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SEPARATION AND QUANTIFICATION OF HISTONE H1 SUBTYPES AND HIGH-MOBILITY-GROUP PROTEINS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY: PROTEIN LEVELS IN RAT TISSUES DURING POSTNATAL DEVELOPMENT

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SUMMARY

The rapid separation and quantification of histone H1 subtypes and high-mobility-group (HMG) chromatin proteins by reversed-phase liquid chromatography on a butylsilica-based column is described. The proteins were fractionated by means of a multi-step acetonitrile gradient containing 0.1% trifluoroacetic acid. This system is capable of resolving the four main HMG proteins (1, 2, 14 and 17), HMG I, protein P1 with HMG 18 and HMG 19 (in one peak) and five histone H1 subtypes in a single 33-min analysis. This method was used to study levels of these chromosomal proteins in nuclei of rat liver, spleen, testis and thymus during postnatal development from 1 to 20 weeks of age. Although no clear tissue specificity of the HMG proteins was apparent, there were significant differences in the relative amounts of these proteins in different tissues. The relative amount of HMG 1 increased from 1 to 12 weeks of age and decreased thereafter, whereas those of HMG 14 and HMG 17 remained almost unchanged. Marked quantitative differences were observed in the five histone H1 subtypes in different tissues. The largest changes in their levels during development were found in the liver and the smallest changes in the thymus. The changes in the spleen and testis were intermediate. These results suggest that the changes in the relative amounts of histone H1 subtypes and HMG proteins observed during postnatal development of the rat may result from differences in the structure of chromatin in these tissues and thus reflect the activity of molecular mechanisms involved in replication and differentiation of the cells.

INTRODUCTION

The structure of chromatin and the regulation of gene expression are determined by a variety of interactions between nucleic acid and protein components of the genome. A predominant class of chromosomal protein is histones [1]. Histone H1 has a dual role in determining the structure of chromatin. It binds to DNA at points of entry into and exit from the nucleosome affecting nucleosome conformation. In addition, H1 is essential for the formation and maintenance of

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higher-order coiling of nucleosome chains [2]. Various subtypes of histone H1 differ in their ability to condense DNA and small chromatin fragments [3,4]. Therefore, they have the potential to cause different parts of chromatin to be condensed to different extents. The proportions of the various H1 subtypes vary from organ to organ in adult animals and even within the same organ during development [4–6].

High-mobility-group (HMG) proteins, named according to their migration behaviour in acetic acid–urea polyacrylamide gels, are the best characterized non-histone proteins (for a review, see ref. 7). The four main proteins of this group (HMG 1, 2, 14 and 17) are present in most eukaryotic cells. The amino acid sequences of these proteins are relatively well conserved and contain several functional domains, which enable them to interact with DNA, histones and other components of the genome. The high-M_r HMG proteins HMG 1 and HMG 2 (M_r ca. 30 000) bind to single-stranded DNA and affect the superhelicity of DNA [8,9]. They are also able to distinguish between various types of single-stranded regions of the genome [10]. The low-M_r HMG proteins HMG 14 and HMG 17 (M_r ca. 10 000) have been implicated in modifying the structure of transcriptionally active genes [11]. In addition, some minor components (HMG 18, 19A and 19B) from calf thymus [12] and two new low-M_r proteins (HMG I and HMG Y) from proliferating cells have been characterized [13].

Both histone H1 and HMG proteins are extractable from nuclei with 5% perchloric acid (PCA) [14]. We have applied a reversed-phase high-performance liquid chromatographic (HPLC) system to analyse these PCA-soluble nuclear proteins from various tissues of the rat in order to determine whether the same H1 subtypes and HMG proteins exist in these tissues, whether the proportions of these proteins are different in different tissues and what changes occur as the proportion of non-dividing cells increases in tissues during development.

