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Histones H1 and H5: one or two molecules per nucleosome?

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ABSTRACT

We have determined histone stoichiometries in nuclei from several sources by a direct chemical method, with the particular aim of quantitating histone H1 and, in chicken erythrocytes, H5, and of distinguishing between one and two molecules per nucleosome. The four histones H3, H4, H2A and H2B are found in equimolar amounts, as expected for the core histone octamer. The molar ratio of H1 in lymphocyte and glial nuclei is 1.0 per octamer, and in liver nuclei from three species 0.8 per octamer. These results suggest that each nucleosome has one H1 molecule; nucleosomes could acquire two molecules of H1 only at the expense of others containing none. The stoichiometry of H5 in chicken erythrocyte nuclei is similar to that of H1 in other nuclei, being about 0.9 molecules per nucleosome; the H1 also present in these nuclei amounts to 0.4 molecules per nucleosome.

INTRODUCTION

It is now well established that the histone core of the nucleosome is an octamer of composition $(H3)_2(H4)_2(H2A)_2(H2B)_2$ (1-3); equimolar stoichiometries have been determined for the four core histones in chromatin from several sources by reliable methods (4-6). The working assumption has been that there is one H1 per nucleosome, as originally proposed on the basis of approximate molar amounts relative to the other histones after extraction from chromatin (1), and supported by analysis of mono- and dinucleosomes (7). The H1 is at least partly associated with the linker DNA that connects one nucleosome core and the next (7-9). It plays a critical structural role in the nucleosome in sealing two complete turns of DNA around the core histone octamer (10,11), and its presence appears to be essential for the formation of higher order structures (see ref. (12) and references cited therein).

The precise stoichiometry of H1 in the nucleosome has been less thoroughly investigated than that of the core histones. A careful re-evaluation by dye-staining methods of selectively extracted H1 indeed suggested one H1 per nucleosome in rabbit thymus nuclei (13), and the same

value was recently indicated for H1 in chromatin obtained from mouse myeloma cells in which the histones had been intrinsically radiolabelled with [^{14}C]-lysine (14). However, some uncertainty still exists about the stoichiometry of H1; moreover it is not clear whether the stoichiometry is the same in nuclei from all sources. Substantially less than one H1 per nucleosome (0.64) was found in mouse cell nuclei in culture (6); and the molar ratio of (H1 + H5) in nuclei from mature chicken erythrocytes was reported to be about two per nucleosome (15-17). Moreover, evidence was presented for a second H1-binding site in the nucleosome; only one H1 appeared to be bound in vivo, and addition of a second H1 in vitro generated a structure more compact than that of native chromatin (18). However, it was subsequently inferred from dissociation-reassociation experiments that two molecules of H1 or H5 were required per nucleosome in order to regenerate the properties of native chromatin (19).

In view of the uncertainty concerning the stoichiometry of binding of H1 (and H5), and the critical structural roles of these histones in chromatin, we have measured the histone stoichiometry in nuclei from several sources, with the particular aim of defining the stoichiometry of H1 and H5. We have avoided methods involving quantitation of histones by dye-staining methods (20-26), and have taken care to avoid the possibility of differential extraction of histones, both of which probably contributed to errors in earlier measurements of stoichiometry. The method we have used is a general and direct chemical method (27) which has been successfully applied to a number of problems (28-30); brief mention of preliminary studies using this approach for histone stoichiometry was made earlier (31). Histone mixtures are exhaustively modified under strongly denaturing conditions with the radiolabelled imidoester, methyl [^{3}H]acetimidate, and then separated by gel electrophoresis. The radioactivity in the bands is directly related to the relative molar amounts of each histone by the known lysine contents. The abundance of lysine residues in histones, particularly in H1 and H5, and the high specific activity of the reagent, makes the method especially suitable and sensitive for measurement of histone stoichiometries.

MATERIALS AND METHODS

Methyl [^{3}H]acetimidate

Acetonitrile (0.6 ml) was tritiated by exchange with [^{3}H]water (1 ml, 5 Ci/ml, Radiochemical Centre, Amersham) catalysed by 20 mg $Ca(OH)_2$ (32). The [^{3}H]acetonitrile was recovered by vacuum distillation (32) and converted

into methyl [^{3}H]acetimidate as described previously (33). The chemical yield was >95% and the specific activity was about 40 Ci/mole.

