Histone H1^o: its location in chromatin

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ABSTRACT

Histone H1^o is an H5-like protein found in mammals. Its location in chromatin from animal tissues has been studied by micrococcal nuclease digestion and by quantification. It was found that H1^o occurs in the linker region of chromatin, and replaces H1 there as it does so. As much as a third of the H1 becomes replaced, at least in some tissues. The abundance of H1^o was similar in bulk chromatin and in a mononucleosome population which was putatively enriched in transcribed DNA sequences. It was concluded that H1^o probably does not suppress transcription.

INTRODUCTION

Most, if not all eukaryotes possess histone H1. H1 is located on the linker region of chromatin, and has been implicated in the maintenance of compact conformations of chromatin, possibly by maintenance of linker DNA length (reviewed in refs 1 and 2). A protein similar to H1 in some respects, histone H5, is also found in the linker region of chromatin, but only in nucleated erythrocytes. The function of H5 has been suggested as being one of suppression of transcription³ (reviewed in ref. 4). It is generally held that when H5 occurs it replaces H1 (eg. refs 5 and 6) but Weintraub⁷ and Olins et al⁸ have suggested that H5 is present in addition to the normal H1 content (of approximately one H1 molecule per nucleosome).

A protein which is like H1 in overall amino acid content but which has some structural homology with avian H5, is H1^o⁹. The function of this protein has been suggested as being suppression of cell replication¹⁰ or of DNA synthesis¹¹. By analogy with H1 and H5, H1^o may be expected to reside in the linker region of chromatin, perhaps replacing H1 when it does so. In the present work we test these expectations and investigate the association of H1^o with the fraction of chromatin which is sensitive to brief digestion with micrococcal nuclease and which is reputed to be enriched in actively transcribing DNA sequences^{12,13}.

METHODS

Preparation of nuclei and proteins

For nuclease digestion studies, tissue from 2-6 week-old calves was used, for reasons of quantity, and because bovine H1^O was available as a standard. The tissue used was brain, since it has minimal endogenous proteolytic and nucleolytic activities^{14,15}. Brains were obtained from a local abattoir, transported on ice. For quantitative studies the species used was changed to the laboratory mouse (strain CBA/Ca) to ensure freshness of tissue and so a minimum of proteolysis. Mice were killed immediately before use.

Nuclei were prepared from fresh tissue in the presence of protease inhibitor phenylmethylsulphonyl fluoride as described previously¹⁶. Proteins were prepared by thorough extraction of nuclei four times with HCl (0.25M) and by precipitation by addition of acetone (10 vol).

Digestion with nuclease

Freshly-prepared nuclei were suspended to 6-8 mg nucleic acid/ml in sucrose (0.3M), CaCl₂ (1mM), tris (5mM) and cacodylic acid (to pH 7.3), and warmed to 37°C. Micrococcal nuclease (Worthington) was added to a concentration of 50 units/mg nucleic acid. In the case of controls, buffer was added instead of enzyme. Incubation at 37°C continued for 90 sec and then the reaction was stopped and nuclei lysed by cooling on ice, addition of diaminoethanetetra-acetic acid (EDTA, to 1.5mM) and brief homogenization. Suspensions were then centrifuged (90,000 x g, 4°C, 30 min) to yield a supernatant which was suitable for electrophoresis, after dialysis (overnight, 4°C, against triethanolamine (2mM) EDTA (2mM) glycerol (10% v/v) and phenylmethylsulphonyl fluoride (0.5mM). The amounts of perchloric acid (5% w/v)-soluble nucleotides in suspensions of nuclei before and after treatment were estimated by absorbance at wavelength 260 nm. It was found that the treatment with micrococcal nuclease rendered approximately 1.5 - 3.0% of nucleic acids soluble in perchloric acid.

Electrophoresis

Deoxyribonucleoprotein particles were fractionated in "DNP" gels, of 5% acrylamide¹⁷, in slabs of 0.3 x 12 x 12 cm. Electrophoresis (at a constant voltage of 150 V) was continued with cooling until 30 min after the bromophenol blue dye front had emerged from the gel.

