Non-random Acetylation of Histone H4 by a Cytoplastic Histone Acetyltransferase as Determined by Novel Methodology

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During periods of active DNA replication and chromatin assembly, newly synthesized histone H4 is deposited in a diacetylated form. In Tetrahymena, a specific pair of residues, lysines 4 and 11, has been shown to undergo this modification in vitro (Chicoine, L. G., Schulman, I. G., Richman, R., Cook, R. G., and Allis, C. D. (1986) J. Biol. Chem. 261, 1071-1076). Presumably, this reaction is catalyzed, at least in part, by histone acetyltransferases (HAT) of the B type, cytoplasmic enzymes displaying strong preference for free, non-chromatin-bound H4. To investigate which lysines are preferred acetylation sites in H4 from other organisms, a cytoplasmic HAT B activity was prepared from Drosophila embryos and used to acetylate H4 from several species. When H4 or synthetic, NH₂-terminal peptides from Tetrahymena were used as unblocked substrates, direct microsequence analyses showed that [³H]acetate was preferentially incorporated at lysine 11 with little, if any, incorporation at other conserved, acetylatable lysines. Drosophila H4 was chemically deblocked following its acetylation in vitro using conditions that do not deacetylate internal lysines. Direct sequence analysis verified the correct NH₂-terminal sequence of Drosophila H4 and demonstrated that [³H]acetate incorporation occurred preferentially on lysine 12, the residue analogous to lysine 11 in Tetrahymena. These data show remarkable preference for lysine 11/12 by the Drosophila HAT B activity in vitro and provide support for the assertion that this activity functions to acetylate new H4, at least in part, for deposition and chromatin assembly in vitro. Since most H4s, like Drosophila, are blocked at their amino termini by an acetylthreonine or acetylserine, our results demonstrate that this deblocking and microsequencing strategy can be used to study acetylation site utilization in H4 and presumably other core histones NH₂ terminally blocked with these residues.

Although the exact function(s) of histone acetylation has yet to be fully established, some aspects of core histone acetylation have been highly conserved. During periods corresponding to active DNA replication and chromatin assembly, newly synthesized histone H4 is deposited in a diacetylated form. This modification, originally described by Allfrey and co-workers (Ruiz-Carillo et al., 1975), has been reported in a wide range of organisms ranging from protozoa to man (Jackson et al., 1976; Woodland, 1979; Chambers and Shaw, 1984; Giancotti et al., 1984; Allis et al., 1985; Harisanova and Ralchev, 1986; Coupez et al., 1987) and thus, is a highly, perhaps absolutely, conserved feature of histone acetylation.

The steady state level and occupancy of specific acetylation sites result from a balance of two opposing cellular activities, histone acetyltransferases (acetilase or HAT) of both nuclear (type A) and cytosolic (type B) origin and deacetylases. Presumably, deposition-related diacetylation of newly synthesized H4 is catalyzed, at least in part, by HAT B type activities, cytoplasmic enzymes displaying strong preference for free, non-chromatin-bound H4. For example, Weigand and Brütgal (1981) identified a HAT B from early Drosophila embryo cytoplasmic fractions and showed this activity to be highly selective for H4. Since this activity is present in the earliest stages of embryogenesis (0-2 h post fertilization), a stage of rapid cytoskeletal nuclear replication/division and very little transcription, it is likely that this HAT activity functions in histone deposition and/or chromatin assembly.

Since this report, HAT activities of the B class have been described in several organisms (see Mingarro et al., 1993 for references), but relatively few experiments have addressed the site specificity of these activities in vitro or in vivo. Facilitated by the fact that H4 is not blocked at its amino terminus in the ciliated protozoan, Tetrahymena, direct microsequence analyses of newly synthesized H4 (labeled in vivo with [³H]lysine) first demonstrated non-random utilization of H4 acetylation sites (Chicoine et al., 1986). Moreover, this report established that a specific pair of residues, lysines 5 and 12, were utilized during deposition-related H4 diacetylation (Allis et al., 1985; Chicoine et al., 1986). Using free H4 as an in vitro substrate, a crude cytosolic HAT B from Tetrahymena faithfully reproduces a 4/11 pattern of acetylation (Richman et al., 1988) and quite recently, a similar preference for lysines 5 and 12 was reported for pea HAT B (Mingarro et al., 1993). Together, these studies suggest that HAT B-type activities display strong preference for lysines 4/5 and 11/12 in the amino-terminal domain of H4.

