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Subcellular Localization and Nucleosome Specificity of Yeast Histone Acetyltransferases†

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ABSTRACT: We have previously reported [López-Rodas et al. (1989) *J. Biol. Chem. 264*, 19028-19033] that the yeast *Saccharomyces cerevisiae* contains four histone acetyltransferases, which can be resolved by ion-exchange chromatography, and their specificity toward yeast free histones was studied. In the present contribution we show that three of the enzymes are nuclear, type A histone acetyltransferases and they are able to acetylate nucleosome-bound histones. They differ in their histone specificity. Enzyme A1 acetylates H2A in chicken nucleosomes, although it is specific for yeast free H2B; histone acetyltransferase A2 is highly specific for H3, and histone acetyltransferase A3 preparations acetylate both H3 and H4 in nucleosomes. The fourth enzyme, which is located in the cytoplasm, does not accept nucleosomes as substrate, and it represents a canonical type B, H4-specific histone acetyltransferase. Finally, histone deacetylase activity is preferentially found in the nucleus.

A variety of roles have been proposed for reversible acetylation of specific lysines in the N-terminal tails of the core histones, and it has been suggested that acetylation may be a specific signal for (i) histone removal during DNA replication, (ii) histone replacement in some differentiation processes, or (iii) H2A-H2B removal during transcription (Loidl, 1988). In addition to these nuclear acetylation events, a cytoplasmic H4 acetylation, related to histone deposition of newly synthesized histones, has been described [for a review, see Vidali et al. (1988)], and a relationship between the acetylation–deacetylation of newly assembled core histones and H1 deposition has recently been established (Perry & Annunziato, 1989).

It has been determined both by sequencing (Pesis & Matthews, 1986; Chicoine et al., 1986, 1987; Couppez et al., 1987; Richman et al., 1988) and by immunological methods (Turner & Fellows, 1989; Lin et al., 1989; Turner, 1989) that the usage of different lysyl residues of histones for reversible acetylation is not a random process. For instance, it has been proposed that acetylation of lysine 5 of H4 histone (lysines 4 and 11 in *Tetrahymena*) is related to histone deposition, whereas the turnover of acetyl groups at other lysines is connected with other functions (Pesis & Matthews, 1986; Richman et al., 1988). At any rate, the differential usage of sites for histone acetylation may be associated with the diversity of roles played by this histone modification (Loidl, 1988).

The turnover of acetyl groups depends on the activity of two kinds of enzymes, the histone acetyltransferases and the histone deacetylases. The knowledge of the regulation of these enzymatic activities may be a decisive stage in the way to elucidate how the different roles of histone acetylation are performed.

We have recently reported that the yeast *Saccharomyces cerevisiae* contains four histone acetyltransferases that can be resolved by DEAE-Sepharose chromatography (López-Rodas et al., 1989). Because of this multiplicity of enzymes, yeast may be the organism of choice to address the regulation of histone acetylation. When assayed with yeast free histones, two of the *Saccharomyces* histone acetyltransferases are specific for H4, one is specific for H2B, and the fourth one is H3-specific. However, the specificity of histone acetyltransferases may change when nucleosomes are used as substrate (Belikoff et al., 1980; Garcea & Alberts, 1980; Kelner & McCarty, 1984; Sendra et al., 1986), and, therefore, it is first interesting to know the histone specificity in chromatin.

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Present research was undertaken with the aim of casting some light on the role of yeast histone acetyltransferases in vivo, by studying their subcellular localization and specificity toward nucleosomes.

Materials and Methods

Preparation of Crude Enzymatic Extracts. Crude preparations of histone acetyltransferases from *S. cerevisiae* 1383 CECT (ATCC 9763) were obtained by the salt-dissociation/ultracentrifugation method described in a previous paper (López-Rodas et al., 1989).

Subcellular Fractionation. Nuclear fraction was prepared essentially according to Alonso and Nelson (1986). Yeast cells were spheroplasted with Novozym and lysed in 0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM PMSF, 0.8% Triton X-100, 15 mM MES, pH 6.6. The suspension was kept on ice for 30 min, and the spheroplast lysis was followed microscopically. Nuclei were then sedimented at 5000*g* for 10 min. The supernatant was centrifuged at 100000*g* for 1 h, and it was used as a source of cytoplasmic enzymes. The nuclear sediment was suspended in lysis buffer (0.25 mM EDTA, 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.7 mM PMSF, 75 mM Tris-HCl, pH 7.9), and the lysate was made 0.5 M in NaCl and centrifuged at 100000*g* for 1 h. The supernatant was used as a source of nuclear histone acetyltransferases.