Preparation of nuclei

Nuclei were prepared from chicken erythrocytes as described previously (34), and from fresh rat and chicken liver by the method of Hewish and Burgoyne (35). Glial nuclei (provided by E.C. Pearson) were prepared from ox cerebral cortex essentially according to Thompson (36). Pig lymphocytes (provided by J.P. Moore) were prepared as described by Hesketh et al. (37) and the nuclei isolated by the detergent (Nonidet P-40) method described for chicken erythrocytes (34). In each case the nuclei were washed and finally resuspended in 0.34 M sucrose - buffer A (35).

Amidination of histones

Samples were prepared for histone amidination in three different ways: (a) by total precipitation of nuclei with trichloroacetic acid (TCA); (b) by acid extraction of nuclei; and (c) from native chromatin, as follows:

(a) Nuclei (40 A_{260} units, measured in 1 M NaOH) were recovered from buffer A (35) by brief centrifugation (1,000 g for 3 min), resuspended in 1 ml of 5 mM triethanolamine hydrochloride, 60 mM NaCl, 1 mM EDTA, 0.25 mM phenylmethylsulphonyl fluoride (PMSF), pH 7.5, and precipitated with 1 ml 50% (w/v) TCA. After 15 min on ice, samples were recovered by centrifugation (12,000 g for 10 min), washed once with acetone-5 mM HCl, and dried in vacuo.

(b) Histones were extracted from nuclei with 0.2 M sulphuric acid. Nuclei (40 A_{260} units) were centrifuged out of buffer A and resuspended as in (a), and then an equal volume of 0.4 M H_2SO_4 was added. After 30 min at 0°C the precipitate was removed by centrifugation (12,000 g for 10 min) and the pellet re-extracted with 2 ml 0.2 M H_2SO_4. The supernatants containing extracted histones were combined and an equal volume of 50% TCA was added. After 15 min on ice the precipitated histones were recovered by centrifugation (12,000 g for 10 min), washed once with acetone-5 mM HCl, and dried in vacuo.

(c) Native chromatin was prepared from nuclei by brief digestion with micrococcal nuclease (38) and 40 A_{260} units were freeze-dried from 0.2 mM EDTA, 0.25 mM PMSF, pH 7.5, without further purification.

All samples were then dissolved for amidination in 0.5 ml Na borate, pH 10.0, containing 5 M guanidinium chloride (27,39). To ensure complete denaturation and solubility, the samples were heated to 100°C for 3 min. To a sample of 100 µl, 5 µl 0.1 M NaOH was added to neutralise any residual acid. Then 12 µl of a 1 M solution of methyl [^{3}H]acetimidate made up in an equi-

volume mixture of 0.2 M Na borate, pH 10, and 2 M NaOH was added, and the reaction was allowed to proceed to completion for 5 h at 22°C.

Separation and counting of labelled histones

Exhaustively modified histones were recovered from the reaction mixture by precipitation with TCA. A 25 µl portion of the reaction mixture was diluted to 1 ml with water, precipitated with 1 ml 50% TCA and centrifuged (12,000 g for 10 min). The pellet was washed once with 1 ml 25% TCA, then with acetone-5 mM HCl, and dried *in vacuo*. Labelled histones were redissolved in sample buffer for electrophoresis and separated in sodium dodecyl sulphate-18% polyacrylamide gels, 15 x 15 x 0.1 cm (40); 10 - 12 tracks were run for each sample and the load was approximately 5 µg total protein per track.

After fixation and staining (40), the bands of labelled histones were cut from the gel and dried *in vacuo* in scintillation inner vials. The gel pieces were solubilised at 60°C with 0.5 ml H_2O_2 (30% w/v) for 20 h in the unsealed scintillation vials which were wrapped twice with "clingfilm". After cooling, 4 ml Triton-toluene scintillation fluid (27) was added, giving a homogeneous solution which was counted for ^3H in an LKB 1215 Rackbeta counter. Quench corrections were determined by the external standards ratio method.

