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Site Specificity of Pea Histone Acetyltransferase B *in Vitro**

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Histone acetyltransferase B from pea embryonic axes has been purified ≈300-fold by a combination of chromatographic procedures, including affinity chromatography on histone-agarose. The enzyme preparation has been used for the *in vitro* transfer of acetyl groups from [1-^{14}C]acetyl-CoA to non-acetylated pea histone H4. Up to three acetyl groups can be introduced into the histone. The resulting mono-, di-, and triacetylated H4 isoforms were separated and sequenced to determine the acetylated sites. Only sites 5, 12, and 16 were used by histone acetyltransferase B, but no clear preference among them was observed. The absence of modification of other potentially acetylatable sites is another indication that acetylation of the different lysine residues in the N-terminal H4 tail serves as a specific signal in different nuclear processes.

Reversible acetylation of the core histones at the ε-amino groups of lysyl residues is thought to be involved in many functional processes occurring in chromatin (Csordas, 1990; Turner, 1991). Acetylation takes place at the N-terminal histone tails, whose participation in chromatin structure is still ill-defined. Therefore, the possible relationships between histone acetylation and the structural changes leading to the transcriptionally active conformation of chromatin still remain obscure.

Two main hypotheses for the nature of the above-mentioned relationships exist. The first one to be posed emphasized the fact that acetylation reduces the net positive charge of the histones and, therefore, might weaken the histone-DNA interactions. Hyperacetylation of histones, however, only causes subtle changes in nucleosomal structure but may influence chromatin higher order structure (reviewed in Turner, 1991). The best characterized acetylation-induced structural change is the reduction in the linking number change when hyperacetylated histones rather than non-acetylated histones are used to reconstitute nucleosomes *in vitro* (Norton *et al.*, 1989, 1990), but serious doubts as to the relevance of this effect *in vivo* have been recently raised (Lutter *et al.*, 1992). On the other hand, electron microscopy and electron spectroscopic imaging studies allowed Dixon and his co-workers to propose that histone hyperacetylation does not alter *per se* the structure of the nucleosome but weakens the interactions that stabilize it (Oliva *et al.*, 1990).

The second hypothesis envisages histone acetylation as a signal introduced in the histones that would allow the specifically acetylated N-terminal tails to be recognized by a putative (protein) factor. The different functional roles ascribed to histone acetylation may then depend on the specific usage of lysines and on the specificity of these putative factors. The hypothesis is based upon the fact that histone acetylation does not occur at random. Actually, both sequencing studies (Pesis and Matthews, 1986; Chicoine *et al.*, 1986, 1987; Couppez *et al.*, 1987; Richman *et al.*, 1988; Thorne *et al.*, 1990) and the use of specific antibodies (Lin *et al.*, 1989; Turner, 1989; Turner and Fellows, 1989; Turner *et al.*, 1989) have revealed that the potential sites for acetylation are differentially used in the naturally occurring mono-, di-, and triacetylated histone isoforms. Taking into account that there are 26 target sites for acetylation in a nucleosome, the number of different acetylation states is enormous. Although all these possibilities need not to be of functional significance, it is obvious that the different combinations of acetylated and non-acetylated lysines may well provide a functional signal for different processes in chromatin (Loidl, 1988). On the other hand, genetic evidence supports the idea that site-specific acetylation of histone H4 may play a definite functional role in the transcription of yeast genes (Megee *et al.*, 1990; Durrin *et al.*, 1991), whereas acetylation of H3 does not seem to be involved in transcriptional activation (Mann and Grunstein, 1992).

Acetyl groups turn over depending on the activity of two enzymatic activities, histone acetyltransferase and histone deacetylase. Therefore, the acetylation state of a given histone results from the activities of these enzymes. Recent data show that multiple enzymes or enzyme forms exist in several organisms. Several histone acetyltransferase and histone deacetylase activities have been unambiguously identified both in yeast (López-Rodas *et al.*, 1989, 1991*a*) and in plants (Sendra *et al.*, 1988; López-Rodas *et al.*, 1991*b*), and there are indications that this multiplicity of enzymes is related to the diverse functions of histone acetylation (Georgieva *et al.*, 1991).

