

## Evidence that the endogenous histone H1 phosphatase in HeLa mitotic chromosomes is protein phosphatase 1, not protein phosphatase 2A

James R. Paulson\*, Jason S. Patzlaff† and Amy J. Vallis‡

Department of Chemistry, University of Wisconsin-Oshkosh, Oshkosh WI 54901-8645, USA

\*Author for correspondence (e-mail: paulson@vaxa.cis.uwosh.edu)

†Present address: Department of Biochemistry, University of Minnesota, Minneapolis, MN 55455 USA

‡Present address: Medical College of Wisconsin, Milwaukee, Wisconsin 53226 USA

### SUMMARY

Histone H1 is highly phosphorylated in mitotic HeLa cells, but is quickly dephosphorylated *in vivo* at the end of mitosis and *in vitro* following cell lysis. We show here that okadaic acid and microcystin-LR block the *in vitro* dephosphorylation of H1 and that they do so directly by inhibiting the histone H1 phosphatase rather than by some indirect mechanism. The concentrations of microcystin and okadaic acid required for inhibition strongly suggest that the histone H1 phosphatase is either PP1 or an unknown protein phosphatase with okadaic acid-sensitivity similar to PP1. The histone H1 phosphatase is predominantly located in chromosomes with at most one copy for every 86 nucleosomes. This tends to support its identification as PP1, since localization in mitotic chromosomes is a characteristic of PP1 but not of the other known okadaic acid-sensitive protein phosphatases.

We also show that treatment of metaphase-arrested HeLa cells with staurosporine and olomoucine, inhibitors of p34<sup>cdc2</sup> and other protein kinases, rapidly induces reassembly of interphase nuclei and dephosphorylation of histone H1 without chromosome segregation. This result indicates that protein kinase activity must remain elevated to maintain a mitotic block. Using this as a model system for the M- to G<sub>1</sub>-phase transition, we present evidence from inhibitor studies suggesting that the *in vivo* histone H1 phosphatase may be either PP1 or another phosphatase with similar okadaic acid-sensitivity, but not PP2A.

Key words: Chromatin, Microcystin-LR, Mitosis, Okadaic acid, Protein phosphorylation

### INTRODUCTION

Histone H1 becomes highly phosphorylated at the onset of mitosis in mammalian cells and is normally dephosphorylated after anaphase (Gurley et al., 1978). Although H1 remains highly phosphorylated in metaphase-arrested cells, it becomes dephosphorylated rapidly after the cells are lysed, for instance during isolation of metaphase chromosomes (D'Anna et al., 1978; Paulson, 1980).

Phosphorylation of histone H1 at the beginning of mitosis has been extensively studied and the mitotic histone H1 kinase (p34<sup>cdc2</sup>) has been identified as a component of MPF, the protein which triggers mitosis and meiosis in a variety of systems (Norbury and Nurse, 1992). By contrast, dephosphorylation of H1 at the end of mitosis has been studied very little, and the phosphatase involved has been neither identified nor characterized. As has been pointed out (Brautigan, 1994; Shenolikar, 1994), it is often assumed that protein phosphatases (including the H1 phosphatase) are non-specific and constitutive, and therefore less interesting than protein kinases, but there is no evidence for this.

The aim of our work is to identify the phosphatase which dephosphorylates histone H1 at the end of mitosis *in vivo* and in isolated metaphase chromosomes *in vitro*, and to understand

how, if at all, the phosphatase is controlled. For *in vitro* studies, in order to have the best chance of detecting the *in vivo* 'mitotic histone H1 phosphatase', we use histone H1 in mitotic chromatin as substrate (i.e. a substrate as close as possible to the *in vivo* substrate) and mitotic cells as the source of enzyme. This approach is very different from that used in previous studies of histone H1 dephosphorylation (e.g. Sola et al., 1991; Kinoshita et al., 1991; Agostinis et al., 1992; Ferrigno et al., 1993; Mayer-Jaekel et al., 1994).

In the work reported here, we have tested the ability of okadaic acid, microcystin-LR, and other protein phosphatase inhibitors to prevent H1 dephosphorylation following lysis of metaphase-arrested HeLa cells. These reagents are well known as inhibitors of protein phosphatases 1 and 2A (PP1 and PP2A) (Bialojan and Takai, 1988; Cohen et al., 1990; MacKintosh et al., 1990; Honkanen et al., 1990), and genetic and biochemical studies have demonstrated roles for PP1 and PP2A in mitosis (e.g. Cyert and Thorner, 1989; Axton et al., 1990; Fernandez et al., 1992; Vandre and Wills, 1992). However, in most cases the substrates are not known.

Our results show that both okadaic acid and microcystin-LR, at appropriate concentrations, inhibit virtually all of the histone H1 phosphatase activity in metaphase chromosomes and lysates. This clearly demonstrates involvement of PP1, PP2A

or another microcystin- and okadaic acid-sensitive phosphatase. A study of the concentrations of the two inhibitors required to prevent H1 dephosphorylation suggests that PP1 accounts for the majority of the H1 phosphatase activity *in vitro*.

We also show that H1 dephosphorylation is induced *in vivo* by treating metaphase-arrested HeLa cells with the protein kinase inhibitors staurosporine and olomoucine. Dephosphorylation of H1 in these cells is likely to be occurring by a similar process as at the end of normal mitosis, since it is accompanied by reassembly of nuclei, and so we have used this as a model system to begin to characterize the *in vivo* H1 phosphatase.

## MATERIALS AND METHODS

### Materials

Okadaic acid and calyculin A (Moana BioProducts, Honolulu, HI) were obtained in sealed vials as 100 µg/ml solutions in dimethylformamide and methanol, respectively, and stored at 2°C. Okadaic acid was used within one month of purchase and the sealed vial was not opened until immediately before use. Microcystin-LR (Sigma Chemical Co., St Louis, MO) was prepared as a 1 mM stock solution in 50 mM Tris-HCl, pH 7.0, and stored frozen in aliquots at -20°C. Cantharidin (Sigma) was dissolved at 0.2 M in dimethylformamide and stored at 2°C. Trifluoperazine (Sigma) was dissolved in lysis buffer immediately before use. Stock solutions of staurosporine (Sigma) and olomoucine (6-benzylamino-2-(2-hydroxyethylamino)-9-methylpurine) (Apex Organics, Honiton, Devon, UK) were prepared in dimethyl sulfoxide at 1 mM and 0.4 M, respectively, and stored at -20°C. Tissue culture media and components were obtained from Gibco BRL (Grand Island, NY), and all other reagents were obtained from Sigma unless otherwise noted.

### Cell culture and metaphase arrest

A mycoplasma-free strain of suspension-adapted HeLa cells, designated H-HeLa (Medappa et al., 1971), was obtained from R. Rueckert at the University of Wisconsin-Madison and grown in Eagle's MEM with Earle's salts, synchronized by treatment with thymidine, and arrested in metaphase with nocodazole as previously described (Paulson et al., 1994). Cell viability was determined using Trypan blue (Paulson et al., 1994). To determine mitotic index, 100 µl of a culture was mixed with 100 µl of water containing 20 µg/ml Hoechst 33342. The cells were allowed to swell for 5 minutes at room temperature, treated with 20 µl of freshly prepared fixative (3 volumes methanol, 1 volume acetic acid), and viewed by epifluorescence. Cells containing dispersed, individual condensed chromosomes were scored as mitotic; cells in which the chromatin exhibited a smooth border indicative of a nuclear envelope were scored as interphase. In the staurosporine experiments, it was determined that the amount of nuclear reassembly which occurs during the 5 minute swelling step is no more than that which occurs during 2 minutes of additional incubation of the culture at 37°C.

### Preparation of lysates and metaphase chromosome clusters

At 18-20 hours after release from a thymidine block, metaphase-arrested cells were chilled on ice, pelleted, washed, lysed at 10<sup>7</sup> cells per ml in cold lysis buffer (10 mM Hepes, pH 7.4, 10 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.5 M sucrose and 0.1% Nonidet P40), and disrupted with a Dounce homogenizer as in the isolation of crude chromosome clusters (Paulson, 1982). This crude homogenate was used directly for studies of histone dephosphorylation in lysates.

For rapid isolation of metaphase chromosomes, cells were lysed in

the cold (as above) and chromosome clusters (Paulson, 1982) were pelleted (1,400 g, 4 minutes in a swinging bucket rotor) through a 10 ml layer of 10 mM Hepes, pH 7.4, 10 mM NaCl, 5 mM MgCl<sub>2</sub>, 1.2 M sucrose and 0.1% Nonidet P40 at 2°C. Chromosome clusters were resuspended in cold resuspension buffer (10 mM Hepes, pH 7.4, 10 mM NaCl, 5 mM MgCl<sub>2</sub>, and 0.1% Nonidet P40), usually at a concentration corresponding to 10<sup>7</sup> cells/ml. Lysates and chromosome clusters were kept on ice and used within 10 minutes after cell lysis for dephosphorylation experiments.

