Micronuclei and the Cytoplasm of Growing *Tetrahymena* Contain a Histone Acetylase Activity Which Is Highly Specific for Free Histone H4

Ronald Richman, Louis G. Chicoine, M. P. Collini, Richard G. Cook,* and C. David Allis

The Verna and Marrs McLean Department of Biochemistry, and *The Howard Hughes Medical Institute and Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77030

Abstract. Salt extracts prepared from purified micronuclei and the cytoplasm of growing *Tetrahymena* contain a histone acetylase (also referred to as histone acetyltransferase) activity which is highly specific for H4 when tested as a free histone. With both extracts, H4 is acetylated first at position 4 (monoacetylated) or positions 4 and 11 (diacetylated), sites diagnostic of deposition-related acetylation of newly synthesized H4 in vivo. As the concentration of cytosolic extract is decreased in the in vitro reactions, acetylation of H3 is also observed. Neither activity acetylates histone in a chromatin form.

These activities are distinct from a macronuclear acetylase which acetylates H3 and H4 (macro- or micronuclear) equally well as free histones and which acetylates all four core histones when mononucleosomes are used as substrate. As well, the micronuclear and cytoplasmic activities give similar thermal-inactivation profiles which are different from that of the macronuclear activity. In situ enzyme assays demonstrate a macronuclear-specific activity which acetylates endogenous macronuclear chromatin and an independent micronuclear-cytosolic activity which is able to act upon exogenously added free H4. These results argue strongly that an identical acetylase is responsible for the micronuclear and cytoplasmic activity which is either modified or altogether distinct from that in macronuclei.

STUDIES in vivo have demonstrated that at least two general systems of histone acetylation exist in the ciliated protozoan, *Tetrahymena*. One system, which we will refer to as "transcription-related" acetylation, is postsynthetic and affects all of the inner (non-H1) histones in transcriptionally active macronuclei. This system does not appear to exist in transcriptionally inactive micronuclei (Vavra et al., 1982; Chicoine and Allis, 1986). In contrast, a second system, which we will refer to as "deposition/replication-related" acetylation, requires protein synthesis and affects histones H3 and H4, regardless of whether they are destined for macro- or micronuclei (Allis et al., 1985).

In an attempt to understand further the biological function of histone acetylation as it relates to both transcription and chromatin assembly, we have begun to analyze the enzyme system(s) responsible for carrying out histone acetylation in *Tetrahymena*. Recently, we described results which suggest that a single histone acetylase (also referred to as histone acetyltransferase) extracted from purified macronuclei catalyzes both transcription- and deposition-related acetylation, depending upon the substrate used in the reaction (Chicoine et al., 1987). When mononucleosomes are used as a substrate for this activity, all four core histones are acetylated in a manner indistinguishable from postsynthetic transcription-related acetylation. However, when free histones are used as a substrate, the same activity acetylates mainly H3 and H4 in a manner surprisingly similar to deposition/replication-related acetylation.

Although having both of these biologically important acetylations carried out by a single macronuclear enzyme simplifies somewhat the picture of histone acetylation in *Tetrahymena*, it leaves unanswered the question of how micronuclear histones become acetylated during their synthesis and deposition onto the DNA. Since the vast majority of micronuclear histone is unacetylated during vegetative growth and fails to become acetylated when isolated micronuclei are incubated in the presence of [³H]acetyl-coenzyme A ([³H]acetyl-CoA)[1] (Vavra et al., 1982), it is reasonable to propose that micronuclei do not contain any histone acetylase activity. Clear precedents are known for differences in the protein compositions of macro- and micronuclear chromosomal proteins (for a review, see Gorovsky, 1986) and, therefore, specific "targeting" of histone acetylase to macronuclei would be consistent with the above data. It would likely follow that micronuclear histones are acetylated for "deposition" function(s) in the cytoplasm before their entry into the nucleus. Alternatively, micronuclear chromatin or even micronuclear histones themselves may contain

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1. *Abbreviation used in this paper:* CoA, coenzyme A.

unusual features (inhibitors, conformational changes, etc.) which prevent histone acetylation even in the presence of acetylase.

In this report, we have directly tested some of these possibilities by comparing the ability of defined macro- and micronuclear substrates to be acetylated in vitro by extracts prepared from highly purified populations of macro- or micronuclei. Evidence is presented that micronuclei contain an extractable acetylase activity that appears to be identical to that contained in the cytoplasm of growing cells. Its high specificity for H4 when tested with free histone, its utilization of unique deposition acetylation sites in H4, its unusual temperature "inactivation" profile, and, importantly, its inability to acetylate histone in a chromatin form, all suggest that this acetylase activity is different from the macronuclear activity recently described (Chicoine et al., 1987). In many ways, the "micronuclear-cytosolic" acetylase activity resembles histone acetyltransferase B from higher organisms.

Materials and Methods

Cell and Culture Conditions

Tetrahymena thermophila (strain Cu 428, Chx/Chx[cy-S]VII provided by P. Bruns, Cornell University, Ithaca, NY) were grown axenically to densities 2–5 × 10⁵ cells/ml in 1% enriched proteose peptone as described (Gorovsky et al., 1975).

Isolation of Highly Pure Macro- and Micronuclei

Macro- and micronuclei were prepared according to published procedures (Gorovsky et al., 1975) with the exception that phenylmethylsulfonyl fluoride (PMSF; 1 mM) replaced spermidine in the isolation buffer. Micronuclear and macronuclear purity was evaluated quantitatively by flow microfluorometry (Allis and Dennison, 1982). The above nucleus isolation method uses octanol and produces a floating "skin" after each centrifugation step. No attempt has been made to recover a "cytoplasmic" acetylase activity from cells lysed in this fashion.

Preparation of Crude Nuclear and Cytoplasmic Fractions from Detergent-lysed Cells

To avoid difficulties with the octanol used in the nucleus isolation method of Gorovsky et al. (1975), a detergent-based lysis method was used. Growing cells were pelleted and immediately lysed at a concentration of 1 × 10⁷ cells/ml by gentle Dounce homogenization in cell lysis buffer (10 mM Tris, 0.1 M sucrose, 0.1% spermidine, 10 mM MgCl₂, 2% gum arabic, and 1 mM PMSF, pH 7.5) containing 0.1% NP-40. This lysate was overlayered onto a cushion of cell lysis buffer containing 0.5 M sucrose and "total" nuclei (macro- and micronuclei) were collected at 7,000 g for 15 min. The entire supernatant above this pellet (including the cushion) was recentrifuged at 25,000 g for 1 h to remove any remaining nuclei and most of an undefined fibrous material. Routinely, this supernatant has been used as a convenient starting source of cytoplasmic acetylase. In one experiment, however, the 25,000 g supernatant was recentrifuged again at 100,000 g for 1 h without any significant loss of acetylase activity or change in substrate specificity. Therefore, we feel that the majority of the cytoplasmic activity we have described in this report is "soluble" as defined by these centrifugation steps.