The availability of different histone acetyltransferase forms opens the possibility of studying their distinctive properties, including site specificity. These studies have to be carried out *in vitro*, but this approach has certain advantages over those carried out with naturally occurring histone isoforms *in vivo*, as the latter may reflect the result of the concomitant activity of several histone acetyltransferases and/or histone deacetylases. In the present paper we address this question by analyzing the positions at which acetyl groups are incorporated into non-acetylated H4 by pea histone acetyltransferase B, highly specific for H4 (Salvador *et al.*, 1985). B-type histone acetyltransferases are localized in the cytoplasm, and they are involved in the modification of H4 prior to chromatin assembly (Vidali *et al.*, 1988).

MATERIALS AND METHODS

Preparation of Enzymatic Fractions—Seeds of pea (*Pisum sativum* cv. Lincoln) were germinated in the dark at 28 °C over moistened

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cotton. Embryos were harvested after 62 h, and the cotyledons were excised and discarded. Crude enzymatic extracts were prepared as described elsewhere for histone deacetylase extraction (Sendra *et al.*, 1988). Briefly, the embryonic axes (80 g) were placed in 2 volumes of cold buffer A (0.5 M NH_4Cl, 10 mM 2-mercaptoethanol, 0.25 mM EDTA, 80 mM $NaHSO_3$, 15 mM Tris-HCl, pH 7.9) and ground with acid-washed sand (80 g) in a mortar. The homogenate was filtered, clarified by centrifugation at 27,000 × *g* for 15 min, and centrifuged at 100,000 × *g* for 1 h. The delipidized supernatant was then dialyzed against two changes of buffer B (0.25 mM EDTA, 10 mM NH_4Cl, 5 mM 2-mercaptoethanol, 20% (v/v) glycerol, 15 mM Tris-HCl, pH 7.9). The resulting solution was then fractionated by ion-exchange chromatography on a DEAE-Sepharose CL-6B column (14 × 2.5 cm), eluted with a 10–350 mM NH_4Cl gradient as described previously (López-Rodas *et al.*, 1985). The fractions containing histone acetyltransferase B activity, which eluted at about 300 mM NH_4Cl (Salvador *et al.*, 1985), were pooled, and solid $(NH_4)_2SO_4$ was slowly added to obtain a 50% saturation. The mixture was gently stirred for 30 min at 4 °C, and the precipitate was removed by centrifuging at 30,000 × *g* for 30 min. Solid $(NH_4)_2SO_4$ was then added to the supernatant to obtain an 80% saturation, and the precipitate was recovered as before, dissolved in 10 ml of buffer C (as buffer B, but containing 100 mM NH_4Cl), and dialyzed against three changes (2 liters each) of the latter buffer. We have found previously that the histone acetyltransferase B preparations routinely recovered after the DEAE-Sepharose step were specific for free H4 either from pea or from chicken and that the preparations were absolutely free from any nucleosome-acetylating activity (Sendra *et al.*, 1986). The dialysate was loaded onto a column (8 × 1 cm) of histone-agarose (Sigma) equilibrated with buffer C. The column was washed with 3 bed volumes of buffer C and eluted with a linear NH_4Cl gradient (0.1–1.0 M) in buffer B. Histone acetyltransferase B eluted at about 0.8 M NH_4Cl, and the fractions containing the enzyme were pooled and dialyzed against buffer C. The dialysate was directly used in the subsequent experiments. The procedure typically gave a yield of 48% and a 300-fold purification (see below). Histone acetyltransferase activity was assayed with [1-^{14}C]acetyl-CoA (Amersham, 52 mCi/mmol) as described previously (López-Rodas *et al.*, 1985).

Isolation of Pea Non-acetylated H4—Pea seeds were grown as described by Ull and Franco (1985). 6-Day-old seedlings were harvested and the chromatin isolated (Sendra *et al.*, 1991). Whole histones were obtained by acid extraction of chromatin and fractionated on a Bio-Gel P-100 column (100 × 3 cm) by the method of Lagenbuch *et al.* (1983). Fractions containing H4 were pooled, desalted with Sephadex G-25 equilibrated with 10 mM HCl, and lyophilized.

