Volume 253, number 1,2, 141-145 FEB 07466 August 1989

Histone H4 acetylation in human cells

Frequency of acetylation at different sites defined by immunolabeling with site-specific antibodies

Bryan M. Turner, Laura P. O'Neill and Irene M. Allan

School of Basic Medical Sciences, Anatomy Department, University of Birmingham Medical School, Birmingham B15 2TJ, England

Received 13 June 1989

Histone H4 can be reversibly acetylated at lysine residues 5, 8, 12 and 16. It is possible that acetylation of individual residues will exert specific effects on chromatin function, but this hypothesis is difficult to test with present techniques for analysis of acetylation. To address this problem, we have prepared antibodies which distinguish H4 molecules acetylated at each of the sites used in vivo. By electrophoresis and immunolabeling we have shown that, in H4 from human cells, the four lysine residues are acetylated in a preferred, but not exclusive order, namely lysine 16, followed by 12 and 8, followed by 5.

Histone H4; Acetylation site; Synthetic peptide; Site-specific antibody; (HL60 cell)

1. INTRODUCTION

The core histones in eukaryotic cells are all subject to cyclical, enzyme catalyzed acetylation and deacetylation at defined lysine residues [1]. This modification can influence chromatin structure [2] and evidence is accumulating that histones associated with actively transcribed genes are more highly acetylated than those from non-transcribed regions [3–5].

Histone H4 can be acetylated at up to four lysine residues in its amino-terminal domain. As this region is identical in most eukaryotic organisms [1], it seems probable that each residue has a defined and significant role and that modification of particular lysines by acetylation will exert specific effects on chromatin structure and function. However, it has proved difficult to test this possibility because, until recently, identification of specific acetylated lysines has required sequencing and/or peptide analysis [6,7], techniques which require purified material from relatively large numbers of cells. We have attempted to develop an alternative approach more suited to functional studies by preparing antibodies which recognise H4 molecules acetylated at specific sites [8]. In this publication we describe antisera which distinguish H4 molecules acetylated at each one of the four possible sites. These antisera have enabled us to define the order in which these sites are used in human cells.

Correspondence address: B.M. Turner, Anatomy Department, University of Birmingham Medical School, Vincent Drive, Birmingham B15 2TJ, England

2. MATERIALS AND METHODS

2.1. *Preparation of antisera*

Peptides were prepared by the University of Birmingham Macromolecular Analysis Service as outlined previously [8]. Peptides were conjugated to ovalbumin (Sigma) with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce Chemical Co.) as described by Lee et al. [9]. New Zealand White rabbits were injected subcutaneously at two sites with peptide-ovalbumin conjugate (100 μg/site) in complete Freund's adjuvant. 24 days later animals were injected in the

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

same way with free peptide or with peptide ovalbumin conjugate. Blood was taken from the ear vein at approximately two weekly intervals thereafter.

2.2. *Solid-phase immunoassay*

Polystyrene microtitre plates (Dynatech) were coated overnight at 4°C with 10 μg/ml histone in phosphate buffered saline (PBS). Plates were blocked for 1 h with 1% bovine serum albumin in PBS. Rabbit antiserum, appropriately diluted in blocker, was added at 50 μl/well. After 1 h at 4°C plates were washed sequentially with PBS/0.1% Tween 20/1 M NaCl, PBS/0.1% Tween 20 and PBS alone. Peroxidase-conjugated goat anti-rabbit IgG (Sigma), diluted 1000-fold in blocker, was added at 50 μl/well and the plates incubated for 1 h at room temperature. Plates were washed twice in PBS/0.1% Tween 20 and four times in distilled water before addition of 100 μl/well *o*-phenylenediamine dihydrochloride solution (0.4 mg/ml in 24 mM citric acid, 51 mM Na_2HPO_4, 0.012% H_2O_2, pH5). After 10 min at room temperature the reaction was terminated with 100 μl/well 5% H_2SO_4. Absorbance at 492 nm was read on a Multiscan Plus plate reader. Inhibition assays were carried out as previously described [8] by mixing varying concentrations of synthetic peptide with a dilution of antiserum giving 60–80% maximum binding. Mixtures were tested against histones from butyrate-treated HL60 cells.