Extraction of Acetylase Activity from Different Cellular Compartments

Equal amounts of purified macro- and micronuclei (correcting for the difference in DNA between the two nuclei) were extracted twice with 0.5 M NaCl in our basic acetylase buffer (50 mM Tris, 0.25 M sucrose, 0.1% spermidine, 15 mM MgCl₂, 10 mM sodium butyrate, 1 mM PMSF, 1 mM dithiothreitol, pH 8.0) exactly as described previously (Chicoine et al., 1987). In cases where total nuclear extracts were to be compared with cytoplasmic extracts prepared from detergent-lysed cells, nuclei were resuspended in 0.5 M NaCl in cell lysis buffer containing 0.1% NP-40 to control for the detergent that is present in the cytoplasmic supernatants. As well, the cytoplasmic supernatants were converted to 0.5 M salt, even though salt is only required in the extractions made from nuclei. All of the salt extracts described in this paper (from macronuclei, micronuclei, total nuclei, or cytoplasm) were dialyzed overnight into acetylase buffer (minus spermidine) and recentrifuged at 25,000 g for 10 min to remove any precipitate that forms during the dialysis step (see Chicoine et al., 1987). All extracts were used immediately after their preparation.

Preparation of Acetylase-free Mononucleosomes

Macronuclei were digested with micrococcal nuclease as described previously (Chicoine et al., 1987) and the digest was fractionated on 5–20% sucrose gradients. Individual fractions from the gradient corresponding to mononucleosomes were identified and assayed for endogenous acetylase activity as described previously (Chicoine et al., 1987). Only those fractions that were essentially free of acetylase activity were pooled and frozen for use as substrate in subsequent experiments.

Acid Extraction of Macro- and Micronuclear Histone

Acid-soluble protein from macro- and micronuclei was extracted as described previously (Allis et al., 1979). For routine acetylase assays using macronuclear histone (see below), histone H1 was removed by extraction with perchloric acid (Glover et al., 1981).

Assay for Histone Acetylase Activity

In comparisons between macro- and micronuclear extracts, equal volumes (typically 100–150 µl) of extract (normalized to total protein) were reacted in the presence of acid-soluble histone (macro- and micronuclear) or sucrose gradient mononucleosomes and 1 µM [³H]acetyl-coenzyme A. In comparisons between extracts from total nuclei and cytoplasm of detergent-lysed cells, equal volumes (150 µl except in Fig. 6 C) of extract prepared from the same cells were assayed as above, although in this case the concentration of total protein in the cytoplasmic extract is considerably higher than that of corresponding nuclear extracts. In some experiments, aliquots of the "crude" cytoplasmic extract were preincubated at various temperatures (usually 45°C) for 15 min immediately before the enzyme assay. The copious precipitate that forms during this pretreatment at temperatures above 40°C was always removed by centrifugation at 25,000 g for 10 min before assaying the activity that remains soluble. All reactions were carried out at 30°C for 30 min before precipitating the reaction products with 20% TCA. Each washed and dried precipitate was dissolved directly in electrophoresis sample buffer and 10% was removed for liquid scintillation counting. Although we recently published a convenient "dot" enzyme assay to monitor the activity of the macronuclear acetylase after it is immobilized on nitrocellulose (Allis et al., 1986a), we have been unable to apply this assay to the nuclear or cytoplasmic activities generated from detergent-lysed cells. Preliminary experiments suggest that the acetylase activity is not being bound by the nitrocellulose under these conditions. For this technical reason, we have performed liquid enzyme assays exclusively in this report.

Gel Electrophoresis

Protein 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, 1980a,b). Gels were stained, photographed, and processed where appropriate for fluorography.

Protein Elution and Automated Sequencing Procedures

[³H]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. (1986b). 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., 1986b; Chicoine et al., 1986). Material collected at each cycle was dried and processed for scintillation counting (Chicoine et al., 1986).

In Situ Acetylase Enzyme Assays

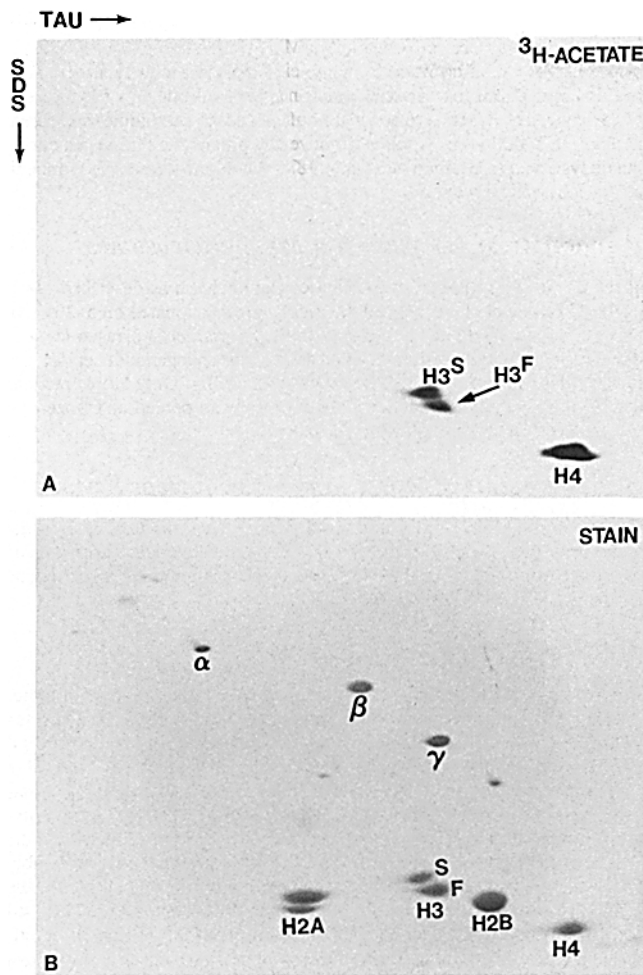Protein synthesis was blocked in growing cells for 5 min with cycloheximide

Figure 1. Gel analysis of micronuclear histones after an in vitro reaction with macronuclear acetylase. After an in vitro reaction with macronuclear acetylase extract, acid-soluble micronuclear histones were recovered and separated on a two-dimensional gel (triton-acid-urea by SDS). Gels were analyzed by staining (*B*) and by fluorography (*A*). The arrow in *A* points to H3^F, a micronuclear-specific core histone, which is derived from H3^S by proteolytic processing (Allis et al., 1980a). Exposure time, 2 d.

(10 µg/ml) before the cells were lightly flattened under siliconized coverslips on polylysine-treated microscope slides and were quickly frozen in liquid nitrogen. In one experiment, cells were killed with Schaudin fixative (Karrer, 1983) before their immobilization on slides. While still frozen, the coverslips were removed and the slides were immediately flooded with acetylation buffer (containing sucrose and spermidine) containing [^{3}H]acetyl-coenzyme A (1 µm) and either no exogenous substrate or 10 µg/ml of H4, H2B, or BSA. H4 and H2B were purified by chromatography on a P60 column. After a 30-min incubation at 30°C (in a moist chamber), the slides were washed twice in acetylation buffer (each for 5 min), washed once in 95% ethanol, dried, and processed for autoradiography. After suitable exposures, slides were developed, stained with diamioinophenylindole DAPI (Schweitzer and Nagl, 1976), and examined under bright field and UV optics.

