

Phosphorylation of Linker Histone Is Associated with Transcriptional Activation in a Normally Silent Nucleus

Melody T. Sweet,* Karrie Jones,* and C. David Allis[‡]

*Department of Biology, Syracuse University, Syracuse, New York 13244; and[‡]Department of Biology, University of Rochester, Rochester, New York 14627

Abstract. Previous studies have suggested that micronuclear linker histones are phosphorylated by cAMP-dependent protein kinase (PKA) in *Tetrahymena* (Sweet, M.T., and C.D. Allis. 1993. *Chromosoma*. 102: 637–647). In this study, we report that a rapid and dramatic phosphorylation of the micronuclear linker histone, δ , occurs early in the sexual pathway, conjugation. Phosphorylated isoforms of δ are detected as early as 30 min after mixing cells of different mating types; blocking pair formation abolishes this induction completely. Phosphorylation of δ is stimulated by the addition of N6-benzoyladenine 3':5' cyclic monophosphate to starved (nonmating) cells, suggesting that a

PKA/cAMP signal transduction pathway is involved. Maximal phosphorylation of δ is observed during meiotic prophase, a period when micronuclei become transcriptionally active. In situ staining, using phospho-delta-specific antibodies combined with [³H]uridine autoradiography, shows that decondensed micronuclear chromatin undergoing active transcription is enriched in phosphorylated δ isoforms. In contrast, condensed inactive micronuclear chromatin is enriched in dephosphorylated δ . These results strongly suggest that phosphorylation of linker histone plays an important and previously unsuspected role in establishing transcriptional competence in micronuclei.

An intimate relationship exists between the transcriptional apparatus and the chromosomal environment in which that machinery must function. Core histones, for example, serve to package eukaryotic DNA into nucleosomal arrays, and mutations in specific core histones have been shown to modulate gene expression directly (for reviews see Workman and Buchman, 1993; Wolffe, 1994). Several levels of organization are required, however, to permit the packaging of eukaryotic genes within nuclei. Beyond the nucleosomal thin filament, a second level of folding occurs that gives rise to higher order chromatin structures, such as the 30-nm chromatin fiber (reviewed by van Holde, 1989; Wolffe, 1995). Proteins of the H1/H5 family (Zlatanova and van Holde, 1996) facilitate this folding and thus appear to play a fundamental role in determining this structure and in mediating chromatin condensation in vitro (Thoma et al., 1979) and in vivo (Shen et al., 1995), although the exact details of this involvement remain unclear (Clark and Kimura, 1990).

Given its fundamental role in higher order chromatin structure, the presence or absence of H1 or postsynthetic modifications affecting H1 and its interaction with DNA are likely to play a pivotal role in regulating gene expression. Indeed, numerous studies have suggested that H1 is

missing, reduced, or modified in genes undergoing active transcription (reviewed by Garrard, 1991; Zlatanova and van Holde, 1992; Wolffe, 1994). Moreover, specialized forms of linker histones appear to keep entire genomes transcriptionally inactive, such as in fowl erythrocyte nuclei, sperm, and *Tetrahymena* micronuclei (for review see Roth and Allis, 1992). From these observations, H1 is generally thought to exert a negative influence on gene expression. Other data, however, have demonstrated that this role of H1 in transcription cannot be generalized (Zlatanova and van Holde, 1992; Sandaltzopoulos et al., 1994), and in some cases, a differential and highly selective role in transcriptional regulation has been observed (Bouvet et al., 1994; Shen and Gorovsky, 1996).

The functional role of linker histone phosphorylation is poorly defined, even though it is well established that fluctuations in the level and sites of phosphorylation change dramatically during the cell cycle (for review see Bradbury, 1992). Hyperphosphorylation of H1 during mitosis, for example, has led to the long-standing hypothesis that H1 phosphorylation is required for condensation of mitotic chromosomes. However, the functional relationship, if any, of H1 hyperphosphorylation to chromosome condensation remains unresolved (for references see Guo et al., 1995). H1 is phosphorylated at other times during the cell cycle and in nuclei that do not divide mitotically (Roth and Allis, 1992). Mitotic chromosome condensation can occur in vivo without linker histone (Shen et al., 1995) and in extracts lacking H1 (Ohsumi et al., 1993; Dasso et al., 1994),

Address all correspondence to C. David Allis, Department of Biology, University of Rochester, 402 Hutchison Hall, Rochester, NY 14627-0211. Tel.: (716) 275-7586; Fax: (716) 275-2070. e-mail: cda@allis.biology.rochester.edu

and it appears that other nonhistone proteins play an important, previously unrecognized role in this process (reviewed by Peterson, 1994; Hirano, 1995; Koshland, 1996). Using antibodies highly selective for phosphorylated isoforms of H1 (Lu et al., 1994), enrichment of phosphorylated H1 in transcriptionally active chromatin has been observed (Lu et al., 1995; Chadee et al., 1995), lending support to the hypothesis that H1 phosphorylation actually causes chromatin decondensation.

The ciliated protozoan, *Tetrahymena thermophila*, provides an ideal model for unraveling relationships between linker histone phosphorylation and gene activation. Each vegetative cell contains two functionally distinct types of nuclei, a germline micronucleus that is most often transcriptionally silent (see below) and a somatic macronucleus that is actively transcribed (for review see Gorovsky, 1980). The diploid micronucleus divides by a typical mitosis during vegetative growth, while the polyploid macronucleus divides by an amitotic process that does not involve spindle formation or chromosome condensation. While the micronucleus is highly condensed and transcriptionally inactive during most of the life cycle, a brief period of transcriptional activity has been reported during the early stages of conjugation corresponding to meiotic prophase (Sugai and Hiwatashi, 1974; Martindale et al., 1985).

Both micro- and macronuclei contain a similar complement of core histones; however, the polypeptides associated with the linker region of these nuclei differ dramatically (Gorovsky, 1986; van Holde, 1988). Although macronuclei divide amitotically, they contain an H1 that resembles vertebrate H1s in several properties, including phosphorylation by a Cdc2-like protein kinase (Roth et al., 1991). In contrast, micronuclei do not contain a linker histone of the type found in macronuclei, but instead, contain four distinct polypeptides, α , β , γ , and δ (Allis et al., 1979, 1984) that, like macronuclear H1, are extensively phosphorylated during vegetative growth (Allis and Gorovsky, 1981). Because micronuclei divide mitotically, it is surprising that none of these micronuclear linker polypeptides contain a recognition sequence for Cdc2 kinase (Wu et al., 1994), and that none are phosphorylated by p34^{Cdc2} in vitro under conditions where macronuclear H1 is phosphorylated (Sweet and Allis, 1993). However, all four of these polypeptides contain at least one canonical phosphorylation site for cAMP-dependent protein kinase (PKA)¹ (Wu et al., 1994), and all are phosphorylated well by PKA in vitro (Sweet and Allis, 1993).

In this report, we present data that cell-cell interactions that are required for entry into the sexual pathway in *Tetrahymena* stimulate a cAMP/PKA signal transduction pathway that leads to the hyperphosphorylation of the linker histone, δ . The induction of δ phosphorylation is rapid and remains high throughout meiotic prophase. In situ immunofluorescent analyses, using antibodies highly selective for phosphorylated isoforms of δ , suggest that δ phosphorylation colocalizes closely with decondensed regions of micronuclear chromatin that are active in transcription. These

data lend strong support to the hypothesis that phosphorylation of linker histones acts as a first-step mechanism that promotes chromatin decondensation and the establishment of transcriptional competency.

Materials and Methods

Cell Culture

Tetrahymena thermophila strain CU428 (Chx/Chx-[cy-S]VII) was grown in 1% enriched protease peptone under standard conditions as described previously (Gorovsky et al., 1975). Cells were starved by harvesting log-phase vegetatively growing cells at ~500 g, washing with 10 mM Tris, pH 7.4, and incubating overnight in 10 mM Tris, pH 7.4, at a concentration of 250–500,000 cells/ml, unless otherwise indicated. Mating cells were produced by crossing *T. thermophila* strains CU428 and CU427 (Mpr/Mpr [6 mp-s] VI) according to the method of Bruns and Brussard (1974) with modifications described by Allis and Dennison (1982). In some cases, mating cells were treated with cycloheximide (10 μ g/ml) immediately after mixing cells of the opposite mating type.

