A Single Histone Acetlytransferase from *Tetrahymena* Macronuclei Catalyzes Deposition-related Acetylation of Free Histones and Transcription-related Acetylation of Nucleosomal Histones

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Abstract. A salt-extracted histone acetyltransferase activity from *Tetrahymena* macronuclei acetylates mostly histone H3 and H4 when free histones are used as substrate. Free histone H4 is acetylated first at position 11 (monoacetylated) or positions 11 and 4 (diacetylated). This activity strongly resembles in vivo, deposition-related acetylation of newly synthesized histones. When acetylase-free mononucleosomes are used as substrate, all four core histones are acetylated by the same extract, and H4 is acetylated first at position 7 (monoacetylated) or positions 7 and 4 (diacetylated). In this respect, the activity of the extract is indistinguishable from postsynthetic, transcription-related histone acetylation that occurs in vivo or in isolated nuclei. Heat inactivation curves with both substrates are indistinguishable, and free histones compete with chromatin for limiting amounts of enzyme activity. These results argue strongly that two distinct, biologically important histone acetylations, one deposition related and one transcription related, are carried out by a single acetyltransferase.

Most eukaryotic DNA is packaged in a fundamental unit, the nucleosome core particle, composed of two each of the four core histones, H2A, H2B, H3, and H4, and 146 bp of DNA (Igo-Kemenes et al., 1982). Underlying this scheme for DNA packaging is a remarkable degree of evolutionary conservation of the four core histones. However, it is also well established that compositional heterogeneity of nucleosomes can be generated by the association of histone primary sequence variants or specific nonhistone proteins with subpopulations of nucleosomes, as well as by the presence of numerous postsynthetic covalent modifications (acetylation, phosphorylation, methylation, ADP-ribosylation, ubiquitination, and proteolytic processing), which affect many histone and nonhistone proteins (see Reeves, 1984).

Quantitatively, acetylation of specific, often highly conserved internal lysines is the major modification affecting nucleosomal histones. Correlational studies suggest a role for histone acetylation in transcription (Allfrey, 1977; Chicoine and Allis, 1986; Mathis et al., 1980; Vavra et al., 1982), in histone deposition/chromatin assembly (Allis et al., 1985; Annunciato and Seale, 1983; Jackson et al., 1976; Louie and Dixon, 1972; Ruiz-Carrillo et al., 1975), and in replacement of histones by protamines during spermatogenesis (Christensen and Dixon, 1982; Christensen et al., 1984). However, the mechanisms by which histone acetylation affects these processes are unknown.

One approach to understanding the biological function of histone acetylation is to characterize the major enzymatic activities responsible for the acetylation of histones. Numerous histone acetyltransferases activities (EC 2.3.1.48) have been described. Based primarily upon subcellular localization and substrate specificity, two major forms of histone acetyltransferase have been characterized. Histone acetyltransferase activity type A reacts with all core histones to varying degrees and is presumably chromatin bound (Garcea and Alberts, 1980; Sures and Gallwitz, 1980; Belikoff et al., 1980; Travis et al., 1984). It is a candidate for a role in transcription-mediated acetylation. Type B activity is believed to be a cytoplasmic enzyme responsible for the specific acetylation of histone H4 before deposition (Garcea and Alberts, 1980; Sures and Gallwitz, 1980; Weigand and Brutlag, 1981).

The ciliated protozoan *Tetrahymena thermophila* offers several advantages for studying histone acetylation as it pertains to transcription- and deposition-related functions. Earlier studies (Chicoine et al., 1986; Gorovsky et al., 1973, 1978; Vavra et al., 1982) have shown that macronuclear core histones are extensively acetylated and incorporate significant amounts of acetate (in vivo or in vitro) in the absence of protein synthesis. Micronuclear histones, on the other hand, are unacetylated and incorporate little if any acetate postsynthetically. Since macronuclei are transcriptionally active while micronuclei are transcriptionally inert, a strong correlation exists between this type of histone acetylation and transcriptional activity.

Recently, a second system of histone acetylation has been

described in *Tetrahymena* (Allis et al., 1985). Newly synthesized H3 and H4 destined for either macro- or micronuclei are deposited in acetylated forms. Since micronuclei replicate but do not transcribe, an independent role for acetylation of H3 and H4 in histone deposition is strongly suggested. Interestingly, unique acetylation sites are utilized in H4 when it is acetylated for transcription- versus deposition-related purposes. (Chicoine et al., 1986).

In this report, we have used a salt extract containing macronuclear histone acetyltransferase activity to compare the substrate specificity and the sites of acetylation (in H4) when either free histone or chromatin-bound histone is used as substrate. The results obtained with free and chromatin-bound histone are different and mimic what is observed in vivo with deposition- versus transcription-related acetylation, respectively. Furthermore, we provide evidence strongly suggesting that a single acetyltransferase catalyzes both activities in macronuclei.

Materials and Methods

Cell Culture and Nucleus Isolation

Tetrahymena thermophila (strain Cu 428, Chx/Chx[cy-S] VII provided by P. Bruns, Cornell University, Ithaca, NY) were grown axenically to densities of 4–6 × 10^5 cells/ml in 1% enriched proteose peptone as described (Gorovsky et al., 1975). Highly purified macronuclei were prepared as described (Gorovsky et al., 1975) except that phenylmethylsulfonylfluoride (PMSF, 1 mM) replaced spermidine in the isolation buffer. Macronuclei were either used immediately or stored as a pellet under nucleus isolation buffer at −80°C. No differences have been observed between extracts from fresh or frozen macronuclei.

Salt Elution of Histone Acetyltransferase from Macronuclei

Macronuclei were thawed, washed twice in modified microsomal medium (MM)[1] (modified MM = 10 mM Tris, 0.25 M sucrose, 0.1% spermidine, 15 mM $MgCl_2$, 10 mM sodium butyrate, 1 mM PMSF, 1 mM dithiothreitol, pH 8.0), and resuspended in modified MM containing 500 mM NaCl (at 3–4 × 10^8 nuclei/ml). Nuclei were extracted with gentle agitation for 30 min at 0°C. Salt-insoluble material was collected by centrifugation at 175,000 *g* for 30 min and typically reextracted as described above.