Chromatographic Procedures. DEAE–Sepharose chromatography was carried out by any of the two procedures previously reported (López-Rodas et al., 1985, 1989). These procedures only differ in that the second one makes use of a shallower NH_4Cl gradient, which results in a better resolution of peaks I and II, although the chromatographic procedure was slower.

Ultrogel AcA 34 chromatography was carried out as previously reported (Salvador et al., 1985). Preparative high-performance liquid chromatography was carried out on CM-Accell as previously described (López-Rodas et al., 1989). In some instances DNA–cellulose chromatography was used instead of CM-Accell to resolve the H3-specific enzyme. Chromatograms were developed in 1 × 8 cm columns filled with DNA–cellulose (Sigma), which were equilibrated with chromatographic buffer (0.25 mM EDTA, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol, 15 mM Tris-HCl, pH 7.9) and eluted with a linear gradient (10–500 mM) of NH_4Cl in the same buffer, at a flow rate of 20 mL/h.

Enzymatic Assays. Histone acetyltransferase assays with free histones were conducted as previously described (López-Rodas et al., 1985).

Enzymatic assays with nucleosomes were carried out with chicken instead of yeast nucleosomes due to the instability of the latter (Lee et al., 1982). Oligonucleosomes were first prepared as described elsewhere (Sendra et al., 1988) and dialyzed against 35 mM NH_4Cl-containing chromatographic buffer (see above). The dialysate was centrifuged at 10000*g* for 15 min, and the supernatant was loaded onto a column of Ultrogel AcA 34 (1.8 × 120 cm), equilibrated and eluted with the same buffer. Fractions containing mononucleosomes (as determined by agarose gel electrophoresis of the DNA) were pooled and concentrated by ultrafiltration through a 30000-MW cutoff membrane (Amicon) to a concentration of ≈1.7 mg of DNA/mL. The nucleosomal preparation was then mixed in 150-μL aliquots with [^{14}C]acetyl-CoA (0.4 μCi) and 1 mL of the corresponding enzymatic extract (the appropriate pooled chromatographic fractions, see Results). The mixture was then incubated at 32 °C for 30 min, and the reaction was stopped by chilling the mixture on ice. The mixture was loaded in the cold on 11 mL of a linear sucrose gradient (7–30%) in 1 mM EDTA, 25 mM Tris-HCl, pH 8.0, and spun at 40000 rpm for 18 h in a Beckman SW41 rotor. After centrifuging, the tubes were bottom-punctured, and fractions of 1.1 mL were recovered, unless otherwise stated. The absorbance at 260 nm was determined, and the radioactivity incorporated was measured in 100-μL aliquots of the fractions.

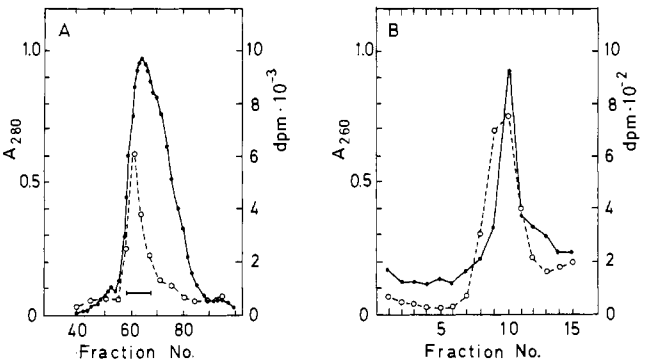

FIGURE 1: (A) Acetylating activity toward nucleosomes of the histone acetyltransferases present in peaks I and II of a DEAE–Sepharose chromatogram. Peaks I and II (López-Rodas et al., 1989) were pooled and rechromatographed on Ultrogel AcA 34. The absorbance at 280 nm (●) and the histone acetyltransferase activity with chicken erythrocyte nucleosomes (○) is plotted. (B) Fractions pooled from Ultrogel AcA 34 chromatography, as indicated by the bar in (A), submitted to gradient ultracentrifugation. After the bottoms of the tubes were punctured, fractions of 750 μL were recovered and the absorbance at 260 nm (●) and the radioactivity (○) of each fraction were measured.

The fractions corresponding to the nucleosomal peak were pooled and precipitated with trichloroacetic acid (25% final concentration). The proteins present in the precipitate were separated by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970), and the gels were submitted to fluorography (Laskey & Mills, 1975).