Results

Micronuclear Histones H3 and H4 Can Be Acetylated In Vitro with Acetylase Activity from Macronuclei

During vegetative growth, micronuclear histones are largely unacetylated as extracted from purified nuclei and fail to incorporate ^{3}H-acetate postsynthetically under a variety of in vivo and in vitro labeling conditions (Vavra et al., 1982). To determine whether micronuclear histones are somehow altered in a way which renders them unable to be acetylated, we asked whether they would become acetylated if incubated in the presence of macronuclear acetylase. It is clear from this analysis (Fig. 1) that acid-extracted micronuclear H3 and H4 are acetylated well under these in vitro conditions. As when "free" macronuclear histones are tested with this activity, only H3 and H4 are acetylated (see Chicoine et al., 1987 for these data). Little, if any, ^{3}H-acetate is incorporated into H2A and H2B or any of the micronuclear-specific linker histones: α, β, and γ. That the acetylated H3 and H4 shown in Fig. 1 *A* do not result from macronuclear contamination within the preparation of micronuclei is clear in the case of H3^F (see *arrow* in Fig. 1 *A*). H3^F is a micronuclear-specific form of H3 which is derived from H3^S by proteolytic processing (Allis et al., 1980a). Furthermore, very little macronuclear-specific histone (H1, hv1, and hv2) is evident in the staining profile of the micronuclear histones used in this experiment (Fig. 1 *B*). We conclude that micronuclear histones (at least H3 and H4) are perfectly good substrates for the macronuclear acetylase during in vitro "deposition-related" acetylation.

Purified Micronuclei Contain an Activity Which Acetylates H4 as a Free Histone, but Fails to Acetylate Any Histone in a Chromatin Form

Having established that micronuclear histones can be acetylated with a macronuclear acetylase (Fig. 1), we were interested to see whether purified micronuclei contain any extractable activity that could acetylate macronuclear histones. Macronuclear histone (both DNA free and chromatin) was chosen as an initial substrate for these experiments (as opposed to micronuclear histone) since it is easier to prepare large quantities of these substrates from macronuclei and since these substrates have been used recently to characterize an acetylase activity salt-extracted from purified macronuclei (Chicoine et al., 1987). As shown in Fig. 2, a salt-extracted activity from macronuclei acetylates primarily H3 and H4 in a DNA-free form (Fig. 2 *A*, lane 2), but acetylates all of the core histones when presented to the enzyme in a chromatin form (Fig. 2 *B*, lane 2). In contrast, highly purified micronuclei (>96% pure by flow microfluorometric measurements; data not shown), contain a salt-extracted activity that acetylates H4 exclusively when reacted with DNA-free histone (Fig. 2 *A*, lane 4). Significantly, the micronuclear activity fails to acetylate any of the core histones when tested in a chromatin form (Fig. 2 *B*, lane 4). It is also noteworthy that the micronuclear extract seems to have abolished the low level of endogenous acetylase present in the preparation of mononucleosomes used in this experiment (Fig. 2 *B*, compare lanes 4 and 6). The unique properties of this micronuclear activity ([*a*] specific acetylation of H4 as free histone and [*b*] a striking inability to acetylate a chromatin substrate) clearly distinguish this activity from that extracted from purified macronuclei (Fig. 2 and Chicoine et al., 1987) and make it extremely unlikely that this activity results from low level macronuclear contamination. These data suggest that micronuclei contain a unique or modified acetylase activity.
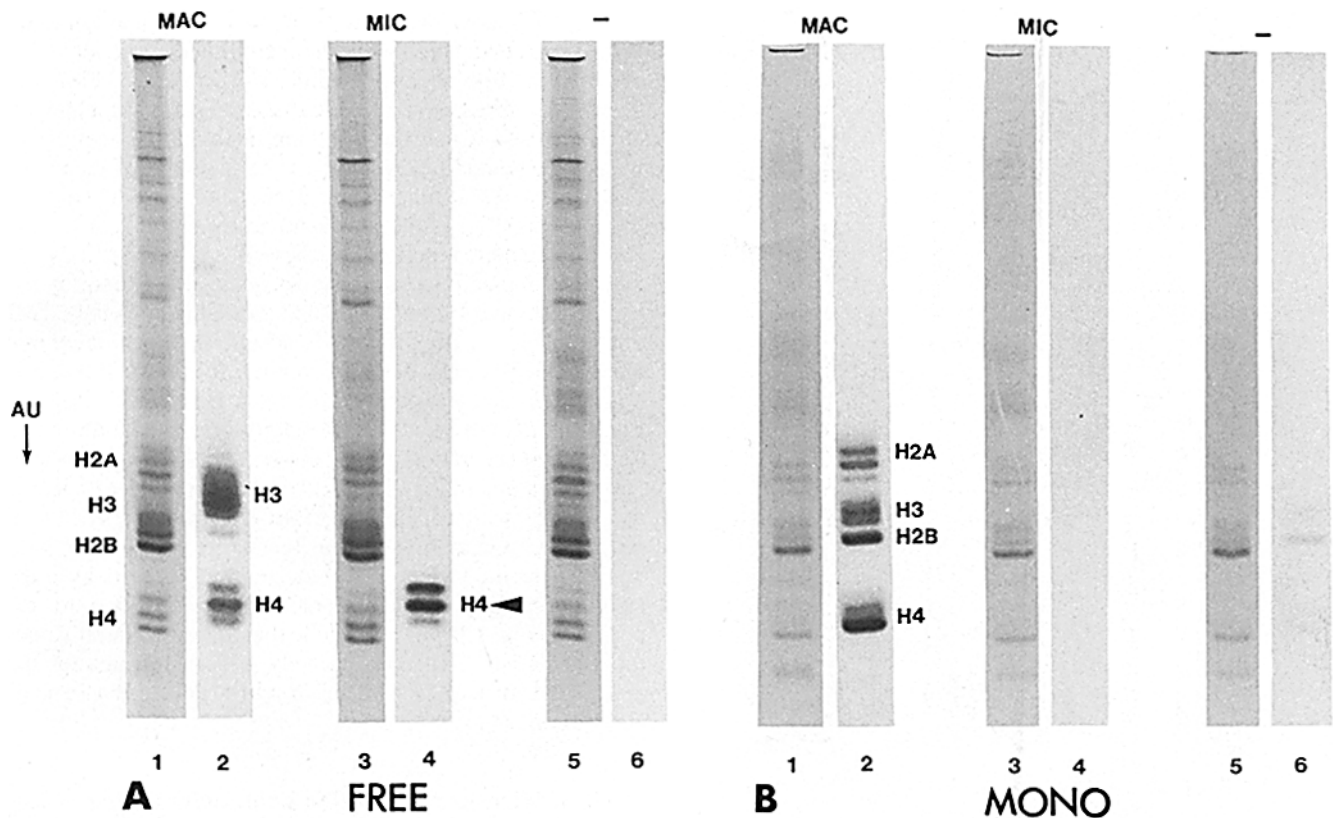
Figure 2. Micronuclei contain an acetylase which is specific for H4 as a free histone and which fails to acetylate histone in chromatin particles. Acid-soluble macronuclear histone (the substrate in *A*) or sucrose gradient–purified mononucleosomes from macronuclei (the substrate in *B*) were reacted with equal volumes of macronuclear acetylase extract (*MAC*, lanes *1* and *2*), micronuclear acetylase extract (*MIC*, lanes *3* and *4*), or a buffer alone control (–, lanes *5* and *6*) in standard liquid acetylase reactions. Reaction products were collected, separated on a one-dimensional acid-urea gel, and analyzed by staining (*odd* lanes) and fluorography (*even* lanes). Micro- and macronuclei used in this experiment were isolated from the same cells by the method of Gorovsky et al. (1975). Micronuclei were found to be >96% pure by flow microfluorometry (Allis and Dennison, 1982). All exposures, 2 d.