### In vitro dephosphorylation experiments

For the experiments shown in Figs 1 and 2, portions of lysates or chromosome preparations were diluted with prewarmed (37°C) resuspension buffer containing various amounts of inhibitors, incubated at 37°C, and agitated at frequent intervals to keep chromosomes in suspension. At various times, samples (each containing the chromosomes from 4×10<sup>6</sup> cells) were removed, treated with 0.8 ml cold resuspension buffer containing 2 mM *p*-chloromercuriphenyl sulfonate to stop further dephosphorylation (Paulson, 1980) and placed on ice. After 15 minutes on ice, chromosomes in these samples were pelleted, washed with resuspension buffer, and extracted with 40 µl 0.2 M HCl (Paulson et al., 1992). Extracts were neutralized and the phosphorylation state of histone H1 analyzed on Hepes/histidine minigels containing 10% acrylamide, 0.267% piperazine diacrylamide (Pierce, Rockford, Illinois) and 8 M urea as previously described (Paulson et al., 1992).

The experiment shown in Fig. 5 was similar, except that each sample consisted of the chromosomes from 1-1.5×10<sup>7</sup> cells and samples were treated with correspondingly more *p*-chloromercuriphenyl sulfonate to stop the reaction. After pelleting the chromosomes, histones were extracted with 1 ml 0.2 M H<sub>2</sub>SO<sub>4</sub>, precipitated overnight with 4 ml ethanol at -20°C (Paulson, 1980), and analyzed by electrophoresis on acid-urea gels (16 cm long × 0.75 mm thick) containing 15% acrylamide, 0.1% *N,N'*-methylene bisacrylamide and 2.5 M urea (Panyim and Chalkley, 1969).

All gels were stained with 0.1% Coomassie brilliant blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid and destained in 5% (v/v) methanol and 10% (v/v) acetic acid.

### Dephosphorylation experiments with chromosomes containing <sup>32</sup>P-labelled proteins

For the experiments shown in Figs 3 and 4, metaphase chromosome clusters were isolated rapidly in the cold, as described above, and proteins were labelled by incubating the isolated chromosome clusters for 3 minutes in resuspension buffer with 30 µCi/ml carrier free [γ-<sup>32</sup>P]ATP. In this procedure, histones H1 and H3 become labelled at the same sites phosphorylated in mitotic cells *in vivo* (Paulson and Taylor, 1982). After a 3 minute labelling period, chromosomes were diluted into prewarmed (37°C) resuspension buffer containing 1.5 mM ATP, with or without 1.5 µM microcystin. Samples were taken at various times and treated with *p*-chloromercuriphenyl sulfonate to block further dephosphorylation. Histones were extracted with 0.2 M H<sub>2</sub>SO<sub>4</sub>, precipitated with ethanol, and analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli and Favre, 1973) on gels 16 cm long containing 18% acrylamide and 0.12% *N,N'*-methylene bisacrylamide. After staining with Coomassie blue R250 and destaining, the relative extent of phosphorylation was determined by counting Cerenkov radiation in the excised bands and correcting for the amount of protein. The relative amount of protein was determined by extracting the Coomassie blue stain in the excised band with 1 ml dimethyl sulfoxide at 37°C for 20 hours and then measuring the absorbance at 560 nm. Alternatively, autoradiography was performed using intact stained gels that had been dried between cellophane sheets.

### Estimation of the extent of phosphatase inhibition and determination of parameters

Experiments similar to that shown in Fig. 1 were carried out using

various okadaic acid and microcystin-LR concentrations and time points of 4, 8, 15, 30, 60 and 120 minutes. For each inhibitor concentration, a delay factor was obtained by estimating the number of time points by which dephosphorylation was delayed relative to a control run at the same time. For example, if dephosphorylation was delayed from 15 to 120 minutes (i.e. 3 time points), the delay factor would be 8 ( $=120/15$ ). When the delay clearly fell between two time points, the delay factor was calculated as a geometric average. Residual phosphatase activity was determined as the reciprocal of the delay factor (in the above example, 0.125; i.e. phosphatase activity is assumed to be one eighth of the control since dephosphorylation took 8 times as long). Error bars in Fig. 2 reflect the fact that the delay can only be estimated to the nearest time point.

To obtain values for  $E_0$ , the total enzyme concentration, and  $K_i$ , the apparent dissociation constant, we used the equation:

$$I_0 = f(r) = \left( E_0 + \frac{K_i}{r} \right) (1-r),$$

where  $I_0$  is the total inhibitor concentration and  $r$  is the residual phosphatase activity as a fraction of the control. This equation follows from the fact that  $K_i = (E \times I) / EI$ ,  $r = E / E_0$ ,  $E + EI = E_0$ , and  $I + EI = I_0$ , where  $E$ ,  $I$  and  $EI$  are the concentrations of free enzyme, free inhibitor, and enzyme-inhibitor complex, respectively.

SigmaPlot Windows (Version 1.02, Jandel Scientific, San Rafael, California) was used to fit the function  $f(r)$  to the data for  $I_0$ , using a least squares method and a weighting factor of  $1/I_0^2$ . Since delay factors of less than 1.4 or more than 15 could not be determined, data points with  $r > 0.70$  or  $r < 0.06$  were excluded from the calculation.

### In vivo dephosphorylation experiments

Treatments of metaphase-arrested cells with staurosporine and olomoucine (cf. Figs 6, 7 and 8) were carried out using 20 ml suspension cultures in small Erlenmeyer flasks as described by Paulson et al. (1994). When a protein phosphatase inhibitor was used, it was added to the empty flask first and its solvent evaporated. Then, a measured volume of stock staurosporine or olomoucine solution was added to the flask before adding the cells. Immediately before the treatment was to begin, the cell concentration of the parent culture was adjusted to  $5 \times 10^5$  cells/ml, and a measured volume of the culture was added to the small flask, which was then placed at 37°C with magnetic stirring.

For extraction of histones, 20 ml culture aliquots containing  $10^7$  cells were diluted in 30 ml ice-cold 0.9% NaCl and the cells were immediately pelleted at 2°C. For extraction of histone H1 with 5% perchloric acid, the cell pellet was immediately resuspended in 500  $\mu$ l of 5% (w/v) perchloric acid. The extract was further processed and analyzed on Hepes-histidine gels as described by Paulson et al. (1994). For extraction of total histones, cell pellets were immediately suspended and lysed in lysis solution containing 2 mM *p*-chloromercuriphenylsulfonic acid (Paulson, 1980). After 15 minutes on ice, crude chromosomes were pelleted and histones were extracted with 0.2 M  $H_2SO_4$  (Paulson, 1980) and analyzed by electrophoresis on acid-urea gels (16 cm long  $\times$  0.75 mm thick) containing 15% acrylamide, 0.1%  $N,N'$ -methylene bisacrylamide and 2.5 M urea (Panyim and Chalkley, 1969). All gels were stained with Coomassie blue.

## RESULTS

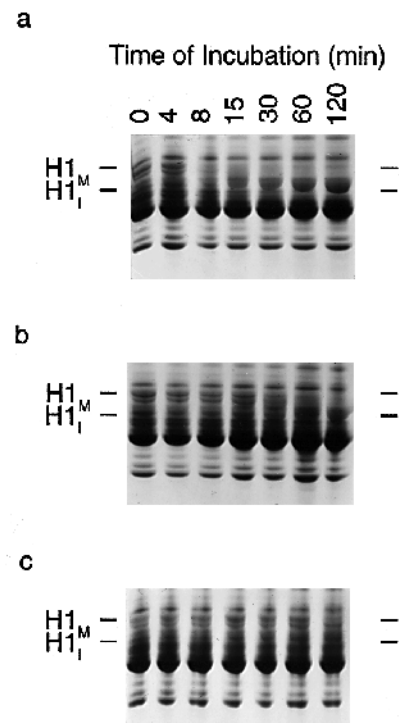
### Okadaic acid and microcystin-LR block dephosphorylation of histone H1 in vitro

Histone H1 is rapidly dephosphorylated during the isolation of metaphase chromosomes (D'Anna et al., 1978; Paulson, 1980). To characterize the endogenous phosphatases involved, we studied the effects of various specific phosphatase inhibitors on H1 dephosphorylation, both in crude lysates of metaphase-

arrested HeLa cells and in isolated metaphase chromosome clusters (Paulson, 1982).

Fig. 1 shows that dephosphorylation of histone H1 in crude lysates of metaphase-arrested cells ( $5 \times 10^5$  cells/ml) is blocked by microcystin-LR, an inhibitor of PP1 and PP2A (MacKintosh et al., 1990; Honkanen et al., 1990) and related protein phosphatases (Honkanen et al., 1991; Brewis et al., 1993; Chen et al., 1994). In the control (Fig. 1a) H1 shifts from the mitotic (phosphorylated) to the interphase (dephosphorylated) position by 8 or 15 minutes. (It will be shown below that this mobility shift is not due to proteolysis.) However, in the presence of 7.5 nM microcystin, dephosphorylation is delayed to 30 or 60 minutes (Fig. 1b), and with 20 nM microcystin it is blocked completely (Fig. 1c). Similar results are seen with isolated chromosomes.