Histone H4 was further fractionated, according to the acetylation level, on a sulfopropyl-Sephadex C-25 column following the procedure of Couppez *et al.* (1987), except that the guanidine hydrochloride gradient was 0.43–0.65 M. Fractions containing non-acetylated H4, as judged by AUT-PAGE[1] (Alfageme *et al.*, 1974), were pooled and desalted as before. The resulting solution was lyophilized, and the material obtained was used as substrate for histone acetyltransferase B.

Enzymatic Acetylation of H4—To determine the extent of the enzymatic acetylation with homologous histone acetyltransferase B, 80 μg of pea non-acetylated H4 were mixed with 100 μl of the enzymatic preparation and 0.01 μCi of [1-^{14}C]acetyl-CoA, and the volume adjusted to 130 μl with buffer C. The mixture was incubated at 37 °C, and aliquots were removed at different time periods (see "Results"). The reaction was stopped by adding 100% trichloroacetic acid to a final concentration of 25%. The resulting precipitate was spun down, washed twice with acetone, 37.5% HCl (100:1, v/v), three times with acetone, and dried under vacuum. The acetylated histones were electrophoresed on AUT gels and the incorporation of labeled acetyl groups was detected by fluorography (López-Rodas *et al.*, 1985). To check the extent of chemical transfer of acetyl groups to histone, control samples were sham-incubated with heat-denatured (10 min at 70 °C) histone acetyltransferase B.

Determination of Acetylation Sites—The sites of histone acetyltransferase B-catalyzed enzymatic acetylation of H4 were determined after scaling up the above procedure. To do this, 2 mg of pea non-acetylated H4 were mixed with 1.5 ml of the enzymatic extract and 1 μCi of [1-^{14}C]acetyl-CoA in a final volume of 2.0 ml (adjusted with buffer C). The mixture was incubated at 37 °C for 80 min, and the reaction was stopped by adding enough 5 M HCl to obtain pH 3.0. The excess of labeled acetyl-CoA was removed by passing the mixture through a PD-10 column equilibrated with 6 M urea, 100 mM 2-mercaptoethanol, 430 mM guanidine hydrochloride, pH 3.0. The excluded peak was directly loaded onto a sulfopropyl-Sephadex C-25 column (5 × 1 cm), which was eluted with 70 ml of the above-mentioned gradient at a flow rate of 1 ml/h. Fractions of 1 ml were collected, and 50-μl aliquots were mixed with 5 ml of Normascint-22 (Scharlau) and counted to determine the radioactivity. The histone concentration was measured by a turbidometric assay (Couppez *et al.*, 1987). Fractions corresponding to mono-, di-, and triacetylated H4 (further referred to as H4Ac1, H4Ac2, and H4Ac3, respectively) were pooled and individually loaded on a C-18 Sep-Pak cartridge (Waters), previously equilibrated with 0.1% trifluoroacetic acid. After washing the cartridge with the same solvent, the histone was eluted with a mixture of water/acetonitrile (40:60) containing 0.1% trifluoroacetic acid and lyophilized.

The free ε-amino groups of H4Ac1, H4Ac2, and H4Ac3 were acetylated to completion with acetic anhydride in order to prevent any unspecific cleavage at lysyl residues in the subsequent treatment with endoprotease Arg-C (see below). To do this, histones were solved at ≈2 mg/ml in 50 mM sodium borate buffer, pH 9.0, a 100-fold excess of acetic anhydride was added, and the solution was allowed to stand for 1 h at 0 °C. A fresh 100-fold excess of acetic anhydride was then added; the solution was incubated as before and, finally, it was allowed to stand at room temperature for an additional period of 2 h. Histones were then purified by using C-18 Sep-Pak cartridges under the above conditions.