2.3. *Cells, histone extraction, electrophoresis and Western blotting*

Human promyeloid HL60 cells [10] were grown in RPMI 1640 medium, 10% foetal calf serum in an atmosphere of 5% CO_2 in air. Hyperacetylation of histones [11,12] was induced by growing the cells for 4–6 h in medium supplemented with 5–10 mM sodium butyrate (Sigma). Histones were extracted from nuclear pellets with 0.2 N HCl as described previously [8]. Sodium butyrate (2 mM) was present throughout the preparation procedure. Electrophoresis on acid/urea/Triton gels [13], transfer to nitrocellulose filters [14], immunolabeling and scanning of gels and autoradiographs, were carried out as previously described [8].

3. RESULTS AND DISCUSSION

3.1. *Preparation of antisera*

Sixteen rabbits were immunized with synthetic peptides corresponding to sequences of ten or more amino acids in the amino-terminal region (1–18) of histone H4. Peptides contained acetyllysine (aK) at all four possible sites (peptide PaK_4), at a single site (PaK_15, etc.) or at none (PaK_0, see table 1). Thirteen of the immunized animals produced anti-H4 antibodies. The six antisera used for the experiments described are listed in table 1.

3.2. *Characterization of antibody specificity*

The specificity of each antiserum for individual acetylated sites was tested by measuring the ability of synthetic peptides to inhibit antibody binding to histones in a solid-phase immunoassay. Results are shown in fig.1. Animals immunized with peptides acetylated at lysines 8, 12 and 16 respectively produced anti-histone antibodies which were inhibited by the immunizing peptide, but not by other mono-acetylated peptides (fig.2A, B and D). In contrast, of the two animals immunized with PaK_15, one failed to respond while the other (R17) produced antibodies which recognised both PaK_15 and PaK_112 (fig.1C). However, an antiserum specific for peptides acetylated at lysine 5 was obtained by immunization with the tetra-acetylated peptide PaK_4, as previously described [8]. In what follows the antisera will be referred to by numbers which define both the particular animal and site specificity, i.e. R6/5, R12/8, etc.

Three of the site-specific antisera R6/5 [8], R20/12 and R14/16 (fig.2B and D) were effectively inhibited by PaK_4. Thus, binding of these antibodies was not influenced by acetylation at adjacent sites, from which it can be concluded that the epitopes recognised do not incorporate adjacent lysine residues (table 1). In contrast, antiserum R12/8 was not inhibited by this peptide (fig.2A), showing that the antibodies in this serum recognise an epitope which requires acetylated lysine 8 together with lysine 5 and/or 12 in the non-acetylated form.

Thus, the inhibition experiments define both antibody specificities and, in some cases, the maximum extent of the epitopes involved. Our conclusions are summarised in table 1. Despite the similarity between three of the four acetylation sites (sites 5, 8 and 12 all contain the Gly-Lys-Gly tripeptide), all but one of the sera tested contain antibodies directed against a single acetylated site and against a narrowly defined epitope. A dual specificity was found only in antiserum R17/5,12 and was confined to the sites at lysines 5 and 12, which share the tetrapeptide Gly-Lys-Gly-Gly.

All four mono-acetylated peptides contain sequences of five or more amino acids which are identical to sequences in non-acetylated H4 (table 1). The results in fig.1B and D show that the non-acetylated peptide could inhibit binding to HL60 histones by 10–20%, suggesting that some sera contain antibodies to non-acetylated H4. These could be completely removed by absorption with immobilised non-acetylated peptide (not shown)

Table 1

Antiserum	Immunizing peptide[a]			Epitope	
	Sequence		Name[b]	Determinant[c]	Extent
	1 5 10 15				
R6/5	AcS G R G * G G * G L G * G G A * R H Y C		PaK_4	aK5	N-term to G7
R17/5,12	S G R G * G G K G L Y C		PaK_15	aK5/12	G*GG
R12/8	S G R G K G G * G L G K Y C		PaK_118	aK8	includes K5 or K12
R20/12	G K G L G * G G A K Y C		PaK_112	aK12	G9 to A15
R14/16	G L G K G G A * R H Y C		PaK_116	aK16	G13 to H18
R15/0	S G R G K G G K G L G K G G A K R H Y C		PaK_0	not known	not known

[a] Single letter code, for emphasis acetyl lysine (aK) is shown as *
[b] These peptides were previously designated Y600, Y632, etc. [8]
[c] Antisera bound only to peptides acetylated at these residues (fig.1)

and have not proved to be a problem in Western blotting experiments, even with non-absorbed serum (see below).