Pilot experiments indicated that acetyltransferase activity is reversibly inhibited by moderate-high concentrations (100–500 mM) of monovalent salt, but was unaffected by moderate concentrations of divalent cation (10–15 mM $MgCl_2$). Both salt-soluble supernatants (S_1 and S_2) were, therefore, dialyzed against modified MM (minus sucrose and spermidine) for 18–24 h at 4°C to reactivate the enzyme and to precipitate any released chromatin. After complete dialysis, the copious precipitate is removed by centrifugation at 16,000 *g* and the cleared supernatants ($S_{1'}$ and $S_{2'}$) are stored in aliquots containing 10% glycerol at −80°C. Acetylase activity remains stable under these conditions for at least 1 mo.

Assays for Histone Acetyltransferase

Standard liquid and a novel nitrocellulose-immobilized dot blot acetylase assays were performed exactly as described (Allis et al., 1986*a*). Where nucleosome fractions were assayed for endogenous acetylase activity (Fig. 4) chromatin from sucrose gradients was simply spotted onto nitrocellulose and the blot incubated directly in buffer containing ^{3}H-acetyl-coenzyme A. All reaction buffers contained 10 mM sodium butyrate to inhibit deacetylase activity present in the salt extract. Using *Tetrahymena* macronuclear histone as a substrate, 1 U of activity is defined as the amount of enzyme required to catalyze the incorporation of 1 nmol of acetate into histone in 30 min under standard reaction conditions.

1. *Abbreviations used in this paper*: MM, microsomal medium.

Chromatin Preparation and Characterization

Macronuclei prepared as described above were washed twice and resuspended in nuclease digestion buffer (10 mM Tris, pH 7.5, 3 mM $MgCl_2$, 10 mM NaCl, 1 mM PMSF), at 4 × 10^8 nuclei/ml (~4 mg DNA/ml) and converted to a final concentration of 1 mm $CaCl_2$. Digestion with micrococcal nuclease (20 U/mg DNA, Worthington Biochemical Corp., Freehold, NJ) for 30 min at room temperature typically rendered 25% of the DNA acid soluble. EDTA was added to a final concentration of 10 mM to terminate the reaction. After chilling on ice, nuclei were vortexed periodically for 15 min at 0°C before being collected at 1,500 *g*, 10 min. The supernatant (S_1) was saved. Nuclei were lysed by vigorous resuspension in 10 mM EDTA at pH 8.0 for 15 min at 0°C and centrifuged at 1,500 *g* for 10 min. This supernatant (S_2) was combined with S_1 and loaded (2 × 10^8 nuclei/gradient) onto 10-ml 5–20% sucrose gradients in 10 mM Tris, pH 7.5, 1 mM EDTA, 1 mM PMSF. Gradients were spun at 150,000 *g* for 12–14 h at 2°C. After manual fractionation, an aliquot of each fraction was taken for DNA, protein, and acetyltransferase assays. DNA prepared according to Ryoji and Worcel (1985) was sized on 1.5% agarose gels containing TEA buffer (Helling et al., 1974). Regions of the gradient corresponding to mononucleosomes that were found to be free of endogenous histone acetylase activity were pooled and stored at −20°C for further use.

Histone Extraction and Gel Electrophoresis

Histones were extracted from nuclei as previously described (Allis et al., 1979). Histone products were precipitated directly from in vitro reactions, by the addition of TCA to a final concentration of 20%. The TCA precipitate was collected, washed with acetone, and dried. Samples were dissolved in acid-urea sample buffer (Allis et al., 1979) and analyzed by one-dimensional (acid-urea or triton-acid-urea) or two-dimensional (triton-acid-urea by SDS) gel electrophoresis (Allis et al., 1979, 1980 *a*, *b*). Where appropriate, gels were stained, photographed, and processed for fluorography. Long acid-urea slab gel electrophoresis was performed according to Laemmli (1970) as modified by Allis et al. (1979).

Protein Elution and Automated Sequencing Procedures

^{3}H-acetate-labeled histone subspecies were eluted and recovered from appropriate positions of lightly stained long acid-urea gels according to the methods of Allis et al., (1986*b*). Before the final acid precipitation step, unlabeled sperm whale myoglobin (4 nmol) was added to radiolabeled samples to act as a carrier and to provide an internal control for the sequence run. Proteins were sequenced on protein sequenator (model 470A; Applied Biosystems, Inc., Foster City, CA) as described previously (Allis et al., 1986*b*; Chicoine et al., 1986). Material collected at each cycle was dried and processed for scintillation counting (Chicoine et al., 1986).

Results

Extraction of a Histone Acetyltransferase from Isolated Macronuclei

A large fraction of the macronuclear genome is transcribed during vegetative growth (Calzone et al., 1983), and histones prepared from macronuclei have been shown to be highly acetylated. Isolated macronuclei contain easily detectable acetylase activity which can transfer ^{3}H-acetyl groups to all core histones from ^{3}H-acetyl-coenzyme A (Gorovsky et al., 1973; Vavra et al., 1982). Essentially all (>95%) of the detectable histone acetyltransferase activity is removed by two extractions with 500 mM NaCl (data not shown).

Fig. 1 shows a typical one-dimensional SDS gel of two successive salt extracts from macronuclei before (S_1, S_2) and after ($S_{1'}$, $S_{2'}$) dialysis. While appreciable amounts of core and linker (H1) histone are released from macronuclei by modified MM containing 500 mM NaCl (Fig. 1, lanes *2* and *5*), essentially all of the histone is removed from the extract in the precipitate which forms after dialysis (Fig. 1, lanes *3* and *6*). These fractions were assayed for histone acetyltransferase activity, reaction products were recovered,