EXPERIMENTAL

Materials

Acetonitrile (HPLC grade) was purchased from Baker Chemicals (Deventer, The Netherlands) and trifluoroacetic acid (TFA) (Uvasol grade) from E. Merck (Darmstadt, F.R.G.). Phenylmethylsulphonyl fluoride (PMSF) and ubiquitin were obtained from Sigma (St. Louis, MO, U.S.A.). Reagents for polyacrylamide gel electrophoresis (PAGE) were purchased from Bio-Rad Labs. (Richmond, VA, U.S.A.). Male rats (Wistar BD-IX) of different ages (1, 4, 8, 12 and 20 weeks) were obtained from the Experimental Animal Centre of the University of Kuopio (Kuopio, Finland). The animals were kept under controlled conditions of temperature (18–22°C), humidity (50–70%), light (from 7 a.m. to 9 p.m.) and fed ad libitum with a standard diet (Hankkija, Turku, Finland). The rats were killed by decapitation and the tissues immediately removed, frozen in liquid nitrogen and stored at −80°C until use.

Preparation of nuclei and isolation of PCA-soluble proteins

All steps were performed at 0–4°C. Tissues (pooled from 5–30 animals) were homogenized with a Potter–Elvehjem homogenizer in three volumes of 0.25 *M*

sucrose, 50 m*M* Tris–HCl (pH 7.5), 25 m*M* KCl and 3 m*M* $MgCl_2$ containing 0.4 m*M* PMSF. The homogenate was filtered through four layers of cheese cloth and centrifuged at 1000 *g* for 10 min. The crude nuclear pellet was homogenized in nine volumes of 2.3 *M* sucrose, 10 m*M* Tris–HCl (pH 7.5) and 3 m*M* $MgCl_2$ containing 0.2 m*M* PMSF and centrifuged at 40 000 *g* for 60 min. The nuclei were washed twice with 0.34 *M* sucrose, 10 m*M* Tris–HCl (pH 7.5) and 3 m*M* $MgCl_2$ containing 0.2 m*M* PMSF and centrifuged at 1000 *g* for 10 min. The HMG proteins and histone H1 subtypes were extracted from the nuclei immediately after preparation with 5% PCA at 0°C. These PCA-soluble proteins were precipitated with six volumes of acidified acetone and processed as described previously [15].

High-performance liquid chromatography and polyacrylamide slab gel electrophoresis

A Hewlett-Packard 1090 liquid chromatograph equipped with a Varian 4270 integrator was used in all determinations. Separations were performed on a Vydac C_4 column (250 mm×4.6 mm I.D., 5 μm particle size, 330 Å pore size). Lyophilized protein samples were dissolved at a concentration of 4 mg/ml in water containing 0.1% TFA and centrifuged in Eppendorf centrifuge at 16 000 *g* for 5 min before analysis. The samples were injected into the column using a 20-μl loop. The proteins were eluted at a flow-rate of 0.5 ml/min at an oven temperature of 37°C using a multi-step linear gradient from 0.1% TFA and 7% acetonitrile (in water) to 0.1% TFA and 70% acetonitrile. Both solvents were filtered through 0.5-μm Millipore FHLP filters before use. The concentration of acetonitrile was increased linearly as follows: from 7 to 19.6% in 8 min, from 19.6 to 26.6% in 2 min, from 26.6 to 36.4% in 18 min and from 36.6 to 70% in 1 min. The eluted proteins were detected by their UV absorption at 214 nm and the peaks were quantified on the basis of their area using Varian 4270 integrator. The lyophilized protein peaks were identified by PAGE in acetic acid–urea slab gels containing 15% acrylamide, 0.1% bisacrylamide and 2.5 *M* urea [16,17]. Purified HMG proteins and ubiquitin from calf thymus were used as markers.

RESULTS

Ion-pair reversed-phase HPLC on a Vydac C_4 column using a multi-step acetonitrile gradient is capable of resolving, in addition to the four main HMG proteins (1, 2, 14 and 17), HMG I, protein P1 with HMG 18 and HMG 19 (in one peak) and five subtypes of histone H1 in a single 33 min analysis (Fig. 1). The proteins from the collected peaks were further analysed by acetic acid–urea PAGE. The analysis of peaks from a thymus sample is shown in Fig. 2. The retention times of the peaks were reproducible to within 0.30 min. Oxidized and reduced forms of HMG 1 and HMG 2 can also be separated by this chromatographic technique (Fig. 1). The oxidized forms (peaks 5 and 8, respectively) disappeared completely when the samples were incubated overnight in the presence of 2 m*M* dithiothreitol and 1 m*M* EDTA at room temperature (not shown).