Histone deacetylase activity was determined with biologically labeled chicken erythrocyte histones as described elsewhere (Sendra et al., 1988).

Other Methods. Standard labeling densities (SLD)[1] were determined by the activity scanning method as previously described (López-Rodas et al., 1989). Briefly, the bands corresponding to individual histones in the fluorograms were integrated and normalized to the histone content, estimated from the Coomassie Blue stained gel.

Results

Yeast histone acetyltransferase activity is resolved into three peaks by chromatography on DEAE–Sepharose, and we have suggested that the third peak probably contains a type B, H4-specific cytoplasmic enzyme, while the first two peaks comprise three activities, specific for yeast free H2B, H3, and H4 (López-Rodas et al., 1989). To determine whether these three activities are able to acetylate nucleosomes, a property shared by all type A histone acetyltransferases, we carried out the experiment shown in Figure 1. First, the fractions corresponding to the first two peaks of a DEAE–Sepharose chromatogram were pooled and chromatographed on Ultrogel AcA 34. Histone acetyltransferase activities eluted as a single, sharp peak, denoting that their molecular weights are in close proximity (Figure 1A). The histone acetyltransferase activity, pooled from the Ultrogel peak as indicated in Figure 1A, was

[1] Abbreviations: SLD, standard labeling density, obtained by dividing the radioactivity incorporated into a given histone band by the staining intensity of the band.

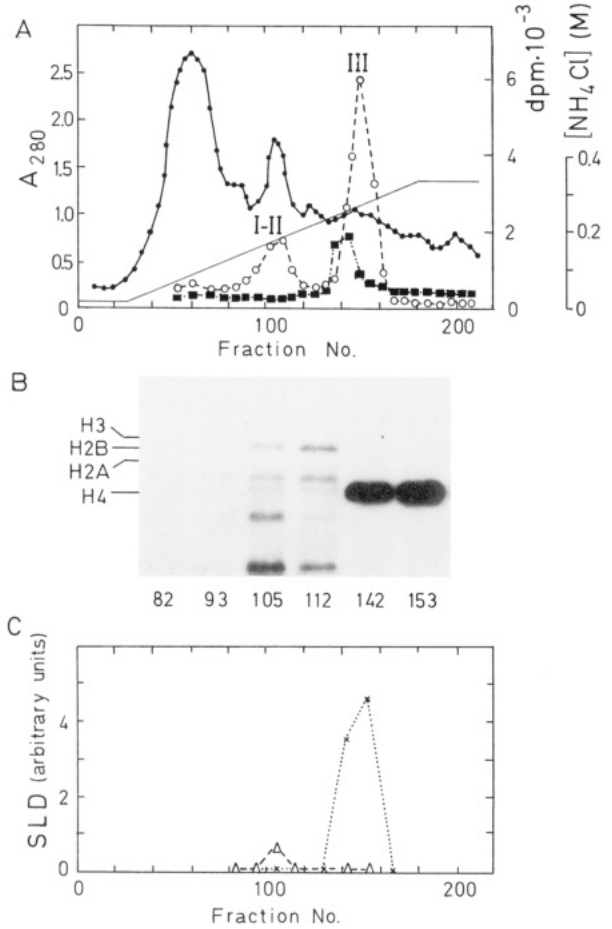

FIGURE 2: Specificity of histone acetyltransferases present in yeast cytoplasm. (A) Chromatographic profile of the histone acetyltransferase separation. A cytoplasmic extract, corresponding to 30 g of yeast cells, was prepared as described under Materials and Methods and resolved by DEAE–Sepharose chromatography (López-Rodas et al., 1989) by using a 3 × 15 cm column, eluted at a flow rate of 60 mL/h with 1200 mL of NH_4Cl gradient, as indicated by the continuous thin line. Fractions of 6 mL were recovered, and the absorbance at 280 nm (●) and the histone acetyltransferase (○) and histone deacetylase (■) activities (in dpm, under the conditions of the standard assay) were determined. (B) Fluorogram obtained after the electrophoretic separation of incubation mixtures of selected chromatographic fractions (as indicated by the numbers below) with yeast histones and labeled acetyl-CoA, to show the specificity of cytoplasmic histone acetyltransferases. (C) Scanning of the acetylating activity toward individual histones. The activity scanning method (López-Rodas et al., 1989) was applied to detect the acetylating activity toward H4 (×) and H2B (△) of the chromatographic fractions shown in (B).

