Separation of rat tissue histone H1 subtypes by reverse-phase h.p.l.c.

Identification and assignment to a standard H1 nomenclature

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H1 histones from rat liver and rat testis were separated by reverse-phase h.p.l.c. Within 40 min six subfractions (H1⁰, H1b, H1a, H1d, H1e + H1c and H1c) and seven subfractions (H1⁰, H1b, H1a, H1d, H1e + H1c, H1c and H1t) respectively were isolated by using a linear acetonitrile gradient. Each individual H1 subtype was identified either by comparing the H1 variants (contained in both tissues but in different quantities) or by SDS/PAGE and acetic acid/urea/PAGE. Moreover, all H1 variants were characterized by amino acid analyses. The amino acid compositions of rat histone subfractions H1⁰, H1b and H1e were determined for the first time. It was possible to classify unambiguously the H1 subfractions obtained by h.p.l.c. by following the standardized H1 nomenclature for electrophoretic systems recommended by Lennox, Oshima & Cohen [(1982) *J. Biol. Chem.* **257**, 5183–5189]. Incorrect assignments that have been made in various publications are discussed.

INTRODUCTION

The very-lysine-rich histones H1 play a fundamental role in organizing the structure of chromatin [1–4]. The H1 histones are located in the region of the exit and entry points of DNA, stabilizing the nucleosome [2,4]. Moreover, the H1 histones participate in forming higher-order chromatin structures [3,4]. Chromatographic and electrophoretic studies demonstrated that the H1 histones can be resolved into several closely related subtypes, which vary in amino acid sequences and molecular size and which, moreover, differ as to their ability to condense DNA (for reviews see refs. [5] and [6]). The microheterogeneity of these histones has important biological consequences (for review see ref. [6]). Unfortunately, no uniform well-defined nomenclature for H1 subtypes has so far been established. Thus, for example, the respective proteins were designated in some instances H1a, H1b, H1c etc. [7–9] whereas in others H1A, H1B, H1C etc. [10–12] or H1.1, H1.2, H1.3 etc. [13,14]. To cite one example, subfraction H1a may or may not be identical with subfraction H1A or subfraction H1.1. Moreover, sometimes the forms designated as H1 subtypes are mixtures and composed of more than one variant [10–12,15].

In their attempt to establish a species-independent nomenclature for H1 subtypes from higher eukaryotes analysed by gel electrophoresis Lennox *et al.* [16] followed the Seyedin & Kistler designation [17] and proposed to standardize the H1 nomenclature as H1a, H1b, H1c, H1d and H1e (and H1⁰ and H5). They separated H1 histones of five mammalian species in two-dimensional gels and correlated each subtype with metabolic and developmental properties [16,18]. At present this nomenclature has been increasingly used for gel electrophoresis.

As has recently been demonstrated, reverse-phase h.p.l.c. is a fast, sensitive and reliable method for analytical and preparative separations of total histones [19–23] and very-lysine-rich histones [11,12,21,22]. The biological results obtained by these different analytical methods cannot readily be compared, however. Thus Kurokawa & MacLeod [24], Karhu *et al.* [11] and Quesada *et al.* [15] tried to set up a correlation between the H1 subfractions

separated by h.p.l.c. and the nomenclature systems of Seyedin & Kistler [17] and Lennox *et al.* [16]. However, several fundamental questions were left open. The present paper attempts to establish the essential facts. We therefore fractionated H1 histones from rat liver and testis by reverse-phase h.p.l.c. Our assignments to the H1 nomenclature following the one proposed for gel electrophoresis by Lennox *et al.* [16] are derived from the different but characteristic contents of H1 variants of two rat tissues, from two types of gel electrophoreses and from amino acid analyses of each H1 subtype.

MATERIALS AND METHODS

Chemicals

H.p.l.c.-grade acetonitrile (type S) was purchased from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland, U.K.), and the water was from Promochem (Wesel, Germany). The trifluoroacetic acid was obtained from Sigma Chemie (Munich, Germany).

