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A cdc2-like kinase phosphorylates histone H1 in the amitotic macronucleus of *Tetrahymena*

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Genetic and biochemical studies have shown that cdc2 protein kinase plays a pivotal role in a highly conserved mechanism controlling the entry of cells into mitosis. It is generally believed that one function of cdc2 kinase is to phosphorylate histone H1 which in turn promotes mitotic chromosome condensation. However, direct evidence linking H1 phosphorylation to mitotic chromatin condensation is limited and the exact cellular function(s) of H1 phosphorylation remains unclear. In this study, we show that mammalian cdc2 kinase phosphorylates H1 from the amitotic macronucleus of *Tetrahymena* with remarkable fidelity. Furthermore, we demonstrate that macronuclei from *Tetrahymena* contain a growth-associated H1 kinase activity which closely resembles cdc2 kinase from other eukaryotes. Using polyclonal antibodies raised against yeast $p34^{cdc2}$, we have detected a 36 kd immunoactive polypeptide in macronuclei which binds to Suc1 (p13)-coated beads and closely follows H1 kinase activity. Since macronuclei divide without mitotic chromosome condensation, these data demonstrate that H1 phosphorylation by cdc2 kinase may be necessary, but is not sufficient to promote mitotic chromatin condensation. The fact that an activity which strongly resembles mammalian cdc2 kinase is active during cell growth in a nucleus which does not undergo mitosis and chromosome condensation suggests that other factors are needed for a true mitotic division to occur. These data also reinforce the notion that H1 phosphorylation has important functions outside mitosis both in *Tetrahymena* and in mammalian cells.

Key words: amitotic macronucleus/cdc2 kinase/chromatin condensation/histone H1 phosphorylation/mitosis/*Tetrahymena*

Introduction

Extensive genetic and biochemical evidence has documented the existence of a highly conserved pathway controlling the entry of cells into mitosis and/or meiosis (see Dunphy and Newport, 1988; Murray and Kirschner, 1989; Lewin, 1990 for recent reviews). One essential gene product in this cascade is a protein kinase commonly referred to as cdc2 or p34 kinase. The *cdc2*$^+$ gene in the fission yeast, *Schizosaccharomyces pombe*, encodes a 34 kd polypeptide which, in association with cyclin regulatory subunits, has protein kinase activity towards several preferred protein substrates (Booher *et al.*, 1989; Moreno and Nurse, 1990). Data suggest strongly that cdc2 kinase is present in all eukaryotic cells pointing to its functioning as a universal mitotic trigger (Lee and Nurse, 1987).

One preferred substrate of cdc2 kinase, at least *in vitro*, is histone H1 and vertebrate H1 has been used routinely in assays of cdc2 kinase activity (Lohka *et al.*, 1988; Arion *et al.*, 1988; Labbe *et al.*, 1988, 1989; Brizuela *et al.*, 1989). Since it has long been recognized that H1 is hyperphosphorylated as cells enter mitosis (Bradbury *et al.*, 1974a,b; Inglis *et al.*, 1976; Chambers *et al.*, 1982), a time in the cell cycle when cdc2 kinase activity also peaks, it is attractive to propose that cdc2 kinase is the activity responsible for H1 phosphorylation at the G_2/M boundary in cycling mammalian cells. Indeed, mammalian growth-associated H1 kinase (Lake and Salzman, 1972; Lake, 1973; Bradbury *et al.*, 1974a; Woodford and Pardee, 1986) has recently been shown to be similar in many respects to yeast and frog cdc2 kinase (Langan *et al.*, 1989).

The consequence of H1 phosphorylation in chromatin during mitosis is not well understood. As first proposed by Bradbury and colleagues (Bradbury *et al.*, 1974a,b), H1 hyperphosphorylation is believed to trigger mitotic chromosome condensation. In support of this proposal, addition of cdc2 kinase (or growth-associated H1 kinase) to cells can cause premature condensation of chromatin in several different cell types (Inglis *et al.*, 1976; Matsumoto *et al.*, 1980; Mueller *et al.*, 1985; Nishimoto *et al.*, 1987; Lamb *et al.*, 1990). Thus, a picture has emerged wherein cdc2 kinase, through its downstream phosphorylation of H1, initiates mitotic chromosome condensation. Presumably this process is reversed as cells exit mitosis by the action of a phosphatase(s) which dephosphorylates H1 causing the chromatin to decondense (see Cyert and Thorner, 1990).

The ciliated protozoan, *Tetrahymena*, provides a useful model system to explore relationships between cdc2 kinase, H1 phosphorylation and mitotic chromosome condensation. Like many protozoa, each vegetative cell contains a diploid germline micronucleus which divides mitotically and a polyploid somatic macronucleus which divides amitotically. Although macronuclei divide without mitotic chromosome condensation, they contain an H1 which is extensively phosphorylated in growing cells (Gorovsky *et al.*, 1974; Allis and Gorovsky, 1981; Roth *et al.*, 1988). Furthermore, steady state levels of H1 phosphorylation in macronuclei are modulated dramatically in response to different physiological settings such as starvation (Allis and Gorovsky, 1981), heat shock (Glover *et al.*, 1981) and conjugation (Roth *et al.*, 1988). Thus, these data force the conclusion that, at least

in *Tetrahymena,* H1 phosphorylation does not play an exclusive role in mitotic chromosome condensation and have led us to suggest that H1 phosphorylation in macronuclei functions as a mechanism to regulate gene expression rapidly and reversibly in that nucleus (Roth *et al.*, 1988). In this report, we have asked whether cdc2 kinase is responsible for H1 phosphorylation in *Tetrahymena* macronuclei.