In this study we have attempted to ask if a cytoplasmic Drosophila HAT B activity (first described by Weigand and Brütgal, 1981) displays the above mentioned site preference in vitro utilizing H4 (or NH₂-terminal synthetic peptides of H4)

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from a variety of homologous and heterologous sources. Using a modification of a method developed by Weller and co-workers (1990), we have successfully deblocked *Drosophila* H4 using conditions that do not deacetylate internal lysine residues and determined the sites of acetylation in *vitro* by *Drosophila* HAT B (DHAT B). As far as we are aware, this is the first application of a method developed to deblock a histone (H4) for the analysis of acetylation sites by direct sequencing.

In all experiments, DHAT B shows a strong preference for acetylation at lysine 11/12 with little, if any, tendency to acetylate any of the other acetylatable lysines in H4. These data strongly suggest that the K11/12 pattern of acetylation exhibited by several HAT Bs has been conserved across widely divergent evolutionary taxa ranging from protozoa to diptera and suggest that acetylation of this specific lysine in H4 plays an important functional role(s) during histone deposition and/or chromatin assembly that has yet to be determined.

**MATERIALS AND METHODS**

Preparation of Cytoplasmic Histone Acetyltransferase—DHAT B was prepared from 0 to 3 hr embryos washed and resuspended in Oregon R embryos, kindly provided by A. Mahowald (University of Chicago, Chicago, IL), as previously described (Wiegand and Brutlag, 1981). Briefly, 10 g of embryos were homogenized in 50 ml of homogenization buffer (0.25 M sucrose, 20 mM Tris-HCl, 1 mM CaCl2, 2 mM EDTA, 100 mM NaCl) at pH 7.5) in a buffer-empiric homogenizer (Pette B). Following the removal of large debris by filtration through a Nitex mesh, nuclei were removed by centrifugation at 2,500 x g for 10 min, and the resulting supernatant was further clarified by sedimentation at 22,500 x g for an additional 35 min. Routine microscopic analysis failed to detect any nuclei in this supernatant under DAPI-UV optics.

This extract (FI) was then treated to remove nucleic acids and endogenous histones by the drop-wise addition of 6.9-diamino-2-ethoxyacridine (final concentration of 0.085%) with constant stirring at 4 °C. Nucleic acid precipitates were removed by sedimentation at 22,500 x g, 4 °C, for 15 min, and this supernatant (FI) was routinely stored at -20 or -80 °C until needed. The FI extract (referred to as DHAT B throughout this report) contains no endogenous histones as determined by reverse-phase HPLC chromatography and Coomassie staining of SDS-polyacrylamide gels.

Preparation of Nuclear Histone Acetyltransferase—Cytoplasmic debris was removed from nuclei (collected in the initial low speed centrifugation described above) by diluting the resuspended nuclear pellet with an equal volume of 2.5 mM sucrose in homogenization buffer and pelleting the nuclei through a 5-ml "heavy" sucrose cushion in buffer B); the interface was stirred gently to form a crude gradient. Gradients were centrifuged at 15,000 x g for 30 min at 0 °C in a Beckman SW 28 rotor. Supernatants were removed by aspiration, and pelleted nuclei were resuspended in buffer C (25% glycerol, 5 mM MgOAc, 50 mM Tris, pH 8.0, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM n-butyrate. 0.1 mM EDTA, pH 8.0) for routine storage at ~80 °C.