The 4 acetylatable lysyl residues found in most eukaryotes (5, 8, 12 and 16) lie between arginines 3 and 17 (Fig. 1), so that all the enzymatically incorporated labeled acetate must be present in a single peptide after endoprotease Arg-C cleavage (peptide 4–17). To this end, H4Ac1, H4Ac2, and H4Ac3 were solved at 4 mg/ml in 0.1% NH_4HCO_3, pH 8.0, and the enzyme (Boehringer) was added (1 μg/50 μg of histone). The solution was incubated at 37 °C for 90 min, and an equal fresh amount of enzyme was added, and the incubation was continued for 90 min. The reaction was then stopped by boiling the samples for 10 min.

Some insoluble products were formed during the endoprotease reaction, and they were removed by centrifugation. We found that all the radioactive material was present in the supernatant. The latter, containing therefore the ^{14}C-labeled peptide 4–17, was lyophilized and stored.

To locate the radioactively labeled lysyl residues, 30 μg of the whole lyophilized material was manually sequenced (Peterson *et al.*, 1972). Mellitin (Sigma), whose size was in the range of peptide 4–17, was added as an internal standard to estimate the yield of the degradation. After each Edman cycle, the anilinothiazolinone derivatives were divided into two aliquots. One of them was used to measure the released radioactivity, and the second one was converted into the respective phenylthiohydantoin derivatives (corresponding to mellitin amino acids, due to the excess of this peptide), which were identified after reverse-phase HPLC on a C-18 Delta-Pak column (Waters) (Lazure *et al.*, 1990). The column was equilibrated with 35 mM sodium acetate (pH 5.0)/acetonitrile (5:1, v/v), and the phenylthiohydantoins were eluted with a linear gradient of isopropanol (0–66.6%, v/v) at a flow rate of 1 ml/min. The elution of phenylthiohydantoins was monitored at 254 nm. The average yield of degradation was 91%, and this value was used to correct the measured released radioactivity according to the following formula (Pesis and Matthews, 1986).

$$\text{Corrected dpm} = (\text{released dpm})/(\text{average recovery})^{(\text{cycle no.})} \quad \text{(Eq. 1)}$$

RESULTS

Purification of Histone Acetyltransferase B—Table I summarizes the yield and purification of histone acetyltransferase B in the last two steps of the procedure described under "Materials and Methods." The enzymatic activity in the crude extracts cannot be confidently determined, because nucleic acids usually inhibit histone acetyltransferase activities (Libby, 1980; Kelner and McCarty, 1984). This effect is especially noticeable in histone acetyltransferase B, and we have shown, for instance, that yeast histone acetyltransferase B activity is reduced to about 50% in the presence of 10 μg/

[1] The abbreviations used are: AUT-PAGE, polyacrylamide gel electrophoresis in the presence of acetic acid-urea-Triton X-100. H4Ac1, H4Ac2, and H4Ac3, histone H4 mono-, di-, and triacetylated isoforms.

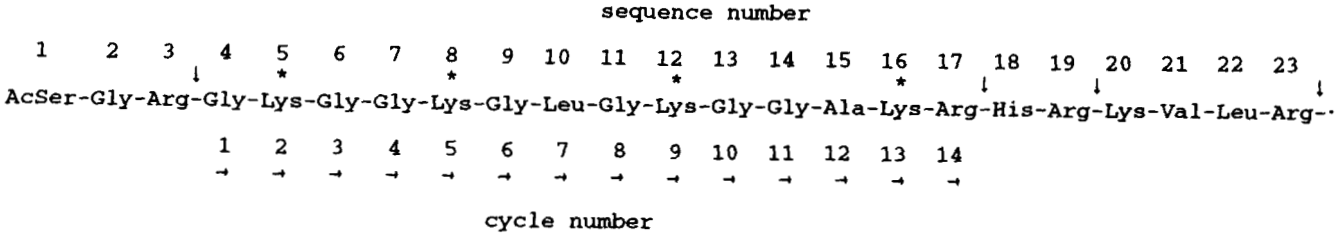

FIG. 1. **Sequence of the N-terminal region of pea H4 (DeLange *et al.*, 1969).** The 4 conserved acetylatable lysines are marked by *asterisks*. *Vertical arrows* show the endoprotease Arg C cleavage sites after blocking lysine side chains with acetic anhydride. The *horizontal arrows* below the sequence indicate the manually sequenced peptide.