Preparation of H1 histones

The rat livers and testes were homogenized in 2 vol. of buffer A [0.25 M-KCl/5 mM-MgCl₂/50 mM-Tris/HCl buffer (pH 7.9)/0.1 mM-phenylmethanesulphonyl fluoride/1 mM-2-mercaptoethanol] in a Potter homogenizer. After centrifugation at 800 g for 5 min, the cells were washed twice with buffer A and then homogenized in buffer A containing 0.1 % Nonidet P40 in a Dounce homogenizer. Nuclei thus obtained were collected by centrifugation at 900 g for 10 min, and the nuclear pellet was washed once with buffer A without Nonidet P40. The pellet was centrifuged down and thereupon extracted with 1 vol. of 10% (w/v) HClO₄ and 2 vol. of 5% (w/v) HClO₄ for 1 h with occasional vortex-mixing. HClO₄-insoluble material was removed by centrifugation at 12000 g for 10 min, and soluble proteins were precipitated overnight at –20 °C by addition of 4 vol. of acetone/conc. HCl (49:1, v/v). The H1 histones were

Abbreviation used: AU/PAGE, acid/urea/polyacrylamide-gel electrophoresis.

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centrifuged down at 20000 *g* for 15 min, washed twice with pure acetone, dissolved in 1 mM-2-mercaptoethanol and freeze-dried. Except for the precipitation by acidified acetone all procedures were done at 0–4 °C.

H.p.l.c.

All h.p.l.c. experiments were performed on a Beckman h.p.l.c. gradient system with two 114 M pumps and a 421 A system controller. The eluate was monitored by the absorbance at 210 nm with a model 165 variable-wavelength u.v.–visible-light detector. The detector signal was documented on a Shimadzu C-R3A integrator utilizing an automatic baseline correction. The histone separations were performed on a Nucleosil 300-5 C₄ column (4 mm internal diam. × 100 mm; 5 µm particle size; 30 nm pore size; end-capped). The freeze-dried proteins were dissolved in water containing 0.1% (v/v) trifluoroacetic acid and 100 µg samples of histones were injected on to the column. The histones were chromatographed within 45 min at room temperature and at a constant flow of 0.5 ml/min, with a linear gradient from 60% (v/v) solvent A/40% solvent B [solvent A, 0.1% (v/v) trifluoroacetic acid in water; solvent B, 0.1% (v/v) trifluoroacetic acid in 70% (v/v) acetonitrile] to 42% (v/v) solvent A/58% (v/v) solvent B.

Gel electrophoresis

H1 histone fractions from the h.p.l.c. runs were collected, freeze-dried and stored at –20 °C. The histones were identified by using both SDS/PAGE by the method of Laemmli [25] (15% polyacrylamide/0.1% SDS) and AU/PAGE as described by Lennox *et al.* [16] (15% polyacrylamide/0.9 M-acetic acid/2.5 M-urea). The gels were stained for 1 h with 0.1% Serva Blue R in 40% (v/v) ethanol/5% (v/v) acetic acid and destained overnight in 20% (v/v) ethanol/5% (v/v) acetic acid.

Amino acid analysis

The proteins of all fractions obtained were rechromatographed, freeze-dried, hydrolysed in 6 M-HCl at 110 °C for 24 h and analysed with an amino acid analyser (model LC 5001 Biotronik; Braun Diessel Biotech, Melsungen, Germany). No corrections for destruction or incomplete hydrolysis were applied.

RESULTS

A special reverse-phase h.p.l.c. system was established to separate H1 histones from rat liver nuclei (Fig. 1a). The fractions corresponding to the six peaks (p1–p6) obtained within 40 min were freeze-dried and analysed by SDS/PAGE (Fig. 1b) with the following result. The histone of peak p1 migrated most rapidly and was thereby identified as pure histone H1⁰. The electrophoretic mobility of the histone of peak p6 was less than that of histone H1⁰ but faster than the ones of peaks p2–p4. Consequently, it could be only the histone H1c according to the Lennox *et al.* terminology for H1 histones [16]. Subfraction p5 consisted of two proteins, i.e. histone H1c and a slower-migrating histone variant, which could be either H1a, H1b, H1d or H1e. By means of the SDS/PAGE analysis alone it was difficult to make a more detailed assignment of this histone, as well as those of peaks p2–p4. A h.p.l.c. fractionation of H1 histones from rat testis nuclei ought to clarify the proper assignment of histones H1a and H1b, because the content of histone H1a is only 4% in rat liver but 36% in rat testis, as documented by earlier studies [26,27]. Moreover, histone H1b is absent [27] or present only in small quantities [11] in rat testis of mature animals. However, rat testis contains the H1 variant H1t, not detected in a variety of other rat organs [17]. The elution profile is shown in Fig. 2(a). In fact, in comparison with Fig. 1(a) a dramatic increase in the