In similar experiments, H1 dephosphorylation is also prevented by okadaic acid, calyculin A and cantharidin, inhibitors of PP1 and PP2A which are structurally unrelated to



**Fig. 1.** Time course of histone H1 dephosphorylation in crude lysates of metaphase-arrested HeLa cells in the presence of (a) no inhibitor (control); (b) 7.5 nM microcystin; and (c) 20 nM microcystin. A nocodazole-arrested culture was lysed at  $10^7$  cells/ml and aliquots were immediately diluted 20-fold into prewarmed (37°C) resuspension buffer containing various concentrations of microcystin. After 0, 4, 8, 15, 30, 60 and 120 minutes at 37°C, 8 ml samples were treated with 0.8 ml resuspension buffer containing 2 mM *p*-chloromercuriphenyl sulfonate to block further dephosphorylation (Paulson, 1980). Histones were extracted with 0.2 M HCl, subjected to electrophoresis on Hepes/histidine minigels (Paulson et al., 1992), and stained with Coomassie blue. In this and subsequent figures, the positions of the mitotic (phosphorylated) and interphase forms of histone H1 are indicated by H1<sub>M</sub> and H1<sub>I</sub>, respectively. Though marked with only one line at the left and right, H1<sub>M</sub> is sometimes resolved into two bands, presumably corresponding to H1A and H1B, the two H1 species found in HeLa cells (Paulson and Taylor, 1982). Only the portions of the gels containing the histones are shown.

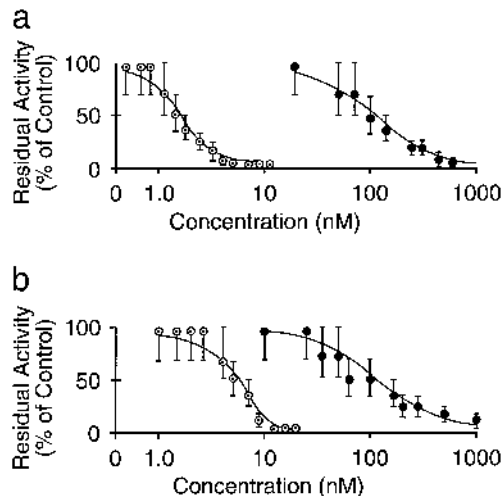
microcystin-LR (Bialojan and Takai, 1988; Cohen et al., 1990; Ishihara et al., 1989; Honkanen, 1993). However, no inhibition has been detected with 200  $\mu$ M trifluoperazine or 1 mM EGTA, specific inhibitors of PP2B (Stewart et al., 1983) (data not shown).

### Effects of various inhibitor concentrations: evidence for involvement of PP1

Microcystin-LR and okadaic acid are known to inhibit several different protein phosphatases, including PP3 (Honkanen et al., 1991), PP4 (Brewis et al., 1993), and PP5 (Chen et al., 1994), as well as PP1 and PP2A. However, okadaic acid has very different dissociation constants for these various enzymes and can thus in principle be used to distinguish between them.

Fig. 2a shows the extent of inhibition of H1 dephosphorylation in isolated chromosomes as a function of inhibitor concentration. The concentration of okadaic acid needed for 50% inhibition is about 50–100 nM, whereas the concentration of microcystin needed for 50% inhibition is only 1–2 nM. The latter result shows that the total concentration of protein phosphatases is at most a few nanomolar, so the high concentration of okadaic acid required to inhibit H1 dephosphorylation is not due to a high concentration of protein phosphatases, but must reflect involvement of a protein phosphatase such as PP1 with a high dissociation constant for okadaic acid. Similar results are seen with crude lysates (Fig. 2b).

The data in Fig. 2 were used to obtain best fit values for the inhibitor dissociation constant,  $K_i$ , and for the total enzyme concentration,  $E_0$ , in the isolated chromosomes and crude



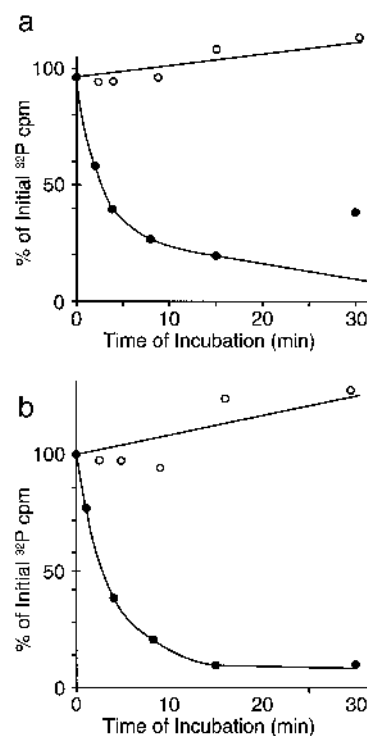
**Fig. 2.** Inhibition of histone H1 dephosphorylation as a function of inhibitor concentration for microcystin-LR (○) and okadaic acid (●). (a) Isolated metaphase chromosomes; (b) lysates of metaphase-arrested cells. Each sample point corresponds to a time course of histone H1 dephosphorylation in resuspension buffer at 37°C with a final concentration of chromosomes corresponding to  $5 \times 10^5$  cells/ml. For each sample, the extent of inhibition was obtained by estimating the extent to which dephosphorylation was delayed relative to a control, which did not contain any inhibitor, run at the same time (see Materials and Methods). The data fit best with a total enzyme concentration of  $E_0 = 2.87 \pm 0.26$  nM in isolated chromosomes and  $E_0 = 10.3 \pm 1.0$  nM in lysates,  $K_i = 0.083 \pm 0.042$  nM for microcystin, and  $K_i = 54.3 \pm 15.8$  nM and  $K_i = 71.5 \pm 13.5$  nM for okadaic acid in chromosomes and lysates, respectively.

lysates. For microcystin the data in Fig. 2a gave  $E_0 = 2.87 \pm 0.26$  nM and  $K_i = 0.083 \pm 0.042$  nM, a dissociation constant which agrees reasonably well with published values (MacKintosh et al., 1990). Using this value for the  $K_i$ , the data for microcystin in lysates (Fig. 2b) fit best with  $E_0 = 10.3 \pm 1.0$  nM. Finally, the values for  $E_0$  in chromosomes and in lysates, determined from the microcystin data, were used to fit the curves for okadaic acid. This gave best fit values of  $K_i = 54.3 \pm 15.8$  nM and  $K_i = 71.5 \pm 13.5$  nM for okadaic acid in chromosomes and lysates, respectively. These values strongly suggest involvement of PP1.

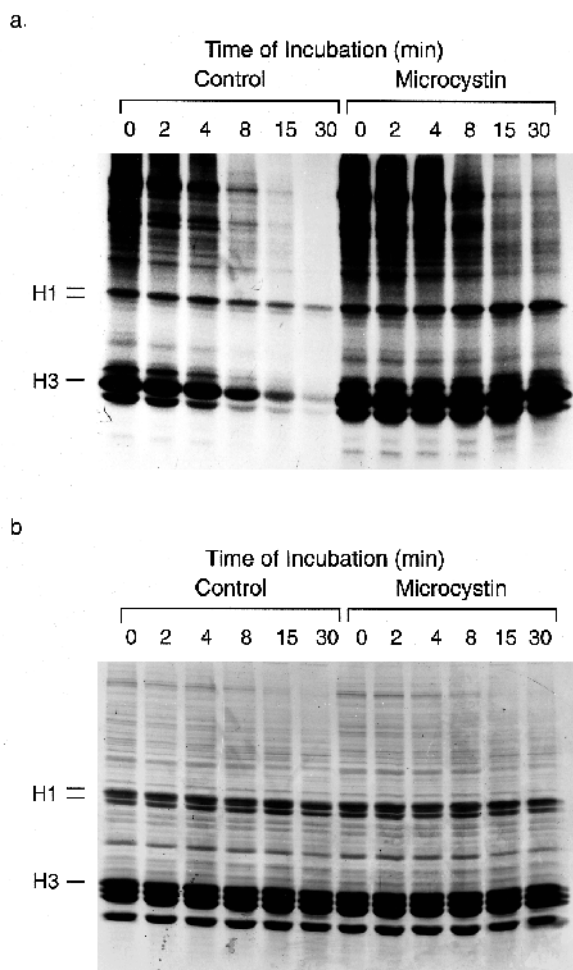
### Okadaic acid and microcystin-LR do not merely stimulate a kinase

Okadaic acid and microcystin-LR are well known protein phosphatase inhibitors, but even so, the fact that they prevent histone H1 dephosphorylation does not necessarily mean that they inhibit the histone H1 phosphatase. It could be, for example, that they cause the stimulation of a histone kinase and thus only appear to block H1 dephosphorylation. To test this possibility, we studied their effect on histone dephosphorylation in metaphase chromosomes containing  $^{32}$ P-labelled proteins.

