Selective use of H4 acetylation sites in the yeast *Saccharomyces cerevisiae*

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The acetylation of specific lysine residues in the histone H4 may play a role in regulating various genes in the yeast *Saccharomyces cerevisiae* [Grunstein (1990) *Annu. Rev. Cell Biol.* 6, 643–678]. The detailed consideration of this possibility has been hampered by the lack of information on the frequency with which different H4 lysine residues are acetylated in yeast. In this paper, we use Western blotting from acid/urea/Triton gels and immunostaining with antisera specific for H4 molecules acetylated at

particular lysine residues to show that 70–80% of H4 molecules in *S. cerevisiae* contain one or more acetylated lysines, and that lysines-5, -8, -12 and -16 are acetylated in an ordered, non-random fashion. The monoacetylated isoform (H4Ac₁) is acetylated predominantly at lysine-16 (rarely at lysine-12), H4Ac₂ is acetylated at lysine-16 and at either lysine-12 or at -8, while lysine-5 is acetylated frequently only in H4Ac₃ and in H4Ac₄.

INTRODUCTION

Histones that are associated with transcriptionally active chromatin are frequently more highly acetylated than those in the bulk chromatin (Allegra et al., 1987; Ridsdale and Davie, 1987; Hebbes et al., 1988; Boffa et al., 1990), and it has often been suggested that histone acetylation is an integral part either of the process by which chromatin is prepared for transcription or of the transcription mechanism itself (reviewed by Turner, 1991). The ways in which histone acetylation may influence gene expression are not known, but recent results from two very different experimental systems strongly indicate that the functional effects are exerted not simply through changes in the overall level of histone acetylation (that is through a general reduction in net positive charge), but through modification of specific lysines in individual histones.

One experimental approach that has led to this conclusion involves the immunolabelling of polytene chromosomes from insect larvae with antisera that are specific for H4 molecules acetylated at each one of the four acetyltable lysines (that is residues 5, 8, 12 and 16). H4 molecules acetylated at different lysines were found to have characteristic patterns of distribution through the interphase genome (Turner et al., 1992). Particularly striking was the finding that H4 acetylated at lysine-16 is associated almost exclusively with a single chromosome, namely the transcriptionally hyperactive X in male larvae (Turner et al., 1992).

Further evidence for the functional significance of site-specific acetylation stems from a series of genetic experiments that have been carried out in the yeast *Saccharomyces cerevisiae* (Grunstein, 1990). These experiments have shown that mutants carrying deletions of the N-terminal domain of H4 (but not of other histones) show inappropriate expression of the normally silent mating-type genes, and a consequent reduction in mating efficiency (Kayne et al., 1988; Johnson et al., 1990; Megee et al., 1990; Park and Szostak, 1990). Substituting a neutral amino acid (but not a positive one) for lysine-16 also substantially reduces mating efficiency, whereas substitution, individually or collectively, for lysines-5, -8 or -12 causes only a minor phenotypic change (Johnson et al., 1990; Megee et al., 1990; Park and

Szostak, 1990). It has been suggested that the neutralization of lysine-16 *in vivo* by acetylation may serve to induce the expression of the mating-type genes when required (Johnson et al., 1990; Park and Szostak, 1990), but direct evidence for this is lacking.

It has been known for some years that the steady-state level of H4 acetylation in yeast chromatin is greater than that which is found in higher eukaryotes (Nelson, 1982). However, the frequency with which individual lysines are acetylated in yeast (the pattern of H4 acetylation) is not known. Knowledge of this pattern would be useful in interpreting the properties of the various H4 deletion and substitution mutants, and in formulating models for the effect of H4 acetylation in yeast. A method has been described for defining patterns of H4 acetylation by Western blotting from acid/urea/Triton (AUT) gels and immunostaining with antibodies that are specific for H4 acetylated at different lysine residues (Turner et al., 1989; Munks et al., 1991). The Western-blotting approach has a major advantage over protein sequencing (the only other way of addressing this question) of requiring only small amounts of unpurified histones. The extreme conservation of the N-terminal domain of H4 means that the same antisera can be used to study a wide range of species. In this paper we describe the application of the Western-blotting technique to determine the pattern of H4 acetylation in *S. cerevisiae*.

