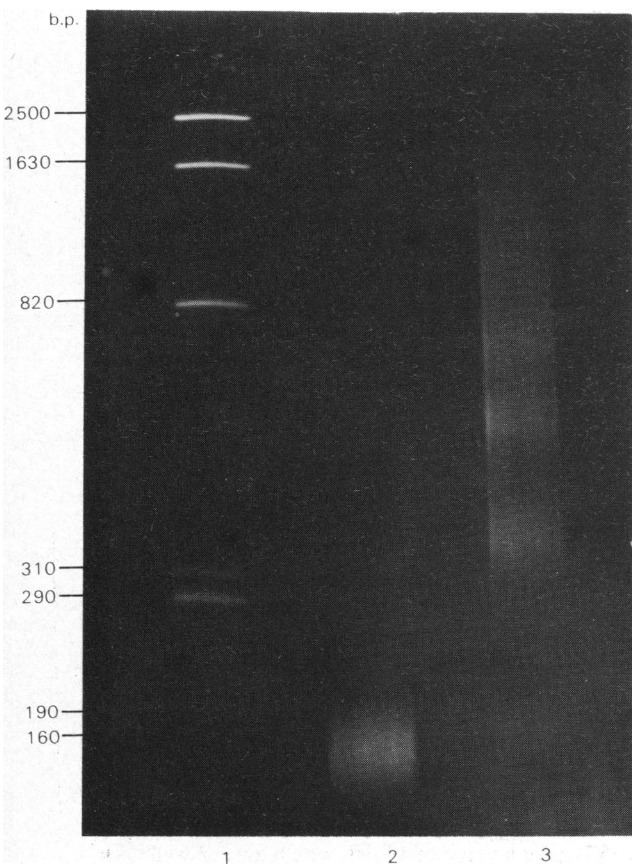

Fig. 2. *Electrophoretic patterns of the DNA fragments on polyacrylamide gels*

The DNA fragments from the nucleosome core and from the oligonucleosome preparations were electrophoresed on non-denaturing 3.5%-acrylamide gels as described in the Materials and methods section, with the *Hae*III digest of phage-M13 DNA as molecular-size markers. Abbreviation: b.p., base-pairs. Slot 1, *Hae*III digest of phage-M13 DNA (bands corresponding to DNA fragments of 190 and 160b.p. were too faint to be visible in the photograph, although their mobilities could be measured; DNA fragments corresponding to 120 and 70b.p. have run off the gel); slot 2, DNA fragment from the core particles; slot 3, DNA fragment from the oligonucleosomes.

(*b*) Analysis on a non-denaturing 3.5%-polyacrylamide gel of the DNA extracted from such core particles gave a single band (Fig. 2). Fig. 2 also shows the mobilities of *Hae*III restriction fragments of the phage-M13 DNA. By comparing the mobility of the nucleosome core DNA with those of phage-M13 DNA fragments, the size of the core DNA was calculated to be 151 ± 5 base-pairs, which is close to the actual size of the nucleosomal core DNA, 146-base-pairs (Lutter, 1978; Thoma *et al.*, 1979).

Fig. 2 also shows the pattern of the DNA isolated from the pellet obtained after the nuclease digest was adjusted to 0.1 M salt concentration with 0.5 M-NaH_2PO_4. The DNA pattern revealed a typical ladder profile corresponding to that given by di-, tri- and oligo-nucleosomes, with the conspicuous absence of the DNA fragments corresponding to those of mononucleosomes or of the core particles, which obviously proved the efficiency of the fractionation procedure used here. Calculations of the sizes of DNA in these oligonucleosomal fragments indicated that they were multiples of approx. 200 base-pairs.

Analysis of the total acid-soluble proteins isolated from the nucleosome core particles and oligonucleosomes

The electrophoretic patterns of the acid-soluble proteins isolated from the oligonucleosome preparation and from the total testis chromatin on acid/urea/15%-polyacrylamide gels given in Fig. 3(*a*) show that the oligonucleosome preparation had the full complement of histones corresponding to those found in total chromatin. On the other hand, similar analysis of the acid-soluble proteins of the nucleosome core particles revealed the complete absence of both histones H1 and TH1 (Fig. 3*b*), which would suggest that both histones are bound to linker DNA. The association of histones H1 and TH1 with the linker DNA was further substantiated by their susceptibility to extraction with 0.6 M-NaCl. The electrophoretic patterns of the acid-soluble proteins of the total testis chromatin and of the proteins that were extracted from the chromatin with 0.6 M-NaCl are presented in Fig. 3(*c*). It is clear that treatment of the chromatin with 0.6 M-NaCl led to a quantitative and selective extraction of histones H1 and TH1, whereas the residue left after the salt extraction had only traces of these histones.