The results shown in Fig. 3 demonstrate that microcystin indeed prevents the loss of phosphate groups from histones. Radioactivity is rapidly lost from both H1 (Fig. 3a) and H3 (Fig. 3b) in the control, but not in the presence of microcystin.



**Fig. 3.** Microcystin-LR blocks dephosphorylation of (a) histone H1 and (b) histone H3 in metaphase chromosomes labelled with [ $^{32}$ P]phosphate. Chromosomes containing  $^{32}$ P-labelled histones were incubated in the presence (○) or absence (●) of microcystin. Samples were taken at various times, extracted with acid, and separated on an SDS-polyacrylamide gel. Individual bands were excised from the gel and the relative amounts of radioactivity in each protein were determined (see Materials and Methods).



**Fig. 4.** Microcystin-LR inhibits dephosphorylation of histone H1, histone H3, and other  $^{32}\text{P}$ -labelled chromosomal proteins. Chromosomes containing  $^{32}\text{P}$ -labelled proteins were incubated in the absence (control) or presence of microcystin. Samples were taken at various times, extracted with acid, and separated on an SDS-polyacrylamide gel which was then dried and subjected to autoradiography. (a) Autoradiogram; (b) Coomassie staining of the same gel.

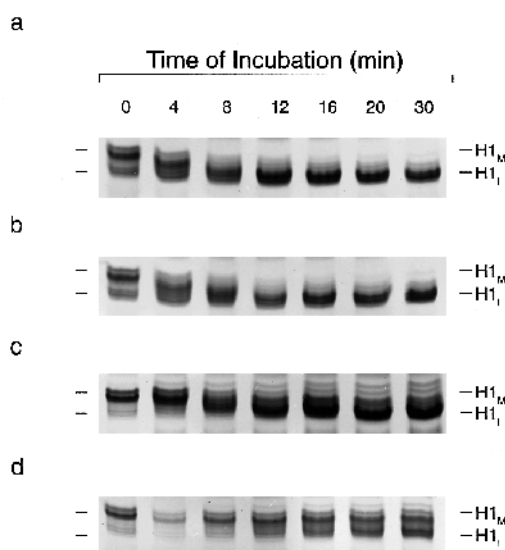
If microcystin merely stimulated a kinase,  $^{32}\text{P}$  should have been lost from the histones even in the presence of inhibitor.

Autoradiography of SDS-polyacrylamide gels (Fig. 4a) shows that microcystin also blocks dephosphorylation of several other unidentified phosphoproteins in isolated chromosomes. Several high molecular mass bands disappear from the autoradiogram at the later time points even in the presence of microcystin (Fig. 4a), but these proteins also disappear from the Coomassie stained gel (Fig. 4b) suggesting that their disappearance may be due to proteolysis rather than dephosphorylation. On the other hand, no proteolysis of histones is detectable after incubation at  $37^\circ\text{C}$  for 30 minutes (Fig. 4b). This confirms that the mobility shift of histone H1 with time in Fig. 1a is due to dephosphorylation, not proteolysis.

#### Okadaic acid and microcystin directly inhibit the histone H1 phosphatase *in vitro*

In the experiments described so far, okadaic acid and micro-

cystin were present from the beginning of the incubation at  $37^\circ\text{C}$ . One could argue that these inhibitors block H1 dephosphorylation by preventing *activation* of the H1 phosphatase. To test this possibility, chromosomes were isolated rapidly in the cold and then diluted into resuspension buffer at  $37^\circ\text{C}$ . At each time point, two samples were taken. The first was treated immediately with *p*-chloromercuriphenyl sulfonate to block further dephosphorylation (Fig. 5a). The second was treated with microcystin and then incubated for another 30 minutes at  $37^\circ\text{C}$  before being treated with *p*-chloromercuriphenyl sulfonate (Fig. 5b). If microcystin only blocked the activation of the histone phosphatase, then once dephosphorylation of H1 had begun (indicating activation of the phosphatase) addition of microcystin would not have prevented further dephosphorylation. In fact, no sample in Fig. 5b is noticeably more dephosphorylated than the corresponding sample in Fig. 5a,



**Fig. 5.** Evidence that microcystin-LR directly inhibits the histone H1 phosphatase rather than merely preventing its activation, and evidence that the enzyme is predominantly located in chromosomes. Time courses of dephosphorylation were carried out at  $37^\circ\text{C}$  and in all four incubations each 1 ml contained the chromosomes from  $5 \times 10^5$  cells. In a and b, metaphase chromosome clusters were isolated rapidly in the cold and diluted 20-fold into resuspension buffer ( $37^\circ\text{C}$ ) at  $t=0$ . Samples were taken at the indicated times and either (a) immediately treated with *p*-chloromercuriphenyl sulfonate to halt further dephosphorylation (Paulson, 1980), or (b) treated with 100 nM microcystin-LR, incubated for a further 30 minutes at  $37^\circ\text{C}$ , and then treated with *p*-chloromercuriphenyl sulfonate. In c, metaphase-arrested cells were lysed at  $10^7$  cells/ml in lysis buffer and then diluted 20-fold into resuspension buffer ( $37^\circ\text{C}$ ) at  $t=0$ . Samples were treated with *p*-chloromercuriphenyl sulfonate at the indicated times. In d, metaphase chromosome clusters were isolated in the presence of 2 mM *p*-chloromercuriphenyl sulfonate to inactivate their endogenous histone phosphatases (Paulson, 1980), washed extensively to remove unreacted mercurial, and then at  $t=0$  diluted into prewarmed ( $37^\circ\text{C}$ ) resuspension buffer to which fresh lysate supernatant from sample (a) (equivalent to  $5 \times 10^5$  cells/ml) had been added. Samples were treated with *p*-chloromercuriphenyl sulfonate at the indicated times. In all cases, histones were finally extracted with 0.2 M  $\text{H}_2\text{SO}_4$  and analyzed on acid/urea gels (Panyim and Chalkley, 1969). Only the portions of the gels containing histone H1 are shown.

indicating that microcystin directly inhibits the histone H1 phosphatase.

### Localization of the histone H1 phosphatase in isolated chromosomes

Dephosphorylation of histone H1 proceeds at essentially the same rate in lysates with  $5 \times 10^5$  cells/ml (Fig. 5c) and in isolated chromosome preparations containing the same concentration of chromatin (Fig. 5a). This suggests that the histone phosphatase is a chromosomal component rather than a soluble enzyme in the lysate. To further test this possibility, chromosomes were isolated in the presence of *p*-chloromercuriphenyl sulfonate to inactivate their endogenous phosphatases (Paulson, 1980; Paulson et al., 1992), washed extensively to remove unreacted mercurial, and then diluted into prewarmed (37°C) resuspension buffer containing lysate supernatant (from the chromosome preparation used in Fig. 5a) to give a final concentration equivalent to  $5 \times 10^5$  cells/ml. Dephosphorylation of H1 was then followed as a function of time (Fig. 5d).

It is clear that dephosphorylation is much slower in Fig. 5d than in any of the other samples. For example, at the 12 minutes time point, dephosphorylation of H1 is nearly complete in Fig. 5a,b and c, but it has barely begun in Fig. 5d. This confirms that most of the H1 phosphatase activity pellets with the chromosomes and relatively little activity remains in the supernatant.