All these proteins, except histone H1° in thymus (peak 7a), were present in nuclei from the four tissues examined (liver, spleen, testis and thymus). HMG

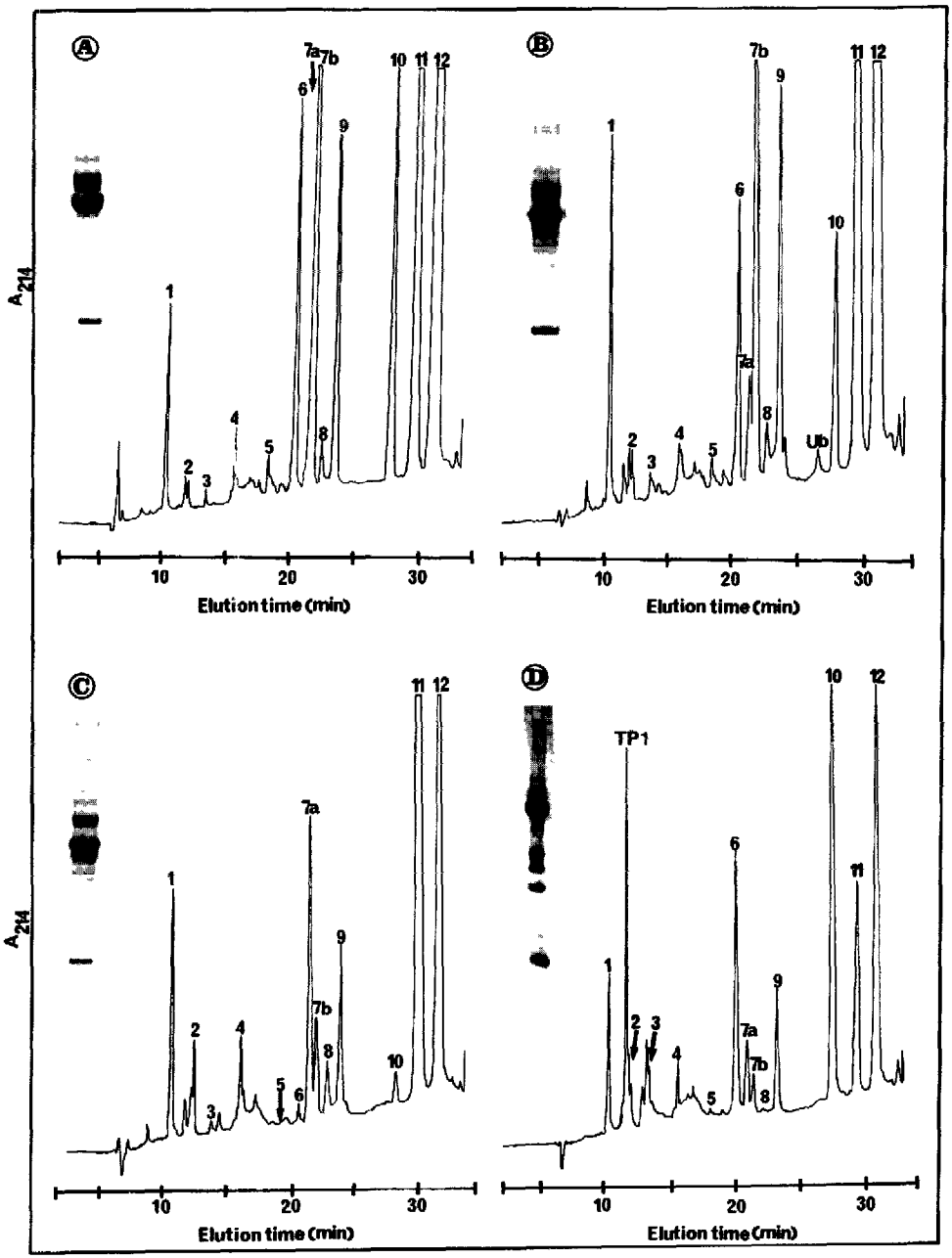

Fig. 1 HPLC elution profiles (absorbance at 214 nm) of PCA-soluble nuclear proteins (HMG proteins and histone H1 subtypes) from 12-week-old rat thymus (A), spleen (B), liver (C) and testis (D). Acetic acid–urea polyacrylamide gel of each sample is shown on the left of the elution profile. Peaks: 1 = HMG 17; 2 = HMG 14; 3 = HMG I; 4 = protein P1 with HMG 18 and HMG 19; 5 = oxidized HMG 2; 6 = HMG 2; 7a = histone H1°; 7b = histone H1A; 8 = oxidized HMG 1; 9 = HMG 1; 10 = histone H1B; 11 = histone H1C; 12 = histone H1D. In addition to the these peaks, the spleen sample contained ubiquitin (Ub) and the testis sample TP 1-protein.