MATERIALS AND METHODS

Antisera

Antibodies against H4 acetylated at particular lysine residues were raised in New Zealand White rabbits by immunization with synthetic peptides as described previously (Turner and Fellows, 1989; Turner et al., 1989). Antisera are referred to by numbers that define both the individual animal and the acetylated lysine residue that the antiserum recognizes. Thus, R6/5 is from rabbit 6 and recognizes H4 that is acetylated at lysine-5.

Cultured cells

S. cerevisiae strain A364A (adenine-dependent) was grown to mid-log phase in medium containing 1% (w/v) yeast extract, 2% (w/v) bacto tryptone, 2% (w/v) glucose and supplementary

Abbreviation used: AUT, acid/urea/Triton.

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adenine (50 mg/ml). Liquid culture (1 ml) was inoculated into 1 litre of medium in a 2 litre flask and grown with shaking at 28 °C until there were 5×10^9 – 10×10^9 cells/l (about 18 h).

Human HL-60 promyeloid cells were grown in RPMI-1640 medium containing 8 % (v/v) fetal-calf serum in an atmosphere of 5 % CO₂ in air. Histones were hyperacetylated by growing the cells in a medium containing 5–10 mM sodium butyrate for 4–16 h, and extracted in 0.4 M HCl as previously described (Turner and Fellows, 1989).

Preparation of yeast histones

Yeast nuclear proteins were extracted from nuclei using the methods described by Nelson (1982) with only minor adjustments. Lyticase (Sigma Chemical Co.; 50 units/g of wet cells) was used to break down cell walls and to produce spheroplasts. Subsequent steps in nuclear isolation were carried out at 0–4 °C. Either 5 mM spermidine or 0.5 mM spermidine plus 10 mM sodium butyrate was added to all the buffers throughout the entire preparation procedure, to minimize the activity of histone deacetylases (Vu et al., 1987).

To partially purify yeast histones, we used a modification of the procedure of Nelson and Alonso (1983). Nuclear pellets were resuspended in 2 % (w/v) NaCl in 75 % (v/v) ethanol and dialysed for 2 h at 4 °C against 100 vol. of the same solution. The suspension was stored overnight at 4 °C without dialysis. A small amount of particulate material was removed by centrifugation (1300 rev./min, 300 g, for 10 min, at 4 °C in an MSE Chilsplin) and the supernatant made 0.2 M in H₂SO₄ and dialysed for 4 h against 0.2 M H₂SO₄. After centrifugation as above, the extract was dialysed for 3 h against 95 % (v/v) ethanol. Precipitation was completed by adding 5 vol. of absolute ethanol and leaving overnight at –20 °C. The precipitate was vacuum-dried and redissolved in 0.2 M HCl.

Histones from *S. cerevisiae* strain RS912 were prepared by Dr. T. Chen-Cleland (Laboratory of Cell Biology, Rockefeller University, 1230 York Avenue, New York, U.S.A.) by extraction of chromatin prepared by the method described by Chen-Cleland et al. (1993) with 0.2 M H₂SO₄. Histones were precipitated with 95 % (v/v) ethanol, dried with ether and then transported as a dry powder.

Electrophoresis and Western blotting

Proteins were resolved according to their net charge on AUT gels (Alfageme et al., 1974) as described previously (Turner and Fellows, 1989). Two-dimensional electrophoresis used AUT gels in the first dimension and SDS/15 % PAGE gels (Laemmli, 1970) in the second. This procedure has been described in detail by Davie (1982). Protein-stained gels were scanned using an LKB laser densitometer. Western blotting (Towbin et al., 1979) and immunolabelling with antibodies to acetylated H4 and autoradiography were carried out as previously described (Turner and Fellows, 1989; Turner et al., 1989). To ensure the complete specificity of antibody labelling (that is to minimize any possibility of cross-reaction with inappropriate acetylated lysines), the labelling mixture for each antiserum contained the three synthetic peptides (2–5 µg/ml) corresponding to the sites that are not recognized by that antibody.

