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Histone H4 acetylation in *Drosophila*

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

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Electrophoresis, Western blotting and immunostaining with antibodies specific for histone H4 acetylated at lysines 5, 8, 12, or 16, were used to define patterns of H4 acetylation in cell lines from humans (HL60) and the fruit fly *Drosophila* (S2, Kc). In human cells, the mono-acetylated isoform $H4Ac_1$ is acetylated predominantly at just one of the four possible lysine residues, lysine 16. This is the first step in the progressive acetylation of H4. In contrast, in *Drosophila*, $H4Ac_1$ is acetylated at lysines 5, 8, or 12 with approximately equal frequency. Fundamental differences appear to exist in control of H4 acetylation in different species, despite the evolutionary conservation of acetylation sites.

Histone H4; Acetylation site; Western blottting; Antibody; *Drosophila*

1. INTRODUCTION

In all animal species examined so far, 4 lysines in the amino-terminal domain of histone H4 are subject to cyclical, enzyme-catalyzed, acetylation and deacetylation, the net result of which is that each one has its own characteristic, steady-state level of acetylation. Changes in histone acetylation are associated with changes in the transcription, replication and packaging of DNA and this modification may play a central role in the control of chromatin function (reviewed in [1,2]).

In the few species studied so far, lysines appear to be acetylated in a fixed order. For example, in cuttlefish testis the mono-acetylated isoform $H4Ac_1$ is acetylated exclusively at lysine 12, with the more acetylated isoforms being generated by addition of acetate groups to lysines 5, 16 and 8 in strict sequence [3]. In humans and other mammals the situation is rather different. Acetylation at a single site, lysine 16, predominates in $H4Ac_1$, but thereafter site usage is more flexible, with isoforms $H4Ac_2$ and $H4Ac_3$ containing a mixture of molecules acetylated at lysine 16 and lysines 8, 12 and 5 [4,5].

We have recently prepared a panel of antisera which can distinguish H4 molecules acetylated at different lysine residues [4,6]. These antibodies are being used to test the hypothesis that acetylation at each lysine has specific effects on chromatin structure and function. An approach which is proving particularly valuable to this end is to use site-specific antisera to immunolabel the giant polytene chromosomes found in some insect cells [7]. By this means it is possible to define both the distribution of acetylated H4 through the interphase genome and its association with functionally different chromatin types. However, in order to interpret such experiments, it is important to know both the steady-state level of H4 acetylation in the species under study and the frequency with which different lysine residues are acetylated. For this reason we have investigated the use of H4 acetylation sites in cells from the fruit fly *Drosophila* using a new approach based on electrophoresis in acid/urea/Triton gels, Western blotting and immunolabelling.

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2. MATERIALS AND METHODS

2.1. *Antisera*

Rabbit antisera against H4 acetylated at specific lysines were prepared by immunization with synthetic peptides and characterized as described previously [4,6]. Antisera are numbered so as to define both the source of the serum and the specificity. Thus, antiserum R6/5 is from rabbit 6 and recognizes H4 molecules acetylated at lysine 5.

2.2. *Cells and tissues*

Drosophila embryo cell lines S2 and Kc [8] were obtained from Dr W.G.F. Whitfield (CRC Eukaryotic Molecular Genetics Research Group, University of Dundee, UK) and grown at 24°C in Schneider's Drosophila medium supplemented with 8% foetal calf serum, penicillin, and streptomycin (all from Gibco). The human promyeloid

cell line HL60 [9] was grown at 37°C in RPMI 1640 medium, 10% foetal calf serum, penicillin and streptomycin, in 5% CO_2 in air. Histone hyperacetylation was induced by growth for 4–16 h in medium supplemented with 5–10 mM sodium butyrate [10,11].