RESULTS

Histone stoichiometries have been determined for nuclei from several sources (Fig. 1). In the case of chicken erythrocyte nuclei, three different methods of sample preparation for amidination were investigated in order to eliminate problems caused by incomplete extraction or selective losses of histones. The results are calculated as shown in Table 1. The incorporated radioactivity (in dpm, column 2) is divided by the number of amino groups per histone (column 3). Since each amino group is equally labelled under the reaction conditions, the quotient shown in column 4 is proportional to the number of moles of the labelled protein present. The molar ratios are calculated so that the mean value of the core histones is 1.00 (column 5), and the results are then less susceptible to random errors in a single histone. The stoichiometry of H1 and H5 can thus readily be related to the core histone octamer (column 6). The amino-acid compositions of H1 and H5 lend themselves particularly well to this method of analysis as illustrated by the results in Table 1. Although the amount of H1A, for example, is only about 10% of that of H4, it contains 50% as much radioactivity as H4. Thus the minor linker histone variants can be quantitated with the same accuracy as the core

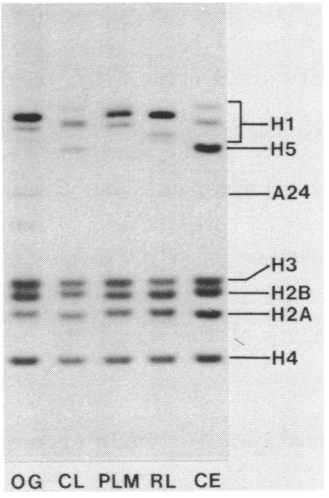

Figure 1. Histone composition of nuclei from various sources.
OG: ox glia; CL: chicken liver; PLM: pig lymphocytes; RL: rat liver; CE: chicken erythrocytes. Exhaustively [^{3}H]amidinated histones from TCA-precipitated nuclei were separated in an SDS-18% polyacrylamide gel. Staining was according to the modified procedure which enhances the staining of H1 [34].

Table 1. Calculation of histone stoichiometry.

	cpm[1]	dpm[2]	Amino groups[3]	Relative moles[4]	Molar ratio[5]	Stoichiometry[6]
H1A	4122	23333	62	376	0.10	1.34
H1B	5104	29936	62	483	0.13	
H5	12323	70507	45	1567	0.43	
H3	8352	48558	14	3468	0.96	8.00
H2B	12825	75497	21	3595	1.00	
H2A	9711	52372	14	3741	1.04	
H4	7684	39951	11	3632	1.01	

[^{3}H]amidinated histones were from TCA-precipitated chicken erythrocyte nuclei. H1A and H1B are the slower and faster migrating H1 subtypes.
1) Uncorrected for background (144 cpm).
2) Corrected for background.
3) Amino groups per mole histone (lysine residues + free N-terminus in H5, H3, H2B). Amino acid sequences are known for chicken H5, H3 and H2A; for H2B and H4 the sequences of calf thymus histones, and for H1 that of rabbit thymus histone (RTL-3) have been used. All the sequence information is summarised in ref. (43).
4) Relative moles (dpm ÷ amino groups).
5) Calculated so that the mean of the four core histones is 1.00.
6) Molecules per core histone octamer.

histones, since the counts are comparable.

Histone stoichiometries of chicken erythrocyte nuclei are shown in Table 2 for three methods of sample preparation. The results are the means and SEMs of numerous determinations calculated as in Table 1, and are identical within the statistical precision of the measurements. As expected, the core histones are present in equimolar amounts, but the (H1 + H5) together constitute about 1.3 moles per mole of core octamer, of which H5 contributes about 0.9 moles and the two H1 subtypes about 0.4 moles. Since the values determined for the stoichiometry are independent of the method of sample preparation, we conclude that they are a true measure of histone stoichiometry in

Table 2. Histone stoichiometries in chicken erythrocyte nuclei.

		Molar ratio[1]	(SEM)[2]	Stoichiometry[3]	(SEM)
Nuclei (9)*	H1A	0.09	(0.00)	1.35	(0.02)
	H1B	0.13	(0.00)		
	H5	0.45	(0.01)		
	H3	0.91	(0.03)	8.00	(0.09)
	H2B	1.06	(0.03)		
	H2A	1.03	(0.01)		
	H4	1.01	(0.02)		
Histones (11)*	H1A	0.09	(0.00)	1.35	(0.02)
	H1B	0.13	(0.00)		
	H5	0.46	(0.01)		
	H3	0.96	(0.01)	8.00	(0.07)
	H2B	1.05	(0.02)		
	H2A	1.06	(0.01)		
	H4	0.93	(0.02)		
Chromatin (11)*	H1A	0.08	(0.00)	1.28	(0.02)
	H1B	0.12	(0.00)		
	H5	0.44	(0.01)		
	H3	0.93	(0.03)	8.00	(0.08)
	H2B	1.03	(0.02)		
	H2A	1.04	(0.01)		
	H4	1.00	(0.02)		

Nuclei were TCA-precipitated directly for amidination; histones were acid-extracted from nuclei; chromatin was prepared by nuclease digestion and freeze-dried (see Materials and Methods).