### Abundance of the histone H1 phosphatase in isolated metaphase chromatin

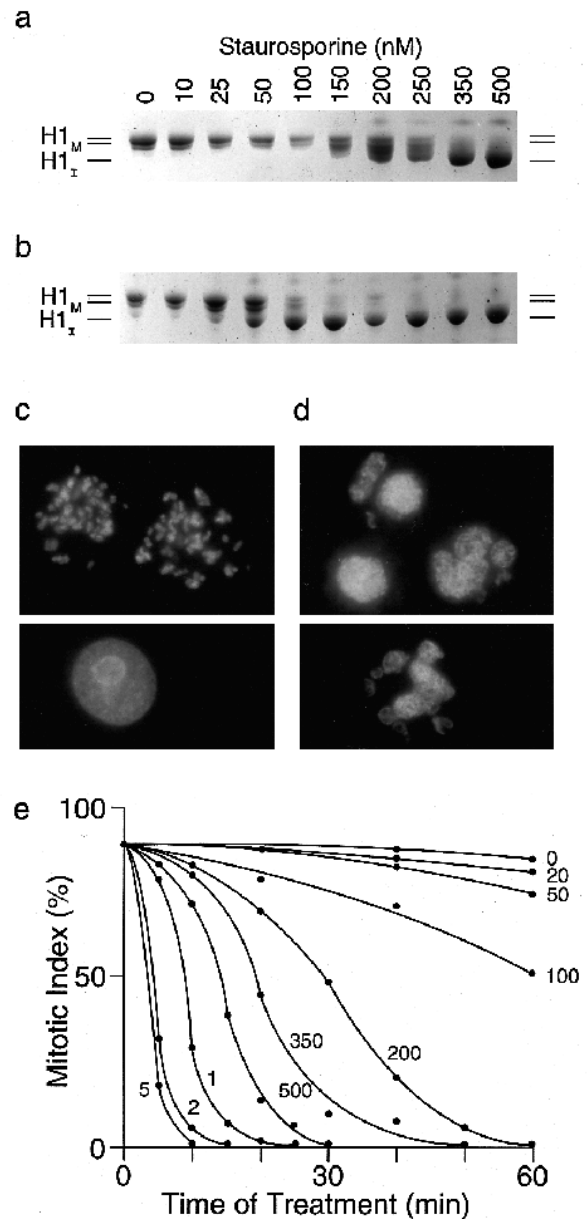
In order to estimate the amount of histone H1 phosphatase in chromosomes, an experiment similar to that in Fig. 2a was carried out using microcystin and a concentrated suspension of isolated chromosomes containing 1 mg/ml DNA (20  $A_{260}$  units/ml) (data not shown). Assuming a  $K_i$  for microcystin of 0.083 nM (see Fig. 2a), the best fit value for  $E_0$  was  $75 \pm 32$  nM. Assuming 660 Daltons per base pair and 186 base pairs per nucleosome,  $E_0 = 75$  nM corresponds to one microcystin binding site for every 86 nucleosomes (16 kb of DNA).

### Staurosporine and olomoucine induce reassembly of nuclei and dephosphorylation of histone H1 in metaphase-arrested cells in vivo

Fig. 6a and b show that dephosphorylation of histone H1 is rapidly induced in metaphase-arrested HeLa cells by treatment with staurosporine, an inhibitor of p34<sup>cdc2</sup> and other protein kinases (Gadbois et al., 1992). Significant dephosphorylation is seen after a 30 minute treatment with 350 nM or more (Fig. 6a), and complete dephosphorylation is seen after a 5 hour treatment with as little as 100 nM staurosporine (Fig. 6b).

Staurosporine treatment of metaphase-arrested cells also induces reassembly of nuclear envelopes. Before treatment (Fig. 6c), fluorescence microscopy shows typical metaphase figures and a few interphase nuclei. After treatment, all cells contain nuclei, and some have multiple micronuclei (Fig. 6d). With 5  $\mu$ M staurosporine, virtually all cells contain nuclei within 10 minutes (Fig. 6e). The extent to which nuclei have reassembled after 30 minutes correlates with the extent of H1 dephosphorylation (compare Fig. 6a and e), and the same is true at other time points.

Nuclear reassembly and H1 dephosphorylation are also induced in metaphase-arrested cells by the purine analogue



**Fig. 6.** Treatment of metaphase-arrested HeLa cells with staurosporine leads to dephosphorylation of histone H1 and reassembly of nuclei. Aliquots of a nocodazole-arrested culture (mitotic index, 90%) containing  $5 \times 10^5$  cells/ml were treated with staurosporine and at various times samples were taken for determination of mitotic index and extraction of histone H1. (a) Analysis of H1 phosphorylation on Hepes/histidine gels (Paulson et al., 1992) after 30 minutes treatment with various concentrations of staurosporine. (b) Analysis of H1 phosphorylation after 5 hours treatment with staurosporine using the same concentrations. Only the portions of the gels containing histone H1 are shown. (c) Examples of cells in a metaphase-arrested culture before treatment with staurosporine. Cells were hypotonically swollen, fixed, stained with Hoechst 33342 and viewed by epifluorescence microscopy. Note two metaphase cells above and an interphase cell below (final magnification,  $\times 580$ ). (d) Examples of cells after treatment for 12 minutes with 5  $\mu$ M staurosporine (final magnification,  $\times 580$ ). (e) Mitotic index as a function of time after treatment with 0 (control), 20, 50, 100, 200, 350 and 500 nM staurosporine, and with 1, 2, and 5  $\mu$ M staurosporine.

olomoucine (6-benzylamino-2-(2-hydroxyethylamino)-9-methylpurine). However, much higher concentrations are required to have the same effects, consistent with the higher dissociation constants of olomoucine for p34<sup>cdc2</sup> and other protein kinases (Vesely et al., 1994). Time courses of mitotic indices after treatment with 1.0 and 2.0 mM olomoucine (data not shown) are similar to those seen with 50 and 100 nM staurosporine (Fig. 6e). Because of limitations on the solubility of olomoucine, only staurosporine was used for further experiments.

### Inhibition of staurosporine-induced effects by protein phosphatase inhibitors

To obtain information about the protein phosphatases involved in reassembly of nuclei and dephosphorylation of histone H1 *in vivo*, the staurosporine-induced effects were used as a model system in which to test calyculin A, cantharidin, and okadaic acid. Microcystin was not tested because of its inability to penetrate living cells.

Fig. 7 shows that 40 nM calyculin completely blocks reassembly of nuclei (Fig. 7a) and dephosphorylation of H1 (Fig. 7c) during a 30 minute treatment with staurosporine, as does 65-100  $\mu$ M cantharidin (data not shown). In parallel experiments carried out *in vitro* in lysates using the same temperature, the same chromatin concentration, and portions of the same metaphase-arrested cultures, very similar inhibitor concentrations were required to prevent H1 dephosphorylation.

By contrast, okadaic acid only partially blocks the staurosporine-induced effects at 10  $\mu$ M, the highest concentration it was practicable to test (Fig. 7b and d), whereas 0.2  $\mu$ M okadaic acid was sufficient to block H1 dephosphorylation in parallel *in vitro* experiments, in keeping with the results in Fig. 2.

### Calyculin A directly inhibits the phosphatase(s) responsible for nuclear reassembly and histone H1 dephosphorylation following staurosporine treatment

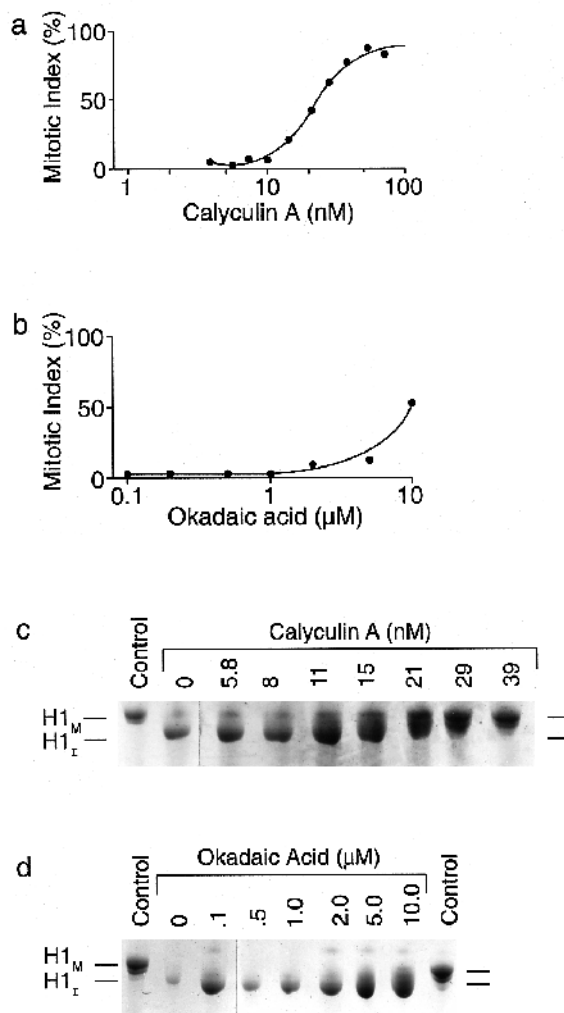
Does calyculin A directly inhibit the phosphatase(s) which dephosphorylate nuclear lamins (or other proteins involved in the reassembly of nuclear envelopes) and histone H1, or does it block the action of these phosphatases indirectly, perhaps by inhibiting another phosphatase which is required for their activation? In the experiments described above, phosphatase inhibitors were present from the beginning of the staurosporine treatment, and so these two possibilities could not be distinguished.

To answer the question, we performed an *in vivo* experiment analogous to the *in vitro* experiment shown in Fig. 5a and b. A metaphase-arrested culture was treated with 500 nM staurosporine and at each time point two samples were taken. The first was used immediately for determination of mitotic index and extraction of histones, while the second was treated with 100 nM calyculin A and incubated for an additional 45 minutes at 37°C before determining the mitotic index and extracting histones. The results are shown in Fig. 8. If calyculin only prevented *activation* of the phosphatases involved in nuclear reassembly and H1 dephosphorylation, then once those processes had begun calyculin would not be able to prevent them from going to completion during the additional 45 minute incubation. But Fig. 8 shows that calyculin A *can* halt these processes even when they are already under way.