Proteins were analysed in "SDS" gels of 15% acrylamide and sodium dodecylsulphate (0.1%)¹⁸, in 0.1 x 12 x 12 cm slabs. Electrophoresis (at a constant current of 20 mA) was continued with cooling until the bromophenol blue dye front reached the bottom of the gel.

DNA was analysed by electrophoresis in "DNA" gels of 4% acrylamide and

sodium dodecylsulphate (0.1%)¹⁹ in 0.3 x 12 x 12 cm slabs. Electrophoresis was continued at room temperature at a constant voltage of 20 V, until the bromophenol blue dye front had neared the bottom of the gel.

Two-dimensional gels were of two types: (a) DNP gel in the first dimension, SDS gel in the second; (b) DNP gel in the first dimension, DNA gel in the second. Strips from the first gel (DNP) were equilibrated in appropriate buffers before application to the top of the second gel.

Double stranded nucleic acid was stained with ethidium bromide after washing the gel with methanol/water (50/50, v/v)¹⁹ and visualized by irradiation with ultraviolet light. Proteins were visualized routinely by staining with Coomassie Brilliant Blue R250¹⁶. However, we have found that this stain is unsatisfactory for quantitative staining of protein - it does not bind to the protein quantitatively, it varies in its ability to stain from batch to batch, and it exhibits metachromicity. Procion Navy MXRB (I.C.I. Ltd.) suffers none of these drawbacks, although being perhaps two or three-fold less sensitive²⁰. Thus, Procion Navy was used to quantitatively stain both one- and two-dimensional gels. Core histones and H1 were previously shown to be quantitatively stained²⁰, as is H1⁰ (data not shown). However, the amount of stain taken up by H1⁰ is slightly less (approximately 80%) than that by homologous H1, on a weight basis. One-dimensional gels stained with Procion Navy were scanned at 580 nm as described previously²⁰, and two-dimensional gels were cut into narrow strips which were each scanned in the same way. Proteins in two-dimensional gels were quantified by summing appropriate peaks in the various scans.

Standard methods of statistical analysis were employed where appropriate.

RESULTS

The location of H1⁰ in chromatin

Digestion of calf brain nuclei with micrococcal nuclease yielded monomer and oligomer nucleosome fractions, but in control experiments there was no generation of nucleosomes, in agreement with the previously observed lack of endogenous nuclease in brain^{14,15}. Small amounts of some protein species were found in supernatant fractions in control experiments, presumably of ribonucleoprotein particle origin. These proteins had lesser mobilities than that of H1 in SDS gels, and so did not interfere with analysis of the proteins of interest from the various nucleosomal species. This was done by two-dimensional electrophoresis (DNP-SDS), in which H1 and H1⁰ were identified by co-electrophoresis of standard proteins.

Fig. 1a shows that like H1, H1⁰ was present in those fractions which retain linker DNA (mononucleosome type MN2²¹, and larger particles) but not in the linker-less core particle ("MN1"²¹). This suggests that H1⁰ resides on the linker region of chromatin.

Inspection of H1 and H1⁰ bands in the MN2 region shows that they are not exactly coincident (see fig. 1a, and inset). This indicates that there exist particles (here called MN2⁰) which bear H1⁰ but not H1, and suggests that H1⁰ can replace H1 in chromatin.

Analysis of the DNA in the digested calf brain chromatin fractions (fig. 1b) did not reveal any diversity of DNA lengths which may have corresponded to the diversity of MN2 types. However, it may be beyond this technique to resolve very small differences in DNA lengths.