TABLE I

Purification of pea histone acetyltransferase B

The purification and recovery of the enzyme are given for the last steps of the preparation procedure. As commented in the text, nucleic acids inhibit histone acetyltransferase activities (Libby, 1980; Kelner and McCarty, 1984), thus precluding the accurate determination of enzymatic activity in the crude extracts. The table shows the results of a typical experiment starting from 70 g of 62 h embryonic axes.

Step	Total protein	Total activity	Specific activity	Purification
	mg	*units*	*units/mg*	*-fold*
DEAE-Sepharose	335.9	127.6	0.38	1
$(NH_4)_2SO_4$	32.4	80.4	2.48	6.5
Histone-agarose	5.6	61.0	10.9	28.7

ml DNA (López-Rodas *et al.*, 1985). A ≈29-fold purification was achieved by ammonium sulfate precipitation and histone-agarose chromatography (Table I). Taking into account this figure and that the pooled histone acetyltransferase B fractions collected from the DEAE-Sepharose chromatography contained only 10% of the loaded protein (on an A_{280} basis), a ≈300-fold overall purification may be roughly estimated. As commented before, crude enzymatic preparations, after DEAE-Sepharose chromatography, were systematically free from other histone acetyltransferase activities. In the present instance, we carried out an AUT-PAGE and fluorography of total pea and chicken histones incubated with [1-^{14}C]acetyl-CoA in the presence of the purified enzymatic extract. As expected, only free H4 was accepted as substrate (results not shown).

Extent of H4 Acetylation by Histone Acetyltransferase B—Pea H4, prepared as described under "Materials and Methods," consists of a major non-acetylated isoform and a minor monoacetylated isoform. The latter accounts for about 5% of total H4, as estimated after sulfopropyl-Sephadex chromatography (Fig. 2*A*). This result agrees with the early estimation of DeLange *et al.* (1969). The good resolution between the non-acetylated H4 and H4Ac1 isoforms allowed us to isolate non-acetylated H4, which was used in the subsequent experiments.

To determine the number of target lysyl residues in the reaction catalyzed by histone acetyltransferase B, non-acetylated H4 was incubated with an enzymatic preparation. Aliquots were taken from the reaction mixture at various times and analyzed by polyacrylamide gel electrophoresis (Fig. 3). The amount of H4Ac1, H4Ac2, and H4Ac3 steadily increased during the incubation, but the total acetate incorporated in the three isoforms was found in roughly comparable proportions. No evidence for the presence of H4Ac4 was found. The existence of non-enzymatic incorporation of acetate under our assay conditions was ruled out after an incubation with heat-denatured enzyme (see lane marked *C* on Fig. 3). Chromatographic analysis (Fig 2*B*) also failed to detect H4Ac4.