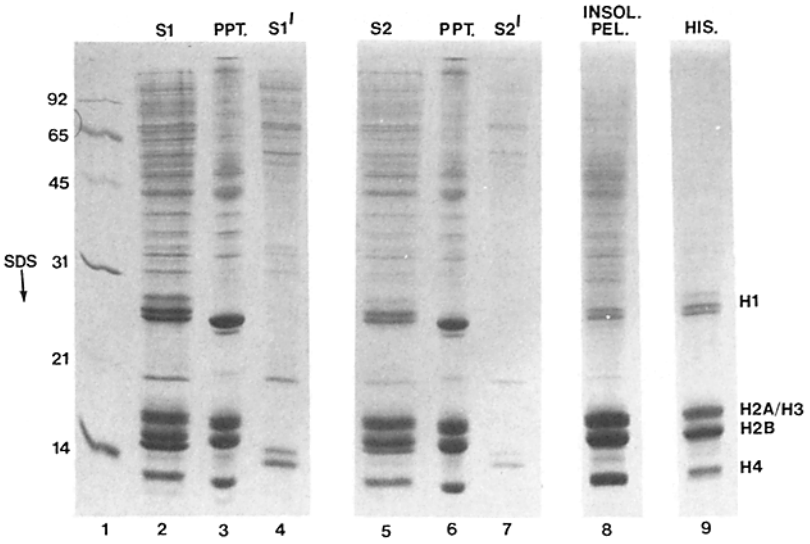

Figure 1. SDS gel analysis of individual fractions during salt elution of acetylase from macronuclei. Equal aliquots of the various fractions generated by the 0.5 M salt elution and subsequent dialysis of histone acetyltransferase activity from isolated macronuclei (see Materials and Methods for details) were subjected to analysis on one-dimensional SDS polyacrylamide gels followed by staining with Coomassie dye. (Lane *1*) Molecular mass markers in kilodaltons; (*2*) first salt extraction before dialysis; (*3*) precipitate from first extraction after dialysis, (*4*) supernatant from first extraction after dialysis; (*5*) second salt extraction before dialysis; (*6*) precipitate from second extraction after dialysis; (*7*) supernatant second extraction after dialysis; (*8*) final salt-insoluble pellet; (*9*) acid-extracted macronuclear histones for reference. Note that a significant amount of H1 is dephosphorylated during the overnight dialysis step (Glover et al., 1981).

separated in a one-dimensional acid-urea gel, and analyzed by staining and fluorography (Fig. 2). Histone acetyltransferase activity is released with each salt wash and little activity is lost by dialysis (compare S_1 with $S_{1'}$ and S_2 with $S_{2'}$). Controls, lacking enzyme (lanes *1* and *2*) or added histone (lanes *11–18*), show little activity. Undialyzed samples (lanes *12* and *16*) do not require substrate, probably due to endogenous "chromatin" being acted upon by the acetylase. Interestingly, H2B is acetylated under these conditions (see * next to lanes *4*, *8*, *12*, and *16*) but is not acetylated as a "free" histone (see below). Based on these results, dialyzed, cleared supernatants ($S_{1'}$ and $S_{2'}$) can be pooled to give a salt-free, chromatin-free histone acetyltransferase activity from macronuclei. Typically, pooled supernatants yield an enzyme-

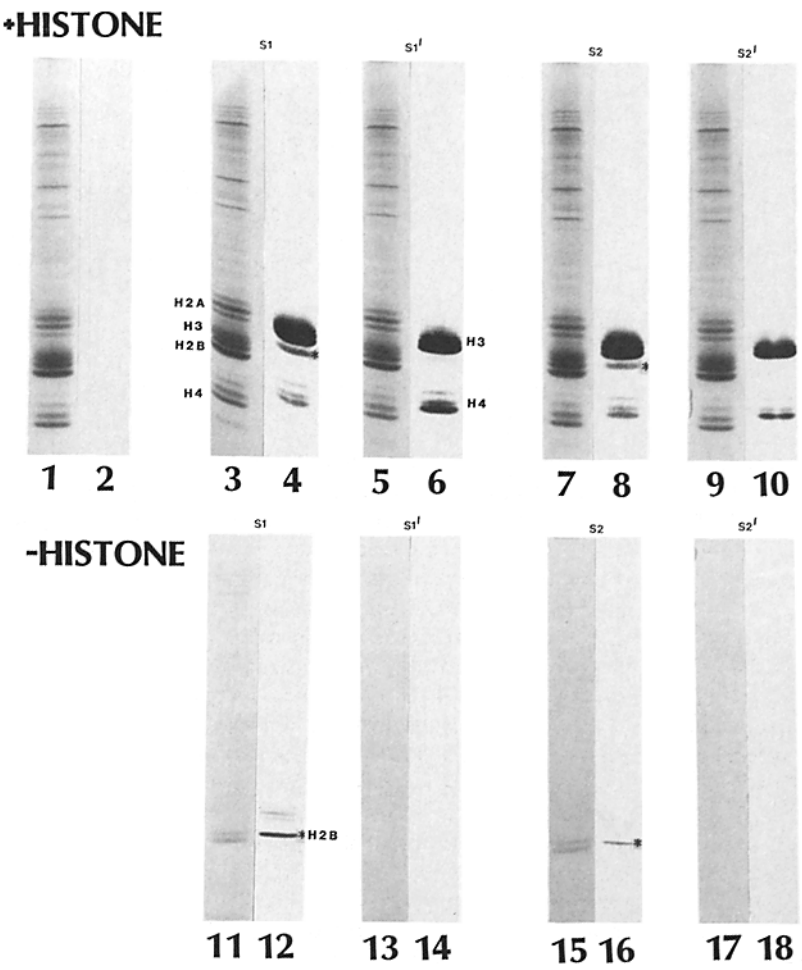

Figure 2. Acetylation profiles of macronuclear histones after incubation with various salt extracts from macronuclei. Equal amounts (40 μl) of both salt extracts shown in Fig. 1, before (S_1 and S_2) and after dialysis ($S_{1'}$ and $S_{2'}$), were adjusted to a final concentration of 0.1 M NaCl in modified MM before being incubated in the presence (lanes *1–10*) or absence (lanes *11–18*) of macronuclear histone (1 mg/ml final concentration) and ^{3}H-acetyl-coenzyme A (2 μM final concentration, 0.1 mCi/ml, 5 Ci/mmole). Reactions (each in 100-μl final volume) were carried out for 45 min at 30°C before the histone was precipitated by the addition of TCA to a final concentration of 20%, washed, and dried. Histone recovered from the in vitro reactions was separated by one-dimensional acid-urea gel electrophoresis and analyzed by staining (*odd-numbered lanes*) and fluorography (*even-numbered lanes*). In one control, acetylase extract was omitted from the complete reaction and replaced with MM buffer (−extract, lanes *1* and *2*). ^{3}H-acetate incorporation into H2B (see *asterisk* next to lanes *4*, *8*, *12*, and *16*) probably results from small amounts of endogenous chromatin in the salt extracts which are removed as a precipitate during the dialysis step (see text for details). Typical counts per minute (10% of each sample) incorporated at the end of such an experiment are: −extract = 4,000; S_1, 74,000; $S_{1'}$, 70,000; S_2, 68,000; $S_{2'}$, 48,000.