2.3. *Histone extraction, electrophoresis and Western blotting*

Histones were extracted from nuclear pellets with 0.2 N HCl as described previously [6]. Sodium butyrate (2 mM) was present throughout the preparation. Electrophoresis on acid/urea/Triton gels [12], transfer to nitrocellulose filters [13], immunolabelling with site-specific and ^{125}I-conjugated anti-rabbit antibodies and preparation of autoradiographs, were carried out as previously described [4]. As before, we attempted to ensure complete specificity of antibody labelling by including in the labelling mixture for each antibody, the three synthetic peptides (1–2 μg/ml) corresponding to the three sites not recognized by that antibody. Gels stained with Coomassie blue and autoradiographs were scanned and integrated on an LKB Ultrascan laser densitometer.

2.4. *Calculation of frequencies of acetylation at each lysine residue*

The final value for the labelling of each isoform transferred from acid/urea/Triton gels with a given antibody depends on (1) the protein content of the gel band representing that isoform, (2) the extent to which the lysine residue recognized by the antibody is acetylated in that isoform (which is what we are trying to calculate), and (3) experimental factors, such as the concentration of antibody used for labelling and the length of time for which the film is exposed in preparing the autoradiographs.

To calculate the extent to which each lysine residue is acetylated in each isoform, antibody binding to each isoform (ax_n, where x defines the specificity of the antiserum for lysine 5, 8, 12, or 16 and n defines the isoform, 1–4) is first divided by the amount of protein in that isoform (p_n). The antibody binding per unit of protein for each isoform (ax_n/p_n) is then expressed as a proportion of the value for the tetra-acetylated isoform, i.e. $(ax_n/p_n)/(ax_4/p_4)$. This sets the binding per unit of protein to $H4Ac_4$ to 100%, which is what it should be, given that each site must be fully acetylated in this isoform. Expressing the values as a proportion of $H4Ac_4$ also serves to eliminate variation from one antibody to another due to experimental factors (3 above). Ideally, these two operations should give an accurate estimate of the percentage acetylation of each of the four sites in each isoform. In practice, however, two technical factors influence these values.

The first is that the protein content of the tetra-acetylated isoform can be overestimated due to co-migation of non-H4 proteins. (A good example is seen in Fig. 1C.) The effect of this is that values for antibody binding per unit of protein to $H4Ac_4$ tend to be too low and values for other isoforms expressed as a proportion of this therefore too high. This manifests itself as occasional values over 100%. This is not a serious problem and does not effect the relative level of acetylation at each site for a given isoform. Its effects can be minimised by adjusting the % acetylation at each site for each isoform by a common factor, so that values for $H4Ac_1$, $H4Ac_2$ and $H4Ac_3$ add up to 100, 200 and 300%, respectively.

The second problem derives from the inefficient binding of antibody R12/8 to tetra-acetylated H4. We have shown previously that binding of this antibody to acetylated lysine 8 is inhibited by acetylation of one or both of the adjacent lysines [4]. As a result, binding of R12/8 to $H4Ac_4$ will be reduced by an unknown factor. We have allowed for this in calculating the final results by taking binding to $H4Ac_2$ or $H4Ac_3$ (whichever is higher) as 100% for R12/8, rather than binding to $H4Ac_4$. Because of this, values for acetylation at lysine 8 will always be maximum estimates.

3. RESULTS

Histones were prepared in parallel from the *Drosophila* cell line S2 and from human HL60 cells, either untreated or grown overnight in the presence of sodium butyrate to inhibit histone deacetylation. Histones were resolved by electrophoresis on acid/urea/Triton gels, transferred to nitrocellulose filters and immunostained with antisera capable of distinguishing between H4 molecules acetylated at each of the four lysine residues. Scans of Coomassie blue-stained gels and autoradiographs from a representative experiment are shown in Fig. 1.

It is apparent from the Coomassie blue-stained tracks (Fig. 1A–D) that the steady-state level of H4 acetylation is less in *Drosophila* than in human HL60 cells (compare panels A and C). Scanning and integration of the appropriate peaks showed that 40% of HL60 H4 molecules contain one or more acetates, whereas only 18% of S2 H4 is acetylated. Sodium butyrate increased H4 acetylation in *Drosophila* cells, as it does in most other species, but the effect was less dramatic than in human cells (compare panels B and D). Increasing the level of butyrate (to 40 mM) or substituting propionate or valerate, both effective deacetylase inhibitors [11] did not increase the effect. We conclude that either histone acetate groups turn over more slowly in *Drosophila* cells than in human, or a sub-population of *Drosophila* deacetylases is resistant to inhibition by short chain fatty acids.