Kumaroo & Irvin (1980) and Seyedin & Kistler (1980) have indicated that histone TH1, which appears as a single band during electrophoresis in an acid/urea gel system, actually consists of two proteins, H1t and H1a, which can be resolved by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. One of these proteins, H1a, which is a minor species in somatic tissues, shows considerable increase in its concentration in relation to other H1 histones at the time of the appearance of the primary spermatocytes. On the other hand, H1t, which is a testis-specific histone, becomes detectable at this particular stage and subsequently increases until the mid-spermatid stage. Since, in the studies reported here, only trace amounts of histone TH1 were present in the chromatin residue left after

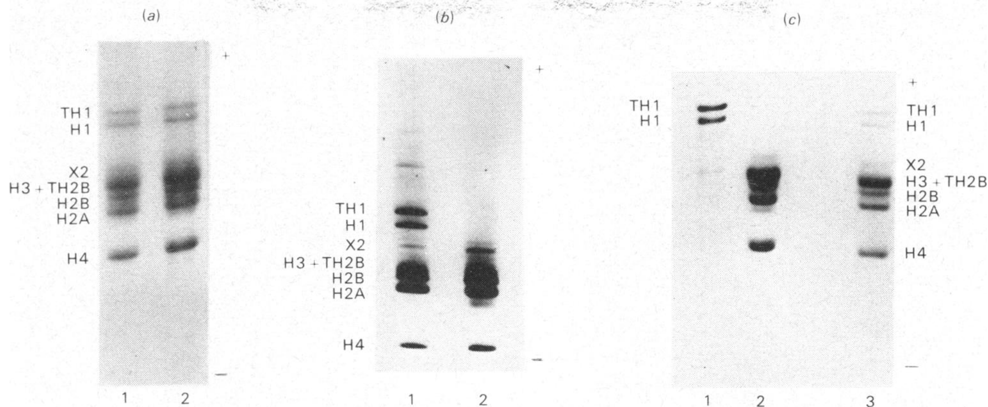

Fig. 3. *Analysis of acid-soluble proteins on acid/urea/polyacrylamide gels*
Electrophoretic analysis on 15%-polyacrylamide/acid/urea gels of the acid-soluble proteins isolated from: (*a*) slot 1, oligonucleosomes; slot 2, total testes chromatin; (*b*) slot 1, total testes chromatin; slot 2, nucleosome core preparation; and (*c*) slot 1, 0.6M-NaCl extract of the chromatin; slot 2, residue left after the salt extraction; and slot 3, total testes chromatin.

0.6M-NaCl extraction and was completely absent from the nucleosome core particles, it is likely that both histones H1a and H1t are bound to the linker DNA.

For analysis of the core histones the one-dimensional gel electrophoresis proved to be inadequate, and hence the two-dimensional electrophoretic separation method described by Candido *et al.* (1974) was used. In this method the separation of the proteins is performed in the first dimension in the regular acid/urea system of Panyim & Chalkley (1969), and separation in the second dimension is effected at acidic pH and in the presence of 7.5M-urea and 0.4% Triton X-100, in which case the hydrophobicity of the proteins also determines their mobilities.

A typical two-dimensional electrophoretic pattern of the acid-soluble proteins of the total rat testis chromatin is shown in Fig. 4(*a*). The positions of the testis-specific histones in the electrophoretogram were ascertained by comparing the gel patterns of the acid-soluble proteins of the chromatin of rat testis with that obtained for the acid-soluble proteins of rat liver chromatin (Fig. 4*b*). The positions of the somatic histones as represented in Fig. 4(*b*) are comparable with those obtained by Goodwin *et al.* (1979) for rabbit thymus histones under similar electrophoretic conditions. The electrophoretic mobility of histone TH2B was also confirmed by running a parallel gel (Fig. 4*c*) of a mixture of histones H2B, TH2B and trace amounts of histone H3 purified from rat testis chromatin on a column of Bio-Gel P-60 by adapting the method of Bohm *et al.* (1973). Analysis on the two-dimensional gel of the acid-soluble proteins obtained from the nucleosome core preparation of rat testis chromatin, which had already been characterized by sedimentation studies and by DNA length, gave the pattern shown in Fig. 4(*d*); histones TH2B and X2 are constituents of the core particle. The subspecies of histones H2A and H3 indicated in Fig. 4 as H2A.1, H2A.2, H3.1 and H3.2 may represent the modified forms that are separated during electrophoresis in the second dimension.

The localization of histone TH1 in the linker region and of histones TH2B and X2 in the core particle, as observed here, raises the question as to whether the testis-specific histones are additional proteins on the same nucleosome. Such a possibility appears unlikely, because the progressive increase in the histone variants is accompanied by a gradual but concomitant decrease in the amounts of the somatic counterparts (Grimes *et al.*, 1975). On the other hand, since each core particle is made up of two molecules of each of histones H2A, H2B, H3 and H4, the question would still remain as to whether the somatic histones and the testis-specific histones coexist in the same core particle or whether the core particles containing the somatic and testis-specific histones are two separate entities.