Using a variety of immunological and biochemical approaches, we present evidence that an activity which strongly resembles cdc2 kinase is responsible for H1 phosphorylation in *Tetrahymena* macronuclei. Furthermore we show that mammalian cdc2 kinase phosphorylates macronuclear H1 *in vitro* with surprising fidelity. Thus, the high degree of evolutionary conservation of the enzyme system(s) responsible for entry into mitosis in mammalian cells has now been extended to protozoa. More importantly, since H1 phosphorylation in *Tetrahymena* macronuclei cannot be related to mitotic chromosome condensation, and yet is catalyzed by an activity which strongly resembles cdc2 kinase from yeast and mammals, we suggest that H1 phosphorylation by cdc2 kinase may be necessary, but is not sufficient for mitotic chromosome condensation to occur. Our data also reinforce the idea that H1 phosphorylation has important functions outside of mitotic chromosome condensation even in mammalian cells.

Results

Tetrahymena macronuclei contain a growth-associated kinase activity with a high degree of specificity for histone H1

To determine whether macronuclei from *Tetrahymena* contain an H1 kinase activity, macronuclei were isolated from growing (50 000−150 000 cells/ml), stationary (500 000−1 200 000 cells/ml) or starved cells (overnight in 10 mM Tris, pH 7.5 at 250 000 cells/ml) and labeled directly with [γ-^{35}S]ATP. Following various incubation times, histones were extracted and analyzed by staining and autoradiography. Figure 1 demonstrates that isolated macronuclei contain an H1 kinase activity which is strongly influenced by the physiological growth state of the cells. As the cell density increases beyond exponential growth (100 000 cells/ml), progressively less H1 kinase activity is observed. Little, if any, H1 kinase activity is observed in cultures starved overnight in Tris (under these conditions cells do not grow or divide).

To determine whether macronuclear H1 kinase activity has properties like those reported for mammalian growth-associated H1 kinase (Lake and Salzman, 1972; Lake, 1973; Bradbury *et al.*, 1974a; Woodford and Pardee, 1986), methods were developed to efficiently extract H1 kinase activity from solubilized macronuclear chromatin (see Materials and methods for details). Whether challenged with free or chromatin-bound H1 as substrate, the extracted macronuclear kinase activity is highly specific for histone H1 under our *in vitro* assay conditions (see Figure 2). Furthermore, like the mammalian H1 kinase, this activity does not require (and is not stimulated by) cyclic nucleotides (cAMP or cGMP) or calcium (data not shown; see Materials and methods for details). Thus, the H1 kinase activity extracted from *Tetrahymena* macronuclei resembles mammalian growth-associated H1 kinase both in its specificity and in at least some aspects of its regulation.

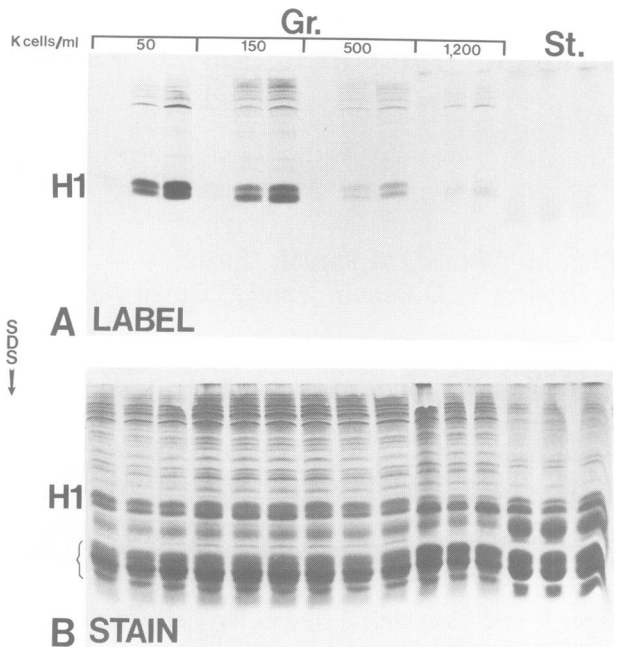

Fig. 1. Macronuclei contain a growth-associated H1 kinase activity. Macronuclei were isolated from growing (Gr.) or starved (St.) cells at the densities indicated (expressed in K cells/ml; starved cells were at 200 to 250 K cells/ml) and were directly assayed for H1 kinase activity. For each group of nuclei, reactions were carried out 0, 5 and 15 min after the addition of labeled ATP. Acid-soluble proteins were fractionated by SDS−PAGE and examined by staining (**B**) and by autoradiography (**A**). The position of H1 is indicated; core histones are indicated by the bracket in (B). Note in the staining profile (B) that H1 becomes progressively dephosphorylated (faster migrating) in stationary and starved cells. Thus, the balance of endogenous H1 kinase to phosphatase activities in macronuclei seems to shift towards being mostly phosphatase in non-growing cells.