Fig. 1. Separation of histones H1 from rat liver nuclei by reverse-phase h.p.l.c. and analysis of the subfractions by SDS/PAGE

(a) Separation of histones H1 from rat liver nuclei by reverse-phase h.p.l.c. The column (4 mm × 100 mm) contained Nucleosil 300-5 C₄ and the flow rate was 0.5 ml/min with the linear acetonitrile gradient (-----) as described in the Materials and methods section. Absorbance was monitored at 210 nm. In order to obtain subfractions p1–p6, 100 µg of histones H1 was injected on to the h.p.l.c. column. Numbered fractions were freeze-dried and subjected to SDS/PAGE analysis. (b) SDS/PAGE of fractions p1–p6 from the separation described in (a). Lane M, total histones H1 (15 µg) from rat liver nuclei. The protein load was 1–4 µg (lanes p1–p6).

histone variant in peak p3 and a small decrease in peak p2 were observed. Thus we assumed that peaks p3 and p2 might contain histones H1a and H1b respectively. Furthermore, an additional peak (p7) with a retention time of about 38 min was detected. As we have shown in a previous study [28], this peak consists of histone H1t, which we had isolated for the first time by h.p.l.c. The peaks eluted between 10 and 14 min were not proteins but impurities caused by traces of 2-mercaptoethanol. For further analysis the seven subfractions (p1–p7) obtained in the h.p.l.c. run (Fig. 2a) were freeze-dried and identified by SDS/PAGE (Fig. 2b) and AU/PAGE (Fig. 2c). Subfractions p1 and p6 were proved once more to be histones H1⁰ and H1c respectively (Fig. 2b). A further identification by AU/PAGE, as shown for the other fractions, was unnecessary. The histone of peak p2 was the slowest-migrating H1 subtype in SDS/PAGE (Fig. 2b) but the second-slowest component in AU/PAGE (Fig. 2c). These electrophoretic mobilities are characteristic for histone H1b [17,29]. The histone of subfraction p3, however, closely followed histone H1c (p6) in SDS/PAGE (Fig. 2b), but in AU/PAGE it was the slowest one (Fig. 2c). These mobilities correspond to histone H1a, as reported previously [27,30]. Moreover, histones

Fig. 2. Separation of histones H1 from rat testis nuclei by reverse-phase h.p.l.c. and analysis of the subfractions by SDS/PAGE and AU/PAGE

(a) Separation of histones H1 from rat testis nuclei by reverse-phase h.p.l.c. The h.p.l.c. conditions were the same as for Fig. 1(a). In order to obtain fractions p1–p7, 100 µg of histones H1 was injected on to the h.p.l.c. column. Numbered fractions were freeze-dried and subjected to SDS/PAGE and AU/PAGE analysis. (b) SDS/PAGE of fractions p1–p7 from the separation described in (a). Lane M, total histones H1 (15 µg) from rat testis nuclei. The protein load was 0.75–4 µg (lanes p1–p7). (c) AU/PAGE of fractions p2–p5 and p7 from the separation described in (a). Lane M, total histones H1 (15 µg) from rat testis nuclei. The protein load was 0.75–4 µg (lanes p2–p5 and p7).