High Speed Supernatants from Detergent-lysed Cells Contain an Acetylase Activity Indistinguishable from that Extracted from Purified Micronuclei

A final cellular compartment that we have checked for acetylase activity is a high speed supernatant prepared from detergent-lysed cells. In this fractionation procedure (see Materials and Methods for details), an initial "nuclear" pellet is obtained that contains essentially all of the expected macro- and micronuclei as well as some contaminating fibrous debris. When salt extracts from both the nuclear pellet and cytoplasmic supernatant are reacted with both free histone and chromatin substrates (Fig. 3), we again observe that the nuclear extract (which is mostly macronuclei by mass) acetylates H3 and H4 well as free histone (Fig. 3 *A*, lane 2) and all of the core histones (except H2B) as chromatin (Fig. 3 *B*, lane 2). The inability of this nuclear extract to acetylate H2B in a chromatin form is not understood, but occurs reproducibly when nuclei are prepared by the detergent lysis method. However, like the extract prepared from purified micronuclei, the crude high speed supernatant acetylates only H4 as free histone (Fig. 3 *A*, lane 4) and fails to acetylate any of the core histones when tested in a chromatin form (Fig. 3 *B*, lane 4). Identical results (i.e., H4 acetylation) are obtained when micronuclear histone is substituted for macronuclear histone (as free histone substrate) in reactions with the cytosolic extract (data not shown). Thus, in agreement with data presented in Fig. 1, micronuclear histones are not refractory to in vitro acetylation.

Micronuclear and Cytoplasmic Acetylase Activities Use the Same "Deposition-related" Acetylation Sites in Free H4

The high specificity of the micronuclear and cytoplasmic acetylase activities for free H4 combined with their inability to acetylate a chromatin substrate is reminiscent of deposition-related acetylation in *Tetrahymena* (Allis et al., 1985). Here, newly synthesized H4 enters micro- and macronuclei in a diacetylated form using a unique pair of acetylation sites (lysines at positions 4 and 11; see Chicoine et al., 1986). To determine which lysines in H4 were being modified by the micronuclear and cytoplasmic activities, each activity was reacted with acid-extracted histone as described in Figs. 2 *A* and 3 *A*. Reaction products were subjected to electrophoresis in a long acid-urea gel, after which mono- and diacetylated H4 was excised, electroeluted, and sequenced as described previously (*Tetrahymena* H4 is not blocked at its amino terminus as it is in most other organisms). The release of ³H-
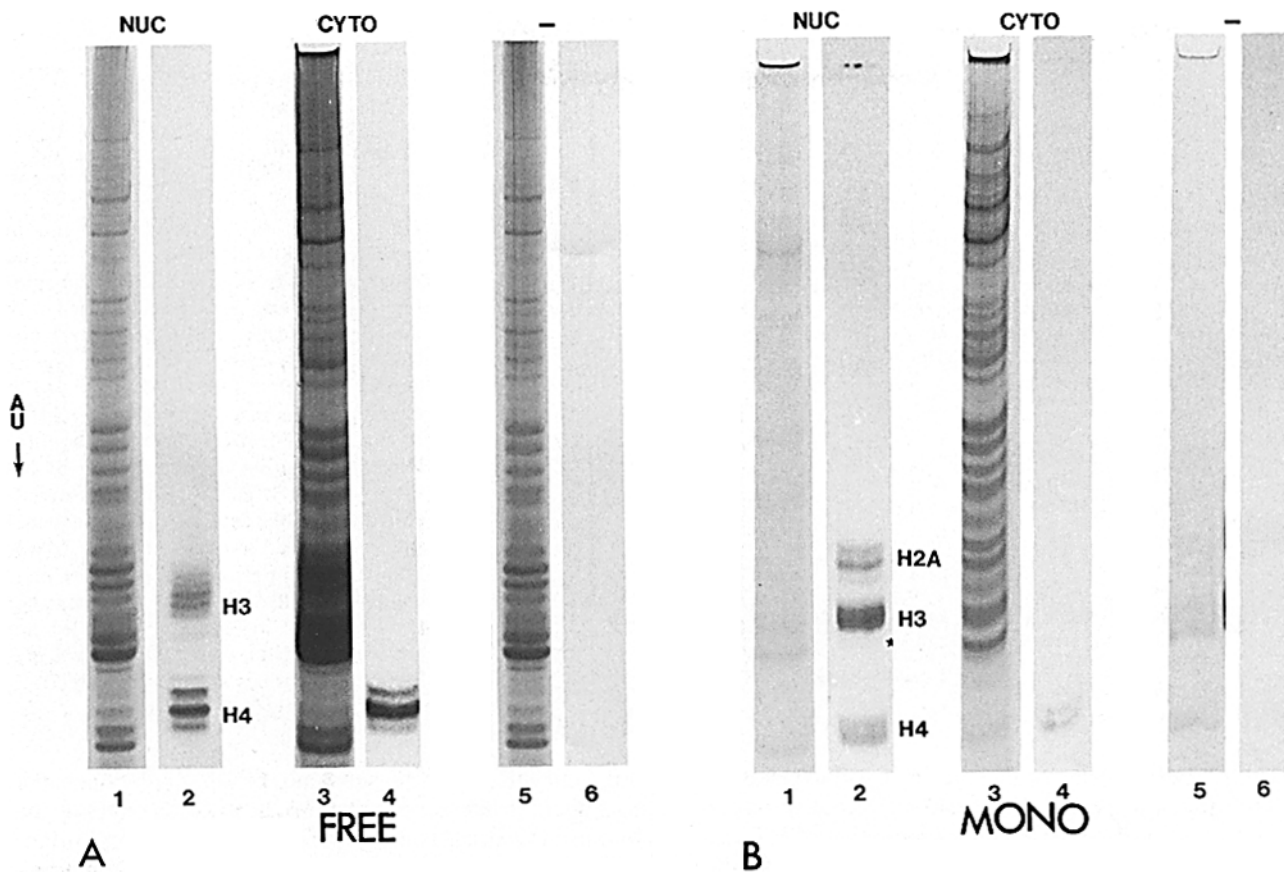
Figure 3. Crude cytoplasmic extracts also contain a free H4-specific acetylase activity which fails to acetylate histone in chromatin. Equal volumes of extract from either crude nuclei (macro- and micronuclei, *NUC*, lanes *1* and *2*), crude cytoplasm (*CYTO*, lanes *3* and *4*) or buffer alone (−, lanes *5* and *6*) were reacted with free histone (*A*) or mononucleosomes (*B*) as in Fig. 2. Reaction products were subjected to electrophoresis in a one-dimensional acid-urea gel and analyzed by staining (*odd* lanes) and fluorography (*even* lanes). In this experiment cells were lysed by the NP-40 detergent method which typically gives a macronuclear extract with very little activity toward H2B (see * next to *B*, lane 2). All exposures, 2 d.