Immunological evidence for cdc2 kinase in Tetrahymena macronuclei

Using polyclonal antibodies raised against recombinant *S.pombe* p34^{cdc2} kinase, we next asked whether an immuno-crossreacting polypeptide could be detected in extracts prepared from *Tetrahymena* macronuclei. Figure 3 shows SDS−gel immunoblots of either 'total' solubilized chromatin from macronuclei (lane labeled T), the chromatin pellet (P) or supernatant fraction (S) following precipitation with magnesium (see Materials and methods and Figure 2 for details). When compared to the yeast recombinant p34 polypeptide (included as a positive control), it is clear that the chromatin supernatant fraction (which is enriched in H1 kinase activity, see Figure 2) contains a polypeptide which reacts strongly with yeast p34 antibodies. This polypeptide is not observed with preimmune control sera. Interestingly, the crossreacting polypeptide from *Tetrahymena* migrates slightly more slowly than yeast p34, roughly at 36 kd. As would be expected if the H1 kinase activity observed in macronuclear extracts is due to a polypeptide which is immunologically related to yeast p34, the immunocross-reactive band at 36 kd is enriched in soluble extracts made from macronuclear chromatin and is greatly diminished in the chromatin pellet which is essentially devoid of H1 kinase activity (see Figure 2). These data indicate that *Tetrahymena* macronuclei contain a polypeptide immunologically related to yeast cdc2 (p34) which is likely to be responsible for the

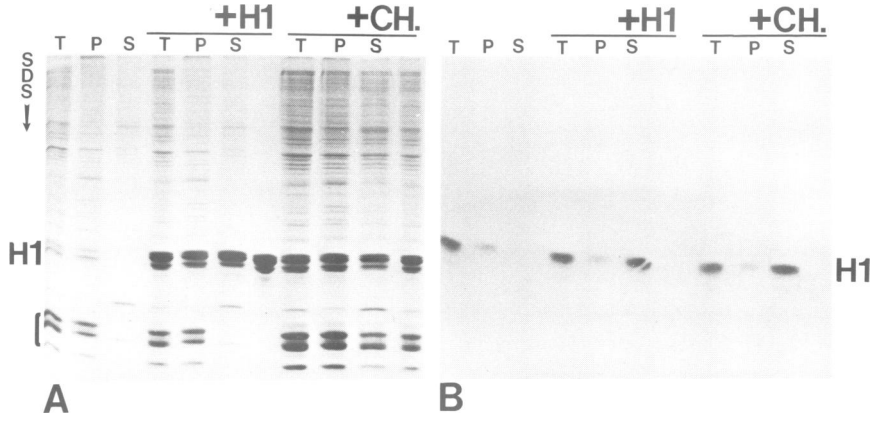

Fig. 2. Extraction of H1 kinase activity from solubilized macronuclear chromatin. Total solubilized chromatin from macronuclei (labeled T) was prepared and further fractionated by precipitation with $MgCl_2$ (see Materials and methods for details). Both the resulting chromatin precipitate (labeled P) and the supernatant (labeled S) were assayed for H1 kinase activity using purified H1 (+H1) or sucrose gradient-purified chromatin (+CH) as substrate. The first three lanes show fractions assayed in the absence of additional substrate. Proteins were fractionated by SDS−PAGE and examined by staining (**A**) and autoradiography (**B**). The position of H1 and core histones (bracket) are indicated.

H1 kinase activity seen in whole macronuclei or in macronuclear chromatin extracts.

Macronuclear H1 kinase interacts with Suc1 (p13)

The *Suc1*$^+$ gene of *S.pombe* encodes a 13 kd polypeptide which has been shown in genetic studies to act negatively upon the cell's ability to enter mitosis (Hayles *et al.*, 1986; Brizuela *et al.*, 1987; Hindley *et al.*, 1987) probably by inhibiting the dephosphorylation and therefore, activation, of cdc2 (Dunphy and Newport, 1989; Morla *et al.*, 1989). From a practical standpoint, p13 has been used as an affinity ligand to purify cdc2 kinase from a wide variety of sources owing to a specific, high affinity interaction between p13 and cdc2 (Brizuela *et al.*, 1987; Arion *et al.*, 1988; Dunphy and Newport, 1989). To investigate whether macronuclear H1 kinase activity is able to interact with p13, p13-coated Sepharose beads were incubated with macronuclear extracts. Following incubation, the beads were collected, washed by low speed centrifugation and both pellet and supernatant fractions were assayed for H1 kinase activity. Figure 4A shows that most of the starting H1 kinase activity can be pelleted following incubation with p13-beads. Sepharose beads coated with BSA fail to pellet H1 activity (data not shown), suggesting that the interaction of the macronuclear H1 kinase with p13 is specific. Finally, we asked if the 36 kd macronuclear polypeptide which crossreacts with cdc2 antibodies (see Figure 3) is pelletable following an interaction with p13 beads. As shown in Figure 4B a significant percentage of the immunoreactive 36 kd polypeptide is detected in the pellet fraction following incubation with the p13 beads. We conclude that the macronuclear H1 kinase activity interacts with p13 in a fashion analogous to cdc2 kinase in other organisms and that the 36 kd polypeptide which reacts positively with the yeast p34 antibodies is likely to be the *Tetrahymena* homolog of cdc2 kinase.