1) Calculated so that the mean of the four core histones is 1.00.
2) SEM is the standard error of the mean for the number of determinations shown.
3) Molecules per core histone octamer.
* Number of determinations.

the nucleus. For convenience all further measurements of histone stoichiometries have been made with TCA-precipitated nuclei.

The results for nuclei from several sources are presented in Table 3. Again the molar ratio of core histones is, within experimental error, 1:1:1:1. The one exception is in ox glial nuclei, where H2A is diminished by about 25%, probably due to conjugation with ubiquitin to give protein A24 (41,6), which we have not quantitated, but which is present in roughly the right amount as judged by staining intensity. The H1 subtypes taken together amount to about 1.0 molecule per octamer in nuclei from pig lymphocytes and ox glia, but liver nuclei from three species all had rather less, about 0.8 molecules. (In the case of chicken liver nuclei, correction was made for contamination by erythrocyte nuclei, revealed by the presence of H5 (Fig. 1), and using the H1:H5 ratio determined for these nuclei.) Since the likely errors are small, these values for liver nuclei are significantly less than 1.00, but it is not clear whether this deficiency in H1 is a general feature of liver nuclei or some preparational artefact. It may be significant that only these nuclei were prepared in the presence of polyamines (spermidine and spermine), although this buffer (35) was routinely used for resuspension of all types of nuclei. The deficiency in H1 may perhaps be related to the transcriptional activity of liver tissue, although glial nuclei which are, if anything, more active (42) have a full complement of H1.

DISCUSSION

The method we have used to determine histone stoichiometries eliminates the possibility of differential extraction of histones or of preferential losses or proteolysis. It also avoids the problems of accurate quantitation in methods based on staining of bands in polyacrylamide gels (20-26), and the possibility of differential histone turnover *in vivo* in methods based on intrinsic radiolabelling of proteins (4-6,14,16). The stoichiometries have been determined to a high degree of statistical precision (an average SEM of 2%) and the only independent information required is the amino acid composition of the histones. For many species this information is available directly from the primary sequence (43), but in cases where this is lacking, we have used the sequence for the closest species available (see footnotes to Tables 1 and 3); in view of the high degree of homology between histones of different species, it is reasonable to assume that this will not be a significant source of error. One potential source of error is the post-synthetic

Table 3. Stoichiometry of histones in nuclei from various sources.

	Molar ratio		(SEM)	Stoichiometry	(SEM)
Chicken[1] erythrocytes (31)*	H1A	0.09	(0.00)	1.32	(0.01)
	H1B	0.13	(0.00)		
	H5	0.45	(0.01)		
	H3	0.93	(0.01)	8.00	(0.05)
	H2B	1.05	(0.01)		
	H2A	1.05	(0.01)		
	H4	0.97	(0.01)		
Chicken[2] liver (10)*	H1A	0.13	(0.00)	0.88	(0.04)
	H1B	0.31	(0.01)		
	H3	0.97	(0.04)	8.00	(0.14)
	H2B	0.96	(0.03)		
	H2A	0.98	(0.03)		
	H4	1.08	(0.03)		
Rat liver (10)*	H1A	0.25	(0.01)	0.79	(0.01)
	H1B	0.09	(0.00)		
	H1C	0.05	(0.00)		
	H3	0.96	(0.03)	8.00	(0.09)
	H2B	1.02	(0.02)		
	H2A	1.02	(0.03)		
	H4	1.01	(0.01)		
Mouse liver (10)*	H1A	0.21	(0.01)	0.74	(0.02)
	H1B	0.10	(0.00)		
	H1C	0.06	(0.00)		
	H3	0.92	(0.02)	8.00	(0.05)
	H2B	1.06	(0.01)		
	H2A	0.94	(0.01)		
	H4	1.08	(0.01)		
Pig lymphocytes (9)*	H1A	0.13	(0.00)	0.97	(0.02)
	H1B	0.20	(0.01)		
	H1C	0.16	(0.00)		
	H3	0.93	(0.03)	8.00	(0.10)
	H2B	1.09	(0.03)		
	H2A	1.00	(0.03)		
	H4	0.97	(0.02)		
Ox glia[3] (9)*	H1A	0.34	(0.02)	1.07	(0.04)
	H1B	0.14	(0.01)		
	H1C	0.06	(0.00)		
	H3	0.91	(0.02)	7.48[3]	(0.09)
	H2B	1.06	(0.03)		
	H2A	0.74	(0.03)		
	H4	1.04	(0.02)		