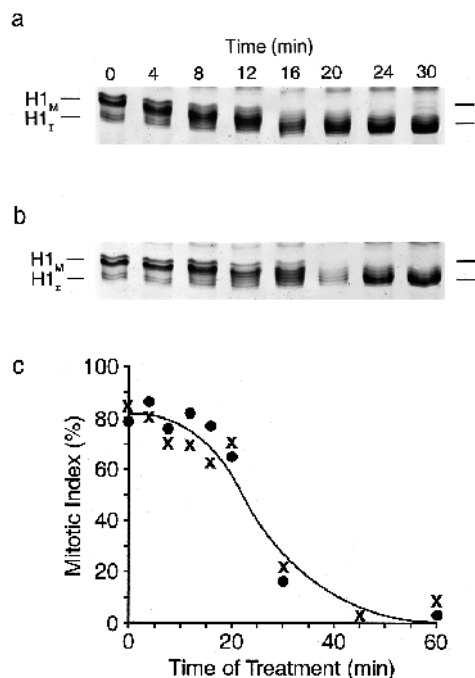
## DISCUSSION

### Okadaic acid and microcystin-LR inhibit the histone H1 phosphatase in HeLa metaphase chromosomes *in vitro*

Our results show clearly that microcystin-LR and okadaic acid block dephosphorylation of histone H1 in isolated HeLa metaphase chromosomes and in lysates of metaphase-arrested cells (Figs 1 and 2). They also prevent dephosphorylation of histone H3 and several other proteins in mitotic chromosomes (Figs 3 and 4). Microcystin may be particularly useful for *in vitro* studies of mitotic phosphoproteins generally (see e.g. Taagepera et al., 1995), since it is effective at much lower con-



**Fig. 7.** Effects of protein phosphatase inhibitors on the staurosporine-induced reassembly of nuclei and dephosphorylation of histone H1. Metaphase-arrested cultures (mitotic index, 85-90%) were treated with 500 nM staurosporine and aliquots were then immediately treated with various concentrations of (a) calyculin A or (b) okadaic acid. After incubation for 30 minutes at 37°C, samples were taken for determination of mitotic index. At the same time, samples were extracted with 5% perchloric acid and histone H1 analyzed by electrophoresis on HEPES-histidine gels (c,d). For controls, H1 was extracted from culture aliquots that were not treated with staurosporine. Only the portions of the gels containing H1 are shown.



**Fig. 8.** Calyculin A can halt staurosporine-induced histone H1 dephosphorylation and nuclear reassembly which are already in progress. A metaphase-arrested culture was treated with 500 nM staurosporine and incubated with magnetic stirring at 37°C. At each time point, two samples were taken. The first was used immediately for determination of mitotic index and histone extraction. The second was treated with 100 nM calyculin A and then incubated with stirring at 37°C for an additional 45 minutes before taking the mitotic index and extracting histones. Histones were extracted with 0.2 M H<sub>2</sub>SO<sub>4</sub> and analyzed by electrophoresis on acid/urea gels (Panyim and Chalkley, 1968). (a) Histones extracted from samples which were taken at the indicated times and treated with *p*-chloromercuriphenyl sulfonate. (b) Histones extracted from samples which were treated with calyculin A at the indicated times and then incubated an additional 45 minutes at 37°C. (c) Mitotic index determined at the indicated time (x) or after an additional 45 minutes incubation in the presence of 100 nM calyculin A (●).

centrations than okadaic acid and can be prepared as a stable aqueous stock solution. However, it may be necessary to use it in conjunction with protease inhibitors (cf. Fig. 4).

One might argue that these compounds do not actually inhibit the H1 phosphatase but act indirectly by stimulating (or preventing inactivation of) a protein kinase, since okadaic acid is known to stimulate p34<sup>cdc2</sup> kinase activity in *Xenopus* oocyte extracts (Cohen et al., 1990). However, this possibility is ruled out by our observation that microcystin prevents dephosphorylation of <sup>32</sup>P-labelled histones (Figs 3 and 4). Alternatively, one might argue that the inhibitors merely block activation of the histone phosphatase. However, this possibility is also ruled out by the demonstration that microcystin can halt dephosphorylation of H1 that is already under way (Fig. 5a and b).

We conclude that okadaic acid and microcystin act directly by inhibiting the histone H1 phosphatase, rather than by some indirect mechanism, and that enzymes which are not sensitive to these reagents do not make a significant contribution to H1 dephosphorylation in vitro.

### Evidence that the histone H1 phosphatase is a protein phosphatase 1 (PP1) located in isolated mitotic chromosomes

Our results strongly suggest that the major histone H1 phosphatase in HeLa metaphase chromosomes and lysates is either PP1 or a previously unknown protein phosphatase with okadaic acid sensitivity similar to PP1. For okadaic acid, the apparent dissociation constant,  $K_i$ , for PP1 has been variously reported as about 10–15 nM (Cohen et al., 1990), 18 nM (Chen et al., 1994), 74 nM (Honkanen et al., 1991), or 100 nM (Bialojan and Takai, 1988), whereas the dissociation constants for PP2A, PP4 and PP5 are all about 1 nM or less (Bialojan and Takai, 1988; Cohen et al., 1989, 1990; Brewis et al., 1993; Chen et al., 1994) and that for PP3 is no more than 5.2 nM (Honkanen et al., 1991). For microcystin-LR, all of these enzymes have  $K_i$  values of 0.1 nM or less (MacKintosh et al., 1990; Honkanen et al., 1990, 1991; Brewis et al., 1993; Chen et al., 1994).

Our data from dilute lysates and isolated chromosomes (Fig. 2) fit best with a  $K_i$  for okadaic acid of 50–75 nM, which is much closer to published values for PP1 than to those for the other protein phosphatases, and a  $K_i$  for microcystin of 0.08 nM, which agrees well with MacKintosh et al. (1990). If dephosphorylation of H1 were due to PP2A, PP3, PP4 or PP5, the okadaic acid curves in Fig. 2 should have been similar to the microcystin curves, and the  $K_i$  for okadaic acid should have been lower by a factor of at least 50. Of course, these other phosphatases may make small contributions to H1 dephosphorylation, since small amounts of inhibition may not have been detected in our study.

Our results also indicate that the histone H1 phosphatase in lysates of metaphase-arrested HeLa cells is predominantly located in chromosomes (Fig. 5). This supports its identification as PP1, since PP1 localizes in chromosomes at mitosis in vivo (Fernandez et al., 1992), whereas PP2A is primarily cytoplasmic (Turowski et al., 1995; Sontag et al., 1995), PP3 appears to be localized in membranes (Honkanen et al., 1991), and PP4 is found in centrosomes (Brewis et al., 1993). PP5 is primarily nuclear but is not found in condensed chromosomes at mitosis (Chen et al., 1994). Localization in mitotic chromosomes presumably puts the H1 phosphatase (PP1) where it needs to be to do its job at the end of mitosis. We find that mitotic chromosomes contain about one microcystin binding site for every 86 nucleosomes. However, this can only be considered an upper limit for the amount of histone H1 phosphatase present, since other phosphatases may account for some of the binding sites.

### Relationship to previous work on histone H1 dephosphorylation

Our evidence that the histone H1 phosphatase may be PP1, but not PP2A, appears to conflict with previous reports that histone H1 and other substrates of p34<sup>cdc2</sup> kinase are primarily dephosphorylated by PP2A (Sola et al., 1991; Agostinis et al., 1992; Ferrigno et al., 1993; Mayer-Jaekel et al., 1994). However, we believe that our work gives a better indication of the true identity of the histone H1 phosphatase in mitotic cells, for two reasons. First, we have studied dephosphorylation of histone H1 in situ in mitotic chromosomes, whereas the previous studies used purified (acid extracted) histone H1 phosphorylated in vitro by p34<sup>cdc2</sup> kinase. Histone H1 in chromosomes

may be more susceptible to PP1 and purified H1 more susceptible to PP2A. A similar phenomenon occurs with myosin. Intact myosin is dephosphorylated *in vivo* by PP1 while purified myosin light chains are dephosphorylated *in vitro* by PP2A (Brautigam, 1994).

Second, we have studied the enzyme in mitotic extracts and chromosomes, whereas the previous studies used interphase extracts or purified phosphatase catalytic subunits. There is increasing evidence that PP1 and other phosphatases are highly regulated (Brautigam, 1994; Hubbard and Cohen, 1993; Shenolikar, 1994; Bollen and Stalmans, 1992). It could be that the mitotic H1 phosphatase requires mitosis-specific regulatory subunits, and therefore is simply not present in interphase nuclei and cytosol. For example, a targeting subunit (Hubbard and Cohen, 1993) could increase PP1 activity toward H1 in mitotic chromosomes in much the same way that the G-subunit increases PP1 activity toward enzymes in glycogen particles (Hubbard and Cohen, 1993; Shenolikar, 1994). This would explain the localization of PP1 in mitotic chromosomes and its greater activity in mitotic versus interphase extracts.