Isotopic Labeling and Autoradiography

Mating cells were labeled for 5 min with 10 μ Ci/ml (50 Ci/mmol) [³H]uridine (New England Nuclear, Boston, MA), fixed in 50% methanol, and spread onto slides. The slides were then processed for autoradiography as described previously (Vavra et al., 1982), except that the slides were incubated twice in 0.02% iodine in 70% ethanol before being dipped into Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, NY). Cells were finally stained with 1 mg 4',6-diamidino-2-phenylindole (DAPI)/ml in 10 mM Tris, pH 7.5, and 150 mM NaCl (TBS).

Measurements of Total cAMP Levels in Starved and Mating *Tetrahymena*

Total cAMP was analyzed by radioimmunoassay, as described by Pasquale and Goodenough (1987). Briefly, 2–5 \times 10⁶ cells were pelleted in a clinical centrifuge, and all but 1 ml of Tris media was removed by aspiration. The cells were frozen in a dry ice/ethanol bath for 10 min, and then thawed by adding 60 μ l of 100% TCA and vortexing. Insoluble debris was pelleted, and the supernatant was removed and extracted five times with 1 ml water-saturated ether. For the cAMP assay, extract from 6 \times 10⁵ cells, ¹²⁵I-labeled succinylated cAMP tyrosine methyl ester, and highly specific antiserum were used (New England Nuclear).

Preparation of Nuclei

Macronuclei and micronuclei were isolated from cells as described by Gorovsky et al. (1975), except that the nucleus isolation buffer contained 10 mM Tris-HCl, pH 7.4, 1 mM iodoacetamide, 1 mM PMSF, and 10 mM sodium butyrate, but not spermidine. To better retain proteins in their most phosphorylated in vivo form, formaldehyde (final concentration = 1%) was added to the cells during homogenization. Alternatively, 0.1 mM rho-chloromercuriphenylsulfonic acid (Sigma Chemical Co., St. Louis, MO) was added to the nuclear isolation buffer; the same results were essentially obtained with both treatments. Micronuclei were further purified from contaminating macronuclei and cellular debris by sedimentation at unit gravity (Allis and Dennison, 1982).

Isolation and Purification of δ

Formaldehyde-fixed nuclei were boiled 30 min in 4 M guanidine HCl, 10 mM EDTA, and 0.5 M β -mercaptoethanol at a concentration of 1–6 \times 10⁸ nuclei/ml to reverse formaldehyde cross-links. After cooling to 4°C, sulfuric acid was added to a final concentration of 0.4 N, and this mixture was dialyzed overnight at 4°C against 0.4 N sulfuric acid with 1 mM PMSF. Acid-soluble micronuclear proteins were then precipitated in 20% TCA and washed sequentially with acidified acetone and acetone (Schulman et al., 1987). Micronuclear linker histones (solubilized in 4 M urea and 5% β -mercaptoethanol) were purified by HPLC using a reverse-phase C8 column with a linear gradient of 5–90% acetonitrile containing 0.1% trifluoroacetic acid (0.425% acetonitrile/min) with a flow rate of 1 ml/min (Sweet and Allis, 1993). Fractions were lyophilized, solubilized in water, and identified by SDS-PAGE.

1. Abbreviations used in this paper: AP, alkaline phosphatase; benzoyl-cAMP, N⁶-benzoyladenine 3':5' cyclic monophosphate; DAPI, 4',6-diamidino-2-phenylindole; IBMX, isobutylmethylxanthine; PKA, cAMP-dependent protein kinase.

Preparation of Polyclonal Antibodies Against δ and Phosphorylated δ Peptide

Rabbits (New Zealand white) were inoculated with two separate injections of HPLC-purified δ (80–100 μ g) or δ peptide (0.5–1.0 mg) synthesized with a phosphorylated serine at one of the two known COOH-terminal sites of in vivo phosphorylation [Thr-Lys-Arg-Lys-Asn-Ser(P)-Lys-Ser]; mapping of in vivo phosphorylation sites in δ will be presented elsewhere (Sweet et al., 1996). Primary injection of protein/peptide in Freund's complete adjuvant (Calbiochem/Novabiochem Corp., La Jolla, CA) was made as an emulsion generated by sonication; 2 wk later, a second injection of emulsified protein in Freund's incomplete adjuvant was injected. Sera were collected at weekly intervals.

General δ antibodies were used without further purification. Antibodies against the phosphorylated δ peptide were affinity purified on a column containing peptide coupled with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) to diaminopropylamine (DAPA) beads. Free amino groups on the peptide were protected before coupling, and protection groups were removed after coupling, as described by Goldberger (1967). Ammonium sulfate (50%)–precipitated antibody was dissolved and bound to the column, and the unbound fraction was eluted with 10 mM Tris, pH 7.5. Bound antibody was eluted with acidified glycine into 1 mM Tris, pH 8, and then dialyzed overnight at 4°C against TBS. The final concentration of antibody was \sim 30 mg/ml.

Alkaline Phosphatase Treatment

Protein from whole-cell SDS lysate was precipitated in 20% TCA. The protein was pelleted by centrifugation and washed with acidified acetone and acetone. The dried pellet was resuspended in 50 μ l of 20 mM Tris, pH 8.0, and 0.5–1.0 μ l of *Escherichia coli* alkaline phosphatase (Sigma Chemical Co.) was added. The reaction mixture was incubated at 37°C for 1–2 h, boosted with another 0.5–1 μ l of enzyme, and then the incubation continued for another 1–2 h. Five times concentrated Laemmli sample buffer (Laemmli, 1970) was added to stop the reaction.

Electrophoresis and Immunoblotting

SDS-PAGE has been described previously (Laemmli, 1970; Allis et al., 1979). In cases where whole-cell SDS lysates were used, aliquots of $1\text{--}2 \times 10^6$ cells were removed from a mating cell culture and prepared as described previously (Guttman et al., 1980). Approximately 2×10^5 cells worth of cellular protein was electrophoresed per gel lane.

Immunoblot analyses were done as described previously (Lin et al., 1989). Balanced protein loads of samples were ensured by staining parallel gels of equivalently loaded samples and by staining immunoblots directly with Ponceau red stain. Blots that were to be probed with whole δ antibody were blocked with 5% nonfat dry milk and incubated with immune sera at a dilution of 1:1,000. Blots that were probed with phospho- δ antibody (diluted 1:100) were blocked with 3% BSA in TBS. Immunoreactivity was detected by alkaline phosphatase (AP)–conjugated secondary antibodies (Pierce Chemical Co., Rockford, IL) or by autoradiography after incubation with 50,000 dpm of 125 I protein A/ml TBS (New England Nuclear).

Indirect Immunofluorescence

Cells were fixed and processed for indirect immunofluorescence as described previously (Wenkert and Allis, 1984). Primary antibodies, δ and phospho- δ antibodies diluted 1:250 and 1:25, respectively, were detected by rhodamine-conjugated goat anti-rabbit secondary antibodies. In cases where in situ δ was competing with peptides for antibody immunoreactivity, the antibody was preincubated with an \sim 250 M excess of peptide for 2 h at room temperature. DNA was finally stained with 1 mg DAPI/ml in TBS.