acetate counts per minute at each successive cycle of automated sequencing is plotted in Fig. 4. It is immediately apparent that both the micronuclear (Fig. 4, *A* and *B*) and the cytoplasmic (*C* and *D*) acetylase activities preferentially use the lysine at position 4 in the population of monoacetylated H4 and lysines 4 and 11 in the population of diacetylated H4. Thus, both of these activities are indistinguishable in their use and order of H4 acetylation sites and mimic precisely the sites recently defined as being diagnostic of in vivo deposition-related acetylation. It is noteworthy that lysines 4 and 11 are also used exclusively in diacetylated H4 when it is reacted with the macronuclear acetylase as free histone (Chicoine et al., 1987). However, unlike both the micronuclear and cytoplasmic activities, the macronuclear activity uses lysine 11 (and not 4) as its preferred site in monoacetylated products.

Thermal Inactivation of Micronuclear and Cytoplasmic Acetylase Activities

In a previous study, the acetylase activity from macronuclei was found to be unstable at temperatures >35°C with 50% of the starting activity being lost after a 30-min preincubation at 45°C (Chicoine et al., 1987). To investigate whether

the micronuclear and cytoplasmic activities would produce temperature-inactivation profiles similar to the macronuclear activity, a similar experiment was performed with all three extracts (Fig. 5). To our surprise, both the micronuclear (Fig. 5, *open diamonds*) and the cytoplasmic (*solid squares*) acetylase activities are "activated" by a 15-min pretreatment of the extract at 45°C. In the case of the cytoplasmic extract, the 45°C treatment causes a copious precipitate (probably representing heat-denatured protein) which contains roughly 75% of the initial protein. The acetylase activity which remains soluble after the 45°C treatment is typically "increased" 2–10-fold as compared with equal amounts of untreated extract (also see Fig. 6) and thus, a substantial increase in specific activity is obtained by this simple procedure. To investigate whether the specificity of the cytoplasmic acetylase changes after the 45°C activation, free histone was reacted with crude extract as well as one-time and two-time 45°C–treated extracts. Fig. 6, *A* and *B* shows that in all cases the high specificity for histone H4 is maintained. Furthermore, 45°C activation of the cytoplasmic extract does not change its inability to acetylate mononucleosome substrates (data not shown). If progressively less 45°C–treated extract is used in a standard in vitro reaction with free histone
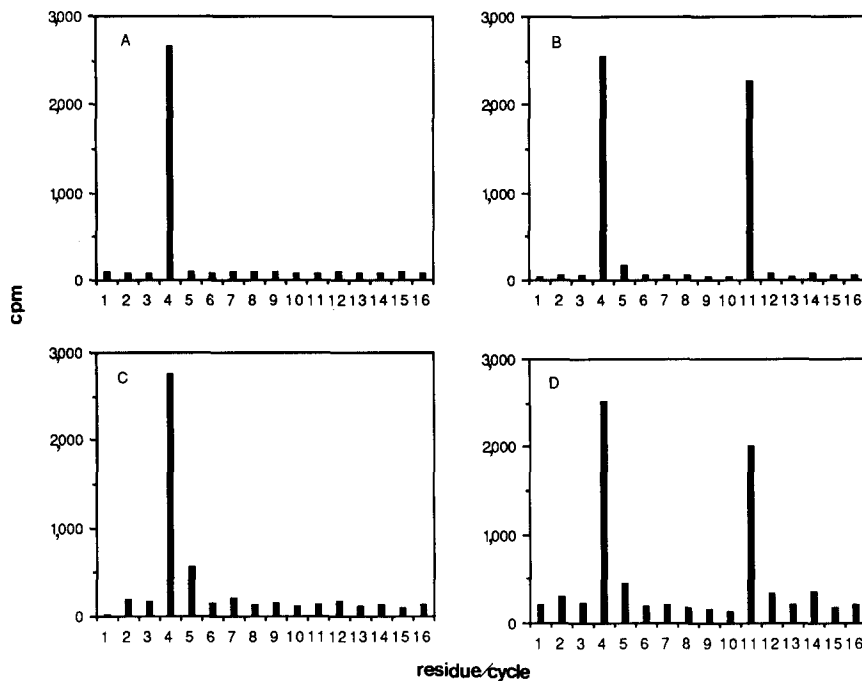
Figure 4. Sites of acetylation in H4 after in vitro reactions with the micronuclear and cytoplasmic acetylase extracts. Acid-soluble macronuclear histone was reacted with the micronuclear (*A* and *B*) or cytoplasmic (*C* and *D*) extracts as described in Figs. 2 and 3. Recovered histone was subjected to electrophoresis in a long acid-urea gel (Allis et al., 1986*b*). After a brief staining, protein corresponding to mono- (*A* and *C*) and diacetylated (*B* and *D*) H4 was excised, eluted, and subjected to automated sequencing as described in Chicoine et al. (1986). Shown are the [³H]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 known acetylation sites (lysines at 4, 7, 11, and 15).

(Fig. 6 *C*), fewer acetate counts are incorporated into the overall reaction as expected. However, we were surprised to detect a proportional increase in the acetylation of H3 (and other nonhistone chromosomal proteins) as the final concentration of extract is decreased in the reaction. Thus, while the cytosolic extract typically displays a marked preference for free H4, H3 is also used (more or less as well as H4) under dilute extract conditions. While it is not clear whether this is an effect of the 45°C treatment or is instead an effect on the enzyme itself, the substrate, or other components of the extract (or interactions therein), acetylation of H3 by the cytoplasmic extract is a significant result since deposition-related acetylation of H3 has been observed in vivo in *Tetrahymena* (Allis et al., 1985). Whether or not the micronuclear activity behaves similarly under dilute conditions has not been tested.

New In Situ Enzyme Assay Documents the Existence of the Micronuclear and Cytoplasmic H4 Acetylase

Several lines of evidence suggest that the acetylase activity extracted from purified micronuclei is the same as that detected in high speed supernatants of detergent-lysed cells. Both activities display high specificity for H4 as free histone, use the same acetylation sites in H4, fail to acetylate a mononucleosome substrate, and are activated by a 15-min pretreatment at 45°C. While unlikely (see the Discussion), it is possible that the preparation of micronuclei is sufficiently contaminated by cytoplasmic debris (or vice versa) that the "micronuclear-cytoplasmic" activity does not actually reside in both compartments, but is instead a preparative artifact from cross-contamination. To address this difficult problem, we sought to obtain autoradiographic evidence to substantiate both a micronuclear and a cytoplasmic acetylase activity.