Mammalian cdc2 kinase phosphorylates macronuclear H1 in vitro

Data presented in Figures 1−4 strongly suggest that *Tetrahymena* macronuclei contain an activity very similar to cdc2 kinase from other organisms. Since macronuclei are amitotic and since macronuclear H1 is missing some of the

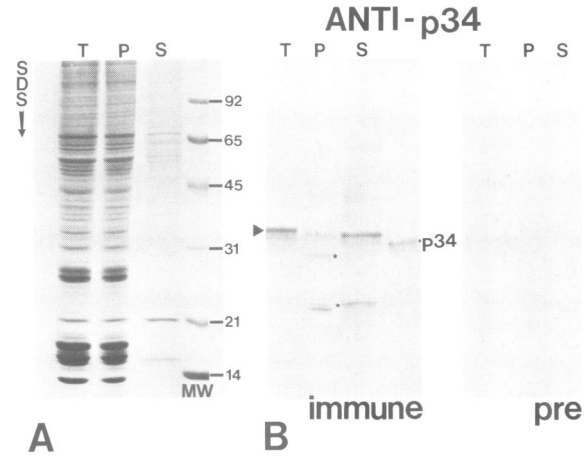

Fig. 3. Immunoblotting of fractions containing *Tetrahymena* macronuclear H1 kinase activity with antibodies specific to yeast $p34^{cdc2}$. Total solubilized chromatin from macronuclei (labeled T) as well as the chromatin precipitate (P) and supernatant (S) fractions following precipitation with $MgCl_2$ (see Figure 2) were electrophoresed on a 12% SDS−polyacrylamide gel and either stained directly (**A**) or were transferred to nitrocellulose and reacted with immune or preimmune sera raised against yeast $p34^{cdc2}$ (**B**). A sample of yeast p34 polypeptide was included in lane 4 of B as a positive control. Relative to mol. wt standards, the major immunoreactive polypeptide from *Tetrahymena* (see right-pointing arrowhead) is judged to have an apparent mol. wt of 36 kd. The identity of the faster migrating peptides in the pellet and supernatant reactions (stars) is not known, but may result from proteolysis of the 36 kd protein during sample preparation.

features of vertebrate H1s including a conserved globular domain (Wu *et al.*, 1986; Hayashi *et al.*, 1987), we were curious whether mammalian cdc2 kinase will phosphorylate macronuclear H1. H1 purified by reverse phase HPLC was reacted *in vitro* using extensively purified preparations of HeLa cdc2 kinase. Several core histones, two abundant HMG-like proteins (HMGB and HMGC, see Schulman *et al.*, 1987 for details), and two polypeptides of unknown function (here labeled Y and E) were also extracted from macronuclei, purified by HPLC, and reacted separately with HeLa cdc2 kinase under identical conditions to test the

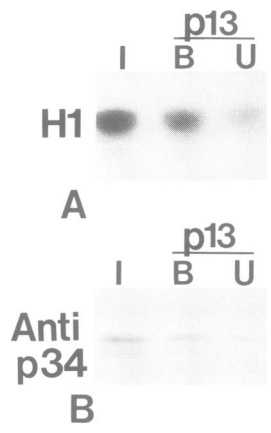

Fig. 4. Macronuclear H1 kinase activity and the 36 kd immunoreactive polypeptide from *Tetrahymena* macronuclei bind to Suc1 beads. The supernatant fraction following chromatin precipitation with $MgCl_2$ was used to test binding of macronuclear H1 kinase activity to Suc1 beads (this is the same as the fraction labeled S in Figures 2 and 3, but is labeled here as I = Input). Following a 1 h incubation with Suc1 (p13)-coated beads, beads were collected by low speed centrifugation (B = Bound) and the supernatant above the beads (U = Unbound) was saved. After washing the beads twice in kinase assay buffer, all fractions were assayed for H1 kinase activity under standard conditions. Shown in (**A**) is the autoradiograph of the H1 following *in vitro* kinase reactions with these fractions. Shown in (**B**) is an immunoblot of these fractions using anti-yeast p34 antibodies. Only the region of the SDS–gel containing the 36 kd polypeptide is shown.

specificity of this reaction. As shown in Figure 5, H1 is extensively phosphorylated under these assay conditions, while little, if any, radiolabelled phosphate is incorporated into most of the other polypeptides tested. Since the polypeptides labeled Y and E are extensively phosphorylated *in vivo* (C.D.Allis, unpublished observations), these data suggest that mammalian cdc2 kinase is somewhat specific for macronuclear H1 under the *in vitro* conditions used. However, a significant amount of phosphorylation is observed with H2B even though H2B is not phosphorylated appreciably *in vivo* (Allis and Gorovsky, 1981). The significance of this result is unclear, but it serves to illustrate the difficulty in accurately identifying *in vivo* substrates of any protein kinase. Thus, other criteria need to be met to establish that macronuclear H1 is an *in vivo* substrate for cdc2 kinase (see Langan *et al.*, 1989; Moreno and Nurse, 1990; Pines and Hunter, 1990).