Results

δ Is Hyperphosphorylated during Early Stages of Conjugation

Micronuclei were isolated from vegetatively growing, starved, and 2-h mating cells under conditions that minimize phosphatase activity. When total nuclear protein was examined

directly on a 10% SDS-polyacrylamide gel, a prominent band was observed immediately above δ in 2-h mating cells (indicated with an asterisk) that was only weakly visible in growing cells and was not detected in starved cells (Fig. 1 A). Several lines of evidence suggested that the slower migrating band might actually be a phosphorylated isoform of δ : (a) micronuclear linker histones, including δ , are phosphorylated in vivo (Allis and Gorovsky, 1981; Sweet and Allis, 1993); (b) micronuclear linker histones are generally less phosphorylated in starved cells than in growing cells (Allis and Gorovsky, 1981); and (c) the mobility of phosphorylated macronuclear H1 is retarded when compared to that of dephosphorylated H1 on SDS-polyacrylamide gels (Glover et al., 1981). We reasoned that, depending upon physiological conditions, δ may be posttranslationally modified, migrating as a “doublet” with the slower species being more phosphorylated and the faster form representing dephosphorylated δ .

To confirm that the slower migrating band of the “doublet” was δ , immunoblotting analyses were conducted using polyclonal antibodies generated against reverse-phase HPLC-purified δ . In whole-cell or whole nuclear immunoblots, this antiserum is highly selective for δ and its precursor polyproteins, α or MicLH (data not shown). With 2-h mating cells, both bands of the δ “doublet” react strongly with the anti- δ antibody (Fig. 1 B). Phosphorylation of the slower band (indicated by an asterisk in Fig. 1 B, left) was confirmed by treating an identical sample with AP. Upon this treatment, the “doublet” visible in the untreated sample ($-$ AP) collapses to a single, faster migrating band ($+$ AP, right). Therefore, analogous to macronuclear H1, the slower migrating band of the doublet appears to be a phosphorylated isoform of δ .

Similar immunoblots were done to determine the state of δ phosphorylation throughout various time points of conjugation, the sexual pathway of *Tetrahymena* (Fig. 1 C). A single, fast-migrating δ -positive band is observed in starved and early mating cells, up to 45 min after mixing cells of opposite mating types. A δ doublet is first observed in \sim 1-h mating cells and is maintained for up to 4 h after mixing cells of opposite mating types. After 5–6 h, most of the δ is converted back to a faster migrating (presumably dephosphorylated) isoform. δ is maintained in this state from 7 up to 25 h (data not shown).

δ Phosphorylation Requires Pair Formation

During the first hour after mixing cells of opposite mating types, *Tetrahymena* undergo a defined series of cell–cell interactions that lead to pair formation (Bruns and Brusard, 1974), and it is during this interval that δ phosphorylation is first observed. Entry into the sexual pathway in *Tetrahymena* requires the following: (a) sexually mature cells must be “initiated” by starvation under appropriate ionic conditions. 10 mM Tris was used in these experiments; higher ionic strengths (>50 mM) block the initiation process. (b) Cell–cell interactions between initiated cells of opposite mating types must take place in a process referred to as costimulation. Cell–cell interaction is optimal in stationary cultures and is operationally blocked by fast shaking. After both conditions (initiation and costimulation) are met, pair formation takes place.

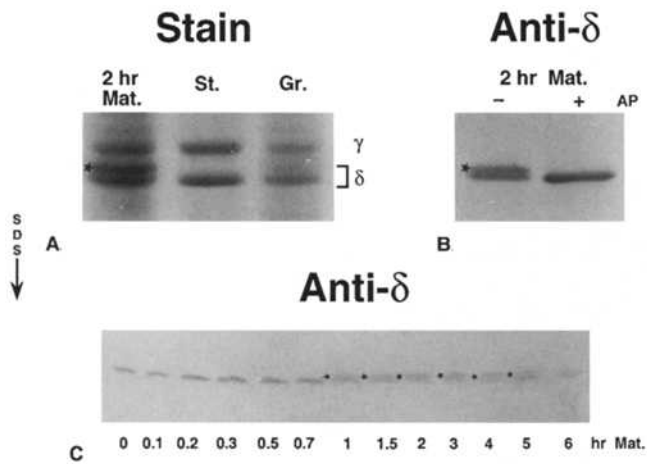


Figure 1. Micronuclear linker histone δ is hyperphosphorylated during meiotic prophase. (A) Acid-soluble micronuclear proteins from 2-h mating, starved, and vegetatively growing cells were resolved on a 10% SDS-polyacrylamide gel and stained with Coomassie blue. Only the region of the gel corresponding to the mobility of linker histones γ and δ is shown. (B) Whole-cell lysates from 2-h mating cells were TCA precipitated and incubated in the presence or absence of AP (\pm AP). An immunoblot of these samples is shown after probing with general δ antibodies. (C) Whole-cell lysates of cells collected at intervals during conjugation were subjected to immunoblot analysis using general δ antibodies. Stars indicate the position of a phosphorylated isoform of δ , which has a decreased mobility relative to the dephosphorylated form of the protein.

To determine whether induction of δ phosphorylation requires the steps that lead to pair formation, initiation and costimulation were blocked experimentally (Fig. 2). Initiation was blocked by starving cells in 60 mM Tris, pH 7.4 (Bruns and Brussard, 1974), after which equal numbers of cells of opposite mating types were mixed and allowed to conjugate for up to 4 h. After this treatment, no pairs are detected, and δ is not phosphorylated (Fig. 2, B and C, lanes 5–7, respectively). Costimulation was obstructed by physical means, fast shaking (200 rpm) initiated cells of opposite mating types. When costimulation is blocked in this fashion, cells are unable to form pairs (Fig. 2 B), and δ is not phosphorylated (Fig. 2 C). However, when these cells are then allowed to sit undisturbed after the 4 h of shaking, pair formation and δ phosphorylation are observed (Fig. 2, B and C). Taken together, these results demonstrate that proper initiation and costimulation are required for the induction of δ phosphorylation.

Pair formation also requires protein synthesis, and the protein synthesis inhibitor, cycloheximide, has previously been shown to inhibit pair formation and possibly costimulation in *Tetrahymena* (Allewell et al., 1976). Cycloheximide (10 μ g/ml) was added to cells that were initiated in 10 mM Tris, mixed, and allowed to remain stationary for up to 4 h. Cells under these conditions do not form pairs and δ does not undergo phosphorylation (Fig. 2, B and C). From these results, we suggest that cell–cell interactions between initiated cells of opposite mating types trigger an increase in an intracellular second messenger that leads to the phosphorylation of δ .

δ Phosphorylation Is Induced by Addition of a cAMP Analogue

Previous studies have indicated that PKA or a PKA-like activity is responsible for the phosphorylation of δ in vivo (Sweet and Allis, 1993). If so, an increase in cAMP level might be expected to activate PKA early in the sexual pathway. Direct measurement of the total intracellular level of cAMP confirmed that a modest but reproducible increase in cAMP occurs during costimulation. Roughly 1 h after mixing cells of opposite mating types, a statistically significant increase (average increase is \sim 25% for three separate matings) in the cAMP level was observed relative to starved, nonmated cells. cAMP levels decline to basal levels as stable pairs form (data not shown).

The above results suggest the involvement of cAMP as a second messenger in a conjugation-induced signal transduction pathway that leads to the phosphorylation of δ . We reasoned that phosphorylation of δ might be induced in nonmating cells by reagents that increase intracellular cAMP levels or by cAMP analogues. The plasma membrane of a related ciliate *Paramecium* is permeable to the cAMP analogue N6-benzoyladenosine 3':5' cyclic monophosphate (benzoyl-cAMP), and addition of this compound leads to increased phosphorylation of ciliary proteins, as well as increased ciliary motion and cell motility (Bonini et al., 1986).

In agreement with the above results, motility (data not shown) and δ phosphorylation increase reproducibly upon addition of benzoyl-cAMP to *Tetrahymena* starved in 10 mM Tris, pH 7.5 (Fig. 3). Time course analyses at an optimal concentration of benzoyl-cAMP (1–2 mM) indicate that phosphorylation of δ is maximal between 1/2 to 1 h of incubation, after which δ phosphorylation begins to decrease (data not shown). In these experiments, N2-benzoylguanosine 5'-monophosphate (benzoyl-GMP) was used as a negative control. When used under identical conditions as for benzoyl-cAMP, benzoyl-GMP does not increase cell motility or influence the level of δ phosphorylation.