Quantification of H1 and H1⁰ in various chromatin fractions

Calf brain nuclei were digested to 1.5 - 3.0% acid-soluble nucleotides by micrococcal nuclease. The amounts of H1⁰ and H1 (relative to core histones) in MN2 and dimer fractions were determined from quantitatively-stained two-dimensional electrophoretograms (DNP-SDS). These gels are slightly less able than are one-dimensional gels to resolve protein bands which run close together, so results of quantification are slightly less accurate than those from one-dimensional gels. The MN2 and dimer fractions contained approximately the same amounts of core histones. Table 1 gives the results together with similar quantifications (from one-dimensional gels) of two preparations from undigested calf brain nuclei (which were insignificantly different from each other at the 5% level, with respect to histone/histone ratios). Both MN2 and dimer fractions seemed to be slightly depleted in H1 and perhaps slightly enriched in H1⁰. The ratio (H1 + H1⁰)/core histones was less than that in whole chromatin. This may have been due to replacement of H1-containing nucleosomes by nucleosomes bearing non-histone(s), although no such protein was obvious on our gels. The increase in ratio H1⁰/H1, probably mainly due to loss of H1, is analogous to the increased H5/H1 ratio in oligonucleosomes (compared with that in whole chromatin), which are produced by digestion of chicken erythrocyte chromatin by micrococcal nuclease²².

Fig. 1.

Two dimensional electrophoretograms of micrococcal nuclease-digested calf brain nuclei: (a) DNP-SDS electrophoresis, gel stained with Procion Navy. Inset: enlarged portion (of MN2 region bearing H1 and H1⁰) from a sister gel stained with Coomassie Brilliant Blue R250; (b) DNP-DNA electrophoresis, gel stained with ethidium bromide.

Table 1: Quantification of H1 and H1^o in various calf brain chromatin fractions: histone/histone ratios determined from electrophoretograms stained by Procion Navy

Chromatin fraction	n, no. of samples *	Ratios: Mean (standard deviation), 2 sig. fig.			
		H1/CH [†]	H1 ^o /CH	(H1+H1 ^o)/CH	H1 ^o /H1
MN2	2	0.14(0.049)	0.040(0.00028)	0.18(0.0049)	0.30(0.11)
Dimer	2	0.14(0.0050)	0.044(0.031)	0.18(0.024)	0.33(0.25)
Whole (a)	16	0.20(0.047)	0.033(0.014)	0.23(0.055)	0.16(0.060)
Whole (b)	14	0.23(0.049)	0.029(0.0077)	0.26(0.047)	0.14(0.062)

*: each sample itself an average of two or more scans.

†: CH = core histone

(a), (b) = two separate preparations, insignificantly different (at 5% level)

The results indicate that there is neither a great increase nor a decrease in H1^o (relative to core histones) in these monomer and dimer fractions.

Quantification of H1 and H1^o in various animal tissues

The species used for this purpose was mouse. That the protein of interest in this species was in fact H1^o was confirmed by the following:

- it was soluble in HCl (0.25M) and perchloric acid (5% w/v), as is bovine H1^o 16;
- its mobility in SDS electrophoresis was identical to that of bovine H1^o;
- its cleavage by cyanogen bromide generated a major peptide of identical electrophoretic mobility, in SDS gels, to the corresponding peptide from bovine H1^o (see ref.16);
- its tissue distribution was as expected for H1^o - less in tissues which are more active in DNA synthesis (see ref 10 and table 2);
- its behaviour was as expected for H1^o (by analogy with regenerating liver²³ and pancreas¹¹) when mouse salivary gland cells were stimulated to enter the cell cycle by administration of isoproterenol²⁴ - levels of the putative H1^o were lower in dividing cells than in cells which were unstimulated and in G₀ phase (data not shown).