3.3. *Site usage in human HL60 cells*

Histones from HL60 cells, either untreated or treated with sodium butyrate to induce histone hyperacetylation [11,12], were resolved by electrophoresis on acid/urea/Triton gels and either stained with Coomassie blue or transferred to nitrocellulose filters and immunostained with site-specific antisera. Results are shown in fig.2.

Antisera raised against the acetylated peptides labeled only the acetylated forms of H4 (e.g. fig.2A, tracks 3,4). In contrast, R15/0, raised against the non-acetylated peptide PaK_0, labeled $H4Ac_{0-2}$ from both butyrate-treated and untreated cells (tracks 5,6) but did not label $H4Ac_3$ and $H4Ac_4$. This result is consistent with the inability of R15/0 to bind to PaK_4 in the inhibition ELISA (not shown) and shows that, as might be expected, the epitopes recognised by this antiserum are masked by acetylation. Labeling of H4 with pre-immune serum was insignificant (tracks 7,8).

In order to ensure complete specificity for only a single acetylated site, antisera were routinely pre-absorbed with all peptides other than that corresponding to the target acetylation site (see legend to fig.2). Antibodies affinity purified on peptide-Sepharose columns (unpublished results) were used for some experiments (R12/8 and R14/16, fig.2B). As an additional test of the specificity of antibody labeling, the inhibitory effect of the peptide corresponding to the target acetylation site (i.e. the immunizing peptide) was tested for each antiserum in the Western blotting-immunostaining assay. Scanning of autoradiographs showed that these peptides, at concentrations of 1–2 μg/ml, reduced antibody binding by at least 90%.

The results of immunolabeling H4 from untreated and butyrate-treated cells with pre-absorbed antisera to acetylated H4 are shown in fig.2B. Only R14/16 (tracks 7,8) gave strong labeling of H4 from untreated cells. These sera labeled the mono-acetylated isoform ($H4Ac_1$) more intensely than the di-acetylated isoform ($H4Ac_2$) and gave very weak labeling of $H4Ac_3$. Butyrate treatment resulted in increased labeling of $H4Ac_{2-4}$ (track 7). In contrast, antisera R6/5, R12/8 and R20/12 weakly labeled $H4Ac_1$ from both butyrate-treated and untreated cells in comparison with the labeling of the more acetylated isoforms. Thus, in both butyrate-treated and untreated cells, most $H4Ac_1$ molecules are acetylated at lysine 16, with relatively small few acetylated at lysines 5, 8 or 12, instead.

R12/8 labeled $H4Ac_4$ very weakly (fig.2B, track 3). As all four sites must be acetylated in $H4Ac_4$, binding of this antiserum to lysine 8 must be inhibited by acetylation of adjacent sites (i.e. lysines

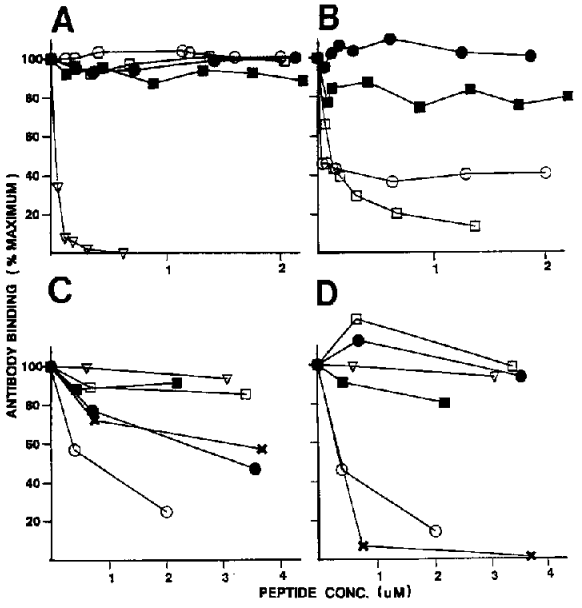

Fig.1. Inhibition by synthetic peptides of antibody binding to HL60 histones. Antisera tested are as follows: (A) R12/8, (B) R14/16, (C) R17/5, (D) R20/12. Inhibiting peptides are represented by the following symbols: PaK_4 (○), PaK_15 (●), PaK_18 (▽), PaK_112 (×), PaK_116 (□), PaK_0 (■). All four sera were tested against all six peptides. Where, for clarity, results for a particular peptide are not shown inhibition by that peptide was negligible.

5 and/or 12). This conclusion is entirely consistent with the failure of the tetra-acetylated peptide PaK_4 to inhibit binding of this antiserum in ELISA assays (fig.1).