The level of δ phosphorylation induced by benzoyl-cAMP is not as high, however, as that observed in 2-h mating cells. The inability of benzoyl-cAMP to produce a strong upper band in the δ "doublet" may have been caused by metabolic degradation of the analogue by endogenous phosphodiesterases. To test this possibility, the cAMP phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) was used. IBMX alone does not stimulate δ phosphorylation, but an increase in cell motility is observed. When IBMX is combined with benzoyl-cAMP, however, δ phosphorylation reproducibly increases over that of benzoyl-cAMP alone (Fig. 3). Although the cAMP analogue and phosphodiesterase inhibitor combination does not produce as strong an upper band in the δ doublet as observed in mating cells, these results suggest that δ phosphorylation depends on an increase in intracellular cAMP.

Measurement of total intracellular cAMP may not reflect the relevant pool of cAMP levels as it applies to the induction of PKA and δ phosphorylation. *Tetrahymena* motility, like that of *Paramecium* (Bonini et al., 1986), is dramatically affected by both benzoyl-cAMP and IBMX. However, only benzoyl-cAMP and IBMX plus benzoyl-cAMP, but not IBMX alone, causes a modest increase in δ

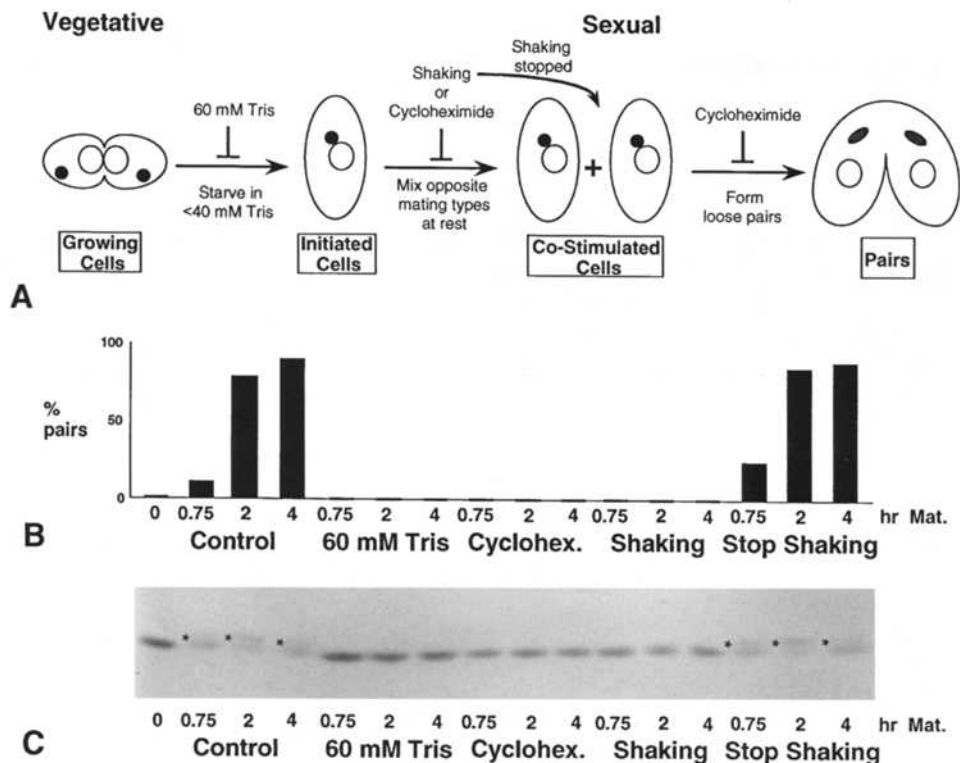


Figure 2. Phosphorylation of δ requires initiation and costimulation, two steps required for pair formation in *Tetrahymena*. (A) Schematic drawing of early events that inhibit or promote pair formation in *Tetrahymena* (Bruns and Brussard, 1974). Following this scheme, cells were either induced to form pairs (control) or manipulated to block pair formation (60 mM Tris, cycloheximide, or fast shaking; see text for details). After 4 h of fast shaking, cells were incubated at rest for an additional 4 h. (B) The percentage of cells in pairs was scored at each time point for up to 4 h. (C) Whole-cell lysates from each of the time points in B were subjected to immunoblot analysis using general δ antiserum. Stars indicate the slower migrating, phosphorylated isoform of δ .

phosphorylation in the absence of cell-cell interactions. Protein synthesis is required for early events in conjugation (Allewell et al., 1976), regulation of PKA activity in *Tetrahymena* (Majumder et al., 1975), and induction of δ phosphorylation seen early in conjugation (Fig. 2). We suspect, therefore, that other factors, perhaps newly synthesized, as well as compartmentalization of cAMP/PKA, play an important role in the conjugation-induced phosphorylation of δ .

Generation and Characterization of an Antibody against Phosphorylated δ

Previous studies have shown that δ is phosphorylated exclusively on serine residues contained within a COOH-terminal, cyanogen bromide-generated peptide (Sweet and Allis, 1993). Two optimal consensus sequences for cAMP-dependent phosphorylation exist in this fragment (Wu et al., 1994), and we recently mapped both of the in vivo phosphorylation sites (Sweet et al., 1996). Antibodies highly selective for phosphorylated H1 isoforms have been gener-

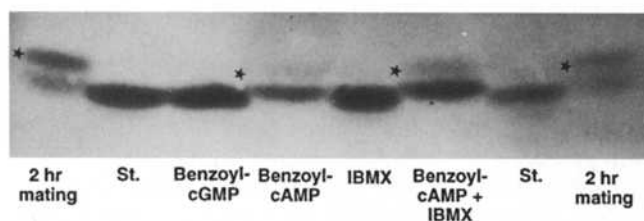


Figure 3. The cyclic AMP analogue, benzoyl-cAMP, induces δ phosphorylation in starved, nonmating cells. Starved cells were incubated with cGMP or cAMP analogues and/or IBMX for 45 min, after which whole-cell lysates were prepared and analyzed by immunoblotting using general δ antiserum.

ated and localized in situ to regions of decondensed macronuclear chromatin (Lu et al., 1994, 1995). An attempt was made to produce a comparable antibody to the phosphorylated isoforms of δ .

The sequence of one in vivo phosphorylation site in δ [Thr-Lys-Arg-Lys-Asn-Ser(P)-Lys-Ser] was used to synthesize a phosphopeptide, and this peptide was used to generate and affinity purify antibodies specific for phosphorylated δ isoforms. Immunoblotting analyses demonstrate that the resultant antibodies react specifically with δ in total micronuclear extracts (Fig. 4, lane 2); none of the proteins in micronuclei react with the phosphodelta antibody (Fig. 4, lane 1). Immunoreactivity was abolished by preincubating the antibody with the phosphorylated δ peptide, but not with a nonphosphorylated peptide that contains the same amino acid sequence (compare Fig. 4, B and C, respectively). δ identity was confirmed by probing a parallel blot with HPLC-purified δ with general δ antibodies (Fig. 4 D).

The phosphodelta antibody is specific for the phosphorylated isoforms of δ (Fig. 5, phosphodelta is identified by stars). As expected, δ from 2-h mating cells reacts strongly with the phosphodelta antibody, while δ from starved cells does not (Fig. 5 B). In addition, this antibody fails to recognize δ from 2-h mating cells when the protein is treated with AP (Fig. 5 B, +AP). δ identity was again confirmed by reprobing the membrane with antibody against general δ (data not shown).