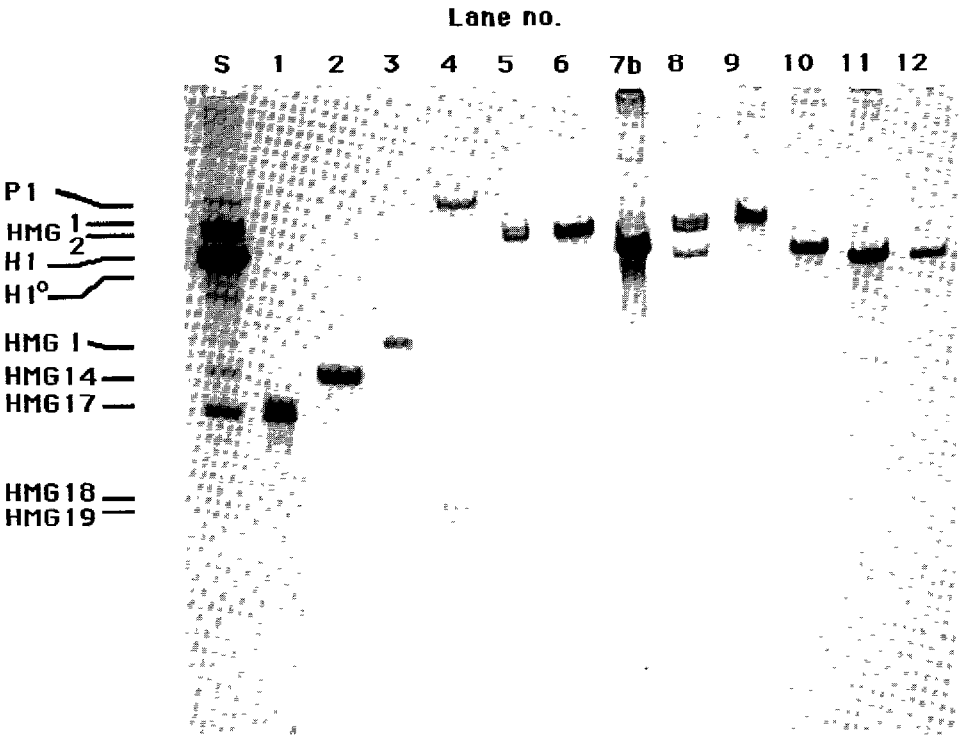

Fig. 2 Identification of HPLC-purified HMG proteins and H1 subtypes from 12-week-old rat thymus by acetic acid–urea gel electrophoresis. The numbers of the lanes in the gel correspond to those of the peaks in Fig. 1A. Lane S: PCA-soluble proteins from rat thymus nuclei.

14 from the testes of animals older than 4 weeks coeluted from the column with a testis-specific protein, putatively identified as TP 1 protein [18] and, therefore, HMG 14 in testis was not quantified in this study. This testis-specific protein seems to be associated with spermiogenesis, as it was not found in nuclei from testes of 4-week-old rats but was present in those from of 8-week-old animals. Two other testis-specific PCA-soluble proteins, TP 2 and TP 3 [18], eluted with HMG I (Fig. 1C). These proteins obscured the region containing HMG I in acidic gels, and the presence of HMG I in the testes of the animals older than 4 weeks, although likely, could not be equivocally proved in this study. HMG 14 from liver, spleen and thymus (and testes of 4-week-old animals) was resolved into two peaks, which were not separated by acetic acid–urea gel electrophoresis.

The relative levels of HMG 14 and HMG 17 remained almost unchanged during postnatal development of the rat until the age of 20 weeks (Fig. 3). However, the amount of HMG 1 in the liver and thymus doubled from 1 to 12 weeks of age, and thereafter the relative amount of HMG 1 decreased in the tissues studied. The level of HMG 2 was much lower in the liver than in other tissues. There was a decline in the level of HMG 2 in the spleen after the age of 4 weeks and in the thymus and testis after the age of 12 weeks, whereas in the liver its level remained almost constant throughout the experiment.