RESULTS

Identification of yeast H4 on AUT gels

Electrophoresis of acid-extractable yeast nuclear proteins on AUT gels and staining with Coomassie Blue gave a complex pattern of bands, amongst which even the major histone species

could not be readily identified (Figure 1). The presence of high concentrations of acid-extractable, non-histone proteins in yeast nuclei has been noted previously (Nelson and Alonso, 1983). Western blotting of yeast nuclear proteins and immunostaining with antisera to acetylated H4 revealed a discrete group of antibody-labelled bands migrating more slowly than did H4 bands in extracts from human cells (Figure 1). In experiments with several different yeast-histone preparations, the antibody-labelled bands consistently migrated only 40–45 % as far as human H4. To confirm that these slow-moving bands did indeed correspond with yeast H4, we carried out two-dimensional electrophoresis, with an AUT gel in the first dimension and SDS/15 % PAGE in the second. The results are shown in Figure 2. Coomassie Blue staining revealed a faint cluster of bands migrating with the mobility expected of H4 in the second dimension, but more slowly than H4 from higher eukaryotes in the first dimension. (These bands, intensified by silver staining, are arrowed in Figure 2a). Western blotting confirmed that this cluster of bands was recognized by antisera to acetylated H4 (Figure 2b). Reduced mobility of yeast histones on AUT gels has been noted before (Nelson, 1982) but the reason for it has not been established.

An enlarged print of the region of the two-dimensional gel containing yeast H4 is inset in Figure 2(a). Five bands can be distinguished, although none of the bands is completely resolved from the rest. On the basis of Western blotting (Figure 2 and below) these bands can be identified as the non-acetylated isoform (labelled 0) and the four acetylated isoforms (1–4) respectively. The results closely resemble the pattern described by Davie (1982) using a very similar two-dimensional gel system and confirm the high steady-state level of H4 acetylation in yeast (Nelson, 1982). The mono-, di- and tri-acetylated isoforms (H4Ac₁, H4Ac₂ and H4Ac₃) are particularly prominent, but the tetra-acetylated isoform (H4Ac₄) is weak. By two-dimensional laser densitometry, we estimate that 70–80 % of yeast H4 is acetylated at one or more lysines (as against 30 % or less in human cells in culture), although the incomplete separation of the non-acetylated and monoacetylated isoforms makes accurate quantitation difficult.

It is interesting to note that antisera to acetylated H4 detected a small amount of material migrating in the first (AUT) dimension with a mobility more closely resembling that of H4 from higher eukaryotes (Figure 2). Nelson and Alonso (1983) also detected a group of faint, more rapidly migrating bands on AUT gels after labelling with [³H]acetate and fluorography.

Patterns of acetylation of yeast H4

To determine the frequencies with which different lysine residues are acetylated in yeast H4, proteins electroblotted from AUT gels were immunolabelled with antisera specific for H4 isoforms acetylated at lysine-5, -8, -12 or -16. The specificities of these antisera have been described elsewhere (Turner et al., 1989; Munks et al., 1991). It is important to note that the binding of these antibodies, with one exception noted below, is not significantly influenced by the state of acetylation of adjacent lysines. For example, R20/12 will bind to all isoforms acetylated at lysine-12, irrespective of whether or not lysines-8 and -16 are acetylated. The one exception to this is R12/8, which binds poorly to tetra-acetylated H4, probably reflecting reduced binding to H4 molecules acetylated at lysine-5 (Turner et al., 1989).