Phosphodelta Is Enriched in Transcriptionally Active Chromatin in Micronuclei

Given the immunoblotting analyses shown in Fig. 1, strong staining of micronuclei is expected with the phosphodelta antibodies during the early stages of conjugation corre-

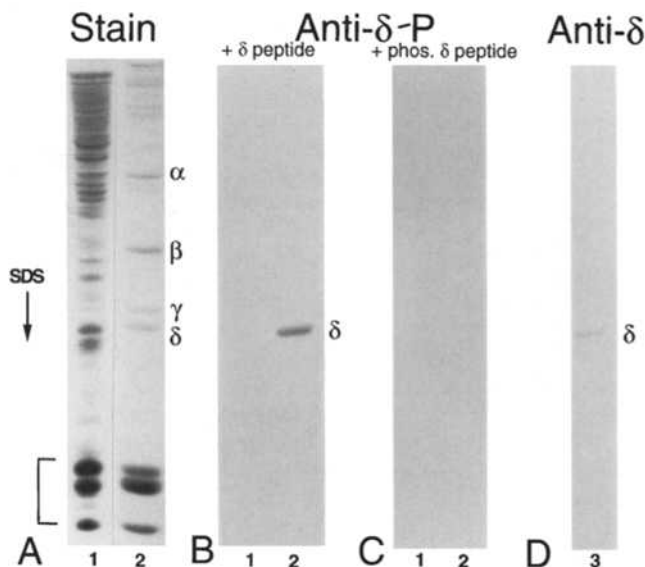


Figure 4. Generation of antibodies highly selective for phosphorylated isoforms of δ . (A) Coomassie stain of acid-soluble nuclear protein from highly purified preparations of micro- and macronuclei isolated from 2.5-h mating cells that were resolved on a 12% SDS-acrylamide gel. Lanes: 1, total macronuclear protein; 2, total micronuclear protein. (B–D) Immunoblot analyses of nuclear proteins using an affinity-purified antibody against a synthetic phosphorylated δ peptide [Thr-Lys-Arg-Lys-Asn-Ser(P)-Lys-Ser]. Phosphodelta antibody was incubated with either a 250-M excess of either nonphosphorylated peptide (B) or phosphorylated peptide (C) for 2 h at room temperature before incubation with the blot. To confirm the identity/mobility of δ , a parallel blot of HPLC-purified δ (lane 3) was probed with general δ antiserum (D). All immune reactions were detected with AP-conjugated secondary antibody. Each of the micronuclear-specific polypeptides α , β , γ , and δ are labeled. Bracket denotes core histones.

sponding to meiotic prophase (1–4 h). As shown in Fig. 6, affinity-purified phosphodelta antibodies consistently stain the periphery of micronuclei weakly in starved cells (*left*), while micronuclei in 2-h mating cells are brightly stained (*center left*). Consistent with our immunoblotting analyses (Fig. 4), preincubating the phosphodelta antibody with phosphorylated peptide blocks essentially all immunoreactivity (Fig. 6, *center right*), while preincubation with non-phosphorylated peptide has no effect (data not shown). As expected, the general δ antibody stains starved and 2-h mating micronuclei more or less equally (Fig. 6, *right*). Thus, in agreement with our immunoblotting data (Fig. 1), the immunofluorescence data shown in Fig. 6 demonstrate that a significant increase in the phosphorylation of δ occurs during the early stages of conjugation.

While the micronucleus is highly condensed and transcriptionally inactive during most of the life cycle, a brief period of transcriptional activity has been reported during the early stages of conjugation corresponding to meiotic prophase (Sugai and Hiwatashi, 1974; Martindale et al., 1985). We were curious if phosphorylation of δ could be positively correlated with transcriptional activation in micronuclei during this period. To investigate this issue, 2-h mating cells were pulse labeled with [3 H]uridine for 5 min, processed for autoradiography, and stained with DAPI

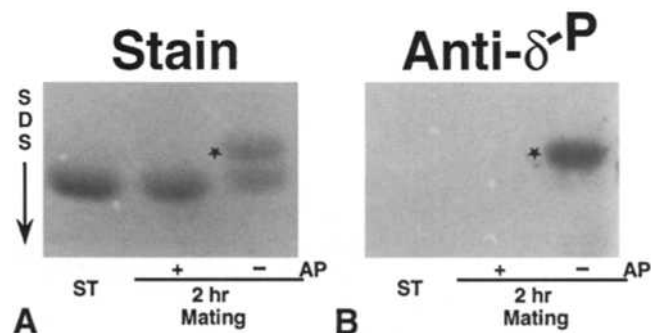


Figure 5. Antibodies against the phosphodelta peptide recognize only the phosphorylated isoform of δ . δ from micronuclei isolated from starved or 2-h mating cells was purified by reverse-phase HPLC and, where indicated (2-h mating cells), was additionally incubated with (+) or without (–) AP. Proteins were resolved on a 10% SDS-polyacrylamide gel and either stained (A) or subjected to immunoblot analysis using the phosphodelta antibody (B). As in Fig. 4, reprobing of the blot shown in B with general δ antibodies confirmed the evenness of sample loading and transfer (not shown). Stars indicate the slow migrating, phosphorylated δ isoform.

(Fig. 7, *middle row*). In agreement with the results first reported by Sugai and Hiwatashi (1974), we find that micronuclei in specific stages of meiotic prophase (primarily stages II and III, Martindale et al., 1982) are transcriptionally active. Unlike the previous study, however, our results indicate that transcriptional activity is not uniform throughout appropriately staged micronuclei. We have consistently observed that the area of more intense DAPI staining (indicative of more condensed DNA, see *arrows* in Fig. 7) is relatively devoid of silver grains, while in contrast, the more extended regions of lighter DAPI staining (indicative of less condensed DNA) contain essentially all of the 3 H-uridine radiolabel observed in 2-h mating micronuclei. These data strongly suggest that more decondensed DNA of micronuclei is transcribed during this time. In parallel, micronuclei in similar stages of meiotic prophase were stained with affinity-purified antibodies against phosphorylated δ (Fig. 7, *top row*). Strong immunoreactivity is consistently observed over extended, less condensed regions of micronuclei during the meiotic stages. In contrast, little, if any, staining is observed over the more condensed “center” of the micronuclear chromatin (see *arrows* in Fig. 7). The absence of immunoreactivity of phosphodelta antibodies in the more condensed region of meiotic micronuclei is not a trivial artifact of antibody inaccessibility, since general δ antibodies stain these regions well, closely mirroring the DAPI staining (Fig. 7, *bottom row*).

As predicted, if the phosphorylation of linker histone is a necessary prerequisite for chromatin decondensation (Roth and Allis, 1992), the onset of micronuclear staining with phosphodelta antibodies (stage I) precedes the onset of transcriptional activity in these nuclei, which becomes evident as soon as the micronuclei begin to elongate. Detectable levels of transcription cease largely by the time micronuclei are fully elongated (stage IV), after which the staining of micronuclei by phosphodelta antibodies is rapidly lost (data not shown). From these data, we argue that phosphorylation of δ and perhaps other micronuclear