Significant tissue differences were observed in the distribution of the five his-

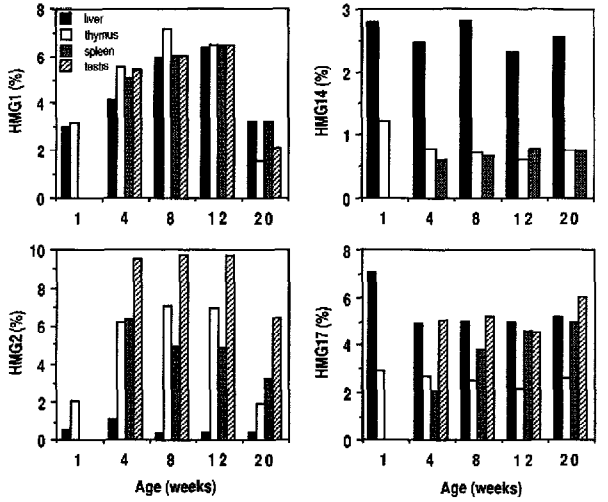

Fig 3. HMG proteins in nuclei from rat liver, spleen, thymus and testis during postnatal development. From 1-week-old animals, only the liver and thymus were studied. The proteins were analysed by reversed-phase HPLC as described under Experimental. The values are expressed as percentages of total PCA-soluble nuclear proteins.

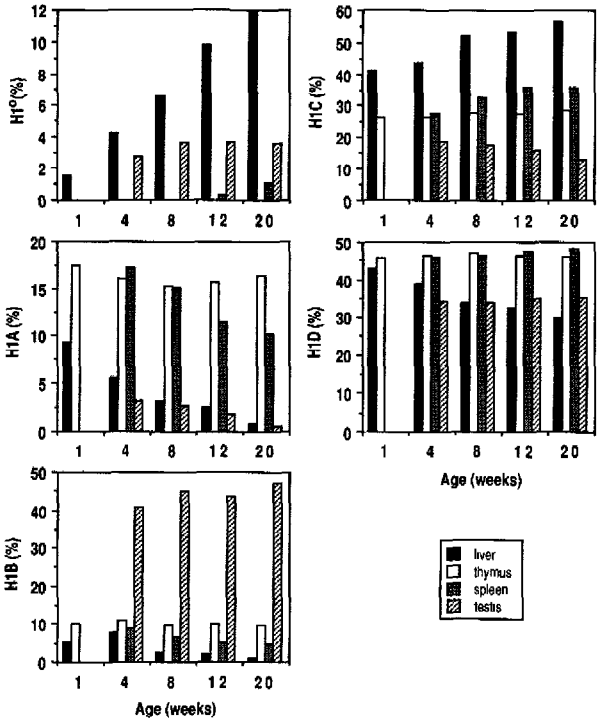

Fig. 4. Quantification of histone H1 subtypes from various rat tissues during postnatal development. From 1-week-old animals, only the liver and thymus were studied. The values are expressed as percentages of total histone H1.

tone H1 subtypes; histone H1° and histones H1A, H1B, H1C and H1D (in order of their elution) (Fig. 4). The amount of histone H1°, which migrates slightly faster than the main histone H1 band in the acetic acid–urea gels, increased about eight-fold in the liver during postnatal development and constituted about 12% of the total histone H1 in 20-week-old rats. The amount of H1A in the liver of 20-week-old rats was one tenth of that in 1-week-old animals but decreased to only a small extent in the spleen and testis during postnatal development. H1B was the main H1 subtype in the testis, constituting nearly half of the total H1, whereas in the liver, spleen and thymus H1C and H1D were the main subtypes. The amount of H1C increased and that of H1D decreased in the liver during growth. In contrast to the liver, the levels of the H1 subtypes in the thymus remained almost unchanged during postnatal development. Treatment of the PCA-soluble nuclear proteins with alkaline phosphatase before HPLC separation did not affect the elution behaviour of the histone H1 subtypes, indicating that they are not differentially phosphorylated variants of histone H1.