Fresh or frozen nuclei were acid extracted in 0.4 M H2SO4 for 2-18 h at 4 °C before the acid-insoluble protein was removed by centrifugation for 15 min at 13,000 x g. Total acid-soluble nuclear protein was recovered by precipitation with 20% trichloroacetic acid, washed with acetylated acetone, acetone, and then dried under vacuum. Protein was stored dry or resuspended in buffer B.

*Tetrahymena* Cell Culture, Nuclear Isolation, and Histone Preparation—Genetically marked strain CU 428, kindly provided by P. Bruns (Cornell University, Ithaca, NY) was grown axenically as described (Gorovsky, 1979). Macronuclei were prepared as described (Glover and Gorovsky, 1979) with minor modifications described in Wang and Allis (1993). In some experiments, *Tetrahymena* histones were labeled in vivo with ['H]sodium in the absence of protein synthesis exactly as described by Vavra et al. (1982). Deacetylated histones were prepared by treating macronuclei in deacetylation buffer overnight at room temperature according to Vavra et al. (1982). Acid-soluble protein was isolated as described above for *Drosophila* with the exception that, where indicated, 5% peracetic acid was used to extract H1 and several abundant high mobility group-like proteins from this fraction.

Reverse-phase (RP) Purification of *Tetrahymena* and *Drosophila* Histones—Total acid-soluble nuclear protein, dissolved in water, was injected onto a DE-52 column (BioRad, 220 x 4.6 mm). Routinely, core histones were separated with a shallow, linear 34-52% acetonitrile (ACN) gradient over 30 min (0.60%/min) with a flow rate of 1 ml/min. All gradient solutions contained 0.1% trifluoroacetic acid. Under these conditions, core histones from *Tetrahymena* elute between 40 and 50% ACN with TH3 and TH4 eluting at 49 and 43% ACN, respectively. *Drosophila* core elute between 40 and 50% ACN with DH3/DH2A coeluting at 43% ACN and DH3 eluting at 48% ACN. Ali fractions were then dried under vacuum and either stored as dry pellets or dissolved in water for storage at ~20 °C. In some cases, H4 was further purified by a second cycle of RP-HPLC using similar gradient conditions.

Synthesis of Unacylated and Acetylated Peptides—Peptides corresponding to the first 18 amino-terminal residues of *Tetrahymena* H4 (Glover and Gorovsky, 1979) were synthesized as previously described (Lin et al., 1989) using either unacylated or acetylated lysine at specific positions during the synthesis of each peptide. Following synthesis, peptides were evaluated by RP-HPLC chromatography, amino acid composition, and direct microsequencing. For each synthetic peptide utilized in this report, the correct amino sequence was obtained. Moreover, because lysine is well separated from acetylated lysine under our HPLC conditions (see Chicoine et al., 1996), the correct position of acetylated *versus* unacylated lysines was verified for each of the synthetic peptides by microsequencing.

*In Vitro (Protein) Acetylation Assay—Reverse-phase-purified core histones or crude mixtures of acid-soluble proteins were labeled in 1 x TEND buffer (20 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, 50 mM NaCl, 1 mM MgOAc, 20-30% acetonitrile, 20-30% trifluoroacetic acid). A lyso- mephine-enriched fraction (type VIII-S) purchased from Sigma. Substrates (typically 1-3 μg of histone) were added to 25-75 μl of [1H]acetylcoenzyme A (1-3 Ci/mmol; Dupont-New England Nuclear) in the presence or absence of *Drosophila* FI extract. In preliminary experiments, nuclear extracts were also examined with crude histones or 4% gradient-purified mononucleosomes prepared as described earlier (Chicoine et al., 1987). Reactions were incubated at room temperature for 40 min and terminated by adding 20 μg of protease sulfate as carrier before adding trichloroacetic acid to a final concentration of 20%. Precipitates were recovered as described, washed in acetone, and dried under vacuum. Reaction products were resuspended in appropriate sample buffers for SDS or acid urea electrophoresis. In some experiments, histones or synthetic peptides were subjected to automated microsequencing following *in vitro* acetylation. In this case, reactions were terminated by a 5-min treatment at 65 °C before being subjected to RP-HPLC.