HeLa cdc2 and Tetrahymena macronuclear H1 kinase phosphorylate H1 with a high degree of fidelity

Recently, the sites of *in vivo* phosphorylation in macronuclear H1 have been described (Roth *et al.*, 1988) and interestingly several of these contain the motif [Thr-Pro-X-Lys], which is often used by cdc2 kinase (Langan *et al.*, 1989; Moreno and Nurse, 1990). To determine how well the sites of H1 phosphorylation *in vitro* (i.e. using HeLa cdc2 kinase and the extracted macronuclear kinase) match the *in vivo* sites of phosphorylation, high resolution tryptic maps were prepared using H1 phosphorylated both *in vitro* and *in vivo*. As shown in Figure 6, excellent agreement is observed in the phosphotryptic maps obtained from H1 phosphorylated *in vivo* (lane 1) and *in vitro* using either *Tetrahymena* (lane 2) or HeLa cdc2 kinase (lane 3) preparations. Furthermore, in all reactions phosphorylation occurred largely on threonine

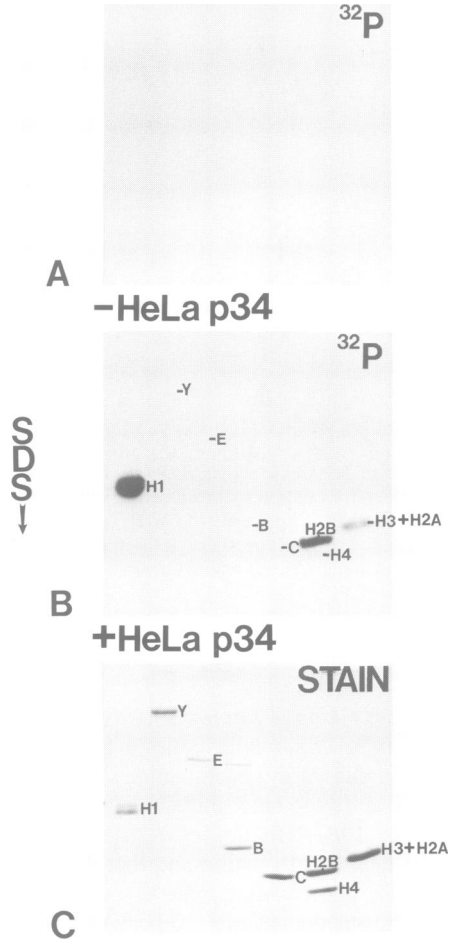

Fig. 5. HeLa $p34^{cdc2}$ kinase phosphorylates macronuclear H1 *in vitro*. Histone and several non-histone chromosomal proteins were extensively purified by reverse phase HPLC and incubated with (**B**) or without (**A**) HeLa $p34^{cdc2}$ kinase under standard H1 kinase assay conditions except that [γ-^{32}P]ATP was used. Following incubation, proteins were precipitated, electrophoresed on an SDS gel and examined by staining (**C**) and autoradiography (A and B). All four of the core histones were purified and assayed individually, but were pooled as pairs (H2B + H4 and H2A + H3) prior to electrophoresis because of lane limitations. One-dimensional SDS–gel analysis of each individual fraction indicated that H3 is the histone which is slightly phosphorylated in the last lane of (**B**). Y and E are two non-histone chromosomal proteins which are extensively phosphorylated *in vivo* (see text for details).

residues in the amino-terminal 'half' of H1 following cleavage with cyanogen bromide (data not shown; a single central methionine is present in *T.thermophila* H1; see Roth *et al.*, 1988, for details and the sequence of macronuclear H1). Although some minor differences in the tryptic maps can be observed (see small arrows in Figure 6), we conclude that mammalian cdc2 kinase and the extracted *Tetrahymena* activity phosphorylate macronuclear H1 *in vitro* with remarkable fidelity.

Discussion

Macronuclei divide without mitotic chromosome condensation. However, macronuclear H1 is extensively phosphorylated during most periods of the *Tetrahymena* life cycle (see Roth *et al.*, 1988). Thus, it was of interest to determine if an activity closely related to cdc2 kinase was

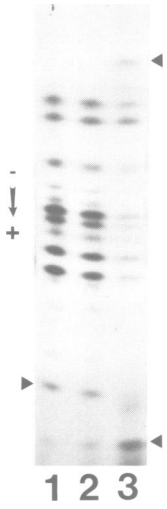

Fig. 6. Tryptic phosphopeptide maps of macronuclear H1 phosphorylated *in vivo* and *in vitro* by HeLa $p34^{cdc2}$ kinase. Macronuclear H1 phosphorylated *in vivo* (lane 1) or *in vitro* by extracted macronuclear H1 kinase (lane 2) or HeLa $p34^{cdc2}$ kinase (lane 3) was recovered and trypsinized as described by Roth *et al.* (1988). Tryptic phosphopeptides were then separated in a 40% alkaline acrylamide gel as described by West *et al.* (1984) and visualized by autoradiography. Arrowheads point to minor differences between samples.