The intensity of labeling of H4 isoforms resolved on acid/urea/Triton gels will depend on the concentration of antibody applied to the filter, the amount of each isoform present and the frequency with which the site recognised by the antiserum is used in each isoform. It is possible to measure both the relative amounts of protein in the different H4 isoforms and the amount of antibody bound, by scanning Coomassie blue-stained gels and autoradiographs, respectively [8]. Antibody binding per unit of protein can then be calculated. This will be directly proportional to the frequency with which a particular site is used in each acetylated isoform. Typical values are shown in fig.3. The results confirm the impression gained by visual inspection of autoradiographs, namely that lysine 16 is the most frequently acetylated site in $H4Ac_1$, with lower levels of acetylation at lysines 8

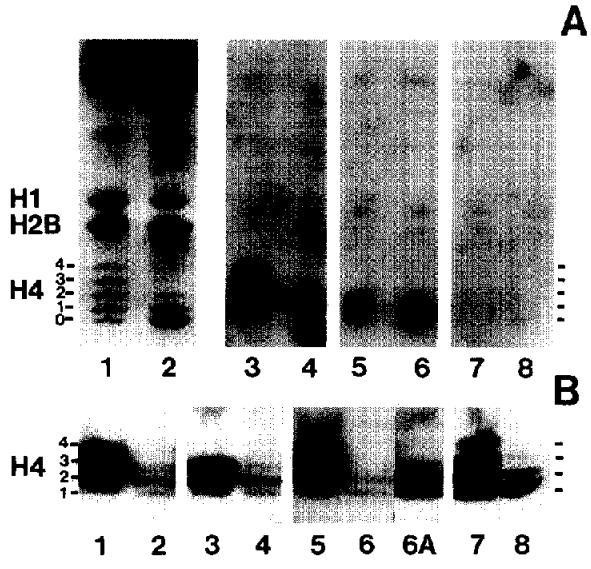

Fig.2. Immunolabeling of HL60 histones separated by electrophoresis on acid/urea/Triton gels. Histones were extracted from butyrate-treated (1,3,5,7) or untreated (2,4,6,8) cells. The number of acetate groups in each H4 isoform is indicated (0–4). (Panel A) Tracks: 1,2, total proteins detected by Coomassie blue; 3–8, proteins transferred to nitrocellulose filters and immunolabeled with R14/16 (3,4), R15/0 (5,6) and non-immune serum (7,8). (Panel B) Tracks 1–8, proteins transferred to nitrocellulose filters and immunolabeled with R6/5 (1,2), affinity purified R12/8 (tracks 3,4), R20/12 (5,6 and 6A, which is a longer autoradiographic exposure of track 6), affinity purified R14/16 (7,8). For the experiment shown in panel B, sera used for labeling were mixed with synthetic peptide PaK_0 plus 1–2 μg/ml of three of the four mono-acetylated peptides shown in table 1, as follows: R6/5 (all except PaK_15), R12/8 (all except PaK_18), R20/12 (all except PaK_112), R14/16 (all except PaK_116).

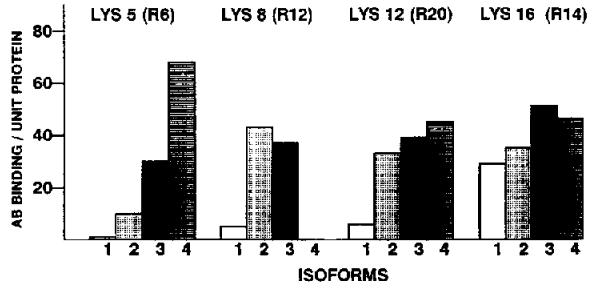

Fig.3. A histogram showing antibody binding (AB) per unit of protein to each of the acetylated isoforms of H4 from human HL60 cells (numbered 1 to 4 on the *x*-axis). Antibody binding was assayed by scanning autoradiographs of immunolabeled filters. Autoradiographs used for scanning were exposed for shorter periods of time than those shown in fig.2, which are overexposed to reveal minor bands.

and 12 and particularly low usage of the site at lysine 5. There is a rapid increase in acetylation at lysines 8 and 12 in $H4Ac_2$ and $H4Ac_3$, whereas use of lysine 5 increases more slowly, matching that of the other acetylation sites only in $H4Ac_4$.