Results of a typical immunolabelling experiment are shown in Figure 1. Yeast and human histones have been run in adjacent tracks and each pair of tracks has been labelled with a different antibody. The human histones provide a landmark for the

Figure 1 Detection of the acetylated isoforms of human and yeast H4 after electrophoresis on AUT gels

Acid extracts of human (HL-60) and yeast nuclei were run in adjacent tracks of an AUT gel. Pairs of tracks were either stained with Coomassie Blue (CB) or electroblotted onto nitrocellulose filters and immunostained with antibodies that are specific for H4 molecules acetylated at lysine-5 (antiserum R6/5), -8 (antiserum R12/8), -12 (antiserum R20/12) or -16 (antiserum R14/16), as indicated. Lanes 1, 3, 5, 7, 9 and 11 contain human nuclear extracts and lanes 2, 4, 6, 8, 10 and 12 contain yeast nuclear extracts. The mobilities of the four acetylated isoforms of human and yeast H4 are labelled 1–4. Note that, despite over-development of the human tracks, cross-reaction with proteins other than H4 is minimal with all antisera. Lanes 2, 4, 6, 8 and 10 contain histones from strain A364A and were all run in parallel. Lane 12 contains histones from strain RS912 and was run in a separate experiment. Identification of the single, strongly labelled band in lane 12 as H4Ac₁ was made by comparison with adjacent tracks labelled with the other antisera to acetylated H4 (see text for details).

Figure 2 Resolution of yeast nuclear proteins by two-dimensional electrophoresis and the identification of acetylated H4

(a) Acid extracts of yeast nuclei were resolved by electrophoresis on an AUT gel in the first dimension and on an SDS gel in the second (SDS). A track from the first-dimension AUT gel, stained with Coomassie Blue, is shown at the top of the figure. An aliquot of purified human histones was placed in a well, formed next to the inserted gel strip, to provide a reference for the migration of yeast histones in the second dimension. Proteins on the second-dimension gel were revealed by silver staining. The arrow indicates the location of yeast H4. This region of the gel is shown enlarged in the top right-hand corner, where the non-acetylated H4 isoform is labelled 0 and the four acetylated isoforms are labelled 1–4. (b) A two-dimensional gel prepared with the same yeast nuclear extract as shown in (a) was electroblotted onto a nitrocellulose filter and immunostained with R12/8, an antiserum that is specific for H4 acetylated at lysine-8. The antiserum recognizes a cluster of bands (left-hand arrow) with the same mobility as human H4 in the second (SDS) dimension, and with the slow mobility that is characteristic of yeast H4 in the first (AUT) dimension. The right-hand arrow indicates a faint cluster of bands with a mobility in the first dimension that more closely resembles that of human H4.

Figure 3 Comparison by SDS/PAGE of histones purified from human and from yeast nuclei

Histones were prepared from the nuclei of human HL-60 cells by acid extraction, and from yeast nuclei by a differential-extraction procedure modified from that of Nelson and Alonso (1983). Proteins were electrophoresed on 15% polyacrylamide gels containing SDS and silver-stained.

accurate alignment of different filter strips and the location of the various yeast isoforms. H4 in the yeast preparations labelled less strongly with all four antisera than its equivalent in human cell extracts, reflecting the fact that H4 represents only a small fraction of the protein in the yeast acid extracts.

R20/12 (Figure 1, lanes 7–8) labelled all four acetylated H4 isoforms, H4Ac₃ being the most intense, and provided a useful standard with which the isoforms that were labelled by the other antisera could be compared. R6/5 (lanes 3–4) labelled H4Ac₃ and H4Ac₄, the latter more strongly despite the low absolute amount of this isoform (Figure 2). R12/8 (lanes 5–6) labelled H4Ac₂ and H4Ac₃. As noted above, R12/8 labels the tetra-acetylated isoform weakly in all species and its absence is not significant, but the absence of labelling of H4Ac₁ suggests that lysine-8 is not acetylated in this isoform. In contrast, R14/16 (lanes 9–10) labelled H4Ac₁ more strongly than the other isoforms, although the labelling of H4 with R14/16, in this yeast preparation, was weaker than that obtained with all other antisera raised to acetylated H4.