The mutations in a regulatory subunit of *Drosophila* PP2A observed by Mayer-Jaekel et al. (1994), may affect H1 phosphatase activity indirectly.

Identification of the H1 phosphatase as PP1, not PP2A, is supported by two additional observations. First, dephosphorylation of H1 in lysates is completely inhibited by sulfhydryl reagents, including mercurial compounds and 5,5'-dithiobis(2-nitrobenzoate) (Paulson, 1980), and it has recently been shown that PP1 is much more sensitive to such reagents than PP2A (Nemani and Lee, 1993). Second, PP1 accounts for more than 80% of the histone H1 phosphatase activity in extracts of fission yeast (Kinoshita et al., 1991).

Although our results strongly suggest that H1 is dephosphorylated by PP1, it will be necessary to isolate the H1 phosphatase from chromosomes to confirm that it is a PP1 and to identify its regulatory subunits.

### Treatment of metaphase-arrested HeLa cells with staurosporine or olomoucine leads to histone H1 dephosphorylation and reassembly of nuclei

Fig. 6 shows that treatment of metaphase-arrested HeLa cells with staurosporine induces reassembly of nuclei and dephosphorylation of histone H1 *in vivo*. Similar results are seen with olomoucine. This shows that protein kinase activity must remain elevated to maintain a mitotic block.

It is intriguing to consider the possibility that these effects could be due to inhibition of MPF, since staurosporine and olomoucine are known to be inhibitors of p34<sup>cdc2</sup> as well as other protein kinases (Abe et al., 1991; Gadbois et al., 1992; Vesely et al., 1994). If this is correct, it could be that inactivation of MPF is the 'trigger' for events in the M- to G<sub>1</sub>-phase transition, just as activation of MPF is the trigger for events in the G<sub>2</sub>- to M-phase transition. MPF inactivation is known to be *necessary* for the M- to G<sub>1</sub>-phase transition (Murray et al., 1989; Luo et al., 1994; Rimmington et al., 1994), but our hypothesis is that for some events, including nuclear envelope reassembly and H1 dephosphorylation, it may also be *sufficient*. We suggest that in normal mitosis the 'anaphase trigger' signals (among other things) activation of the cyclin degradation pathway and thus inactivation of MPF; that protein phosphatases then dephosphorylate histones, nuclear lamins and

other proteins; and that nuclear reassembly (e.g. Glass and Gerace, 1990), and possibly chromosome decondensation and other events follow as a consequence. Staurosporine and olomoucine would induce some of the same events artificially, in the absence of the anaphase trigger, by inhibiting MPF. Similarly, low concentrations of okadaic acid would induce H1 dephosphorylation in metaphase-arrested cells (Paulson et al., 1994) by inhibiting PP2A and thus triggering cyclin degradation and MPF inactivation (Lorca et al., 1991, 1992).

Staurosporine's effects on metaphase-arrested cells have also been studied by Nakamura and Antoku (1993a,b) and Th'ng et al. (1994). Nakamura and Antoku did not examine histone phosphorylation, but observed that all the cells were multi-nucleate following staurosporine treatment. We have also observed multiple small nuclei in many cells (e.g. Fig. 6d), but certainly not in all. The difference could be due to the use of suspension cultures versus attached cells or the use of different cell lines. While our work was in progress, Th'ng et al. (1994) reported that staurosporine induces histone H1 dephosphorylation and argued that H1 dephosphorylation is the cause of chromosome decondensation. However, in our experiments the reassembled nuclei appear to contain condensed chromosomes at first and only later come to resemble normal interphase nuclei, well after H1 is dephosphorylated. This suggests that complete chromatin decondensation may require reassembly of the intranuclear matrix, resumption of transcription, or other events in addition to H1 dephosphorylation.

### Staurosporine-induced H1 dephosphorylation as a model system: characterization of the H1 phosphatase *in vivo*

Staurosporine should be a useful tool for studying the M- to G<sub>1</sub>-phase transition since it can induce certain events of this transition synchronously in large populations of cells, and we have used it in this way to study the protein phosphatases involved. The fact that staurosporine treatment induces nuclear envelope reassembly as well as H1 dephosphorylation makes it seem likely that this treatment reproduces some of the same events as at the end of normal mitosis, and involves the same phosphatases.

Our results show that H1 dephosphorylation *in vivo* must be due to either PP1, PP2A, PP3, PP4, PP5 or another protein phosphatase sensitive to the same class of inhibitors, since calyculin A directly inhibits the *in vivo* H1 phosphatase (Figs 7c, 8a,b). Similarly, one of these phosphatases must be required for the reassembly of nuclear envelopes (e.g. in the dephosphorylation of nuclear lamins) (Figs 7a, 8c).

To distinguish among these phosphatases, we tested okadaic acid, as in the *in vitro* work. Okadaic acid apparently does not enter the cells efficiently, since only 500 nM is sufficient to inhibit PP1 (the phosphatase which is least sensitive to okadaic acid) *in vitro* (Fig. 2b) whereas not even 5  $\mu$ M is able to inhibit H1 dephosphorylation *in vivo* (Fig. 7d). But other work (Lorca et al., 1991, 1992; Paulson et al., 1994) indicates that PP2A (the phosphatase most sensitive to okadaic acid) is inhibited *in vivo* in mitotic HeLa cells by 0.15  $\mu$ M okadaic acid. Since the staurosporine-induced effects are not blocked even by 5 or 10  $\mu$ M okadaic acid (Fig. 7b and d), it seems unlikely that these effects could require PP2A. Nor is it likely that they require PP3, PP4 or PP5, which are similar to PP2A in their sensitiv-

ity to okadaic acid (Honkanen et al., 1991; Brewis et al., 1993; Chen et al., 1994).

All of our results are consistent with the hypothesis that the *in vivo* mitotic H1 phosphatase is the same phosphatase (PP1 or an unknown phosphatase with similar okadaic acid sensitivity) whose action we have observed *in vitro*. Identification of the enzyme as PP1 is further supported by the localization of PP1 in mitotic chromosomes *in vivo* (Fernandez et al., 1992), as discussed above.

### Is the mitotic histone H1 phosphatase constitutive or regulated?

This question cannot yet be answered, but our results place some important constraints on possible answers. Histone H1 remains phosphorylated for many hours in metaphase-arrested cells, but it is rapidly dephosphorylated when the cells are treated with staurosporine (Fig. 6) or when they are lysed (Fig. 1). If the H1 phosphatase is constitutively active in mitotic chromosomes, our results imply complete turnover of phosphate in histone H1 every 15 minutes or less (cf. Fig. 1a) which would seem to be very wasteful. On the other hand, if the H1 phosphatase is inactive in mitotic chromosomes, our results show that it must be capable of being activated very rapidly by factors present, along with the phosphatase itself, in isolated chromosomes.

A constitutive H1 phosphatase cannot be ruled out, and would provide the simplest explanation for H1 dephosphorylation following inactivation of the H1 kinase (MPF). However, there is increasing evidence that protein phosphatases are regulated (Hubbard and Cohen, 1993; Brautigam, 1994; Shenolikar, 1994), and regulation of PP1 by p34<sup>cdc2</sup> has recently been discovered (Dohadwala et al., 1994; Yamano et al., 1994). We propose that the histone H1 phosphatase (PP1) may be inactivated at the onset of mitosis by phosphorylation on its catalytic subunit by MPF (Dohadwala et al., 1994; Yamano et al., 1994), and reactivated at the end of mitosis by dephosphorylation by itself (Dohadwala et al., 1994) or other endogenous chromosomal phosphatases. Some H1 phosphatase (PP1) activity may remain throughout mitosis because turnover of phosphate in controlling pathways makes it possible for them to be triggered rapidly (Brautigam, 1994).

This hypothesis is consistent with all available data concerning H1 dephosphorylation *in vivo* and *in vitro*. The H1 phosphatase would remain largely inactive in metaphase-arrested cells as long as MPF remained active. However, activation of the H1 phosphatase and dephosphorylation of H1 would occur rapidly *in vitro* following cell lysis due to MPF inactivation or dilution of ATP. Similarly, the H1 phosphatase would be rapidly activated *in vivo* whenever MPF activity fell substantially, for example as a result of cyclin degradation or inhibition by staurosporine. (Dephosphorylation of nuclear lamins and other proteins at the end of mitosis could be explained similarly.)

The hypothesis makes several testable predictions. First, it predicts that the PP1 catalytic subunit should be phosphorylated in metaphase-arrested cells but rapidly dephosphorylated after cell lysis. Second, it predicts some turnover of phosphate in histone H1 during metaphase arrest, but significantly less than would be expected if the H1 phosphatase were constitutively active. Finally, it predicts that site-directed mutagenesis to eliminate the phosphorylation site in the PP1 catalytic

subunit (Dohadwala et al., 1994) should be lethal, because it will prevent cells from entering mitosis.