Scans from autoradiographs prepared from S2 and HL60 histones labelled with site-specific antisera are shown in Fig. 1E–L. All results shown are from the same experiment in which *Drosophila* and human histones were run on adjacent tracks and transferred and immunolabelled together. There are clear dif-

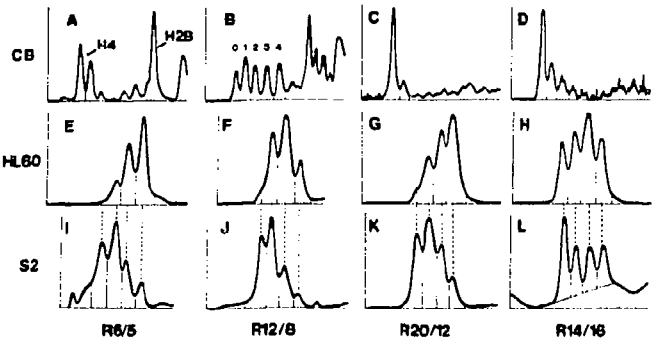

Fig. 1. Scans of histones separated on acid/urea/Triton gels and stained with Coomassie blue (panels A–D) or transferred to nitrocellulose and immunolabelled with antisera to acetylated H4 (panels E–L). A,B and E–H: Histones from human HL60 cells treated overnight with butyrate (B, E–H), or untreated (A). C,D and I–L: Histones from *Drosophila* S2 cells treated overnight with butyrate (D, I–L) or untreated (C). Tracks containing S2 or HL60 histones and labelled with the same antiserum (R6/5, R12/8, R20/12 or R14/16) have been placed one above the other and corresponding peaks aligned (dotted lines). These antisera recognize only the acetylated isoforms. The number of acetate groups in each H4 isoform is indicated in panel B. Vertical lines define the peaks used for integration. In one case (panel L) a combination of high background and weak labelling made it necessary to change the baseline to avoid distortion of peak areas. The baseline used for integration is shown.

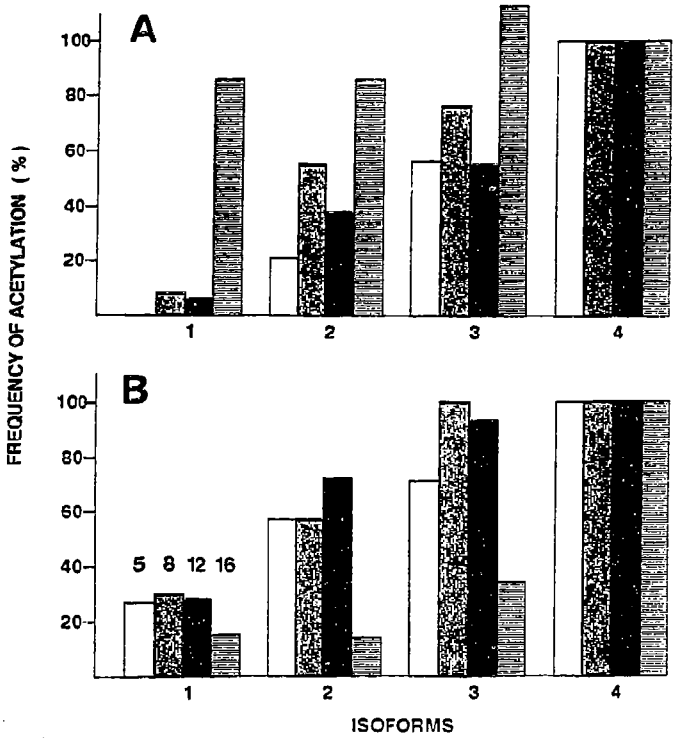

Fig. 2. Frequency of acetylation at lysines 5, 8, 12 and 16 in the mono-, di-, tri- and tetra-acetylated isoforms of H4 from human HL60 cells (A) and *Drosophila* S2 cells (B). The four vertical bars for each isoform show the frequency of acetylation at each of the four sites (5 to 16, left to right as indicated in panel B).