Very similar results were obtained with histones prepared by a different procedure from a different strain of *S. cerevisiae*, RS912 (Chen-Cleland et al., 1993). Antisera raised to H4 acetylated at lysines-8 and -12 labelled H4Ac₂₋₄, while antibodies raised to H4

acetylated at lysine-5 labelled the tri- and tetra-acetylated isoforms most strongly (results not shown). These antisera showed little or no labelling of H4Ac₁. In contrast, antibodies raised to H4 acetylated at lysine-16 (R14/16) labelled H4Ac₁ much more strongly than the other isoforms and, in this histone preparation, unlike those tested earlier, H4 labelled just as strongly with R14/16 as with the other antisera (Figure 1, lanes 11–12). Possible reasons for this difference are discussed below.

Differential extraction of yeast histones

We were concerned that the immunolabelling results, particularly the occasionally weak labelling with R14/16, might be influenced by the histone-extraction protocol and by the large amount of non-histone protein present in acid extracts of yeast nuclei. We therefore repeated the experiments with histones that had been prepared by a method that avoids acid extraction, and is designed to separate histones H2A, H3 and H4 from most of the nuclear non-histone proteins (Nelson and Alonso, 1983). The method succeeded in this respect, although in our hands, possibly because of modifications to the original procedure (see the Materials and methods section), the supernatant fraction contained proteins corresponding in electrophoretic mobility with all four core histones, as well as several non-histone proteins of a similar size, and a higher-molecular-mass protein with a mobility similar to that of human H1 (Figure 3). Electrophoresis on AUT gels, Western blotting and immunolabelling with antisera to acetylated H4 confirmed the results obtained with acid-extracted histones (Figure 4). However, the relatively strong labelling of H4Ac₄ with R6/5, the labelling (albeit weak) of H4Ac₄ with R12/8, and the weak labelling of H4Ac₁ with R20/12, all suggest that the level of H4 acetylation in this preparation is higher than the level in previous ones. As before, labelling with R14/16 was confined to the isoforms that were less highly acetylated (in this preparation H4Ac₂ and, to a lesser extent, H4Ac₃), but was still comparatively weak.

DISCUSSION

Because antisera raised to H4 acetylated at lysines-5, -8, -12 and -16 all react with yeast H4, we can conclude that H4 acetylation in *S. cerevisiae* occurs at the same sites as in other species. Furthermore, the finding that each antiserum gives a unique

Figure 4 Detection of the acetylated isoforms of H4 in partially purified histones from human and from yeast cells after electrophoresis on AUT gels

The purified human and differentially extracted yeast histones that are shown in Figure 3 were run in adjacent tracks of an AUT gel. Pairs of tracks were either stained with Coomassie Blue (CB) or electroblotted onto nitrocellulose filters and immunostained with site-specific antibodies to acetylated H4 exactly as described in Figure 1. Only the lower part of each track is shown. The four acetylated H4 isoforms are labelled 1–4. Antisera R6/5, R12/8 and R20/12 all show weak labelling of a yeast protein with a mobility close to that of the diacetylated isoform of human H4. Coomassie Blue staining does not reveal a major protein band in this region, and the identity of this cross-reacting band remains unclear.

pattern of labelling of the various H4 isoforms shows that acetylation at the four possible lysines proceeds in an ordered and non-random fashion. Antibodies raised to H4 acetylated at lysine-12 label all four acetylated isoforms, including, although relatively weakly, H4Ac₁, indicating that this residue can be acetylated early but not usually first. Antibodies raised to H4 acetylated at lysine-8 labelled only H4Ac₂ and more highly acetylated isoforms, suggesting that acetylation at one of the other sites is a prerequisite for acetylation at lysine-8. The finding that H4Ac₄ labels more strongly than the other isoforms with antibodies raised to H4 acetylated at lysine-5, while being rarest in absolute amount (Figure 2), suggests that lysine-5 is usually the last residue to be acetylated. Finally, antibodies to H4 acetylated at lysine-16 labelled the monoacetylated isoform more strongly than the rest. This finding, in conjunction with the results outlined above, shows that H4Ac₁ is acetylated predominantly at lysine-16, which is therefore the first residue to be acetylated.