*In Vitro (Peptide) Acetylation Assays—In vitro acetylation of synthetic peptides (5 μg/reaction) was exactly as described above except that incorporation was determined by collecting *H*-labeled peptides on Whatman P-81 paper (Horvich and Fujimoto, 1975) as modified by Bellido et al. (1980). Briefly, reaction mixtures were spotted on P-81 paper, washed three times in 50 mM NaHCO3, pH 9.0, at room temperature and once in EtOH. Filters were placed in vials and counted in a water-based scintillation mixture.

Several experiments were performed to determine if the major and only readable sequence was that initiated at the NH$_2$ terminus of DH4.

Vacuum, purified DH4 was incubated directly with 100% trifluoroacetic acid until needed. Routinely, gels were stained with Coomassie following electrotransfer to Immobilon samples for automated sequencing. Proteins were transferred from SDS gels as described previously (Lin et al., 1989); proteins were transferred from acid-urea gels for 15 min in 0.28% acetic acid at 0.5 amps using a commercially available transfer apparatus ( Hoefer Scientific, Inc.). Efficiency of transfer and retention was evaluated directly by staining and fluorographing side strips of the same blots used for sequence analysis. Blots were air dried and stored at -20°C in airtight plastic heat-sealed bags until needed. Routinely, gels were stained with Coomassie following transfer to ascertain the cycle/residue where [H]acetate had occurred.

Electrophoresis and Electrophoretic Transfer—SDS and acid-urea gels used in this study were essentially as previously described (Allis et al., 1980). Gels were typically stained with Coomassie Brilliant Blue R, destained, photographed, and processed for fluorography or where indicated, transferred (prior to staining) to Immobilon-PSQ (Millipore) for automated sequencing. Peptides were separated from SDS gels as described previously (Lin et al., 1989); proteins were transferred from acid-urea gels for 15 min in 0.28% acetic acid at 0.5 amps using a commercially available transfer apparatus ( Hoefer Scientific, Inc.). Efficiency of transfer and retention was evaluated directly by staining and fluorographing side strips of the same blots used for sequence analysis. Blots were air dried and stored at -20°C in airtight plastic heat-sealed bags until needed. Routinely, gels were stained with Coomassie following transfer to ascertain the completeness of transfer. Typically, greater than 90-95% of the proteins were transferred from the gel and retained on the membrane. A new band was found for any selective transfer/resolution of specific acetylated histone subpeptides.

Deblocking of Native Proteins for Sequencing—Drosophila H4 labeled as described above was isolated by RP-HPLC. After drying under vacuum, purified DH4 was incubated directly with 100% trifluoroacetic acid (Baxter Healthcare Corp.) for 2 h at 37°C according to methods modified from Wellner and co-workers (1990). Trifluoroacetic acid was removed by vacuum drying and the treated protein was either stored at -20°C for sequencing or redissolved into sample loading buffer for SDS or acid-urea gel electrophoresis. Typically, 1-3 μg of purified protein was treated with 100 μl of trifluoroacetic acid.

Automated Microsequencing Procedures—In vitro [H]acetate labeled H4 subpeptides were recovered by RP-HPLC or from protein transfers and microsequenced on an Applied Biosystems 477A protein sequencer. Since in these experiments the [H]-label is directly in the acetyl modification itself, determination of radioactivity at each position in the sequence defines which residues are acetylated. Samples were solubilized in water and applied to Bio-Rad-treated glass fiber filters or Immobilon samples were loaded into a Blott cartridge; optimized Normal cycles or Blott cycles were used, respectively. In each sequence analysis, 50% of each cycle was routed to the in line AminoLink:18578 and 50% for radioactivity determination. The remaining 50% was partitioned to the sequencer's fraction collector for determination of counts/min; samples were transferred directly to scintillation vials and the radioactivity determined by liquid scintillation counting. Repetitive yields determined for each sequencing run were between 90 and 95%. In all cases, the appropriate NH$_2$-terminal amino acid sequence of DH4 was obtained. In the case where DH4 was deblocked with trifluoroacetic acid, there was evidence for some internal peptide bond cleavage, however, the major and only readable sequence was that initiated at the NH$_2$ terminus of DH4.