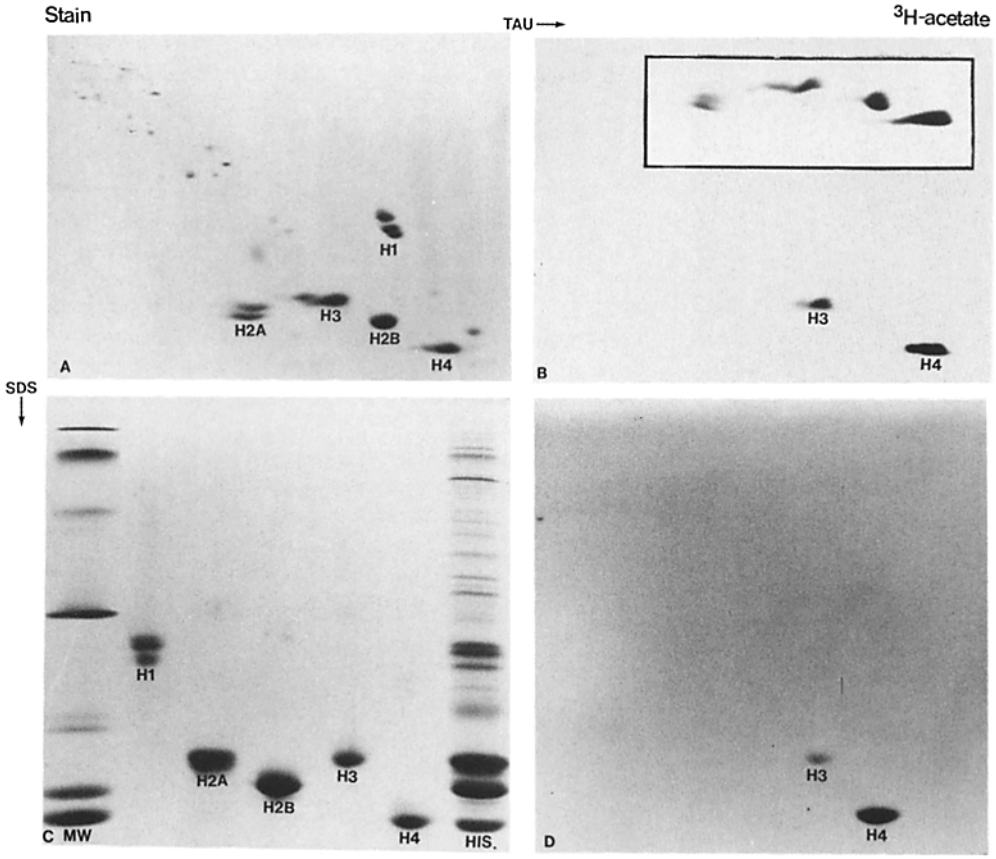

Figure 3. Gel analysis of macronuclear histones after an in vitro reaction with macronuclear acetylase. After complete in vitro reactions with macronuclear acetylase extract (as in Fig. 2), acid-extracted macronuclear histones were recovered and separated on a two-dimensional gel (triton-acid-urea by SDS). Gels were analyzed by staining (*A*) and by fluorography (*B*). The *inset* in *B* shows typical results when isolated intact macronuclei are incubated directly in ^{3}H-acetyl-coenzyme A according to Vavra et al. (1982). Individual macronuclear histones (H2A, H2B, H3, H4, and H1) were excised and eluted from acid-urea gels and reacted separately with macronuclear acetylase activity. After the reaction, products were resubjected to electrophoresis into an SDS one-dimensional gel and were analyzed by staining (*C*) and fluorography (*D*). Exposure times in *B* and *D* are identical.

specific activity of 3–6 U/mg protein, similar to crude yeast or calf thymus chromatin-bound acetyltransferase A (Belikoff et al., 1980; Travis et al., 1984). We shall refer to this as an acetyltransferase activity realizing that, at this point, no evidence has been presented indicating whether it contains one or more enzymes (see below).

Specificity of Macronuclear Acetylase Activity with Free Histone Substrate

When macronuclear histones are used as substrates for the macronuclear acetyltransferase activity and the products are analyzed by two-dimensional gel electrophoresis (Fig. 3, *A* and *B*), the arginine-rich core histones, H3 and H4, are preferentially acetylated. Little, if any, [^{3}H]acetate is associated with H2A, H2B, and or H1. The failure to acetylate H1 in vitro is not surprising since H1 is not acetylated in vivo or in intact nuclei (Gorovsky et al., 1973, 1978; Vavra et al., 1982). Failure to acetylate H2A and H2B is unexpected, however, since both are acetylated in vivo and in the isolated macronuclei from which the extract is prepared (Fig. 3 *B*, *inset*). Failure to acetylate H2A and H2B is not a consequence of the fact that a mixture of core histones is used as substrate in these reactions. If gel-purified macronuclear histones are incubated individually with acetylase activity, [^{3}H]acetate is again incorporated only into H3 or H4 (Fig. 3, *C* and *D*). These data suggest that when acetyltransferase activity is extracted from macronuclei and presented with free histone as substrate, either its specificity is changed or that multiple enzymes are responsible for activity in vivo and in intact macronuclei and only a restricted subset is extracted in active form.