The thymus gland has little or no $H1^O$ (less than 0.5% relative to $H1$ in calf thymus (Smith and Johns, unpubl.)), and the amount of $H1$ in mouse thymus has been estimated to be one molecule of $H1$ per nucleosome²⁰. Therefore, the $H1$ level in the thymus was taken as the maximum, and $H1$ and $H1^O$ levels in other mouse organs compared with it. To enable identical treatment of all tissues studied, young mice (4-7 weeks old) were used, for the thymus atrophies with age. To facilitate scanning of protein bands on stained gels, preparations free of cytoplasmic proteins were made, from nuclei. Preparation of nuclei seems to result in some degradation or other alteration of $H1^O$ ¹⁶, but since the product of alteration is all or virtually all of the molecule and it has a mobility in SDS gels which is almost identical with that of the parent molecule, no correction was made for this. No loss of $H1$ was apparent during isolation of nuclei.

Various histone/histone ratios were determined for young mouse tissues, from scans of replicated, quantitatively-stained, one-dimensional SDS gels. The results are summarised in table 2. The ratio $H1$ /core histone in each of the liver and kidney was significantly lower (at the 5% level) than that of the thymus, but in each case the ratio $(H1+H1^O)$ /core histone was not significantly different. This suggests that in organs where $H1^O$ occurs, it replaces $H1$, so that the level of total lysine-rich histone $(H1+H1^O)$ remains constant at about one molecule of $H1$ or $H1^O$ per nucleosome.

It has been reported that $H1^O$ levels increase with age^{25,26}. Preparations were made from nuclei of tissues from 6 and 9 month-old mice. Quantification of histones in these preparations (Table 2) agrees with this observation, with $H1^O$ levels increasing and stabilizing by 6 months of age. The results from aged mice support the conclusion from more comprehensive data with young animals - that $H1^O$ replaces $H1$.

Upon SDS gel electrophoresis, the $H1$ split into two bands, the relative abundance of which changed from tissue to tissue. However, no firm conclusion could be drawn concerning the preferential replacement of one $H1$ band or the other by $H1^O$ during ageing. Thus, it remains unknown which $H1$ sub-fraction(s) becomes replaced by $H1^O$.

DISCUSSION

The evidence presented here shows that $H1^O$ can replace $H1$ in its position in the linker region, and probably protect a length of DNA not very, if at all different from that protected by $H1$ (about 20 base-pairs)²⁷. The replacement of $H1$ can be quite extensive, so that for old mouse liver

Table 2: Quantification of H1 and H1⁰ in nuclei from various mouse tissues: histone/histone ratios determined from electrophoretograms stained by Procion Navy

Tissue	Age (mths)	No. of preps.	n, total no. of samples *	Ratios: Mean H1/CH [†]	(standard deviation) H1 ⁰ /CH	2 sig. fig. (H1+H1 ⁰)/CH	fig. H1 ⁰ /H1
Thymus	1-2	3	19	0.28(0.053)	-	-	-
Liver	1-2	4	19	0.21(0.058)	0.055(0.018)	0.26(0.070)	0.26(0.034) [‡]
Kidney	1-2	2	13	0.21(0.063)	0.073(0.035)	0.28(0.096)	0.31(0.077) ^x
Thymus	6	1	3	0.28(0.0076)	-	-	-
Liver	6	1	3	0.20(0.0083)	0.090(0.0048)	0.29(0.013)	0.45(0.013)
Kidney	6	1	3	0.17(0.011)	0.071(0.0095)	0.24(0.022)	0.42(0.040)
Liver ⁺	9	1	3	0.20(0.032)	0.082(0.015)	0.28(0.047)	0.41(0.012)
Kidney	9	1	2	0.21(0.028)	0.097(0.016)	0.31(0.044)	0.46(0.015)

*: each sample itself an average of two or more scans.

†: CH = core histones

‡: n = 25

x: n = 16

+: 9 mth.-old thymus not done - insufficient tissue in old mice.

or kidney it was estimated that on stained gels H1⁰/H1 $\approx$ 0.4. Bovine H1⁰ takes up slightly less stain than does bovine H1 (see Methods). Assuming that this is also the case in mouse, and correcting accordingly, on a weight basis H1⁰/H1 $\approx$ 0.5, which is equivalent to about a third of nucleosomes bearing H1⁰ instead of H1.