RESULTS

Histone H4 Is the Preferred Substrate for Drosophila HAT B—Several experiments were performed to determine if the original histone acetyltransferase activity described by Weigand and Brutlag (1981) was indeed cytosolic and distinct from nuclear histone acetyltransferase activity(ies). Nuclear (type A) and cytosolic (type B) activities were compared from the same batch of extracts and tested in parallel with free histones or mononucleosomes. Surprisingly, we did not observe the type A acetylase from the nuclear fraction, a finding also reported by Weigand and Brutlag (1981). In contrast, the FIT extract (DHA T B) strongly labeled H4 (but not H3, H2A, or H2B) as a free histone whether in a crude mixture or in a pure form (see below). Weigand and Brutlag reported that core histones in nucleosomes are not acetylated by an identically prepared activity, and we have confirmed their finding (data not shown). From these tests, we conclude that the FIT extract contains a histone acetyltransferase activity with a strong preference for free H4 (referred to as DHAT B throughout this report).

As shown in Fig. 1, core histones from a variety of homogeneous and heterogeneous sources were tested as in vitro substrates with DHAT B. Free (non-chromatin bound) H4 from Drosophila, Tetrahymena, bovine, and human, are labeled strongly by DHAT B in vitro (tested either as crude mixtures or as homogeneous preparations; Fig. 1B, lanes 1–4, 7, and 8); H3 is also weakly labeled under these conditions, however, further tests showed this labeling to be enzyme-independent. Call thymus (CT) histones are not labeled in reactions lacking DHAT B (as a control for non-enzymatic labeling, Fig. 1B, lane 9). However, when extracts containing DHAT B are labeled in the absence of exogenously added histones (Fig. 1B, lane 10), a polypeptide migrating with an apparent molecular mass of 46 kDa is typically observed; the identity of this polypeptide is not known.

These data demonstrate that DHAT B, as with HAT B activities described from other sources, shows strong preference for free histone H4 in vitro. Moreover, HAT B from Drosophila utilizes H4 from species as widely divergent as ciliated protozoa and humans; a result particularly interesting given that H4 from Tetrahymena differs from other H4s in several positions within its first 20 amino acids (Glover and Gorovsky, 1979 and Fig. 4).

The products shown in Fig. 1B were also analyzed by acid-urea gel electrophoresis to determine the extent of acetylation under our in vitro assay conditions (Fig. 1, C and D). In this gel system, H4 (as well as other core histones) migrate, in part, according to charge and produce an acetylation "ladder" of distinct unmodified and acetylated (mono-, di-, tri-, etc.) subpeptides (each acetylation abolishes one positive charge; see Fig. 1C, lane 2 for a clear example of this ladder). From this analysis, it is apparent that under our in vitro conditions, monoacetylated H4 is the principal product (a small amount of diacylated H4 is observed sometimes; Fig. 1D, lanes 1, 4, 7, and 8).

Incorporation of [H]acetate into NH$_2$-terminal, Synthetic H4 Peptides—Histone acetylation is highly selective for lysines located in the NH$_2$-terminal domain of each of the core histones. Given this, we wonder whether DHAT B could utilize a specific set of synthetic peptides corresponding to the first 18 amino acids of Tetrahymena H4. Five distinct peptides, varying in the degree and/or sites of acetylation, were synthesized and are identified as follows: monoacetylated, monoacetylated (mono-11), diacetylated (di-4,11), diacetylated (di-4,7) and tetraacetylated (teta) (see Fig. 2A for details).