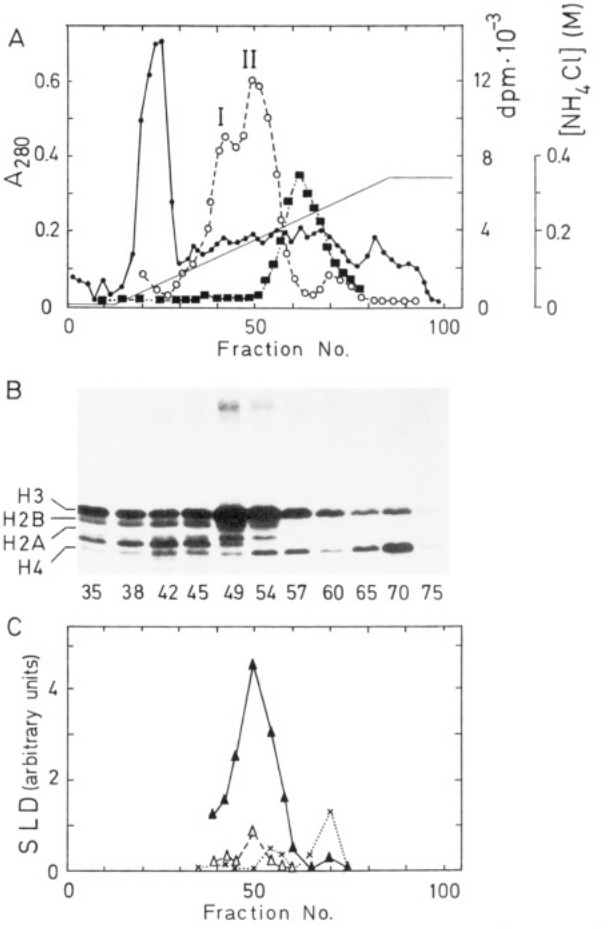

FIGURE 3: Specificity of yeast nuclear histone acetyltransferases. All the symbols and conditions are as in Figure 3 except that the nuclear fraction from 62 g of yeast cells was used to prepare the initial extract and that the H3-acetylating activity (▲) was included in the activity scanning method of panel C. The column was eluted with 400 mL of gradient at a flow rate of 30 mL/h. Fractions of 4 mL were recovered. The labeled band migrating between H2A and H4 histones corresponds to a specific proteolytic product (López-Rodas et al., 1989).

able to catalyze the incorporation of acetyl groups from acetyl-CoA into a preparation of nucleosomes. It may be possible, however, that incorporation took place in some protein stripped from nucleosomes during handling or loosely bound to them rather than in nucleosomal histones. To check this possibility, the substrate nucleosomes were submitted to gradient ultracentrifugation and the incorporated acetate was determined in the gradient fractions. The results of the experiment (Figure 1B) show that nucleosomes were actually acting as substrate for these histone acetyltransferase activities.

It remained to be determined whether all three histone acetyltransferase activities are able to acetylate nucleosomes. The fact that a histone aceyltransferase acetylates nucleosomes would suggest, but not prove, that it is a nuclear enzyme. To solve these questions, the following experiments were planned.

We first analyzed the subcellular distribution of histone acetyltransferase activities. The profile of a DEAE–Sepharose chromatogram of cytoplasmic histone acetyltransferases is given in Figure 2A. Most of the acetylating activity (73%) elutes in peak III. The percentage of histone acetyltransferase eluting in peak III must be still higher because yeast type A enzymes, present in peaks I and II, largely acetylate chromatin proteins other than core histones when chicken erythrocyte histones were used to assay the acetylating activity in the chromatographic fractions (López-Rodas et al., 1985). The fluorogram of Figure 2B, in which the incorporation of acetate into yeast histones is analyzed, shows that the above assumption was correct. Only a small proportion of H2B-acetylating activity was recovered, in strong contrast with the activity of the H4-specific enzyme present in peak III. The former activity probably is due to a contamination due to nuclear breakage. The results of the fluorogram are still clearer in the SLD plot (Figure 2C).