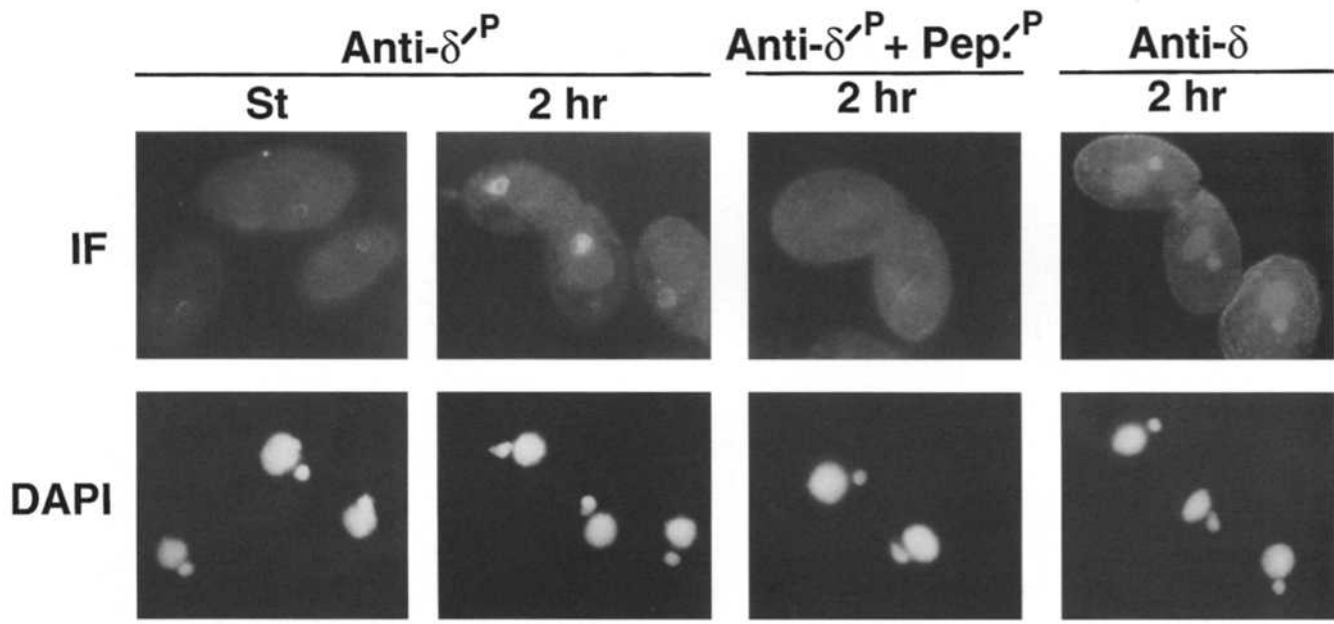


Figure 6. Antibodies against phosphorylated δ strongly stain micronuclei in meiotic prophase. (Top) Starved or 2-h mating cells were fixed and incubated with phosphodelta antibodies (left) or, where indicated, with antibodies against general δ (right). As an additional specificity control, phosphodelta antibodies were preincubated in the presence (center right) or absence (center left) of phosphorylated peptide, as described in Fig. 4. (Bottom) Corresponding DAPI staining. Bar, 1 μ m.

linker histones functions in establishing a decondensed, transcriptionally competent state of micronuclear chromatin in this unique stage of the life cycle.

Discussion

cAMP/PKA Signal Transduction Induces Early Events in Conjugating Tetrahymena

The cAMP/PKA signal transduction pathway is composed of an elaborate cascade of regulatory proteins that functions as one of several second messenger-dependent systems for generating intracellular responses to extracellular signals (for review see Beebe, 1994). In this report, we provide evidence that this signaling pathway is initiated upon cell-cell interactions made early in the sexual pathway in *Tetrahymena*. Our data demonstrate that a modest, but reproducible, increase in total intracellular cAMP (second messenger) occurs during costimulation of properly initiated cells. It is likely that this increase in cAMP, perhaps in a compartmentalized form, activates one or more PKA isoforms that, in turn, lead to the hyperphosphorylation of micronuclear linker histone δ . In general, the physiological substrates of the cAMP/PKA signaling cascade are only beginning to be recognized (Beebe, 1994), and have not been well studied in ciliates. As far as we are aware, the cAMP/PKA-mediated induction of δ phosphorylation in micronuclei provides one of the first clear indications that this pathway stimulates the phosphorylation of a linker histone, an event that correlates spatially and temporally with remodeling the chromatin into a transcriptionally competent form.

Our data suggest, however, that the magnitude and extent of the signaling events in *Tetrahymena* differ consid-

erably from the events in *Chlamydomonas*, where gametes make sex-specific adhesive contacts, triggering a rapid but transient 10-fold elevation in intracellular levels of cAMP (Pasquale and Goodenough, 1987). The magnitude of the cAMP increase in mating *Tetrahymena* differs significantly from that seen in *Chlamydomonas*, and is probably caused by differences in mating characteristics between the two organisms. Initial mating events in *Chlamydomonas* occur rapidly and synchronously, such that within 10 min, \sim 80% of the cells have formed stable pairs (Pasquale and Goodenough, 1987); cAMP levels then decline rapidly to a basal level after pair formation. In *Tetrahymena*, on the other hand, typically 50% of the cells have formed pairs 1 h after mixing opposite mating types (Martindale et al., 1982), and often pairing kinetics differ considerably from one mating to the next. cAMP levels decline to basal levels as stable pairs form (data not shown).

Phosphorylation of Linker Histone Is Associated with Chromatin Decondensation and Transcriptional Activation

We have proposed that linker histone phosphorylation acts as a first-step mechanism to decondense chromatin, which in turn increases the accessibility of *trans*-acting factors to target DNA sequences (Roth and Allis, 1992), and in this report, we have focused our attention on the phosphorylation of the micronuclear linker histone δ . Although δ is largely dephosphorylated during much of the *Tetrahymena* life cycle, δ is hyperphosphorylated during stages of meiotic prophase, when micronuclei decondense and become transcriptionally active, showing a strong temporal correlation with micronuclear transcription. Interestingly, the period of maximal δ phosphorylation (\sim 1–4 h, Fig. 1)