Shown in Fig. 2B is the incorporation profile of [H]acetate label by DHAT B into each of the above peptides over time. Several observations are noteworthy. First, as expected, a significantly higher level and rate of acetylation (3–5-fold) is observed when the unacylated peptide is compared directly with the other peptides used in this assay. Although this result could reflect the fact that the unacylated peptide has 4 lysines potentially available for acetylation, this is not the case (see the sequencing data presented below). Second, little, if any, incorporation is observed with the tetraacetylated peptide (or other controls such as minus extract). This result is expected since no sites are available for acetylation in the tetraacetylated peptide.

Third, most of the other peptides tested in this assay were surprisingly poor substrates for DHAT B displaying incorporation levels barely above that of the negative controls. Unexpected-
Drosophila histone acetyltransferase B (HAT B) is highly selective for histone H4 in vitro. Total acid-soluble proteins from Drosophila (DRO), Tetrahymena (TET), HeLa (HUM, kindly provided by A. Annunziato) nuclei, and calf thymus (Sigma fraction VIII-S, CT) (lanes 1–4) or partially purified (RP-HPLC) histones (lanes 5–8) were incubated with [3H]acetyl-coenzyme A ([3H]AcCoA) and DHAT B in vitro. Reaction products were analyzed by SDS-gel electrophoresis followed by staining with Coomassie Blue (A) and fluorography (B). Lanes 9 and 10 are controls of minus extract (–) and extract (DHAT B) alone, respectively. The region of the gel containing the core histones is bracketed in lane 1 of A. In addition, a 46 kDa band from the extract is consistently labeled by [3H]AcCoA during our in vitro assays (see small arrowhead next to lane 10 of B). Although the identity of this polypeptide is not known, it comigrates on RP-HPLC with fractions containing abundant polypeptides which are visible at 43–45 kDa. Identical aliquots of the same reaction products shown in A and B were also analyzed by acid-urea gel electrophoresis. As in A and B, both the stained gel (C) and its corresponding fluorograph (D) are shown. The bracket next to lane 1 of C denotes the position of the H4 ladder; positions of the un-, mono-, di-, and tri-acetylated H4 subspecies are indicated. The H4 ladder is particularly clear in the Tetrahymena sample, lane 2 of C. For alignment purposes, a dot to the right of each lane in C and D denotes the position of the monoacetylated subspecies.

To test this hypothesis directly, unacetylated peptide was isolated following an in vitro reaction with DHAT B and microsequenced. By determining the counts/minute released at each cycle of sequencing, we determined that essentially all of the [3H]acetate label was incorporated into lysine 11 (Fig. 3A). Under these conditions, only a small amount of label is observed at the positions of the other acetylatable lysines (lysines 4, 7, and 15). These data are consistent with our hypothesis and directly demonstrate a strong preference for lysine 11 by the Drosophila activity.

During periods of rapid DNA replication and chromatin assembly, newly synthesized H4 is deposited into many nuclei in a diacetylated form (see Introduction). At least in Tetrahymena, lysine 4 and 11 in H4 are used exclusively during this process (Chicoine et al., 1986). We were curious if DHAT B could acetylate a peptide at position 4 if site 11 was unavailable for acetylation. To test this possibility, the mono-11 peptide was reacted with DHAT B and microsequenced. The results (Fig. 3B) failed to detect a significant level of incorporation at any of the available lysines, 4, 7, or 15. From these data, we conclude that DHAT B displays a strong, perhaps absolute, preference for lysine 11 at least when assayed with synthetic, NH₂-terminal peptides of H4. Interestingly, lysine 11 is the site of acetylation which distinguishes deposition, from transcription-related acetylation in Tetrahymena in vivo (Chicoine et al., 1986). Lysine 11 Is the Preferred Site of Acetylation in Tetrahymena H4—As shown in Fig. 4, H4 is acetylated on 4 lysines that have...
glycine (19) and cysteine (20) were included in each peptide for coupling. Included, DHAT B (FII extract) alone and unacetylated peptide minus peptide) and were verified by microsequence analysis. Only the first 18 amino acids listed are from the amino-terminal synthetic peptides of Tetrahymena H4; the carboxyl-terminal glycine (19) and cysteine (20) were included in each peptide for coupling to carrier protein and antibody production. Peptides were incubated with DHAT B and [3H]AcCoA and incorporation data over time was determined by a phosphocellulose filter binding assay (Fig. 2). Following purification by RP-HPLC, each of the labeled reaction products were microsequenced. In both A and B, 50% of the material recovered at each cycle of sequencing was used to identify the amino acid (shown on the x axis in one-letter code); the remaining 50% was reserved for determining the H counts/minute released at each position/cycle. Potentially acetylable lysines are indicated by underlined Ks.