The chromatographic profile of nuclear enzymes was completely different (Figure 3A). Most of the enzymatic activity was recovered in peaks I and II, suggesting that the enzymes of these peaks are nuclear. Parts A and B of Figure 3 show that this is true for all three, i.e., H2B-, H3-, and H4-specific, enzymatic activities. We have suggested in a previous paper (López-Rodas et al., 1989) that there are two H4-specific histone acetyltransferases in yeast with different subcellular localization, and present experiments demonstrate that this assumption was correct. In the experiment shown in Figure

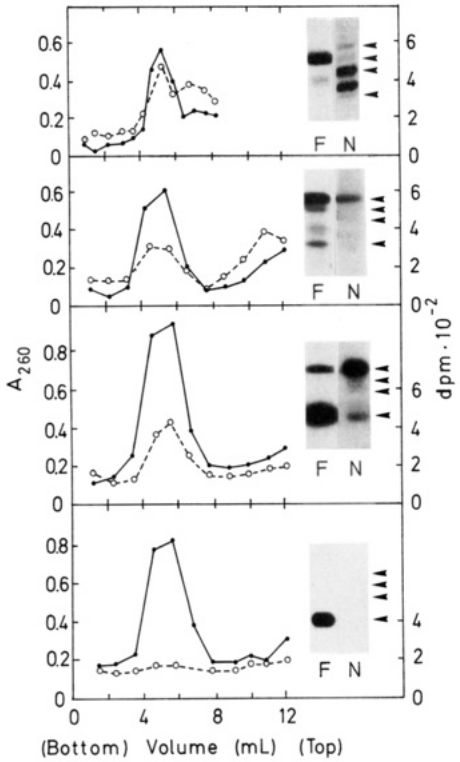

FIGURE 4: Activity of yeast histone acetyltransferases with chicken erythrocyte nucleosomes as substrate. Nucleosomes were incubated as described in the text with labeled acetyl-CoA in the presence of enzymatic extracts specific for (from top to bottom) H2B, H3, H4 (nuclear), and H4 (cytoplasmic). See the text for the preparation of these extracts. After incubation, the nucleosomes were gradient-centrifuged as described under Materials and Methods. Fractions (0.65 mL in the top experiment and 1.1 mL in the rest) were recovered, and their absorbance at 260 nm (●) and radioactivity (○) were determined. The insets show the fluorograms indicating the histone specificity of the different enzymatic fractions in the assay with nucleosomes (N), and they were obtained from electrophoreses carried out with the proteins recovered from the nucleosomal peaks of the gradient. The specificity toward free yeast histones is also shown (F). The arrows mark the position of the core histones H3, H2B, H2A, and H4 (from top to bottom).

3, the SLD maxima of the H2B- and H3-specific enzymes coincide, but this may be due to a poor chromatographic resolution in this particular experiment because it is clear that these histone acetyltransferases may be resolved under appropriate conditions (López-Rodas et al., 1989).

On the other hand, most of the histone deacetylase activity was also found in nuclei (Figure 3A). A discrete activity was present in cytoplasm (Figure 2A), but it remains to be determined whether it corresponds to a cytoplasmic specific enzyme; it is also possible that the "cytoplasmic" activity resulted from breakage of nuclei.

The above experiments suggest that histone acetyltransferases specific for H2B, H3, and H4 are nuclear enzymes. We next examined whether all of them can accept nucleosomes as substrate. The experiment of Figure 4 showed that, in strong contrast to the H4-specific type B histone acetyltransferase, this is indeed the case. We incubated nucleosomes with different enzymatic preparations, namely, (i) a retained fraction from CM-Accell chromatography, specific for yeast free H2B (López-Rodas et al., 1989); (ii) a fraction, largely specific for free H3, prepared by DNA–cellulose chromatography of pooled DEAE–Sepharose peaks I and II from a nuclear extract; (iii) a late fraction from DEAE–Sepharose peak II from a nuclear extract, which contained the H4-acetylating activity with a small contamination of the H3

Table I: Some Properties of the Histone Acetyltransferases from the Yeast *S. cerevisiae*

enzyme	histone specificity toward free histones: yeast	free histones: chicken	chicken nucleosomes	DEAE–Sepharose behavior (peak)	subcellular localization
A1	H2B	H2A	H2A	I	nucleus
A2	H3	H3	H3	I–II	nucleus
A3	H4 (>H3?)	H4	H3 > H4	II	nucleus
B	H4	H4		III	cytoplasm

enzyme; and (iv) peak III from DEAE–Sepharose chromatography. In our hands, yeast nucleosomes were not stable enough against proteolysis unless protease inhibitors were present in all the experimental steps, and the presence of Mg^{2+}-activated endogenous nucleases (Bhargava & Mayalagu, 1990) also may cause trouble. Unfortunately, protease inhibitors also inhibit histone acetyltransferase activity, so we decided to use chicken erythrocyte nucleosomes.