Recently, a novel dot enzyme assay has been developed to assay the macronuclear-specific, transcription-related acetylase while it is immobilized on nitrocellulose (Allis et al., 1986). Along the same lines, we reasoned that it may be possible to carry out in situ enzyme reactions using unfixed whole cells immobilized on microscope slides followed by conventional autoradiography. To test this possibility, we first asked whether we could detect the macronuclear-specific transcription-related acetylase. Growing cells, lightly flattened under a coverslip on a polylysine-treated slide, were quickly frozen in liquid nitrogen. While still frozen, the coverslip was removed and the slides were immediately in-
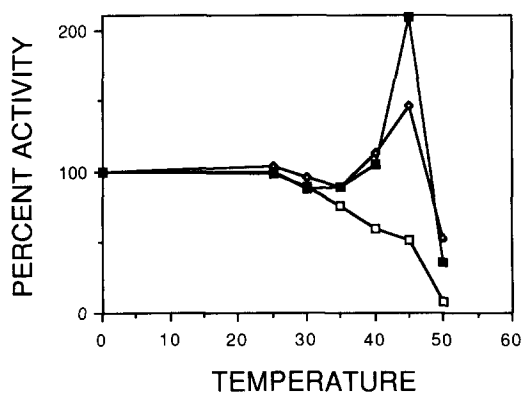

Figure 5. Heat inactivation of macronuclear, micronuclear, and cytoplasmic acetylase activities when tested with free histone. Equal aliquots of macronuclear (□), micronuclear (◇), and cytoplasmic (■) extracts were preincubated at 0, 25, 30, 35, 40, 45, and 50°C for 15 min before being reequilibrated to 30°C for 15 min and assayed with free macronuclear histone. At temperatures above 40°C, a copious precipitate forms in the cytoplasmic extract; this was not noticeable with either the macronuclear or the micronuclear extracts. All samples were spun at 25,000 *g* for 10 min to remove any precipitate that did form. For preincubation at 0°C, counts per minute of each extract was ∿75,000 cpm. These values were set equal to 100% activity.
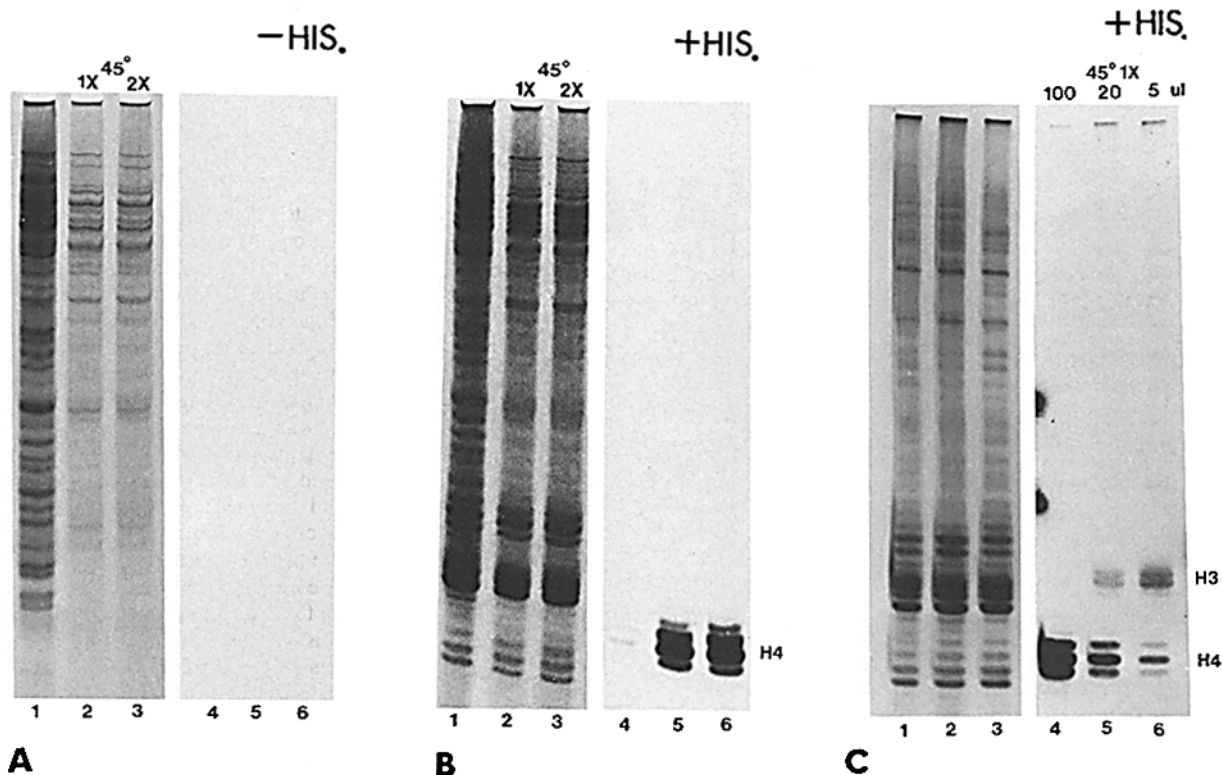
Figure 6. Specificity of the 45°C-treated cytoplasmic extract. Crude cytoplasmic acetylase extract from detergent-lysed cells (*A* and *B*, lane *1*) was preincubated at 45°C for 15 min for one (*1X*; *A* and *B*, lane *2*) or two (*2X*; *A* and *B*, lane *3*) times. After each incubation, any precipitate that formed was removed by centrifugation at 25,000 *g* before supernatants were assayed with free macronuclear histone (+ *HIS.*, *B*). All products were subjected to electrophoresis in a one-dimensional acid-urea gel and were analyzed by staining (lanes *1–3*) and fluorography (lanes *4–6*). The complexity of cytoplasmic extract before and after 45°C treatments is shown in *A* (− *HIS.*). In most reactions, 100–150 µl of cytoplasmic extract (crude or heat treated) is used which gives highly specific acetylation of free H4 (see *C*, lane *4*). However, when 100, 20, or 5 µl of one time 45°C-treated cytoplasmic extract (all adjusted to 100 µl final volume with buffer) is reacted with free histone (in *C* lanes *4*, *5*, and *6*, respectively), one observes a proportional increase in the acetylation of histone H3 and numerous other nonhistone proteins. When only 5 µl of extract is used (*C*, lane *6*), more or less even acetylation of H3 and H4 is observed. Exposure time in *A* and *B*, 1 d; exposure time in *C*, 10 d.

cubated in the presence of acetylase buffer containing only [³H]acetyl-CoA. Autoradiographic analyses of these cells show silver grains localized exclusively over macronuclei; few, if any, silver grains are evident over micronuclei (see *arrows*) or the cytoplasm of these cells (Fig. 7, *A* and *B*). Since isolated macronuclei (but not micronuclei) have been shown to carry out transcription-related acetylation in vitro in the presence of [³H]acetyl-CoA, and since this reaction is highly specific for core histones (Vavra et al., 1982), the silver grains detected over macronuclei in Fig. 7 (*A* and *B*) most likely reveal the in situ localization of the macronuclear-specific acetylase responsible for this acetylation reaction.