Because lysine residues are the same in Tetrahymena and Drosophila except that Tetrahymena lacks an arginine residue at position 3 and therefore, the lysines are in positions n-1 when compared with all other known H4s. In addition, all H4s except Tetrahymena begin with an NH2-terminal acetyl-serine or acetyl-threonine which prevents sequence analysis from the amino terminus. Because Tetrahymena H4 (TH4) is an effective substrate for DHAT B under our in vitro assay conditions (Fig. 1, B and D) and because Tetrahymena H4 begins with a non-blocked alanine and is therefore, readily sequenced, we chose to use this heterologous H4 to investigate site utilization by DHAT B.

When deacetylated TH4 is purified, labeled in vitro with DHAT B, and analyzed by electrophoresis on an acid-urea gel, the vast majority of [3H]acetate label is incorporated into the monoacetylated species (see Fig. 1D). Direct microsequencing of total TH4 following in vitro acetylation is shown in Fig. 6A. Strikingly, essentially all of the [3H]acetate label incorporated into TH4 is at lysine 11 even though all 4 acetylatable lysines were potentially available for acetylation in this substrate. Relative to lysine 11, essentially no label is incorporated at lysines 4, 7, or 15. These results are in excellent agreement with the results obtained with unacetylated H4 synthetic peptide (Fig. 3A) and show that the strong preference for site 11 is not an artifact resulting from the fact that in the latter case, an NH2-terminal peptide was used as substrate for DHAT B.

Deblocking of Drosophila H4—Recently, Wellner and coworkers (1990), published a method for deblocking proteins beginning specifically with NH2-terminal acetyl-serine or acetyl-threonine. A mechanism of deblocking was proposed involving an acid-catalyzed “N to O” shift of the acetyl group onto the hydroxyl oxygen of the blocked serine or threonine residues (see Fig. 4 for details), followed by a β-elimination. If correct, we reasoned that this procedure should not deacetylate internal lysine residues in Drosophila H4 (DH4) under examination in this study.

To examine this issue directly, TH4 (labeled in vivo with [3H]sodium acetate; see Vavra et al. 1982) or DH4 (labeled in vitro with DHAT B, this study) were purified by RP-HPLC and incubated either in water or 100% TFA. Following incubation at 37 °C for 4 h, both samples were dried under vacuum and analyzed by SDS and acid-urea gel electrophoresis (± trifluoroacetic acid, Figs. 5, A and B). If the deblocking mechanism proposed by Wellner and co-workers is correct, the free amino terminus of H4 could be ionized, causing a downward shift in acid-urea gels. This is not observed with TH4 because this histone is not blocked (see Fig. 5B). Interestingly, a downward shift by one is observed when DH4 is treated with trifluoroacetic acid (data not shown). Moreover, both H4s exhibited identical degrees of acetate labeling suggesting that the trifluoro-
acetic acid treatment does not appreciably deacetylate the internal lysines under examination in this study. Although results are only shown for in vitro labeled TH4, identical results were obtained with in vitro labeled TH4 (data not shown).