The three nuclear enzymes are able to acetylate nucleosomes, but the H4-specific histone acetyltransferase present in DEAE–Sepharose peak III, which we have shown to be a cytoplasmic enzyme, is not able to acetylate nucleosomes (Figure 4). The insets of Figure 4 show that the specificity of histone acetyltransferases toward free histones does not always coincide with that toward nucleosome-associated histones. The most remarkable case is that of the nuclear H4-specific histone acetyltransferase, which, though able to acetylate H4 in nucleosomes, strongly prefers H3 when nucleosomes are used as substrate. This enzymatic preparation also acetylated free H3 (see lane F in the corresponding inset). We cannot discard the possibility that the nuclear, H4-specific enzyme also accepts H3, although a small contamination by the enzyme specific for free H3—which possesses a high enzymatic activity (Figure 3)—may account for this behavior. The differences found between the patterns of acetylation of free or nucleosome-bound histones by the H2B-specific enzyme are most likely due to the fact that the enzyme that acetylates H2B in yeast histones catalyzes the transfer of acetate to chicken H2A (López-Rodas et al., 1989).

Discussion

Present results support our previous finding that yeast contains at least four histone acetyltransferases. We have reported, for instance, that the ability to acetylate free H2B and H3 histones resides in different enzyme fractions (López-Rodas et al., 1989), and the fact that these fractions retain their characteristic histone specificity when nucleosomes are used as substrate is further proof that the enzymes that acetylate free H2B and H3 are different. It is also clear from the results presented in this paper that two different enzymes specific for H4 exist in yeast, one nuclear and the second one cytoplasmic. The difference between the nuclear enzymes specific for free H3 and H4 cannot be unambiguously established from the results presented here, but this matter was established by our previous experiments [see, for instance, Figure 3 in López-Rodas et al. (1989)]. Therefore, the existence of three type A acetyltransferases and one type B histone acetyltransferase in the yeast *S. cerevisiae* has been demonstrated. Table I summarizes some properties of the enzymes, for which a nomenclature based on the chromatographic properties is proposed. A quantitative comparison of the activity of the different enzymes may be troublesome, as the actual number of residues that undergo acetylation in yeast histones may only be guessed by analogy to histone from other organisms. Studies are in progress in our laboratory to de-

termine the residue specificity of the yeast enzymes.

The presence of several forms of histone acetyltransferases in yeast contrasts with the multifunctional role proposed for a single enzyme in *Tetrahymena* macronuclei (Chicoine et al., 1987). It is not known whether the different activities of the single *Tetrahymena* enzyme are regulated in different ways. If they were, the situation would be similar to that of *Saccharomyces*.

The results shown in Figure 3 indicate that histone deacetylase activity largely resides in the nucleus, although the presence of a ctyoplasmic form of the enzyme cannot be ruled out. Alonso and Nelson (1986) first described the presence of a histone deacetylase activity in yeast nuclei, and they developed an in situ assay that revealed that the activity removes acetyl groups from all four core histones. Preliminary data from our laboratory (M. M. Sânchez del Pino, unpublished results) showed that a partially purified deacetylase is also able to deacetylate all four core chicken histones, although it has a certain preference for H2A and H2B. The presence of nucleosome-bound histone acetyltransferases (Böhm et al., 1980) and deacetylases (Mold & McCarty, 1987) has been described in animal cells. We have demonstrated that histone acetyltransferase A3 is bound to chromatin (López-Rodas et al., 1989), but more research is needed to ascertain whether this enzyme and the remaining enzymes described in this paper actually are bound to nucleosomes.

Recent genetic analysis suggests that different combinations of the acetylation modes of the four potential lysyl residues of yeast H4 may play different roles (Megee et al., 1990), and these results are consistent with the finding that the different lysyl residues of histones are not acetylated at random (Pesis & Matthews, 1986; Chicoine et al., 1986, 1987; Couppez et al., 1987; Richman et al., 1988; Turner & Fellows, 1989; Lin et al., 1989; Turner, 1989). The acetylation level of a given lysyl residue of a histone molecule depends on the relative activities of histone acetyltransferase and deacetylase *toward that specific residue*. Turner (1989) has shown that, in certain cases, the increase in the frequency of acetylation at a given site may be explained even if histone deacetylase would act at random, but in other instances the situation is not so simple.

The description of the minimal set of histone acetyltransferases in yeast, together with the knowledge of their subcellular localization and histone specificity, may represent the first stage to exploration of the more complex problem of the regulation, at a single-site level, of histone acetylation turnover.

Registry No. Histone acetyltransferase, 9054-51-7; histone deacetylase, 9076-57-7.

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