H1a and H1t (p7) have virtually identical mobilities in AU/PAGE [17], as also clearly shown in Fig. 2(c). Thus the above-mentioned presumption with regard to histones H1a and H1b was confirmed by the electrophoreses. Consequently, fraction p4 could be either histone H1d or H1e. In AU/PAGE the histone of peak p4 migrated just above the upper band of peak p5

Table 1. Assignment of the histone H1 subfractions p1–p7 obtained by reverse-phase h.p.l.c. from rat liver and testis nuclei to the standardized H1 nomenclature recommended by Lennox *et al.* [16]

Fraction ...	Assignment						
	p1	p2	p3	p4	p5	p6	p7
Rat liver	H1 ⁰	H1b	H1a	H1d	H1e+H1c	H1c	–
Rat testis	H1 ⁰	H1b	H1a	H1d	H1e+H1c	H1c	H1t

Table 2. Amino acid compositions of rat H1 histone subtypes

Amino acid analyses were performed as described in the Materials and methods section. The values are given as percentages of amino acid residues detected. No corrections have been made for hydrolytic losses. Results are average values for at least two determinations. The designation 'Trace' indicates an amino acid content of less than 0.5%. Values for cysteine were not determined.

Amino acid	Composition (% of total amino acid residues)						
	H1a	H1b	H1c	H1d	H1e	H1 ⁰	H1t
Asx	2.6	2.1	2.2	1.9	2.9	3.7	4.8
Thr	6.2	6.8	4.7	6.0	5.7	4.9	5.3
Ser	8.6	6.8	7.5	6.3	8.9	7.9	10.7
Glu	5.2	4.6	3.1	3.9	3.8	6.1	6.7
Pro	9.3	8.7	7.6	9.6	8.6	7.9	5.2
Gly	5.8	8.3	7.1	7.1	7.4	5.1	11.2
Ala	17.7	18.7	24.5	25.4	21.5	15.1	14.6
Val	8.7	6.6	5.8	3.3	4.7	6.5	4.7
Met	0	0	Trace	0	0	Trace	Trace
Ile	0.89	0.84	1.4	1.3	1.3	3.3	1.0
Leu	4.2	3.9	4.3	4.2	4.2	2.2	6.5
Tyr	0.62	0.94	0.51	0.55	0.52	1.7	0.65
Phe	0.54	0.62	0.56	0.50	0.51	1.1	1.0
His	Trace	Trace	Trace	0	0	1.3	Trace
Lys	26.7	28.6	28.7	27.1	27.5	28.6	18.6
Arg	2.5	2.8	1.8	2.8	2.4	4.4	7.7

(Fig. 2c). In accordance with the literature cited [18,29] this suggested the presence of histone H1d in fraction p4. Moreover, it has been reported that histone H1d is the main H1 variant in rat liver [26] but not in testis [27]. This fact, as well as the amino acid composition [26], corresponded to our findings (Figs. 1a and 2a and Table 2). Consequently, the histone of peak p5 had necessarily to be histone H1e (contaminated with histone H1c from peak p6), confirmed by gel electrophoreses (Figs. 2b and 2c) according to a previous report [29]. Furthermore, we analysed H1 histones of rat thymus, spleen and kidney by h.p.l.c. The distribution of each subtype (results not shown) was similar to published values [26] and confirmed our designations. Table 1 summarizes the assignments of all histone H1 subfractions p1–p7 according to the nomenclature of Lennox *et al.* [16].

For a further characterization amino acid analyses of all histone subtypes purified by rechromatography were carried out (Table 2). Subfraction p5, primarily a mixture of histones H1e and H1c, was rechromatographed several times. The histone H1e and the other H1 variants finally obtained were free of any contamination, as judged by SDS/PAGE analyses. The amino acid compositions of the histone variants H1a, H1c, H1d and H1t corresponded to the data previously reported [17,26,27]. In addition, the present study reports for the first time a compositional analysis of rat histones H1⁰, H1b and H1e. The amino acid analysis of histone H1⁰ indicated the presence of

methionine and of significant amounts of histidine, and resembled those of other mammalian, including human, H1⁰ histones [31]. Similarly to histone H1a, histone H1b revealed both a higher valine content and a lower alanine content as compared with the variants H1c, H1d, and H1e. In this context it should be noted that the H1 subtypes H1a and H1b of the murine lympho-leukaemic cell line L5178Y also show higher valine content and a lower alanine content [32]. Histone H1e, finally, revealed a higher content, of aspartic acid/asparagine and serine and a lower content of alanine as compared with histones H1c and H1d (Table 2). It should be stressed, however, that the absolute identification of individual subtypes can only be obtained when their amino-acid sequences have been determined.