Meiotic Prophase

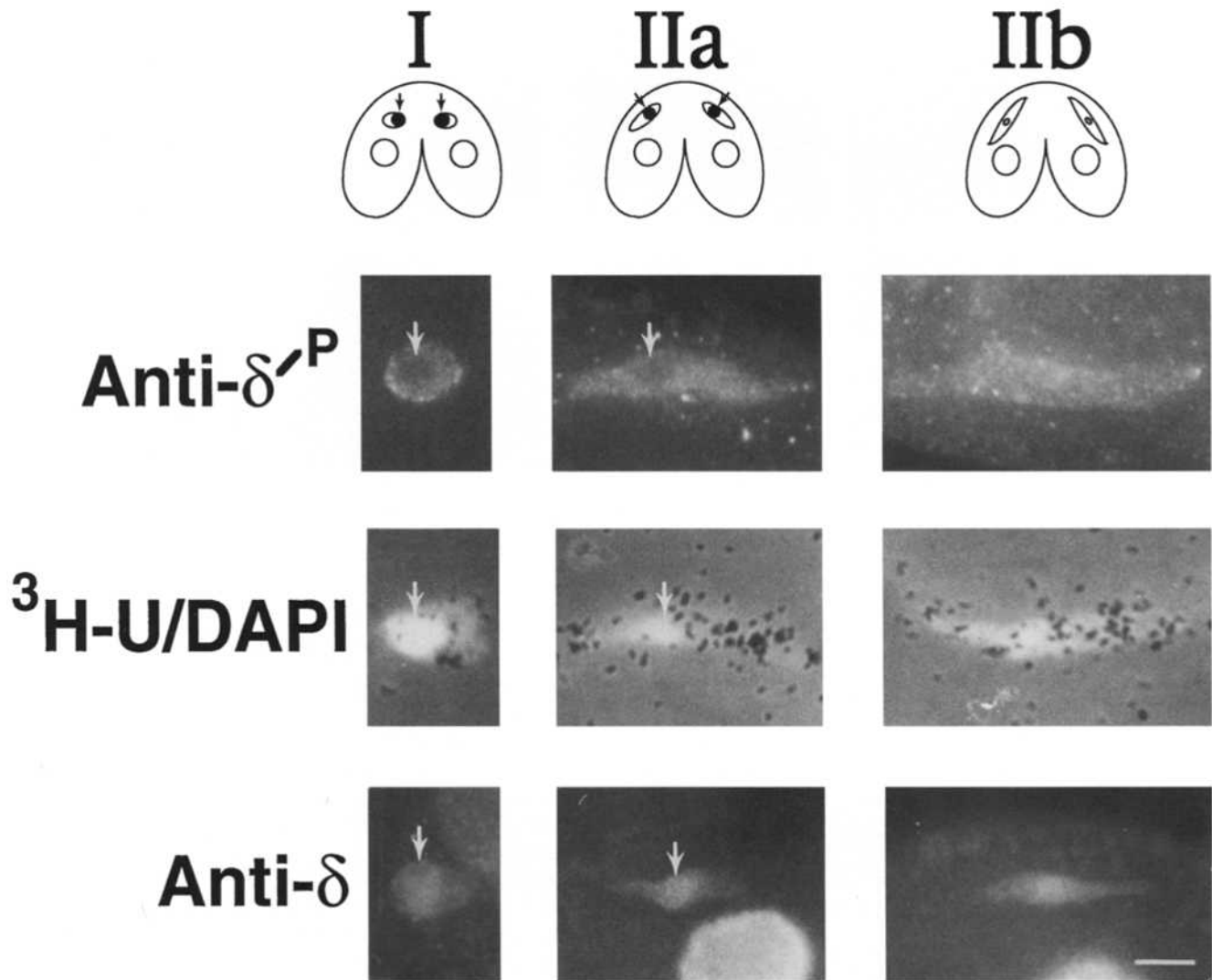


Figure 7. Staining with phosphodelta antibody is localized to regions of decondensed, transcriptionally active chromatin in micronuclei. A schematic drawing of micronuclei in stages corresponding to meiotic prophase I (*left*) and II (*center and right*) is shown (for details see Sugai and Hiwatashi, 1974; Martindale et al., 1985). Darker areas correspond to areas of more condensed chromatin (more intense DAPI staining). Cells were fixed and incubated with antibodies against general δ (*bottom*) or phosphodelta (*top*). In parallel, cells were pulse-labeled with [^3H]uridine for 5 min and prepared for conventional autoradiography, followed by DAPI staining (*middle*). Note that essentially all of the [^3H]uridine label is associated with the less condensed chromatin corresponding to the extended micronuclear "tails"; few, if any, silver grains are found over the more condensed DAPI staining "center" of each micronucleus (*arrows*). Bar, 2 μm .

corresponds closely with the period for optimal efficiency of germline (micronuclear) transformation in *Tetrahymena* (Bruns, P.J., and M.A. Gorovsky, personal communication). It is not clear whether the decondensation of micronuclear chromatin, potentially triggered by δ hyperphosphorylation or another factor(s), is responsible for these effects.

The subnuclear localization of phosphorylated δ in meiotic micronuclei was determined by fluorescent microscopy using phosphodelta antibodies (generated against a phosphopeptide mimicking an *in vivo* phosphorylation site within δ). As expected from our immunoblotting analyses,

transcriptionally inert micronuclei did not stain well during starvation, but stained strongly during the early stages of meiotic prophase. During this period, micronuclei swell, and the highly condensed chromatin normally observed in nonconjugating cells is transformed into a "halo" of less condensed chromatin (Wolfe et al., 1976). Consistent with a change in chromatin structure attributable to the hyperphosphorylation of δ , less condensed regions of micronuclear chromatin stain strongly with phosphodelta δ antibodies while, in contrast, the more condensed chromatin stains well with general δ antibodies. Micronuclei then begin an extended period of elongation and further decon-

densation marked by the onset of transcriptional activity in the early crescent stage (Fig. 7; Sugai and Hiwatashi, 1974; Martindale et al., 1985). The detection of hyperphosphorylated δ in micronuclei precedes the period of transcription, and is rapidly lost during subsequent chromosome condensation and entry into the first meiotic division. Collectively, these data suggest that phosphorylation of δ is more a requirement for establishing transcriptionally competent chromatin than a result of transcription per se.

The hypothesis that phosphorylation of δ in micronuclei is associated with transcriptional activation is supported by reports that TATA-binding protein and an evolutionarily conserved H2A variant, hv1, are detected in micronuclei at or before activation (Stargell et al., 1993; Stargell and Gorovsky, 1994). The Roth-Allis model (Roth and Allis, 1992) predicts that decondensed chromatin is closely associated with phosphorylated linker histone. Lu et al. (1995) have recently shown that phosphorylated H1 is associated with decondensed regions of macronuclear chromatin in close association with hv1. These results are extended here by showing that the phosphorylation of δ is also associated with decondensed chromatin and transcriptional competence in micronuclei. We speculate that the phosphorylation of δ is required to allow for the binding of hv1 and TATA-binding protein during this period of dynamic chromatin reorganization. With the development of techniques for gene replacement and mass transformation (Gaertig and Gorovsky, 1995), along with the mapping of in vivo phosphorylation sites in macro- and micronuclear linker histones (Sweet, M.T., C. Mizzen, and C.D. Allis, unpublished observations), we look forward to a direct test of some of these predictions.