In preparations of histones from strain A364A, which was used for most of our experiments, H4 electroblotted from AUT gels labelled only very weakly with antibodies raised to H4 acetylated at lysine-16 (antiserum R14/16). In all such cases, human H4 in adjacent tracks was strongly labelled. The reason for this weak labelling remains unclear, but it may be significant that H4 from strain A364A labelled as strongly with R14/16 as with other antisera after transfer from SDS-containing gels (results not shown). This, together with the finding that H4 from the strain RS912 labelled strongly with R14/16 after transfer from AUT gels (Figure 1), makes it unlikely that yeast H4 is genuinely acetylated only rarely at lysine-16. It seems more probable that the occasional weak labelling with R14/16 is caused by a selective blocking or alteration of the epitope, possibly as a result of conformational changes. There are eight amino-acid differences between yeast and human H4, all in the central, globular domain (Wu et al., 1984). These could lead to altered folding under the conditions of electrophoresis and transfer that, possibly amplified by changes in detergent binding or even by aggregation, could explain the large difference in electrophoretic mobility between yeast and human H4. The accessibility of lysine-16, the closest of the acetyltable lysines to the globular domain, would be particularly sensitive to such conformational changes. This explanation suggests that the observed differences between H4 from strains A364A and RS912 may be due to the different procedures used for their preparation.

H4 acetylation has been found to proceed in a non-random fashion in all species examined so far and the results presented here for *S. cerevisiae* are no exception. However, the frequency with which particular lysine residues are acetylated varies widely between species. For example, the first lysine to be acetylated (that is, the one that is modified in the monoacetylated isoform) can be any of lysines-16 (humans, other mammals and amphibia), -12 (cuttlefish) and -7 (*Tetrahymena*, equivalent to lysine-8 in other species; Chicoine et al., 1986; Couppez et al., 1987; Turner et al., 1989; Thorne et al., 1990; Sommerville et al., 1993). Subsequent acetylation can be either completely site-specific (Couppez et al., 1987), or flexible, as in mammals in which the monoacetylated isoform is modified almost exclusively at lysine-16 while the diacetylated isoform contains H4 molecules acetylated at either 16 and 8 or 16 and 12 (Turner et al., 1989;

Thorne et al., 1990). The results presented here show that the pattern of usage of H4 acetylation sites in *S. cerevisiae* is remarkably similar to that in mammalian cells. H4 acetylation proceeds rather differently in *Drosophila*, where the monoacetylated isoform can be acetylated at lysine-5, -8, -12 or, to a lesser extent, -16 (Munks et al., 1991). This appears to be due to the location of site-specific acetyltransferases in different genomic domains rather than to random site usage (Turner et al., 1992). The extent to which the pattern of H4 acetylation in *S. cerevisiae* is influenced by the existence of site-specific acetyltransferases that are located in spatially separate genomic domains remains to be established. The fact that the initial acetylation can be either at lysine-16 (usually) or at lysine-12 (rarely) suggests that separate enzymes may exist. Multiple histone acetyltransferases that are separable by conventional chromatographic techniques have been found in yeast (Lopez-Rodas et al., 1989), but H4-specific enzymes have not yet been identified.

We are grateful to Dr. Tom Young for help and advice on the growth and extraction of yeast cells, to Dr. Thelma Chen-Cleland for sending us histones from strain RS912, and to Jayne Lavender for help with AUT gels. This work was supported by grants from the U.K. Cancer Research Campaign and from the Wellcome Trust.

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