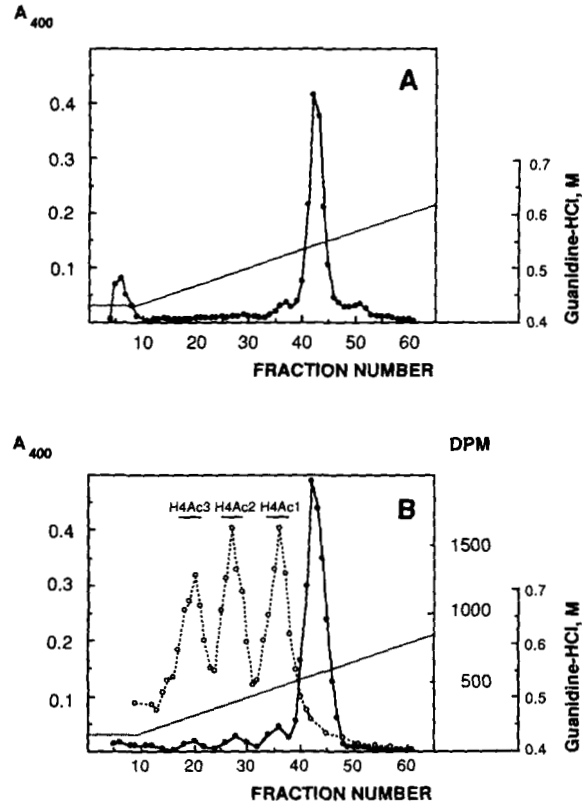

FIG. 2. **Separation of pea H4 isoforms by ion-exchange sulfopropyl-Sephadex chromatography.** The guanidine hydrochloride gradient (*dotted line*) was 0.43–0.65 M. *A*, chromatography of native H4 (10 × 1.8-cm column) to isolate the non-acetylated isoform. *B*, separation of the reaction products after incubation of non-acetylated H4 with [1-^{14}C]acetyl-CoA in the presence of histone acetyltransferase B. A 5 × 1-cm column of was used. ●——●, protein concentration, determined by turbidometry at 400 nm; O- - -O, radioactivity.

Similar results were obtained when chicken erythrocyte H4 was used as substrate (not shown).

Determination of the Positions of Acetylation—The H4Ac1, H4Ac2, and H4Ac3 isoforms were pooled as depicted in Fig. 2*B* and subjected to manual sequencing as described under "Materials and Methods." The sites of H4 acetylation in most organisms are lysines 5, 8, 12, and 16 (Turner, 1991), so radioactivity is expected to be present, under our experimental conditions, in cycles 2, 5, 9, or 13. Fig. 4 shows the results of the sequential analysis. The patterns are similar for the three isoforms and radioactivity was only found in cycles 2, 9, and 13. The radioactivity released in cycle 13 is significantly decreased when compared with that found in cycles 2 and 9. However, this behavior may be artifactually due either to the partial solubility of peptide 4–17 in the solvents used in the

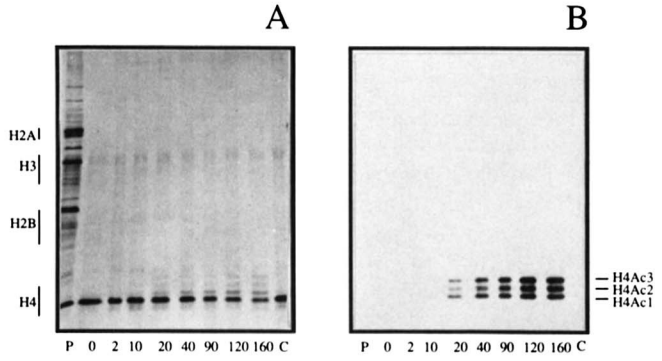

FIG. 3. **Enzymatic incorporation of acetate into non-acetylated H4.** Purified non-acetylated H4 isoform was incubated with [1-^{14}C]acetyl-CoA in the presence of histone acetyltransferase B, and aliquots were taken from the reaction mixture at the indicated times and analyzed by AUT polyacrylamide gel electrophoresis. *A*, Coomassie-stained gel. *B*, Fluorogram. Total unlabeled pea histones were loaded on *lane P*, and a control (*lane C*) was run in which the sample was incubated for 160 min with heat-denatured enzyme to check the extent of non-enzymatic chemical acetylation.

Edman degradation (Pesis and Matthews, 1986) or to errors in determining the repetitive yield, because the values obtained with mellitin may differ from those of H4. Therefore, it remains to be determined whether the sequential preference found among lysines 5, 12, and 16 is significant, but the results are clear enough to draw the conclusion that position 8 is not acetylated at all by histone acetyltransferase B.

DISCUSSION

Most of the studies to investigate the differential usage of acetylation sites in core histones have been carried out with histones labeled *in vivo*. This approach offers the obvious advantage of reflecting the actual acetylation status of histones in the living cell, but, as pointed out before, the level of acetylation of a given lysyl residue results from the action of both histone acetyltransferases and histone deacetylases. Therefore, the study of histones acetylated *in vivo* cannot cast any light on the site specificity of the enzymes involved in acetylation. *In vitro* studies were carried out by the group of von Holt (Thwaits *et al.*, 1976a, 1976b), but they used total nuclear histone acetyltransferases, with a somewhat broad substrate specificity. More recently, Richman *et al.* (1988) also used an *in vitro* assay to study the site specificity of a *Tetrahymena* histone acetyltransferase present in both cytoplasm and micronuclei, but the used substrate, macronuclear, total H4, is naturally acetylated to some extent. The approach followed in this paper circumvent these disadvantages. First, we have used a cytoplasmic purified histone acetyltransferase B highly specific toward H4; previous studies from our laboratory allowed us to conclude that even the crude preparations used here as starting material were free from other histone acetyltransferase (Salvador *et al.*, 1985) or histone deacetylase (Sendra *et al.*, 1988) activities. Second, the substrate was a homologous non-acetylated pea H4 isoform.