modification of histones by acetylation and dimethylation of lysine amino groups, which would render these residues unreactive in our method (monomethyl-lysine would still be reactive). In chicken erythrocytes for example (44), H3 is substantially acetylated (35% monoacetylated, 13% diacetylated); yet this amounts to an overall reduction in free amino groups of less than 5%. The extent of methylation is similarly small, and since the resultant uncertainty arising from these modifications is of the same order as the statistical errors inherent in our method, we have not corrected for them.

As expected, the stoichiometry of the core histones in nuclei from several sources is essentially equimolar, with the single exception of ox glial nuclei, in which the presence of the protein A24 is accompanied by a reduction in H2A of about 25%. The slightly low value (by about 7%) found consistently for H3 (Table 3) could be accounted for by the loss of about one lysine residue per molecule as a result of the post-synthetic modifications already discussed.

Except in chicken erythrocyte nuclei, the H1 stoichiometry is close to one molecule per octamer in all cases, in agreement with earlier values (13, 14). Thus although it may be possible in some circumstances to reconstitute chromatin *in vitro* with two molecules of H1 per nucleosome (19), there clearly cannot be two molecules associated with each nucleosome *in vivo* since there is insufficient H1 in the nucleus. If nucleosomes containing two H1 molecules exist, it can only be at the expense of others containing none.

Both the nucleosome core particle and the core histone octamer possess a dyad axis of symmetry (45,46), which would imply the existence of two

Table 3.

The amino acid compositions of chicken erythrocyte and liver histones were taken to be as indicated in Table 1. For H1 (all subtypes, designated H1A, H1B and H1C in order of increasing mobility) from all species the composition is taken to be that of the sequenced rabbit thymus subfraction RTL-3. The core histones from ox glia, pig lymphocytes, mouse and rat liver were taken to be identical with those of sequenced calf thymus histones, except that mouse and rat liver H2A were assumed to have one lysine residue less, as reported for rat chloroleukaemia cells. All the amino acid sequence information is summarised in ref. (43).

1) Mean of results in Table 2.
2) Results corrected for contamination by chicken erythrocyte nuclei assuming that the H5 (see Fig. 1) derives from erythrocytes.
3) Results calculated such that the mean of H3, H2B and H4 is 1.00, since H2A is partly conjugated with ubiquitin as A24 (see Fig. 1 and text). This accounts for the core histone stoichiometry of less than 8.00.

* Number of determinations.

binding sites for H1. The probable presence of only one H1 per nucleosome in vivo may be explained in terms of mutual steric hindrance for example (18), and the unfilled site may retain a weaker affinity for a second molecule when provided in vitro (18,19). If two H1 sites do indeed exist, then a single molecule must bind asymmetrically in one of two orientations, thus, in effect, distinguishing the two strands of DNA. This may have implications for gene expression as suggested previously (47).

In chicken erythrocyte nuclei, which contain H5 as well as H1, the amount of H5 in the nucleus corresponds to one molecule per nucleosome, and the arguments made above for H1 apply equally to H5. However, the combined (H1 + H5) content amounts to about 1.3 molecules per nucleosome (substantially lower than values of about two determined earlier by dye-staining methods; see Introduction). Thus although there is not enough H5 in the nucleus for each nucleosome to bind two molecules, there is sufficient (H1 + H5) for essentially all the nucleosomes to contain H5 and for 30% of them to contain H1 also, perhaps bound to a second, low-affinity site. However, since we are measuring bulk properties, we can only speculate on the H1/H5 content of individual nucleosomes. Nonetheless, in view of the nuclear H5 stoichiometry, it seems not unreasonable to speculate that each nucleosome binds one H5, and that this, rather than an accumulation of non-core histones (H1 and H5) up to a level of 1.3 molecules per nucleosome, may be the significant feature in relation to the transcriptional inactivity of the mature chicken erythrocyte nucleus.

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