Specificity of Macronuclear Acetylase Activity with a Chromatin Substrate

Based on earlier studies (Gorovsky et al., 1978; Vavra et al., 1982) we would have predicted a priori that macronuclear histone acetyltransferase activity would resemble transcription-related acetylation wherein all core histones are acetylated. The major in vivo substrate for this enzyme is likely to be chromatin, not free histone. We wondered, therefore, what the outcome would be if the same extract was tested with a chromatin substrate. To prepare a chromatin substrate free of endogenous acetylase activity, we took advantage of the observation that histone acetyltransferase activity from yeast is easily displaced from chromatin after digestion with micrococcal nuclease (Travis et al., 1984). Sucrose gradient–fractionated, micrococcal-digested chromatin from macronuclei was assayed for endogenous histone acetylase activity (Fig. 4 *A*) using a recently reported dot blot assay (Allis et al., 1986) and its DNA was sized on agarose gels (Fig. 4 *B*). Substantial acetylase activity is detected in the region of the sucrose gradient containing dinucleosomes and larger sized chromatin oligomers (*arrow*, Fig. 4 *B*). Mononucleosomes are relatively devoid of endogenous histone acetyltransferase, suggesting that acetylase may be located in the linker region of macronuclear chromatin. The results presented in Fig. 4 demonstrate that sucrose gradient-purified mononucleosomes are sufficiently free of endogenous acetylase to serve as substrate for the macronuclear acetyltransferase extract.

Significant amounts of acetate appear to be associated with all the incompletely resolved core histones when an aliquot of mononucleosomes is incubated with the acetylase activity

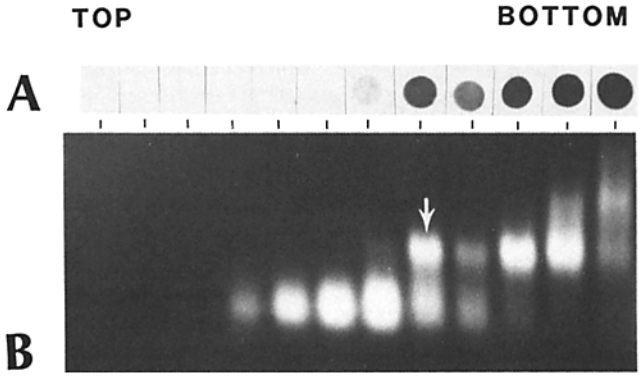

Figure 4. Sucrose gradient analysis of endogenous histone acetyltransferase activity in micrococcal-nuclease–digested macronuclear chromatin. Micrococcal-digested chromatin from macronuclei was fractionated on a 5–20% sucrose gradient. Individual fractions from the entire gradient were analyzed for endogenous acetylase activity (*A*) by spotting the material onto nitrocellulose and performing "dot-blot" enzyme analyses (Allis et al., 1986*a*); exogenous histone was omitted from in the reaction buffer. DNA from the same fractions was also analyzed on agarose gels (*B*). Only the region of the sucrose gradient corresponding to mono-, di-, and trinucleosomes is shown. The arrow in *B* denotes the first fraction containing appreciable amounts of dinucleosomes, which is also the first fraction to show endogenous acetylase activity.

extracted from macronuclei (Fig. 5 *A*, lanes *3* and *4*). This result is confirmed by two-dimensional analysis (Fig. 5 *C*); H2A, H2B, H3, and H4 are acetylated as are the minor histone variants, hv1 and hv2. These observations mimic results obtained when cells are labeled in vivo with [^{3}H]sodium acetate or when isolated macronuclei are incubated in vitro in the presence of ^{3}H-acetyl-coenzyme A. Note, however, that it is the extracted activity that is being assayed since mononucleosomes purified from sucrose gradients (*arrow*, Fig. 5 *B*) incorporate little ^{3}H-acetate when incubated in vitro without extract (Fig. 5 *A*, lanes *1* and *2*). Thus, the extracted macronuclear acetylase activity can react with all core histones when they exist in a chromatin form. This result is in striking contrast to the results obtained when the same acetyltransferase activity is incubated with histones in a free form, where only H3 and H4 are acetylated (Figs. 2 and 3).

Different H4 Acetylation Sites in Free Histone and Chromatin Substrates

The specificity of the acetyltransferase activity for H3 and H4 in a free histone substrate resembles deposition-related acetylation in vivo, while acetylation of all four core histones in chromatin resembles transcription-related in vivo acetylation. Recent studies have demonstrated a clear distinction between the sites in histone H4 that are modified in vivo in association with each type of acetylation (Chicoine et al., 1986). Acetylation at residues 11 and 4 occurs during deposition-related acetylation, while residue 7 is modified exclusively in monoacetylated H4 and residues 7 and 4 in diacetylated H4 in conjunction with transcription-related acetylation. To see if the single extract faithfully carried out both types of acetylation in a substrate-dependent fashion, we analyzed the sites of in vitro acetylation of histone H4.

To analyze the sites on a free histone substrate, unacetylated macronuclear H4 was purified from long acid-urea gels (see Materials and Methods), acetylated in vitro under standard conditions, and reanalyzed on a second long acid-urea gel. Reaction products corresponding to mono- or diacetylated H4 were excised, eluted, and subjected to automated microsequencing (note that *Tetrahymena* H4 does not have a blocked amino terminus as do most other H4s; see Glover and Gorovsky, 1979). It is clear that the lysine at residue 11 is the exclusive site of acetylation in monoacetylated H4 (Fig. 6 *A*) while lysine at position 11 and 4 are preferred in diacetylated molecules. (Fig. 6 *B*). Thus, the sites being used in vitro on a free histone substrate are the same as those used in vivo when newly synthesized histone H4 is deposited in either macro- or micronuclei (Chicoine et al., 1986).

To analyze the acetylation sites on a chromatin substrate, mononucleosomes were purified and acetylated in vitro under standard conditions. Histones were then extracted, subjected to electrophoresis in a long acid–urea gel, and the mono- and diacetylated forms of H4 were eluted and analyzed in the sequenator. When chromatin is used as a substrate, lysine 7 (instead of lysine 11 as in free histone) is the exclusive site of monoacetylation (Fig. 6 *C*) and residues 7 and 4 are acetylated exclusively in diacetylated H4 (Fig. 6 *D*). Thus, the sites being used in vitro on a chromatin substrate differ from those observed with a free histone substrate, and are the same as the sites used in vivo and in intact isolated nuclei for the bulk of postsynthetic, presumably transcription-related, acetylation.