responsible for its phosphorylation. In this study we have shown that macronuclei contain a 36 kd polypeptide which is immunologically related to *S.pombe* p34 and which is extracted from macronuclei using methods that solubilize a significant percentage of the H1 kinase activity from macronuclear chromatin. Also, this polypeptide and the macronuclear H1 kinase activity reacts with suc1 (p13) beads in a manner identical to mammalian cdc2 kinase. Furthermore, we show that mammalian cdc2 kinase will phosphorylate H1 from *Tetrahymena* macronuclei with extremely high fidelity when compared to macronuclear H1 phosphorylated *in vivo*. Taken together, these data strongly suggest that *Tetrahymena* macronuclei contain an H1 kinase closely related to the cdc2 kinase described in yeast, frogs and mammalian cells, and suggest that H1 phosphorylation by cdc2 kinase may have physiological function(s) other than mitotic chromosome condensation.

One attractive alternative function for H1 phosphorylation by cdc2 kinase is a mechanism for the rapid and reversible modulation of gene expression (Roth *et al.*, 1989) during different phases of the cell cycle and under different conditions of cell growth. Histone H1 is known to play a central role in formation of higher order chromatin structures which are thought to be transcriptionally inactive (Thoma *et al.*, 1979; Allan *et al.*, 1980; Allan *et al.*, 1986; Thoma, 1988; van Holde, 1989) and numerous reports have suggested that the amount of H1 is modified, reduced or missing altogether in regions of active chromatin (Weintraub, 1984, 1985; Gross and Garrard, 1987). However, the issue of the presence of H1 in active chromatin remains controversial (see Nacheva *et al.*, 1989; Hill *et al.*, 1989; Wolffe, 1989; Dedon *et al.*, 1991; Ericsson *et al.*, 1990).

The sites of H1 phosphorylation in vertebrate H1 are localized in the basic amino- and carboxy-terminal domains (see, Hohmann, 1983; Langan *et al.*, 1989, for references), which are regions of H1 shown experimentally to be involved in the condensation of chromatin (Allan *et al.*, 1986). Although macronuclear H1 lacks a central globular domain, these basic 'tail' regions are present. Phosphorylation in these domains usually occurs on serine or threonine residues located in a cdc2 kinase consensus motif [Lys-Ser/Thr-Pro-Lys] or [Lys-Ser/Thr-Pro-X-Lys]. Suzuki and associates (Churchill and Suzuki, 1989; Suzuki, 1989a,b and 1990) have suggested that these sequences, when dephosphorylated, may form β-turn secondary structure which binds to the minor groove of DNA. As suggested by Suzuki (1989a), phosphorylation of Ser/Thr in this domain is likely to disrupt this conformation, thereby weakening the interaction of these sequences with DNA. Experimental evidence consistent with this idea has recently been provided (Suzuki *et al.*, 1990; Hill *et al.*, 1990). Whether changes of this type cause chromatin to condense or decondense is not known, but we speculate that such a mechanism could be exploited by the cell to regulate gene expression rapidly and transiently in restricted chromatin domains (see Roth *et al.*, 1988).

Regardless of its exact cellular function(s), the notion that H1 phosphorylation promotes mitotic chromosome condensation needs to be re-evaluated given that an activity which strongly resembles mammalian cdc2 kinase has now been identified in the amitotic macronucleus of *Tetrahymena*. Our data suggest that H1 phosphorylation by a cdc2-like kinase may be necessary for mitotic chromosome condensation, but is not sufficient by itself. Again, differences in the structure of macronuclear H1 and vertebrate H1s could partially explain this difference, but the conservation of the amino- and carboxyl-terminal phosphorylation domains thought to be involved in chromatin condensation make this appear less likely. Clearly, the absence of other yet unidentified cdc2-like kinase substrates in macronuclei and/or altered cdc2-like regulatory subunits (cyclins, for example) in macronuclei, may explain why macronuclei do not display true mitotic divisions and yet have an activity which closely resembles mammalian cdc2 kinase. Along this line we point out that in *S.cerevisiae*, although a spindle-like structure does form, mitotic chromatin condensation and nuclear envelope breakdown are not observed. In addition, H1 has not been positively identified in this yeast (Grunstein, 1990). Nonetheless, CDC 28 kinase is still required for this nuclear division. In the future, it will be interesting to evaluate the situation of cdc2-like kinases in *Tetrahymena* micronuclei since these nuclei are mitotic and since they contain a unique set of phosphorylated, linker-associated histone polypeptides (Allis and Gorovsky, 1981). Thus, *Tetrahymena* maintains two seemingly independent nuclear division cycles, one mitotic and one amitotic, during each vegetative cell cycle. We would like to know how many $p34^{cdc2}$-like kinases (genes) act to coordinate these events.