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References

- Allewell, N.M., J. Oles, and J. Wolfe. 1976. A physicochemical analysis of conjugation in *Tetrahymena pyriformis*. *Exp. Cell Res.* 97:394-405.
- Allis, C.D., and D.K. Dennison. 1982. Identification and purification of young macronuclear anlagen from conjugating cells of *Tetrahymena thermophila*. *Dev. Biol.* 93:519-533.
- Allis, C.D., and M.A. Gorovsky. 1981. Histone phosphorylation in macronuclei and micronuclei of *Tetrahymena thermophila*. *Biochemistry.* 20:3828-3833.
- Allis, C.D., C.D.C. Glover, and M.A. Gorovsky. 1979. Micronuclei of *Tetrahymena* contain two types of histone H3. *Proc. Natl. Acad. Sci. USA.* 76:4857-4861.
- Allis, C.D., J.C. Allen, L.C. Wiggins, L.C. Chicoine, and R. Richman. 1984. Proteolytic processing of H1-like histones in chromatin: a physiologically and developmentally regulated event in *Tetrahymena* micronuclei. *J. Cell Biol.* 99:1669-1677.
- Beebe, S.J. 1994. The cAMP-dependent protein kinases and cAMP signal transduction. *Semin. Cancer Biol.* 5:285-294.
- Bonini, N.M., M.C. Gustin, and D.L. Nelson. 1986. Regulation of ciliary motility by membrane potential in *Paramecium*: a role for cyclic AMP. *Cell Motil. Cytoskel.* 6:256-272.
- Bouvet, P., S. Dimitrov, and A.P. Wolffe. 1994. Specific regulation of *Xenopus* chromosomal 5S rRNA gene transcription in vivo by histone H1. *Genes Dev.* 8:1147-1159.
- Bradbury, E.M. 1992. Reversible histone modifications and the chromosome cell cycle. *Bioessays.* 14:9-16.
- Bruns, P.J., and T.B. Brussard. 1974. Pair formation in *Tetrahymena pyriformis*, an inducible developmental system. *J. Exp. Zool.* 188:337-344.
- Chadee, D.N., W.R. Taylor, R.A. Hurta, C.D. Allis, J.A. Wright, and J.R. Davie. 1995. Increased phosphorylation of histone H1 in mouse fibroblasts transformed with oncogenes or constitutively active mitogen-activated protein kinase kinase. *J. Biol. Chem.* 270:20098-20105.
- Clark, D.J., and T. Kimura. 1990. Electrostatic mechanism of chromatin folding. *J. Mol. Biol.* 211:883-896.
- Dasso, M., S. Dimitrov, and A.P. Wolffe. 1994. Nuclear assembly is independent of linker histones. *Proc. Natl. Acad. Sci. USA.* 91:12477-12481.
- Gaertig, J., and M.A. Gorovsky. 1995. DNA-mediated transformation in *Tetrahymena*. *Methods Cell Biol.* 47:559-569.
- Garrard, W.T. 1991. Histone H1 and the conformation of transcriptionally active chromatin. *Bioessays.* 13:87-88.
- Glover, C.V.C., K.J. Vavra, S.D. Guttman, and M.A. Gorovsky. 1981. Heat shock and deciliation induce phosphorylation of histone H1 in *T. pyriformis*. *Cell.* 23:73-77.
- Goldberger, R.F. 1967. Trifluoroacetylation of ϵ -amino groups. *Methods Enzymol.* 11:317-322.
- Gorovsky, M.A. 1980. Genome organization and reorganization in *Tetrahymena*. *Annu. Rev. Genet.* 14:203-239.
- Gorovsky, M.A. 1986. Ciliate chromatin and histones. In: *The Molecular Biology of Ciliated Protozoa*. J.G. Gall, editor. Academic Press, New York. 227-261.
- Gorovsky, M.A. 1993. Temporal and spatial association of histone H2A variant hv1 with transcriptionally competent chromatin during nuclear development in *Tetrahymena thermophila*. *Genes Dev.* 7:2641-2651.
- Gorovsky, M.A., M.-C. Yao, J.B. Keever, and G.L. Plegler. 1975. Isolation of micro- and macronuclei of *Tetrahymena pyriformis*. *Methods Cell Biol.* 9: 311-327.
- Guo, X.W., J.P. Th'ng, R.A. Swank, H.J. Anderson, C. Tudan, E.M. Bradbury, and M. Roberge. 1995. Chromosome condensation induced by fostriecin does not require p34^{cdc2} kinase activity and histone H1 hyperphosphorylation, but is associated with enhanced histone H2A and H3 phosphorylation. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:976-985.
- Guttman, S.D., C.V.C. Glover, C.D. Allis, and M.A. Gorovsky. 1980. Heat shock, deciliation and release from anoxia induce the same set of polypeptides in starved *Tetrahymena pyriformis*. *Cell.* 22:299-307.
- Hirano, T. 1995. Biochemical and genetic dissection of mitotic chromosome condensation. *Trends Biochem. Sci.* 20:357-361.
- Koshland, D. 1996. Chromosome condensation. *Annu. Rev. Cell Dev. Biol.* In press.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Lin, R., J.W. Leone, R.G. Cook, and C.D. Allis. 1989. Antibodies specific to acetylated histones document the existence of deposition- and transcription-related histone acetylation in *Tetrahymena*. *J. Cell Biol.* 108:1577-1588.
- Lu, M.J., C.A. Dadd, C.A. Mizzen, C.A. Perry, D.R. McLachlan, A.T. Annunziato, and C.D. Allis. 1994. Generation and characterization of novel antibodies highly selective for phosphorylated linker histone H1 in *Tetrahymena* and HeLa cells. *Chromosoma.* 103:111-121.
- Lu, M.J., S.S. Mpoke, C.A. Dadd, and C.D. Allis. 1995. Phosphorylated and dephosphorylated linker histone H1 reside in distinct chromatin domains in *Tetrahymena* macronuclei. *Mol. Biol. Cell.* 6:1077-1087.
- Majumder, G., E. Shrago, and C.E. Elson. 1975. Changes in cyclic AMP-dependent protein kinase activity in *Tetrahymena pyriformis* during the growth cycle. *Biochem. Biophys. Acta.* 384:399-412.
- Martindale, D.W., C.D. Allis, and P.J. Bruns. 1982. Conjugation in *Tetrahymena thermophila*. A temporal analysis of cytological stages. *Exp. Cell Res.* 140:227-236.
- Martindale, D.W., C.D. Allis, and P.J. Bruns. 1985. RNA and protein synthesis during emiotic prophase in *Tetrahymena thermophila*. *J. Protozool.* 32:644-649.
- Ohsumi, K., C. Katagiri, and T. Kishimoto. 1993. Chromosome condensation in *Xenopus* mitotic extracts without histone H1. *Science (Wash. DC).* 262:2033-2035.
- Pasquale, S.M., and U.W. Goodenough. 1987. Cyclic AMP functions as a primary sexual signal in gametes of *Chlamydomonas reinhardtii*. *J. Cell Biol.* 105:2279-2292.
- Peterson, C.L. 1994. The SMC family: novel motor proteins for chromosome condensation? *Cell.* 79:389-392.
- Roth, S.Y., and C.D. Allis. 1992. Chromosome condensation: does histone H1 dephosphorylation play a role? *Trends Biochem. Sci.* 17:93-98.
- Roth, S.Y., M.P. Collini, G. Draetta, D. Beach, and C.D. Allis. 1991. A cdc-2-like kinase phosphorylates histone H1 in the amitotic macronucleus of *Tetrahymena*. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:2069-2075.
- Sandaltzopoulos, R., T. Blank, and P.B. Becker. 1994. Transcriptional repression by nucleosomes but not H1 in reconstituted preblastoderm *Drosophila* chromatin. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:373-379.
- Schulman, I.G., R.G. Cook, R. Richman, and C.D. Allis. 1987. *Tetrahymena* contain two distinct and unusual high mobility group (HMG)-like proteins. *J. Cell Biol.* 104:1485-1494.
- Shen, X., and M.A. Gorovsky. 1996. Linker histone H1 regulates specific gene expression but not global transcription in vivo. *Cell.* 86:475-483.
- Shen, X., L. Yu, J.W. Weir, and M.A. Gorovsky. 1995. Linker histones are not

- essential and affect chromatin condensation in vivo. *Cell*. 82:47–56.
- Stargell, L.A., and M.A. Gorovsky. 1994. TATA-binding protein and nuclear differentiation in *Tetrahymena thermophila*. *Mol. Cell. Biol.* 14:723–734.
- Stargell, L.A., J. Bowen, C.A. Dadd, P.C. Dedon, M. Davis, R.G. Cook, C.D. Allis, and M.A. Gorovsky. 1993. Temporal and spatial association of histone H2A variant hv1 with transcriptionally competent chromatin during nuclear development in *Tetrahymena thermophila*. *Genes Dev.* 7:2641–2651.
- Sugai, T., and K. Hiwatashi. 1974. Cytologic and autoradiographic studies of the micronucleus at meiotic prophase in *Tetrahymena pyriformis*. *J. Protozool.* 21:542–548.
- Sweet, M.T., and C.D. Allis. 1993. Phosphorylation of linker histones by cAMP-dependent protein kinase in mitotic micronuclei of *Tetrahymena*. *Chromosoma*. 102:637–647.
- Sweet, M.T., G. Carlson, R.G. Cook, D. Nelson, and C.D. Allis. 1996. Phosphorylation of linker histones by a protein kinase A-like activity in mitotic nuclei. *J. Biol. Chem.* In press.
- Thoma, F., T. Koller, and A. Klug. 1979. Involvement of histone H1 in the organization of the nucleosome and of the salt-dependent superstructures of chromatin. *J. Cell Biol.* 83:403–427.
- van Holde, K.E. 1989. *Chromatin*. Springer-Verlag, New York.
- Vavra, K.J., C.D. Allis, and M.A. Gorovsky. 1982. Regulation of histone acetylation in *Tetrahymena* macro- and micronuclei. *J. Biol. Chem.* 257:2591–2598.
- Wenkert, D., and C.D. Allis. 1984. Timing of the appearance of macronuclear-specific histone variant hv1 and gene expression in developing new micronuclei of *Tetrahymena thermophila*. *J. Cell Biol.* 98:2107–2117.
- Wolfe, J., B. Hunter, and W.S. Adair. 1976. A cytological study of micronuclear elongation during conjugation in *Tetrahymena*. *Chromosoma*. 55:289–308.
- Wolffe, A.P. 1994. Transcription: in tune with the histones. *Cell*. 77:13–16.
- Wolffe, A. 1995. Structure and function. In *Chromatin*. Academic Press, San Diego, CA.
- Workman, J.L., and A.R. Buchman. 1993. Multiple functions of nucleosomes and regulatory factors in transcription. *Trends Biochem. Sci.* 18:90–95.
- Wu, M., C.D. Allis, M.T. Sweet, R.G. Cook, T.H. Thatcher, and M.A. Gorovsky. 1994. Four distinct and unusual linker proteins in a mitotically dividing nucleus are derived from a 71-kilodalton polyprotein, lack p34cdc2 sites, and contain protein kinase A sites. *Mol. Cell. Biol.* 14:10–20.
- Zlatanova, J., and K. van Holde. 1992. Histone H1 and transcription: still an enigma? *J. Cell Sci.* 103:889–895.
- Zlatanova, J., and K. van Holde. 1996. Linker histones and chromatin structure: new twists. In *Progress in Nucleic Acid Research and Molecular Biology*. 217–259.