A Single Enzyme Catalyzes Deposition-related and Transcription-related Histone Acetylation

With a free histone substrate, the macronuclear extract exhibits a histone acetyltransferase activity that strikingly

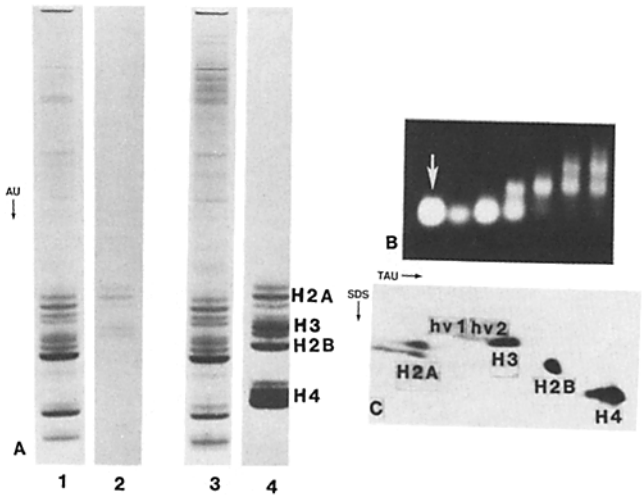

Figure 5. Acetylation profiles of nucleosomal histone after an in vitro reaction with macronuclear acetylase. Mononucleosomes isolated from 5–20% sucrose gradients (*arrow*, *B*) were incubated in vitro in the presence (lanes *3* and *4*) or absence (lanes *1* and *2*) of macronuclear acetylase (as in Fig. 2). After the reaction, histones were extracted and separated on one-dimensional (acid-urea, *A*) or two-dimensional (triton-acid-urea by SDS, *C*) gels. Gels were analyzed by staining (*A*, lanes *1* and *3*) and fluorography (*A*, lanes *2* and *4*, and the two-dimensional gel in *C*). The fluorograph shown in *C* is from a separate complete reaction with the acetylase as in *A*, lanes *3* and *4*.

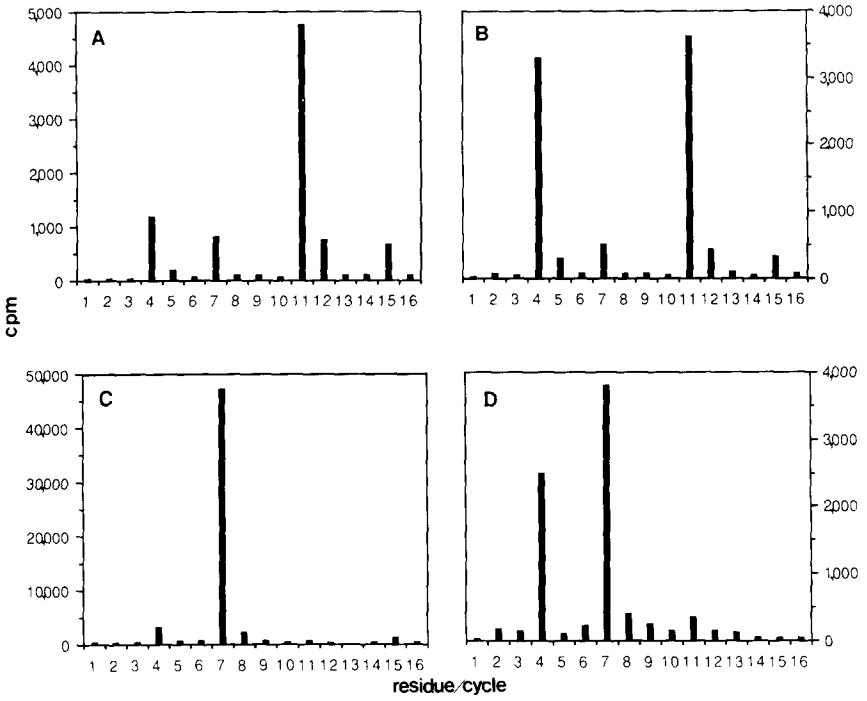

Figure 6. Sites of acetylation in H4 after in vitro reactions on free histone or chromatin substrates. Unmodified H4 was excised and eluted from long acid-urea gels as described (Allis et al., 1986*a*), dissolved in modified MM, and reacted with the macronuclear acetylase as described in Fig. 2. After the reaction, histone was recovered by precipitation with 20% TCA and resubjected to electrophoresis into a second long acid-urea gel. Protein corresponding to mono- (*A*) and diacetylated (*B*) H4 was excised, eluted, and subjected to automated sequencing as described in Chicoine et al. (1986). Shown are the ^{3}H-acetate counts per minute released at each cycle of sequencing. Only the first 16 amino acids in H4 are shown; this includes all of the acetylation sites (lysines at 4, 7, 11, and 15) in *Tetrahymena* H4 (see Chicoine et al., 1986). Mononucleosomes, purified on a sucrose gradient, were reacted as described above. After the reaction, histones were recovered and processed exactly as described above for free histone reactants. The first 16 residues are shown of mono- (*C*) and diacetylated (*D*) H4 using a chromatin substrate.

resembles deposition-related histone acetylation both in selectivity for histones H3 and H4 and in specificity for residues 11 and 4 in H4. With a chromatin substrate, the same extract shows an activity that strikingly resembles transcription-related acetylation in acetylating all four core histones and in specificity for residues 7 and 4 in H4. The question arises as to whether the extract contains two (or more) acetyltransferases or whether a single enzyme can catalyze both activities. One approach to resolving the issue would be to initiate purification of the enzyme(s) and to test all stages of purification with both substrates. However, preparation of chromatin substrate free of endogenous acetyltransferase is difficult and time-consuming, making quantitative assays at every step of purification with this substrate prohibitive. Also, cofractionation of two activities cannot prove that both reside in a single enyzme until complete purification is achieved, a task not yet accomplished for histone acetyltransferase from any source, in spite of numerous attempts. For these reasons, we sought alternative tests of whether we were dealing with one or two enzymes.