It is clear that *cdc2*$^+$/*CDC28* genes regulate progression through the cell cycle at points other than the G_2/M boundary (see, Hartwell and Weinert, 1989; Blow and Nurse, 1990; Pines and Hunter, 1990; for references) and recently, Pines and Hunter (1990) have discussed the issue of multiple $p34^{cdc2}$ M and S phase kinases. It is also becoming clear that cdc2 kinase activity is regulated by a family of cyclin proteins. Mitotic and G_1-specific cyclins have been identified in *S.cerevisiae* which associate with cdc2/cdc28 and control its activity at multiple points in the cell cycle (Wittenberg *et al.*, 1990). Although the presence of mitotic

cyclins appears unlikely in *Tetrahymena* macronuclei, the macronuclear cdc2-like kinase may be regulated by cyclins whose accumulation/degradation accompanies stationary versus active cell growth. We do not at present have any data regarding the existence of such proteins in macronuclei, but the loss of H1 kinase activity that we observe upon starvation and upon increased cell density (Figure 1), conditions under which cell division is halted or slowed, is consistent with some form of regulation. Hyper-phosphorylation of H1 in macronuclei following heat shock, and changes in H1 phosphorylation during conjugation also indicate that this activity may be regulated in response to different physiological conditions (Roth *et al.*, 1988). Significant levels of H1 phosphorylation and H1 kinase activity have been detected in G_1, S, and G_2 phases of the mammalian cell cycle before mitotic hyperphosphorylation begins (see Hohmann, 1983 for review).

Understanding how this phosphorylation is regulated, and in turn, how H1 phosphorylation regulates chromatin structure should provide important insights into the role(s) of H1 phosphorylation. It seems likely from our data that in *Tetrahymena* macronuclei and in mammalian cells, H1 phosphorylation serves functions outside of mitotic chromosome condensation.

Materials and methods

Cell culture and preparation of nuclei

Tetrahymena thermophila strain CU428 (Chx/Chx-[cy-S] VII) were grown under standard conditions as previously described (Gorovsky *et al.*, 1975). To starve cells, growing cultures were collected by centrifugation at 1200 *g*, resuspended in 10 mM Tris−HCl, pH 7.4, and incubated with shaking for 16−24 h at 30°C. Macronuclei were isolated as described by Gorovsky *et al.* (1975) except that the isolation buffer did not contain spermidine but did contain 10 mM Tris−HCl, pH 7.4, 1 mM iodoacetamide, 1 mM PMSF, and 10 mM sodium butyrate.

Assay of kinase activity in isolated nuclei

Isolated macronuclei were incubated in reaction buffer consisting of 0.25 M sucrose, 0.02 M glucose, 0.005 M $MgCl_2$, and 0.01 M Tris−HCl, pH 8.0 (Hayashi *et al.*, 1987) at a density of 1×10^8 nuclei per ml. After 5 min of equilibration at 22°C, reactions were initiated by the addition of [γ-^{35}S]ATP (15 μCi per 0.1 ml reaction) in combination with unlabeled [γ-S]ATP such that the final concentration of [γ-S]ATP in the reaction was 10 μM. At appropriate times, reactions were stopped by mixing with an equal volume of 0.8 N H_2SO_4. Histones and other acid-soluble proteins were extracted by shaking at 4°C for at least 30 min. Nuclear debris was then removed by centrifugation in a microfuge. Total acid-soluble proteins were precipitated by the addition of trichloroacetic acid (TCA) to 20% from a 100% (w/v) stock solution. Alternatively, perchloric acid (PCA)-insoluble proteins were precipitated by the addition of perchloric acid to 5% (v/v) and collected by centrifugation. PCA-soluble proteins, including histone H1, were then precipitated from the resulting supernate with TCA as described above. Extracted proteins were separated by SDS−PAGE as previously described (Laemmli, 1970). Proteins were visualized by staining in Coomassie blue, and labeled proteins were detected by autoradiography.

Extraction of H1 kinase activity from isolated nuclei

Isolated nuclei were resuspended in RSB (10 mM Tris−HCl, pH 7.5, 3 mM $MgCl_2$, 10 mM NaCl, and 1 mM PMSF) containing 1 mM $CaCl_2$ at a density of 2×10^8 nuclei/ml and were digested with micrococcal nuclease (25 units per 10^8 nuclei) at 22°C for 20−30 min. Typically 5−10% of the total nucleic acid was rendered acid-soluble under these conditions. Following digestion, nuclei were collected by centrifugation at 1000 *g* at 4°C for 5 min and were gently resuspended in 10 mM EDTA, pH 8.0. Nuclear lysis occurred during 10 min of incubation in EDTA on ice. Nuclear debris was then removed by centrifugation at 1000 *g* at 4°C for 5 min. The cleared supernate typically contained 50−80% of the total chromatin. Chromatin was precipitated by the addition of $MgCl_2$ to 10 mM from a 1.0 M stock and incubation on ice for 10−15 min. The chromatin precipitate was removed by centrifugation at 1500 *g*. Protein content of the initial solubilized chromatin fraction, the final supernatant and the chromatin pellet (redissolved in 10 mM EDTA) was measured using the BioRad protein assay reagent, and DNA content was measured by absorbance at 260 nm. Typically, more than 95% of the total protein and 100% of the total DNA present in the chromatin fraction was precipitated by the addition of $MgCl_2$, while 50−70% of the H1 kinase activity, assayed as described below, remained soluble.