DISCUSSION

The simultaneous analysis of histone H1 subtypes and HMG proteins has previously been laborious and time-consuming. Gurley et al. [19] fractionated PCA-soluble chromatin proteins from CHO cells into five fractions (HMG E/G, H1°, H1, HMG 2 and HMG 1) using a μBondapak CN column and a 0–50% linear gradient of acetonitrile containing 0.2% TFA in 5 h. Lennox and Cohen [6] used two-dimensional PAGE (acetic acid–urea in the first and SDS in the second dimension) to resolve histone H1 from mouse tissues into five major spots (a, b, c, d and e), and H1°, for which they quantified fractions H1°, H1a+c, H1b and H1d+e. Using ion-exchange chromatography, it has also been possible to separate the five major H1 subtypes [5]. Histone H1 from mouse tumour cells has been separated into four subtypes by RP-HPLC using a C_{18} column and an acetonitrile gradient with 0.1 *M* sodium perchlorate–0.1% phosphoric acid in water [20]. HMG proteins have previously been separated using a C_4 column and a linear gradient from 15 to 50% acetonitrile in 0.1% TFA in 60 min [21]. Using a multi-step acetonitrile gradient as described under Experimental, we were able to separate and quantify the HMG proteins and the five histone subtypes in a single analysis in about 30 min. This provides a fast and sensitive procedure for the simultaneous analysis of the relative levels of these proteins from tissues and cell culture samples. The method can also be used for the micro-scale purification of these proteins from PCA extracts of nuclei in one step.

Previous studies of HMG proteins from calf tissues using CM-Sephadex chromatography and gel electrophoresis have not indicated tissue specifity [22], and our results for rat tissue agree with those findings. Nuclei from all the tissues studied contained the four main HMG proteins (1, 2, 14 and 17), HMG I and a PCA-soluble HMG-like phosphoprotein P1, which was recently described by Østvold et al. [23]. In addition to these proteins, nuclei from rat testis contained

spermatid-specific transition proteins TP 1, TP 2 and TP 3, which may be involved in nuclear condensation and shaping of the sperm head [18].

Although no clear tissue specificity of the HMG proteins was apparent, there were significant differences in the relative amounts of these proteins in different tissues, as nuclei from the liver contained about three times more HMG 14 and HMG 17 than e.g., those from the thymus. Nuclei from testes derived from 20-week-old animals contained about ten times more HMG 2 (μg protein per mg DNA) than those from the liver. The HMG 2-to-HMG 1 ratios in the liver, spleen, thymus and testis of 20-week-old animals were 0.1, 1.0, 1.2 and 3.1, respectively. These values resemble those from whole tissue extracts of adult rats as described by Bucci et al. [24]. Their results demonstrated that the amounts of HMG 2 in non-proliferative tissues are low, whereas the HMG 1 levels did not show a marked organ variability [24]. Our results suggest that the amount of HMG 1 is correlated with the rate of tissue development during postnatal growth, whereas those of HMG 14 and HMG 17 remain almost unchanged. These results also imply that HMG 1 and HMG 2 could have different functions in cell proliferation. Shastri et al. [25] have previously shown that chromatin from rapidly dividing rat hepatoma cells contains eight times more HMG 1 and HMG 2 than chromatin from adult liver. Antibodies against HMG 1 have been shown to inhibit the incorporation of [^{3}H]thymidine into DNA in an in vitro replication system with Ehrlich ascites cell nuclei [26]. Electron microscope immunocytochemistry and autoradiography have revealed that, during SV40 lytic infection, HMG 1 accumulates at sites of active viral DNA replication [27].

Quantitative tissue differences were also observed in the distribution of the histone H1 subtypes. The largest changes occurred in the liver, whereas essentially no changes were found in the thymus during postnatal development. The amount of H1°, which was originally found from mammalian tissues with little cell division [28] and which is associated with repressed α-foetoprotein gene in adult mouse liver [29], increased about eight-fold in the liver in rats from 1 to 20 weeks of age. At the same time the amount of H1A, which probably corresponds to H1a in the nomenclature of Lennox and Cohen [6], decreased to one tenth of the original level. These results resemble those from mouse liver using two-dimensional gel electrophoresis and densitometry of stained spots [6]. The changes in the relative amounts of H1 subtypes and HMG proteins observed during postnatal development of the rat may result from fine differences in the structure of chromatin and thus reflect the activity of molecular mechanisms involved in the replication and differentiation of the cells.

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