The difference in mobilities of MN2 and MN2⁰ particles in DNP gels is of interest. This is analogous to H5-containing mononucleosomes ("MN2E") moving faster than H1-containing MN2 particles in DNP gels²². This could have arisen by MN2E being more negatively charged, because its DNA was 5 to 10 base pairs longer than that in MN2²², although Simpson²⁷ apparently found no difference in DNA lengths between such particles. The nature of the difference between MN2 and MN2⁰ is unclear. There may be a difference in DNA lengths but it must be small if it exists. H1 and H1⁰ contain roughly similar proportions of acidic and basic amino acid residues¹⁶, and the isoelectric point of H1⁰ is probably slightly higher than that of H1²⁸, so differences in charge probably

do not explain the difference in mobilities of MN2 and MN2⁰. The explanation possibly lies in the sizes of the particles, arising perhaps by differences in compaction, but also by differences in molecular weight - H1⁰ probably has a smaller molecular weight than H1 does. This is suggested by the greater mobility of H1⁰ in SDS gels¹⁶, and its lesser mobility upon gel filtration chromatography²⁹.

The high H5/H1 ratio observed in oligonucleosomes from micrococcal nuclease digested chicken erythrocyte chromatin²², is analogous to our result with high H1⁰/H1 ratios in monomer and dimer fractions. Bakeyeva and Bakayev²² suggested that this arose because H5 protected nucleosomes against nuclease attack more efficiently than H1 did (that is, providing greater resistance to further nuclease action which generates MN1 particles). Like H5, H1⁰ may protect against nuclease attack more efficiently than H1 does.

While this paper was in preparation, two relevant papers appeared in print. The first, by D'Anna et al³⁰ reported (contrary to our results from normal tissues) that an H1⁰-like protein accumulated without concomitant loss of H1 in cultured cells blocked in G1 phase by butyrate treatment. It is known, however, that blockage of the cell cycle disrupts the cell's normal functioning and may introduce artifacts (e.g. refs. 31,32). That this may have been the case was suggested by the fact that there was no direct correspondence between the abundance of this protein and the proportion of the population blocked in G1³⁰.

The second relevant paper by Albright et al³³ mentions that "NF" (probably H1⁰) occurs in MN2-type mononucleosomes, agreeing with our observations, as does the report of "IP₂₅" (possibly an H1⁰-like protein described in mouse erythroleukaemia cells) existing in the linker region³⁴.

It has been found that brief digestion of chromatin by micrococcal nuclease generates a mononucleosome population enriched in transcribed sequences^{12,13}. The brain is an organ which is active in transcription³⁵, so it is assumed that in the present work brief digestion yielded a mononucleosome fraction enriched in transcribed sequences. It has also been reported that micrococcal nuclease may preferentially attack nascent DNA³⁶, but although brains from young animals such as those used here synthesize some DNA³⁷, we expect the contribution from this source to be small. Since it was found that H1⁰ was only slightly or not at all enriched in MN2 and dimer fractions, we tentatively conclude that the distribution of H1⁰ is not affected by transcription. This conclusion is in apparent disagreement with a pair of (contradictory) reports. Firstly, the H1⁰-like protein IP₂₅ was found not to be

solubilized by digestion by DNase I, which lead to the suggestion that this protein is associated with repression of gene activity³⁴. Secondly, and to the contrary, it was reported³⁸ that a protein ("20K") with electrophoretic mobilities like those of H1^o was enriched in putative actively transcribing chromatin as isolated by the DNase II/Mg²⁺ solubility procedure from trout testis (an organ which in mammals contains H1^o). Our conclusion is possibly supported by the observation that there is in rat satellite chromatin a putative H1^o protein which is present in quantities not very or at all different from those of bulk chromatin^{39,40}. Satellite chromatin is generally considered to be transcriptionally inactive, although there is one report to the contrary⁴¹.

Our results disfavour the idea of analogy with the structurally-related H5 that H1^o may repress transcription, but probably still allow that it may repress DNA synthesis.

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