Assay of extracted H1 kinase activity

H1 kinase activity in the macronuclear extract was measured by incubating 20 μl of the extract with 10 μl of concentrated reaction buffer (10 mM Tris, pH 8.0, and 50 mM $MgCl_2$) and substrate (20 μg of PCA-soluble, PCA-insoluble, or TCA-insoluble proteins or 5 μg of purified H1). Reaction volumes were adjusted to 100 μl with deionized H_2O and reactions were initiated by the addition of a mixture of labeled and unlabeled [γ-S]ATP such that each reaction contained 15 μCi [γ-^{35}S]ATP and 10 μM [γ-S]ATP. Incorporation of label into H1 was linear under these conditions at 22°C for up to 60 min. Typically, reactions were stopped after 30 min by the addition of 25 μl 100% (w/v) TCA. Histones were prepared as described below and were analyzed by SDS−PAGE. Incorporation of label into H1 was monitored by autoradiography of stained, dried gels as described above. Quantification of total label incorporation was also monitored by collection of TCA precipitates by centrifugation. After washes in 20% TCA, acidified acetone, and acetone, dried precipitates were redissolved, and aliquots were counted with Spectrofluor. All reactions, monitored by autoradiography or by scintillation counting, were compared to a control reaction performed in the absence of extract.

To assay the effects of various compounds on kinase activity, various concentrations of each effector compound were added immediately prior to addition of [γ-^{35}S]ATP under the reaction conditions described above. In experiments involving inhibitors of cAMP-dependent protein kinases, authentic cAMP protein kinase (Sigma) was assayed in parallel experiments using H1 or casein (Sigma) as substrate. Effectors tested included $CaCl_2$ (0.001−100 μM), cAMP and cGMP (0.1−10 000 μM), and the cAMP kinase regulatory subunit (Sigma) (0.1−10 units). Concentrations of effectors tested were chosen based on reported effects on other kinase activities *in vitro*. All reactions were compared to control reactions lacking effector or lacking extract, as described above. Each experiment was repeated at least twice.

Preparation of substrates

PCA-soluble, macronuclear proteins were prepared as described previously (Schulman *et al.*, 1987). *Tetrahymena* H1 was purified from PCA-soluble macromolecular extracts by HPLC using a reverse phase C8 column with a linear gradient of 0−60% acetonitrile. Fractions containing H1 were identified by SDS−PAGE and were subsequently pooled and lyophilized. Chromatin was prepared as substrate by micrococcal nuclease digestion and lysis of macronuclei as described above followed by centrifugation through linear 5−20% (w/v) gradients of sucrose (in 10 mM Tris, pH 7.5, 1 mM EDTA, and 1 mM PMSF) in a Beckman type SW41 rotor at 36 000 r.p.m. for 12−16 h at 4°C. Pilot experiments showed that most of the H1 kinase activity does not cosediment with chromatin oligonucleosomes under these gradient conditions. These results suggest that the H1 kinase activity in *Tetrahymena* is probably not tightly bound to chromatin. Therefore, fractions containing oligonucleosomes, as determined by agarose electrophoresis, were pooled for use as substrate.

Analysis of phosphorylation sites

H1 was labeled by the H1 kinase as described above except that [γ-^{32}P] ATP was used instead of [γ-^{35}S]ATP to facilitate detection of phosphopeptides and phosphoamino acids. *In vivo* labeling of H1, cyanogen bromide cleavage, tryptic mapping phosphorylation sites and phosphoamino acid analysis were performed as described previously (Roth *et al.*, 1988).

Purification of HeLa p34 kinase

HeLa cdc2 kinase was prepared exactly as described by Brizuela *et al.* (1989).

Preparation of antisera and immunoblotting assays

The preparation of affinity-purified anti-34^{cdc2} polyclonal antibodies has been described previously (Draetta *et al.*, 1987). The specificities of preimmune and immune sera were assayed by immunoblotting analyses. Protein samples fractionated on SDS−polyacrylamide gels were transferred electrophoretically to nitrocellulose. Blots were routinely stained with Ponceau red to evaluate transfer efficiency, blocked with non-fat dry milk and incubated with immune or preimmune sera (all at 1/250 dilution). Antibody reactions were detected by peroxidase-conjugated goat anti-rabbit secondary antibodies (1/2000 dilution) followed by a color reaction using 4-chloro-1-napthol as substrate.

Preparation and assays using p13-Sepharose

p13 was purified and conjugated to CNBr-activated Sepharose 4B as dcescribed by Arion *et al.* (1988). Approximately 10 μl of p13 beads were added to 20 μl of the crude macronuclear H1 kinase extract: (generated by the $MgCl_2$ precipitation of solubilized macronuclear chromatin; see Figure 2 and Materials and methods for details). Following a 1 h incubation at 4°C, the beads were collected by low speed centrifugation and the supernatant above the beads was saved. The beads were then washed twice in kinase assay buffer and all fractions (input, pellet and supernatant) were assayed directly for H1 kinase activity. Following the reaction, samples were loaded directly onto a SDS–gel for analysis by autoradiography or by immunoblotting with the yeast p34 antiserum.

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