ferences between the labelling patterns of histones from the two species. Most strikingly, antisera R12/8 and R20/12 labelled the mono-acetylated isoform weakly in human H4 (panels F and G) but relatively strongly in *Drosophila* (panels J and K). In the scans shown in Fig. 1, the *y* axes are set so that the maximum peak height for each scan is the same and they therefore cannot be used to compare the strength of labelling with different antibodies. This can of course be done after integration of peaks or simply by inspection of the original autoradiographs (not shown). In fact, the antisera labelled human and *Drosophila* H4 with similar intensities, although with labelling differently distributed among the isoforms, with the exception of R14/16 which labelled *Drosophila* H4 comparatively weakly.

The extent to which each lysine residue is acetylated in each H4 isoform was calculated as outlined in section 2. The results are presented in Fig. 2. In HL60 cells, $H4Ac_1$ was acetylated predominantly at lysine 16, and $H4Ac_2$ at lysines 16, 8 and, to a lesser extent, 12. These findings are generally consistent with previous results obtained by both immunolabelling and sequencing [4,5]. In contrast, in *Drosophila* S2 cells, $H4Ac_1$ was acetylated at lysines 12, 8 and 5 in roughly equal proportions, with acetylation at 16 lower but still significant. Acetylation at each site increased in $H4Ac_2$, with acetylation at lysine 16 remaining relatively low. Similar experiments have been carried out with the independently derived *Drosophila* cell line Kc [8] and have shown that, in these cells too, $H4Ac_1$ is a mixture of molecules acetylated at all four sites, with acetylation at lysines 8 and 12 occurring most frequently.

Calculation of the frequency of acetylation at each lysine residue can only be carried out if sufficient tetra-acetylated H4 is present to give a measurable signal with each antibody. This can only be achieved by treating the cells with butyrate, which has the potential disadvantage that the inhibitor may distort the normal pattern of H4 acetylation. To test this, we have compared the binding per unit of protein of each antibody to the mono- and di-acetylated isoforms from butyrate-treated and untreated cells. If butyrate treatment causes a shift in the way individual sites are used, then differences in antibody binding should occur. As shown in Table I, within each cell type the values are generally very similar, bearing in mind the wide range of variation possible, and not consistent with a major, butyrate-induced shift in the pattern of site usage. The small effect of butyrate treatment on site usage also shows that acetylation at each site in $H4Ac_1$ arises predominantly by direct acetylation (i.e. $Ac_0 \rightarrow Ac_1$) rather than by deacetylation of $H4Ac_2$ (i.e. via the indirect pathway $Ac_0 \rightarrow Ac_1 \rightarrow Ac_2 \rightarrow Ac_1'$), which would be blocked by butyrate.

Table I

Antibody binding to the mono-acetylated (1) or di-acetylated (2) isoforms of histone H4 from *Drosophila* S2 and human HL60 cells, either treated with sodium butyrate (+) or untreated (−)

Antibody	Site detected	Antibody binding/unit protein					
		Drosophila S2		Human HL60			
		1+	1−	1+[1]	1−	2+	2−
R6/5	lysine 5	11.2	9.8	0.8	0.8	17.0	14.0
R12/8	lysine 8	9.6	15.7	1.1	2.3	17.5	18.4
R20/12	lysine 12	4.0	2.9	0.8	3.1	10.8	18.1
R14/16	lysine 16	11.4	7.1	6.9	9.8	14.0	12.4

[1]Binding of antisera R6/5, R12/8 and R20/12 to this isoform was very low (see Fig. 1) and the values given must be regarded as approximate.

We have carried out some experiments to determine whether the pattern of site usage found in cultured cells from *Drosophila* embryos is also found in the larval salivary glands used for immunofluorescence studies. The experimental results obtained so far suggest that it is, though we have been unable to carry out a complete, quantitative analysis of site usage due to the difficulty of inducing high levels of H4 acetylation in salivary glands. However, antisera to H4 acetylated at lysines 5, 8, or 12 all labelled the mono- and di-acetylated isoforms of H4 from salivary glands, whereas labelling was very weak with antibodies to H4 acetylated at lysine 16.