The first test consisted of a comparison of the heat inactivation of the extracted activity with both substrates. As seen in Fig. 7, pretreatment of the macronuclear extract at various temperatures, before assay with either substrate at 30°C under standard reaction conditions, yields indistinguishable inactivation curves, consistent with the presence of a single enzyme.

The second test is a more rigorous one, consisting of an analysis of the direct competition of the two substrates with each other under conditions of limiting enzyme and of acetyl-coenzyme A excess. In these experiments, a constant amount of mononucleosomes per reaction was mixed with increasing amounts of free histone. Two extreme outcomes of this experiment can be envisioned. First, if two distinct acetylase activities exist, one specific for chromatin and the other specific for free histone, the acetate incorporation profile typical of mononucleosomes, in which all histones are acetylated (see Fig. 5), should be relatively unaffected by increasing amounts of free histone added to the in vitro reaction. On the other hand, if only one activity catalyzes acetylation onto both substrates, a progressive shift in the acetyla-

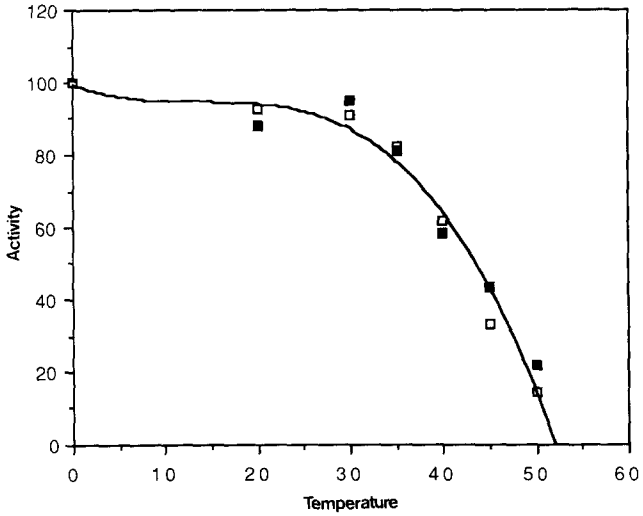

Figure 7. Heat inactivation of macronuclear acetylase tested with free and nucleosomal histones. Equal aliquots of macronuclear acetylase extract were preincubated at 0, 20, 30, 35, 40, 45, and 50°C for 30 min before being reequilibrated to 30°C for 15 min. Half of each aliquot was reacted with free histone (■) and half with sucrose-gradient purified mononucleosomes (□). For preincubation, at 0°C cpm of the free histone reaction was 78,000 cpm and that of the mononucleosome was 74,000. These values were set equal to 100% activity.

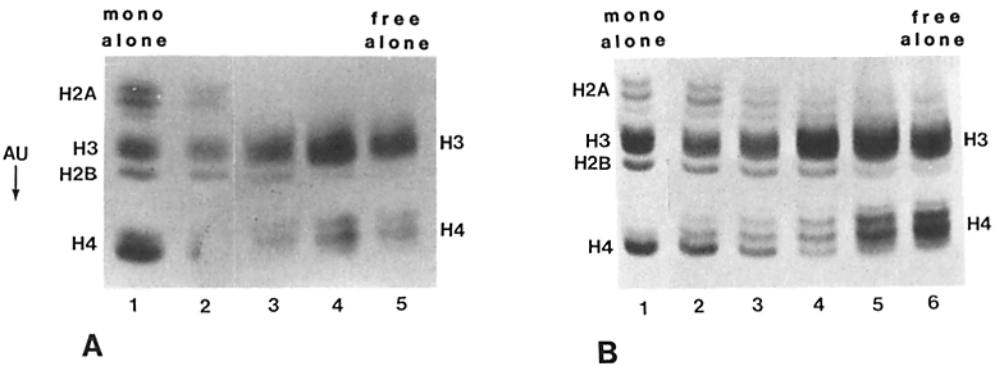

Figure 8. Competition of free and nucleosomal histone for macronuclear acetylase. (*A*) Constant amounts of sucrose-gradient mononucleosomes were mixed with either 0- (lane *1*), 0.5- (lane *2*), 2.0- (lane *3*), or 3.0- (lane *4*) fold amounts of free histone before being incubated with a fixed amount of macronuclear extract and acetyl-coenzyme A. In *A* the final concentration of acetyl-coenzyme A was 2 μM (1:1, labeled/unlabeled). Lane *5* shows free histone alone as a control. Part of the H4 seen in lane *2* was affected by a tear in the gel which caused the H4 acetate signal to seem abnormally low. Only the H2A to H4 region of the fluorograph is shown. Exposure time, 15 h. (*B*) Identical to *A* except that mononucleosomes were mixed with either 0- (lane *1*), 0.25- (lane *2*), 0.5- (lane *3*), 1- (lane *4*) and 2- (lane *5*) fold increasing amounts of free histone, and the reactions were carried out with a final concentration of acetyl-coenzyme A of 12 μM (a 1:11 mixture of labeled and unlabeled). Lane *6* shows a free histone alone control. Exposure time, 4 d. Free and mononucleosome histone amounts were adjusted to the specified ratio by determining the amounts of the two substrates giving similar intensities of core histone staining with Coomassie Blue. All reactions were carried out at 30°C for 30 min before histone reaction products were recovered, subjected to electrophoresis in an acid-urea gels, and examined by fluorography.

tion profile should occur toward that typically observed with free histone in which H3 and H4 are preferentially labeled (see Fig. 3).

In previous studies (Allis et al., 1986*a*), we demonstrated that, under standard reaction conditions with a free histone substrate, the assay was proportional to the amount of extract added (i.e., enzyme, not substrate, is limiting). In other studies (data not shown), the K_m for acetyl-coenzyme A was found to be ~1 μM and the reaction rate was found to be independent of free histone concentrations ≥1 mg/ml. Thus, our standard reaction conditions (2 μM acetyl-coenzyme A, 1 mg/ml histone substrate) fulfill the criteria required to see if the two substrates compete. When increasing free histone is added to a constant amount (equivalent to ~1 mg/ml of histone) of mononucleosomes under standard reaction conditions (Fig. 8 *A*), ^{3}H-acetate incorporation into histones H2A and H2B (which is easily detectable when mononucleosomes alone are used as a substrate) is progressively competed to the point where a 3:1 ratio of free histone/chromatin (lane *4*) is virtually indistinguishable from a reaction containing only free histone (lane *5*).