Under the experimental conditions used by Pesis and Matthews (1986) to determine the H4 acetylation sites by sequencing, partial cleavage with endoprotease Arg-C occurred at lysine 6 as well as incomplete cleavage at arginine 17. This resulted in the contamination of peptide 4–17, which contains all the acetylated lysines, with peptides 6–17, 6–19, or 4–19. Only the presence of the first two peptides may cause trouble in the sequential analysis, but this was also circumvented in our experiments by using endoprotease Arg-C digestion, after blocking the free ϵ-amino groups of unmodified lysines. All

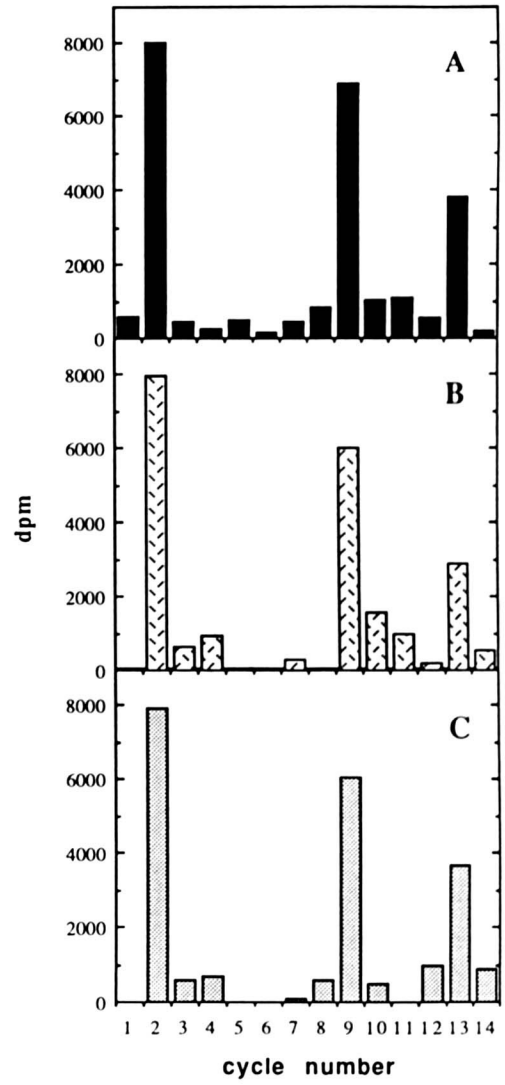

FIG. 4. **Histograms representing the radioactivity recovered by sequencing peptide 4–17 from different H4 isoforms.** Non-acetylated H4 was incubated with [1-^{14}C]acetyl-CoA, and the resulting products separated as indicated in Fig. 2. After blocking free ϵ-amino groups, the corresponding isoforms were digested with endoprotease Arg-C and subjected to manual Edman degradation as described in the text. The radioactivity is plotted against the cycle number for H4Ac1 (*A*), H4Ac2 (*B*), and H4Ac3 (*C*). See Fig. 1 for the equivalence between cycle number in peptide 4–17 and position in the sequence of the histone.

these conditions made it unnecessary to purify the endoprotease Arg-C peptides, which were directly sequenced to determine the cycle in which radioactivity appears. Of course, purification of the peptide 4–17 would be essential if the acetylation were to be detected by analysis of the phenylthiohydantoin derivative of acetyllysine, as done by Thorne *et al.* (1990).