4. DISCUSSION

In humans and other mammals [4,5], cuttlefish [3] and tetrahymena [14], a single, specific lysine residue is the exclusive or predominant site of acetylation in the mono-acetylated H4 isoform ($H4Ac_1$). The results presented here confirm these findings by showing that in human HL60 cells $H4Ac_1$ is acetylated predominantly at lysine 16. In contrast, in *Drosophila* cells tested in parallel, this isoform is acetylated at lysines 5, 8, or 12 with approximately equal probability and less so at lysine 16. This indicates fundamental differences between these species in the control of H4 acetylation.

Initial acetylation at a single, defined lysine residue could be due either to the site specificity of the histone acetylase which attaches the first acetate group, or to better accessibility of a particular lysine residue, leading to preferential acetylation by a relatively non-specific enzyme. The fact that lysine 16 is the first residue to be acetylated in mammalian cells argues against the latter explanation. This residue is closely adjacent to core DNA [15] and is likely to be the least accessible of the four acetylation sites. Also, to explain the fact that the first site to be acetylated differs between species on the basis of accessibility differences, would require fundamental differences in chromatin conformation. Thus, catalysis of the first acetylation step by site-specific acetylases provides the most likely explanation for the observed results, which subsequent acetylation steps depending on enzymes which act preferentially on H4 molecules acetylated at the first site.

If the heterogeneous pattern of $H4Ac_1$ acetylation in *Drosophila* cells is to be explained by site-specific acetylases, then there must be different enzymes acting on different lysines and presumably located in different parts of the genome. (Limited distribution of these enzymes, with large areas of chromatin not subject to their activity, could explain the weak response to sodium butyrate found in *Drosophila* cells.) Alternatively, acetylation by a single enzyme with low site specificity but limited by the accessibility of the four lysines, is also consistent with the results obtained, particularly the fact that lysine 16, probably the least accessible, is relatively under-used. The former explanation predicts that H4 molecules acetylated at different lysines will show different patterns of distribution through the interphase genome, whereas the latter predicts no such differences. Immunolabelling experiments with polytene chromosomes are in progress to test these predictions.

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REFERENCES

[1] Csordas, A. (1990) Biochem. J. 265, 23-38.
[2] Turner, B.M. (1991) J. Cell Sci. 99, in press.
[3] Couppez, M., Martin-Ponthieu, A. and Sautiere, P. (1987) J. Biol. Chem. 262, 2854-2860.
[4] Turner, B.M., O'Neill, L.P. and Allan, I.M. (1989) FEBS Lett. 253, 141-145.
[5] Thorne, A.W., Kmiciek, D., Mitchelson, K., Sautiere, P. and Crane-Robinson, C. (1990) Eur. J. Biochem. 193, 701-703.
[6] Turner, B.M. and Fellows, G. (1989) Eur. J. Biochem. 179, 131-139.
[7] Turner, B.M., Franchi, L. and Wallace, H. (1990) J. Cell Sci. 96, 335-346.
[8] Schneider, I. and Blumenthal, A.B. (1978) in: The Genetics and Biology of *Drosophila*, Vol. 2a, (M. Ashburner and T.R.F. Wright, eds.) pp. 265-315, Academic Press, London.
[9] Collins, S.J. (1987) Blood 70, 1233-1244.
[10] Candido, E.P., Reeves, R. and Davie, J.R. (1978) Cell 14, 105-113.
[11] Sealy, L. and Chalkley, R. (1978) Cell 14, 115-121.
[12] Alfageme, C.R., Zweidler, A., Mahowald, A. and Cohen, L.H. (1974) J. Biol. Chem. 249, 3729-3736.
[13] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.
[14] Chicoine, L.G., Schulman, I.G., Richman, R., Cook, R.G. and Allis, C.D. (1986) J. Biol. Chem. 261, 1071-1076.
[15] Ebralidse, K.K., Grachev, S.A. and Mirzabekov, A.D. (1988) Nature 331, 365-367.