Next, we reasoned that the micronuclear-cytoplasmic acetylase is likely responsible for deposition-related acetylation of newly synthesized H4 and therefore would require free H4 as its substrate. To ensure that the reaction would be dependent on exogenously added substrate, protein synthesis was blocked in these cells with cycloheximide before their freezing and immobilization (cells were also treated with cycloheximide in Fig. 7, *A* and *B*). When cells such as these were incubated in the presence of [³H]acetyl-CoA and column-purified H4, autoradiographic analyses clearly show

silver grains over macronuclei, micronuclei, and the cytoplasm (especially see *inset*, Fig. 7 *C*). If fixed cells are used for these analyses (Fig. 7 *D*) or if nonphysiological protein substrates are added to the reaction buffer in place of H4 (we have tried bovine serum albumin and *Tetrahymena* histone H2B, see Fig. 7 *E*), silver grains are either not detected at all (Fig. 7 *D*) or are detected only over macronuclei (Fig. 7 *E*). These results are consistent with the biochemical data suggesting that a similar if not identical acetylase activity exists in micronuclei and the cytoplasm of growing *Tetrahymena* that prefers free H4 as its substrate. This activity is distinct from a macronuclear-specific activity (Fig. 7, *A* and *B*) which acetylates endogenous chromatin as its primary substrate in these in situ analyses.

Discussion

In this report we have presented biochemical and autoradiographic data documenting the existence of both micronuclear and cytoplasmic acetylase highly specific for free histone H4. At this point, we have not found any property that distinguishes these two activities from one another and, therefore, we will refer to it as a micronuclear-cytoplasmic activity.
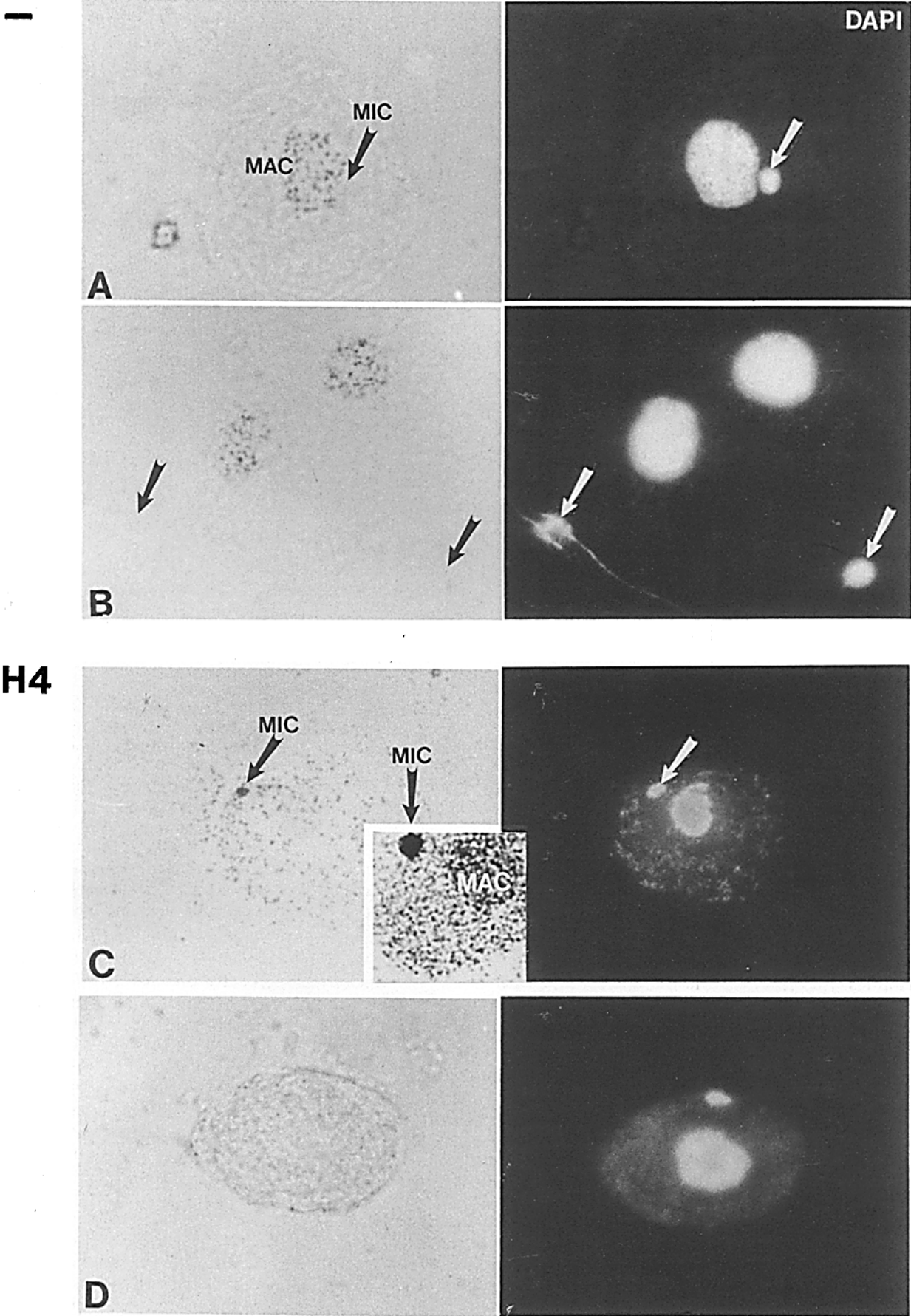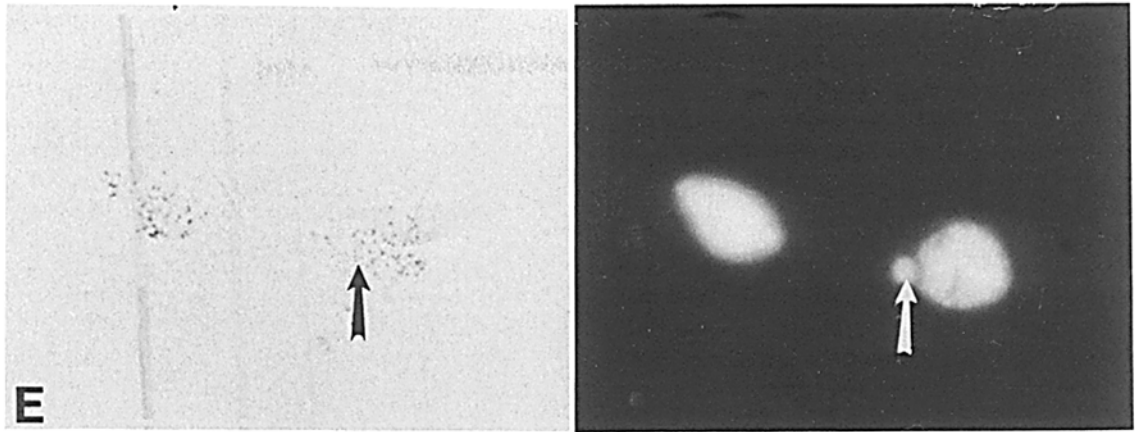
Figure 7. In situ acetylase enzyme assay in growing *Tetrahymena*. Protein synthesis was blocked in growing cells before the cells were flattened, frozen, and incubated immediately in acetylase buffer containing [³H]acetyl-CoA and either no exogenous substrate (*A* and *B*) or column-purified H4 (*C*) or H2B (*E*). In *D* the cell was first killed with fixative before immobilization and incubation with [³H]CoA and free H4. After the reaction, slides were washed and processed for autoradiography (see Materials and Methods for details). All slides were examined by bright-field (*left* panel of each pair) and DAPI-UV (*right* panel) optics. In some panels the positions of micronuclei are indicated by arrows. It is not unusual to find numerous lysed nuclei on each slide in unfixed samples; for example, see the left-hand micronucleus in *B*. Arrows point to some micronuclei. Most exposures, 8 d; the inset shown in *C* was from a 1-mo exposure.