The competition experiment in Fig. 8 *A* argues strongly that the two substrates compete for the same enzyme. However, to completely rule out the possibility that two (or more) acetylases were competing for limiting amounts of acetyl-coenzyme A, the experiment was repeated using six times as much acetyl-coenzyme A (12 μM instead of 2 μM). Again, free histone substrate, where only H3 and H4 are acetylated, effectively competes out the acetylation of H2A and H2B on a chromatin substrate (Fig. 8 *B*). These results, coupled with the heat inactivation studies (Fig. 7), argue strongly that a single macronuclear histone acetylase is responsible for the very different acetylation patterns of free and chromatin-bound histone substrates.

Discussion

In this report, methods are described for the efficient salt elution of histone acetyltransferase activity from highly purified *Tetrahymena* macronuclei. With this activity we have set up an in vitro system capable of acetylating *Tetrahymena* histones. Interestingly, the substrate specificity and sites of acetylation in H4 change depending upon whether free or chromatin-bound histones are acetylated, and these differences mimic exactly what has been described from in vivo studies documenting deposition- and transcription-related acetylation in *Tetrahymena* (Allis et al., 1985; Chicoine et al., 1986; Vavra et al., 1982).

The finding that the specificity of histone acetyltransferase for particular histones can change dramatically depending upon the substrate is not new. Garcea and Alberts (1980) reported differences when free or nucleosomal histones were reacted with highly (but incompletely) purified nuclear acetylase A from calf thymus. Like these authors, we find that the activity from macronuclei displays strong preference for H4 and H3 when reacted with free histones. However, in *Tetrahymena*, approximately equal acetylation levels are observed with all non-H1 histones both in vivo and when the *Tetrahymena* extract is given a mononucleosomal substrate; the extracted acetylase A from calf thymus was less reactive toward H3 when tested on a nucleosomal substrate than was the in vivo acetylase (Garcea and Alberts, 1980).

The *Tetrahymena* acetylase is the first whose activity after extraction has been shown to mimic the major in vivo activity on chromatin both in general substrate choice and in specificity of acetylation sites. As described earlier (see Introduction), this isolated activity on chromatin is likely to represent the major, macronuclear-specific, transcription-related histone acetylation. Unexpectedly, the same macronuclear extract also mimics deposition-related acetylation when tested with free histone substrate as indicated both by its specificity (H3 and H4 only, Figs. 2 and 3) and by the acetylation sites in H4 (lysine 11 rather than 7, Fig. 6). In hindsight, this result is reasonable if one considers that free histone is probably similar to newly synthesized histone before its association with DNA.

Finding two distinct, biologically meaningful activities in a single extract raised the interesting possibility that *Tetrahymena* macronuclei contain a single enzyme responsible for acetylation of both newly synthesized H3 and H4 before

deposition and for transcription-related acetylation of mature, fully assembled histone in macronuclear chromatin. Two independent lines of evidence strongly support this hypothesis. First, identical heat-inactivation profiles are obtained when the extract is tested with free and nucleosomal histones (Fig. 7). Second, free histones can compete efficiently with mononucleosomes when both substrates are mixed and reacted with the same extract (Fig. 8). These data are consistent with the suggestion, first made by Wiktorowicz and Bonner (1982) that a subpopulation of acetylase from rat liver may reside at the periphery of the nucleus to preferentially acetylate newly synthesized histone upon entry into the nucleus. Similarly, the yeast and calf A-type acetyltransferases display kinetic preference for free versus polynucleosome substrates (Garcea and Alberts 1980; Travis et al., 1984), suggesting that these enzymes could also play a role in deposition-related modification. However, it should be noted that there is considerable evidence for diverse histone acetylase activities in metazoans (Belikoff et al., 1980; Sures and Gallwitz, 1980) raising the possibility that two (or more) enzymes have evolved to perform deposition-related and transcription-related acetylations in higher organisms, that additional functions exist for histone acetylation, or that one (or both) of these systems is more complex than indicated by the single *Tetrahymena* enzyme we have studied to date.

Although having two activities in a single enzyme would certainly simplify analysis of histone acetylation in *Tetrahymena*, numerous complexities still remain. Earlier studies have shown that the bulk of micronuclear histone is unacetylated and fails to become acetylated when isolated micronuclei are incubated in the presence of ^{3}H-acetyl-coenzyme A (Vavra et al., 1982). However, micronuclear H3 and H4 do show deposition-related histone acetylation in vivo (Allis et al., 1985). These results could mean that histone acetyltransferase is missing from micronuclei and that deposition-related acetylation of micronuclear histones occurs in the cytoplasm. Alternatively, micronuclei could contain a typical (macronuclear type) acetylase and chromatin structure (conformation, accessibility, etc.) could limit its activity on micronuclear histones; there could also be a unique micronuclear acetylase. The existence of kinetically distinct subcomponents of chromatin containing nucleosomes that are especially susceptible or remarkably inaccessible to acetylase (Garcea and Alberts, 1980) also remains to be explained, as do the precise roles of acetylase and deacetylase in regulating histone acetylation.

In conclusion, in *Tetrahymena* as well as in other eukaryotes, histone acetylation can be separated into reactions occurring on histones before (newly synthesized) and after they have assembled into nucleosomes. Our results suggest that one macronuclear acetylase is capable of carrying out both reactions. These studies, as well as those of Garcea and Alberts (1980), demonstrate that the conformation of the histone substrate plays a critical role in the outcome of these in vitro reactions. In our hands, both substrate specificity and H4 acetylation sites are affected by whether the histone is free or chromatin bound. These differences show remarkable correspondence to in vivo transcription- and deposition-related acetylation in vivo suggesting that, in *Tetrahymena*, a single enzyme carries out both functions.

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