DISCUSSION

In this study the isolation of rat liver H1 histones by reverse-phase h.p.l.c. yielded six subfractions and that of rat testis H1 histones seven subfractions (Figs. 1a and 2a). However, it should be noted that subfraction p5 was not always well separated from subfraction p6. In a previous study Kurokawa & MacLeod [24] fractionated histone H1 subtypes from rat liver by reverse-phase h.p.l.c. by using a RP-300 column and a perchlorate/phosphoric acid/acetonitrile gradient. They resolved five peaks (P1–P5), designating their subfraction P2 as histone H1a. Their assignment was primarily based on an amino acid analysis revealing a higher valine content and a lower alanine content than peaks P3, P4 and P5, similarly to the values found for histone H1a [17,27]. The facts that the amino acid composition of rat histone H1b at that time was unknown and that the h.p.l.c. system applied was incapable of resolving authentic histone H1a may have led these authors to an incorrect interpretation. As we have documented in Table 2, both variants H1a and H1b show higher valine and lower alanine contents compared with the variants H1c, H1d and H1e. In addition, the subfraction P2 obtained by Kurokawa & MacLeod [24] was the slowest-migrating component in their SDS/PAGE system, which is typical for rat histone H1b but not for histone H1a [17]. Thus the present study suggests that the subfraction P2 obtained by Kurokawa & MacLeod [24] (our subfraction, p2) in reality consists of histone H1b. The genuine histone H1a (our subfraction p3), however, is eluted immediately before histone H1d and was not separated by Kurokawa & MacLeod [24]. Similarly, a clear assignment of the questionable peaks referred to by these authors is also possible on the basis of results of the present paper.

Karhu *et al.* studied the concentrations of H1 histones and HMG (high-mobility-group) proteins in nuclei of rat liver, spleen, testis and thymus during postnatal development [11]. For this purpose the chromosomal proteins were separated by h.p.l.c. The resulting H1 subfractions were named H1⁰, H1A, H1B, H1C and H1D. In their AU/PAGE system subfraction H1D appeared to be a pure protein fraction, whereas according to our studies this subfraction H1D consists of two H1 variants, i.e. H1e and H1c. It therefore appears that the AU/PAGE system used by Karhu *et al.* [11] was unable to separate these two variants. Since histones H1e and H1c show functional differences of biological importance [30] (histones H1a and H1c, for example, condense chromatin less than do histones H1b, H1d and H1e [18]), the joint quantification of those two subtypes seems problematic. As to the subfraction H1A, Karhu *et al.* [11] presumed that it may be identical with the histone variant H1a. Our results, however, have shown that this subfraction H1A is identical with histone H1b, whereas subfraction H1B corresponds to histone H1a and subfraction H1C to histone H1d.

More recently, Tchouatcha-Tchouassom *et al.* [33] described a preparative procedure for the isolation of histone H1⁰ and five

H1.1 subfractions (named H1.1a to H1.1e) from adult rat liver by reverse-phase h.p.l.c. On the basis of both the gel-electrophoresis systems (SDS/PAGE and AU/PAGE) and the amino acid compositions the subfractions H1.1a and H1.1c should correspond to the variants H1b and H1d in the nomenclature system of Lennox *et al.* [16]. The high protein load of their preparative column (25 mg) and the resulting effects, however, permits no further correlation of the other subfractions with our analytical method. For example, subfraction H1.1b reveals electrophoretic mobilities similar to those of histone H1a. Nevertheless the amino acid composition shows a very low valine content and a high alanine content, which is in contrast with published and our values for histone H1a. Since subfraction H1.1b is not well separated and is eluted as a shoulder just preceding the main peak H1.1c, we consider that subfraction H1.1b is a mixture of the variants H1a and H1d. This would explain the similar amino acid analyses of subfractions H1.1b and H1.1c.

In the past two-dimensional gel electrophoresis was most frequently applied for analysing H1 histones. This is a laborious, time-consuming and, moreover, unsatisfactory method for precise quantification. On the basis of the clear identification and characterization of all rat H1 subtypes, this paper seeks to demonstrate that now another, simpler, system having the obvious advantages of the h.p.l.c. technique is available.

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