While it is possible that one activity is artifactually created by cross-contamination of one compartment with the other, we feel that this is unlikely for several reasons. First, the cytosolic activity released after detergent lysis remains soluble after high speed centrifugation, a step that should easily remove all intact micronuclei. Furthermore, we can account for essentially all of the expected micronuclei (by counting in the light microscope) in the initial nuclear pellet produced from the detergent lysate. As well, we have tested whether resuspending purified micronuclei in the detergent-lysis buffer causes significant release of acetylase activity from micronuclei. No activity was detected after this treatment. Finally we have presented in situ autoradiographic data which provide direct visual evidence for both micronuclear and cytoplasmic H4–dependent acetylase activities.

Significant amounts of triacetylated H4 are observed in in vitro reactions with both micronuclear and cytosolic extracts, whereas, in vivo, essentially all of the newly synthesized H4 is deposited into micro- and macronuclei in a diacetylated form (Allis et al., 1985; also see Chambers and Shaw, 1984; and Giancotti et al., 1984). While the exact reason for this discrepancy is unclear, it is worth noting that the macronuclear H4 substrate used for these reactions is already modified by acetylation. This is particularly evident in free histone reactions (for example, see Fig. 2 *A*, lane 5). Protein sequencing experiments have shown that lysines 4 and 7 are used exclusively as sites of postsynthetic acetylation in populations of diacetylated H4 extracted from macronuclei (Chicoine et al., 1986). In contrast, lysines 4 and 11 are used exclusively in newly synthesized diacetylated H4. It may be, then, that the micronuclear-cytosolic activity in our in vitro reactions acetylates lysine 11 which is available on preexisting diacetylated H4 molecules, giving rise to triacetylated H4 products. In support of this argument, H4 exists in mostly an unacetylated form in our chromatin samples (see Fig. 2 *B*, lane 5; this is probably due to an endogenous deacetylase activity in our chromatin preparations; Vavra et

al., 1982); here, the in vitro products are mostly mono- and diacetylated H4.

The thermal-inactivation profile displayed by the micronuclear-cytoplasmic activity (Fig. 5) is unusual and is not completely understood. It is curious that the activity is activated so well by a 45°C pretreatment and yet is nearly totally inactivated by pretreatment at 50°C. Furthermore, the activity withstands a second 45°C treatment (Fig. 6). It is conceivable that cytoplasmic activity undergoes a conformational change at 45°C which stably activates the enzyme. Inasmuch as numerous proteins are denatured and precipitated by the 45°C treatment, it seems more likely that an inhibitor (which may or may not be physiologically relevant) is removed, thereby causing the activation. Consistent with this possibility is the fact that the "crude" cytoplasmic extract can also be activated by diluting the extract with acetylase reaction buffer prior to performing the enzyme assay (Allis, C. D., unpublished observation). From a practical standpoint, the 45°C pretreatment is useful since it results in a fairly dramatic increase in the specific activity of the cytoplasmic acetylase (compare lanes *1-6*, Fig. 6, *A* and *B*).

In almost every property examined, the micronuclear-cytoplasmic activity differs from that extracted from macronuclei. Of these differences, the most striking and perhaps the most biologically significant is the total inability of the former activity to acetylate histone in a chromatin form, while the latter gives more or less uniform acetate incorporation into all core histones when reacted with the same chromatin substrate. This is not surprising if one assumes that one function of the macronuclear activity (and not the micronuclear-cytoplasmic activity) is to carry out transcription-related acetylation on macronuclear chromatin. However, recent evidence has suggested that the same macronuclear activity can also catalyze deposition-related acetylation when it is presented with DNA-free histone as substrate (Chicoine et al., 1987). If the macronuclear enzyme can, under appropriate conditions, carry out deposition-related

acetylation of its own histone, what is the function of the micronuclear-cytoplasmic activity?

Recent studies have shown that newly synthesized H3 and H4 entering micronuclei are acetylated in vivo in early (meiotic) stages of conjugation and that these acetyl groups are quickly removed after deposition (Allis et al., 1985). It is, of course, possible that the micronuclear-cytoplasmic activity described here (from vegetative cells) functions to carry out transient deposition-related acetylation of only micronuclear H4 (and H3). Alternatively, it is possible that this activity catalyzes deposition-related H4-H3 acetylation for both macro- and micronuclei in vivo and that the deposition-related activity displayed by the macronuclear acetylase is an artifact produced in vitro by presenting it with a substrate that it does not normally encounter in vivo, namely DNA-free histone. Ironically, the macronuclear activity acetylates H3 and H4 equally well as free histone under a variety of in vitro conditions (see Fig. 1 and Chicoine et al., 1987), while the micronuclear-cytoplasmic activity tends to be more specific for H4 alone. Finally, we point out that the micronuclear-cytoplasmic H4 acetylase may carry out a yet unknown *in vivo* function which is not related to "deposition-related" acetylation at all. Regardless of its exact in vivo function(s), the H4-specific acetylase from *Tetrahymena* strongly resembles an acetylase-type B activity which has been extracted from the cytoplasm of higher eukaryotes (Sures and Gallwitz, 1980; Belikoff et al., 1980; Garcea and Alberts, 1980; Weigand and Brutlag, 1981). In these studies it has been generally assumed that the activity is responsible for "deposition-related" acetylation.

In conclusion, it is clear that in *Tetrahymena* as in other eukaryotes, two distinct systems of histone acetylation exist. One affects newly synthesized histones H3 and H4 and the other affects all core histones after they have assembled into mature nucleosomes. However, it is not yet clear how many different enzymes are involved in carrying out these reactions. At the very least, a seemingly distinct macronuclear and a micronuclear-cytoplasmic activity have now been identified in *Tetrahymena*. Efforts are under way to purify and compare the enzymes responsible for carrying out these biologically important acetylation reactions.

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