The very fact that both somatic and testis-specific histones are present within the pachytene spermatocytes (Platz *et al.*, 1977; B. J. Rao & M. R. S. Rao, unpublished work) would suggest that the appearance of the testis-specific histones might lead to the formation of specific nucleosomal loci with altered properties during the process of meiotic division (Branson *et al.*, 1975). Whatever might be the subtle roles played by these testis-variant histones in the meiotic events, these should be ascribed to the

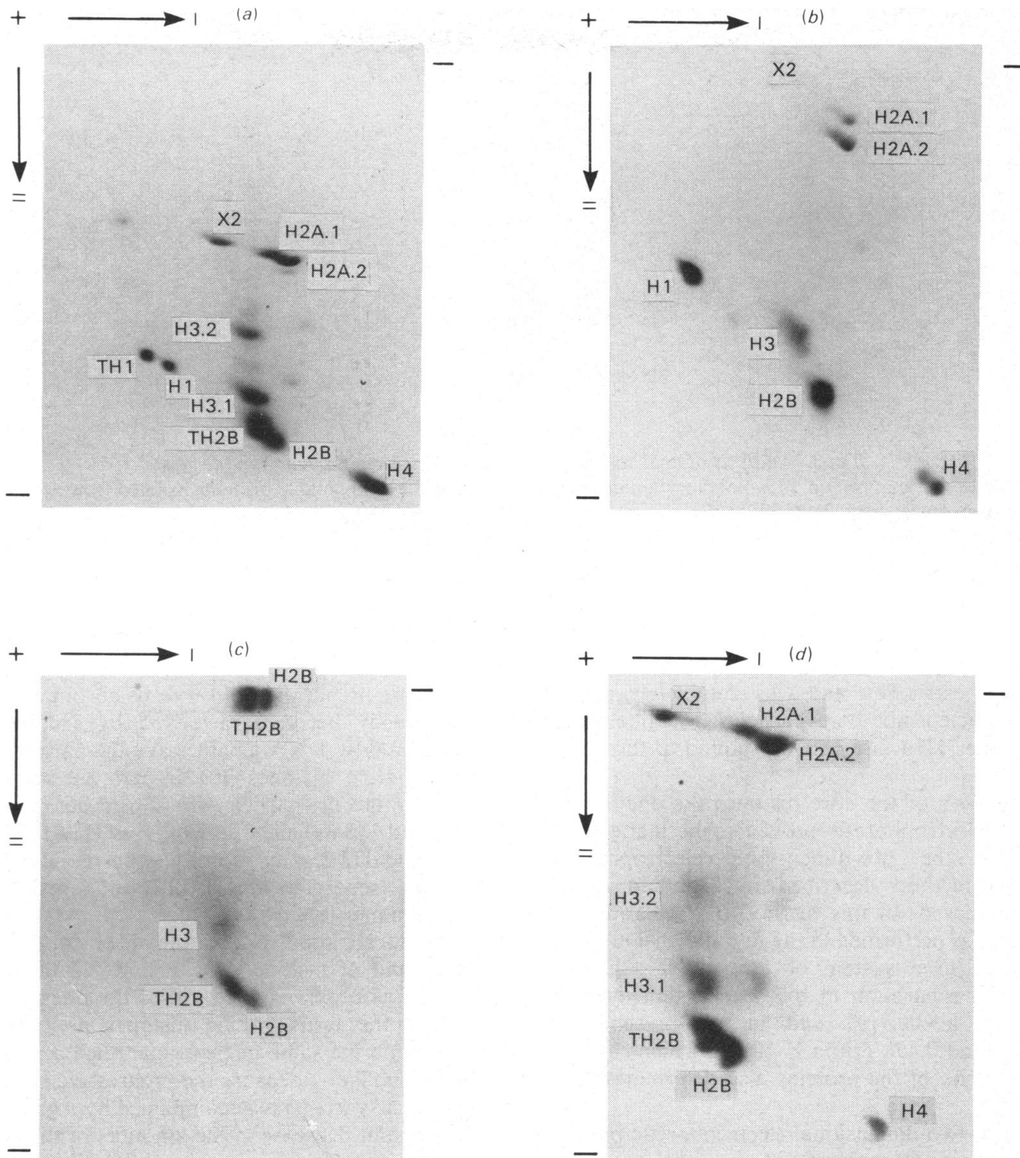

Fig. 4. *Analysis of acid-soluble proteins on two-dimensional polyacrylamide gels*
Two-dimensional electrophoretic analysis of the acid-soluble proteins isolated from (*a*) total rat testes chromatin, (*b*) total rat liver chromatin, (*c*) partially purified H2B, TH2B preparation and (*d*) nucleosome core preparation of rat testes chromatin.

special structural features conferred by them on the organization of the chromatin at the mononucleosomal level or at the higher-order level. Such structural features could be a variation in the length of the linker DNA owing to the tissue-specific histone H1t (Noll, 1976). The appearance of the testis-specific core histone TH2B together with the increase in histone X2 might bring about some changes in the conformational features of the core particles. Indeed, Anguravirutt & Svasti (1981) have observed that, compared with histone H2B, TH2B is eluted at a higher salt concentration from sheared rat testis chromatin that had been adsorbed on hydroxyapatite, which suggested that the strength of the interaction of histones H2B and TH2B with the nucleosomal DNA may be different.

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