The results obtained in the present work clearly show that pea histone acetyltransferase B may introduce up to three acetyl groups in homologous H4. A similar result was obtained by Richman *et al.* (1988) working with *Tetrahymena* cytoplasmic histone acetyltransferase, although taking into account that it had been reported that *Tetrahymena* H4 enters micro- and macronuclei as the diacetylated isoform (Chicoine *et al.*, 1986), they assumed that the presence of a triacetylated form after the *in vitro* acetyltransferase reaction was due to the preexistence of acetylated H4 in the substrate molecules. This is not the case under our experimental conditions, as

care was taken in using the non-acetylated H4 isoform. Several other groups (Jackson *et al.*, 1976; Cousens and Alberts, 1982; Chambers and Shaw, 1984; Shimamura and Worcel, 1989) also found that the newly synthesized H4 binds to DNA as the diacetylated isoform. We do not know whether newly synthesized pea H4 is also bound to DNA as the diacetylated form. If it were, either the specificity of histone acetyltransferase B changes under *in vivo* conditions, or the triacetylated isoform, is the substrate of a deacetylase on its way to DNA.

In a recent paper, Waterborg (1992) reported that lysine 20 from alfalfa H4 is also subject to acetylation, and he related this behavior to the absence of methylation in this residue. We have not found any evidence of histone acetyltransferase B-catalyzed acetylation in pea lysine 20, which is also unmethylated (DeLange *et al.*, 1969). Under our experimental conditions, after cleaving with endoprotease Arg-C, lysine 20 is the N-terminal residue of the tetrapeptide Lys-Val-Leu-Arg. Should this peptide be acetylated, radioactivity would have appeared either in the precipitate, if the peptide was insoluble or in the first Edman cycle if soluble.

The present results also show that lysine 8 of histone H4 is not used at all by pea histone acetyltransferase B *in vitro*. The three remaining sites are occupied at random, in contrast with the results on *Tetrahymena* histone acetyltransferase B (Richman *et al.*, 1988). This fact poses an interesting question on the site specificity of histone acetyltransferases, namely the nature of the sequence signals recognized by the active site of the enzyme. It has been often recalled that three of the four target sites for H4 acetylation, *i.e.* sites 5, 8, and 12, share the sequence motif Gly-Lys-Gly. In our case, however, it is clear that the enzyme does not recognize the sequence Gly-Lys-Gly for site 8, but the sequence Ala-Lys-Arg for site 16.

Our results confirm that histone acetylation represents a specific signaling for different functions. Site 8 is used *in vivo* in mammals and *Drosophila* (reviewed in Turner, 1991), in *Tetrahymena* (Chicoine *et al.*, 1986), and in *Physarum* (Waterborg *et al.*, 1983). Since these data were collected from transcriptionally active cells, it might occur that acetylation at lysine 8 were related in some way to transcribed regions. The recent finding that H4 acetylated at lysine 8 occurs in euchromatin of *Drosophila* polytene chromosomes (Turner *et al.*, 1992) is compatible with this idea. Lysine 8 is not used in cuttlefish testis (Couppez *et al.*, 1987), where acetylation is involved in the replacement of histones by protamines. The presence of an acetyl group at a given lysine residue depends on the site specificity and activity of histone acetyltransferases and deacetylases. The growing evidence on the role of histone N-terminal tails in gene regulation (Grunstein, 1990), together with the recent finding that specificity of maize histone deacetylase is regulated by phosphorylation (Brosch *et al.*, 1992) and the functional role proposed for histone deacetylation (López-Rodas *et al.*, 1993), open some new inroads into the field of structure-function relationships in chromatin.

Finally, if acetylation of histones actually provides a signal for different processes, a mechanism to recognize the signal would be necessary. Genetic evidence obtained by the group of Grunstein (Johnson *et al.*, 1990) suggests that SIR3 protein may be involved in recognizing acetylation at lysine 16 from histone H4, an essential mechanism for the correct function of *Saccharomyces* mating. If, as suggested by the present work, acetylation of lysines 5, 12, and 16 of pea H4 would function as a signal for histone import into the nuclei and/or for chromatin assembly, a component of the translocating and/or assembling machinery must recognize acetylation at those positions.

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