Lysine 11/12 in Drosophila H4 Is a Highly Preferred Site of Acetylation—Encouraged that the acetyl groups on internal lysines in H4 are not being removed by the deblocking treatment with trifluoroacetic acid, DHAT B was used to acetylate homologous DH4 (which is largely deacetylated; see Fig. 1C, lane 7) in vitro. Following partial purification by RP-HPLC, [3H]acetate-labeled DH4 was deblocked with 100% trifluoroacetic acid (37 °C for 2 h), dried under vacuum, electrophoresed in an SDS gel (to separate it from contaminating H2A which coelutes with H4 under our HPLC conditions), electroblotted onto an Immobilon PSQ membrane and microsequenced. Importantly, direct sequence analysis of DH4 following these procedures generated the correct NH2-terminal sequence (listed on the x axis of Fig. 6B) Thus, this indicates not only that the deblocking treatment with trifluoroacetic acid was successful, but also that cleavage of internal peptide bonds in DH4 during trifluoroacetic acid treatment was not a significant problem. Analysis of [3H]acetate label released at each cycle of sequencing demonstrated that lysine 12 is the exclusive site of acetylation in DH4 following in vitro acetylation by DHAT B (Fig. 6B). Little, if any, incorporation is observed on lysines 5, 8, or 16, the other potential acetylation sites in DH4.

**DISCUSSION**

Deposition of newly synthesized H4 in diacetylated form has been reported in a wide range of organisms during periods
corresponding to active DNA replication and chromatin assembly. Until recently, only one study has addressed which pair of lysines in H4 undergo this modification in vivo. Allis and coworkers (Chicoine et al., 1986) demonstrated that in the Tetrahymena system, lysines 4 and 11 are the exclusive sites of diacetylation in newly synthesized H4. Recently, we have extended these results significantly by showing that lysines 5 and 12 are the exclusive sites of diacetylation in newly synthesized human H4. Thus, it appears that the 5/12 pattern of H4 diacetylation is a highly, perhaps absolutely, conserved pattern affecting new H4 as it is deposited in chromatin.

Given these results, it is important to determine to what extent type B acetylases mimic the in vivo pattern of acetylation site utilization. A consistent pattern emerges from the determination of acetylation sites utilized by DHAT B with all of the H4 substrates tested in this study. Lysine 11/12 is the strongly preferred site for one specific substrate, free H4 and as such, may have evolved specialized properties to acetylate new H4 during the rapid, syncytial nuclear divisions made by the Drosophila embryo. If DHAT B is indeed a cytosolic activity, this activity resides in one subcellular compartment and displays a strong preference for one specific substrate, free H4. Thus, studying the acetylation pattern of DHAT B may not give a complete picture of the in vivo situation. Both of the studies referred to above (Chicoine et al., 1986), utilized H4 that was newly synthesized and deposited into nuclei. It is an intriguing possibility that DHAT B (and perhaps other HAT B-type activities) catalyze only the acetylation of free H4 at lysine 11/12 and that a second, distinct activity, catalyzes acetylation at lysine 4/5. It is a formal possibility that acetylation at this second site occurs after H4 has entered the nucleus and begun to be chromatin associated.

Along this line, the HAT extracted from highly purified populations of Tetrahymena macronuclei (operationally, a type A acetylase) acetylates lysines 4 and 11 equally well when free, unacetylated TH4 is used as a substrate (Chicoine et al., 1987). Based upon these and other data, it was concluded that this nuclear activity displays features of both transcription- and deposition-related acetylation. Interestingly, we have consistently observed a 2-fold higher level of acetyl incorporation when the Tetrahymena HAT A is reacted with the mono-11 peptide as compared with the corresponding unacetylated peptide. Although we have not determined what site(s) is(are) being utilized under these conditions, it would be interesting to determine if lysine 4 is a highly preferred site in this situation.

The most significant finding reported here is a remarkably strong preference observed for only one acetylation site in H4 (lysine 11/12) by a DHAT B. Using free H4 as an in vitro substrate, a crude cytosolic HAT B from Tetrahymena (Richman et al., 1988) and quite recently, pea HAT B (Mingarro et al., 1993) also displayed a strong preference for lysine 11/12 in the amino-terminal domain of H4. These findings argue strongly that acetylation of this specific lysine in H4 during periods active in histone synthesis and chromatin assembly plays an important role(s) that has yet to be understood.

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