As outlined elsewhere [8], by expressing antibody binding per unit protein for each isoform as a proportion of binding per unit protein to $H4Ac_4$ (which should give the maximum possible value), one can calculate the proportion of H4 molecules in that isoform which are acetylated at each site. Thus, from the data presented in fig.3, 60–65% of all $H4Ac_1$ molecules are acetylated at lysine 16, 10–15% at lysine 12 and only 1–2% at lysine 5. Use of the sites at lysines 12 and 16 increases to between 70 and 100% in $H4Ac_2$ and $H4Ac_3$ whereas only about 15% and 45%, respectively, are acetylated at lysine 5. These values are typical of several experiments. Such calculations are not possible for the site at lysine 8 because of the weak binding of R12 to $H4Ac_4$. However, use of this site in $H4Ac_2$ and $H4Ac_3$ is clearly much more frequent than in $H4Ac_1$.

The steady-state frequency of use of each acetylation site in H4 is the net result of the actions of acetylating and deacetylating enzymes. Thus, the fact that most $H4Ac_1$ molecules in normal cells are acetylated at lysine 16, could formally be explained by either of two models. (i) Acetylation at lysine 16 is usually the first step in the acetylation pathway, or (ii) a number of sites can be acetylated first, but lysine 16 is relatively resistant to subsequent deacetylation. The second model predicts an increase in the use of sites other than lysine 16 after inhibition of deacetylases by butyrate. The fact that this did not occur (figs 2 and 3), shows that acetylation at lysine 16 normally precedes acetylation at other sites. Thus, we can conclude that in human cells, as H4 molecules move to progressively more acetylated forms, the four acetylation sites are used in a preferred, but not exclusive order, namely lysine 16, followed by lysines 8 and 12, followed by lysine 5.

We have obtained similar results with human HL60 cells, HeLa cells and peripheral blood lymphocytes and with Chinese hamster ovary fibroblasts. Our findings are also in general agreement with the amino acid sequencing of monoacetylated calf thymus H4 [7,15], which detected acetyllysine at position 16. The use of H4 acetylation sites in mammalian cells is clearly different from that in two other experimental systems studied to date, namely cuttlefish testis [7] and the macronucleus of *Tetrahymena* [6]. In both these organisms sites are used in a strict order, namely 12, then 5, then 16 for cuttlefish and 7, then 4, then 11 or 15 for *Tetrahymena* (which lacks the arginine residue at position three of other species [1]). Whether these differences are a reflection of evolutionary distance or the different requirements of the tissues involved remains to be established, as does the possible functional significance of the alternative orders of site usage which appear to be possible in at least some human cell types.

Acknowledgements: We thank Patricia Pitters for skilled technical assistance and Alison Orchard for photographic work. This work was supported by grants from the Wellcome Trust and the UK Cancer Research Campaign.

REFERENCES

[1] Wu, R.S., Panusz, H.T., Hatch, C.L. and Bonner, W.M. (1984) CRC Crit. Rev. Biochem. 20, 201–263.
[2] Simpson, R.T. (1978) Cell 13, 691–699.
[3] Allegra, P., Sterner, R., Clayton, D.F. and Allfrey, V.G. (1987) J. Mol. Biol. 196, 379–388.
[4] Ridsdale, J.A. and Davie, J.R. (1987) Nucleic Acids Res. 15, 1081–1096.
[5] Hebbes, T.R., Thorne, A.W. and Crane-Robinson, C. (1988) EMBO J. 7, 1395–1403.
[6] Chicoine, L.G., Schulman, I.G., Richman, R., Cook, R.G. and Allis, C.D. (1986) J. Biol. Chem. 261, 1071–1076.
[7] Couppez, M., Martin-Ponthieu, A. and Sautiere, P. (1987) J. Biol. Chem. 262, 2854–2860.
[8] Turner, B.M. and Fellows, G. (1989) Eur. J. Biochem. 179, 131–139.
[9] Lee, A.C.J., Powell, J.E., Tregear, G.W., Niall, H.D. and Stevens, V.C. (1980) Mol. Immunol. 17, 749–756.
[10] Collins, S.J. (1987) Blood 70, 1233–1244.
[11] Candido, E.P., Reeves, R. and Davie, J.R. (1978) Cell 14, 105–113.
[12] Sealy, L. and Chalkley, R. (1978) Cell 14, 115–121.
[13] Alfageme, C.R., Zweidler, A., Mahowald, A. and Cohen, L.H. (1974) J. Biol. Chem. 249, 3729–3736.
[14] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[15] De Lange, R.J., Fambrough, D.M., Smith, E.L. and Bonner, J. (1969) J. Biol. Chem. 244, 319–334.