We are grateful to R. E. Moore for assistance with the curve fitting and to D. Gatz for assistance in preparation of the figures. This work was supported by the University of Wisconsin-Oshkosh Faculty Development Board and by grants GM-39915 and GM-46040 from the National Institutes of Health, US Public Health Service.

### REFERENCES

- Abe, K., Yoshida, M., Usui, T., Horinouchi, S. and Beppu, T. (1991). Highly synchronous culture of fibroblasts from G2 block caused by staurosporine, a potent inhibitor of protein kinases. *Exp. Cell Res.* **192**, 122-127.
- Agostinis, P., Derua, R., Sarno, S., Goris, J. and Merlevede, W. (1992). Specificity of the polycation-stimulated (type-2A) and ATP, Mg-dependent (type-1) protein phosphatases toward substrates phosphorylated by p34<sup>cdc2</sup> kinase. *Eur. J. Biochem.* **205**, 241-248.
- Axton, J. M., Dombradi, V., Cohen, P. T. W. and Glover, D. M. (1990). One of the protein phosphatase 1 isoenzymes in *Drosophila* is essential for mitosis. *Cell* **63**, 33-46.
- Bialojan, C. and Takai, A. (1988). Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. *Biochem. J.* **256**, 283-290.
- Bollen, M. and Stalmans, W. (1992). The structure, role, and regulation of type 1 protein phosphatases. *Crit. Rev. Biochem. Mol. Biol.* **27**, 227-281.
- Brautigam, D. L. (1994). Protein phosphatases. *Recent Prog. Hormone Res.* **49**, 197-214.
- Brewis, N. D., Street, A. J., Prescott, A. R. and Cohen, P. T. W. (1993). PPX, a novel protein serine/threonine phosphatase localized to centrosomes. *EMBO J.* **12**, 987-996.
- Chen, M. X., McPartlin, A. E., Brown, L., Chen, Y. H., Barker, H. M. and Cohen, P. T. W. (1994). A novel human protein serine/threonine phosphatase, which possesses four tetratricopeptide repeat motifs and localizes to the nucleus. *EMBO J.* **13**, 4278-4290.
- Cohen, P., Klumpp, S. and Schelling, D. L. (1989). An improved procedure for identifying and quantitating protein phosphatases in mammalian tissues. *FEBS Lett.* **250**, 596-600.
- Cohen, P., Holmes, C. F. B. and Tsukitani, Y. (1990). Okadaic acid: A new probe for the study of cellular regulation. *Trends Biochem. Sci.* **15**, 98-102.
- Cyert, M. S. and Thorner, J. (1989). Putting it on and taking it off: Phosphoprotein phosphatase involvement in cell cycle regulation. *Cell* **57**, 891-893.
- D'Anna, J. A., Gurley, L. R. and Deaven, L. L. (1978). Dephosphorylation of histones H1 and H3 during the isolation of metaphase chromosomes. *Nucl. Acids Res.* **5**, 3195-3207.
- Dohadwala, M., Da Cruz e Silva, E. F., Hall, F. L., Williams, R. T., Carbonaro-Hall, D. A., Nairn, A. C., Greengard, P. and Berndt, N. (1994). Phosphorylation and inactivation of protein phosphatase 1 by cyclin-dependent kinases. *Proc. Nat. Acad. Sci. USA* **91**, 6408-6412.
- Fernandez, A., Brautigam, D. L. and Lamb, N. J. C. (1992). Protein phosphatase type 1 in mammalian cell mitosis: Chromosomal localization and involvement in mitotic exit. *J. Cell Biol.* **116**, 1421-1430.
- Ferrigno, P., Langan, T. A. and Cohen, P. (1993). Protein phosphatase 2A<sub>1</sub> is the major enzyme in vertebrate cell extracts that dephosphorylates several physiological substrates for cyclin-dependent protein kinases. *Mol. Biol. Cell* **4**, 669-677.
- Gadbois, D. M., Hamaguchi, J. R., Swank, R. A. and Bradbury, E. M. (1992). Staurosporine is a potent inhibitor of p34<sup>cdc2</sup> and p34<sup>cdc2</sup>-like kinases. *Biochem. Biophys. Res. Commun.* **184**, 80-85.
- Glass, J. R. and Gerace, L. (1990). Lamins A and C bind and assemble at the surface of mitotic chromosomes. *J. Cell Biol.* **111**, 1047-1057.
- Gurley, L. R., D'Anna, J. A., Barham, S. S., Deaven, L. L. and Tobey, R. A. (1978). Histone phosphorylation and chromatin structure during mitosis in Chinese hamster cells. *Eur. J. Biochem.* **84**, 1-15.
- Honkanen, R. E., Zwiller, J., Moore, R. E., Daily, S. L., Khatra, B. S., Dukelow, M. and Boynton, A. L. (1990). Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases. *J. Biol. Chem.* **265**, 19401-19404.
- Honkanen, R. E., Zwiller, J., Daily, S. L., Khatra, B. S., Dukelow, M. and Boynton, A. L. (1991). Identification, purification, and characterization of a novel serine/threonine protein phosphatase from bovine brain. *J. Biol. Chem.* **266**, 6614-6619.

- Honkanen, R. E.** (1993). Cantharidin, another natural toxin that inhibits the activity of serine/threonine protein phosphatases types 1 and 2A. *FEBS Lett.* **330**, 283-286.
- Hubbard, M. J. and Cohen, P.** (1993). On target with a new mechanism for the regulation of protein phosphorylation. *Trends Biochem. Sci.* **18**, 172-177.
- Ishihara, H., Martin, B. L., Brautigan, D. L., Karaki, H., Ozaki, H., Kato, Y., Fusetani, N., Watabe, S., Hashimoto, K., Uemura, D. and Hartshorne, D. J.** (1989). Calyculin A and okadaic acid: Inhibitors of protein phosphatase activity. *Biochem. Biophys. Res. Commun.* **159**, 871-877.
- Kinoshita, N., Yamano, H., Le Bouffant-Slavecsek, F., Kurooka, H., Ohkura, H., Stone, E. M., Takeuchi, M., Toda, T., Yoshida, T. and Yanagida, M.** (1991). Sister-chromatid separation and protein dephosphorylation in mitosis. *Cold Spring Harbor Symp. Quant. Biol.* **56**, 621-628.
- Laemmli, U. K. and Favre, M.** (1973). Maturation of the head of bacteriophage T4: I. DNA packaging events. *J. Mol. Biol.* **80**, 575-599.
- Lorca, T., Fesquet, D., Zindy, F., LeBouffant, F., Cerruti, M., Brechot, C., Devauchelle, G. and Doree, M.** (1991). An okadaic acid-sensitive phosphatase negatively controls the cyclin degradation pathway in amphibian eggs. *Mol. Cell. Biol.* **11**, 1171-1175.
- Lorca, T., Labbe, J. C., Devault, A., Fesquet, D., Capony, J. P., Cavadore, J. C., LeBouffant, F. and Doree, M.** (1992). Dephosphorylation of cdc2 on threonine 161 is required for cdc2 kinase inactivation and normal anaphase. *EMBO J.* **11**, 2381-2390.
- Luo, Q., Michaelis, C. and Weeks, G.** (1994). Overexpression of a truncated cyclin B gene arrests *Dictyostelium* cell division during mitosis. *J. Cell Sci.* **107**, 3105-3114.
- MacKintosh, C., Beattie, K. A., Klumpp, S., Cohen, P. and Codd, G. A.** (1990). Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett.* **264**, 187-192.
- Mayer-Jackel, R. E., Ohkura, H., Ferrigno, P., Andjelkovic, N., Shiom, K., Uemura, T., Glover, D. M. and Hemmings, B. A.** (1994). *Drosophila* mutants in the 55 kDa regulatory subunit of protein phosphatase 2A show strongly reduced ability to dephosphorylate substrates of p34<sup>cdc2</sup>. *J. Cell Sci.* **107**, 2609-2616.
- Medappa, K. C., McLean, C. and Rueckert, R. R.** (1971). On the structure of rhinovirus 1A. *Virology* **44**, 259-270.
- Murray, A. W., Solomon, M. J. and Kirschner, M. W.** (1989). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature* **339**, 280-286.
- Nakamura, K. and Antoku, S.** (1993a). Staurosporine induces multinucleation following chromosome decondensation in colcemid-arrested cells. *In Vitro Cell Dev. Biol.* **29A**, 525-527.
- Nakamura, K. and Antoku, S.** (1993b). Protein kinase inhibitor, staurosporine, prevents okadaic acid- or caffeine-induced chromosome condensation. *In Vitro Cell Dev. Biol.* **29A**, 760-762.
- Nemani, R. and Lee, E. Y. C.** (1993). Reactivity of sulfhydryl groups of the catalytic subunits of rabbit skeletal muscle protein phosphatases 1 and 2A. *Arch. Biochem. Biophys.* **300**, 24-29.
- Norbury, C. and Nurse, P.** (1992). Animal cell cycles and their control. *Annu. Rev. Biochem.* **61**, 441-470.
- Panyim, S. and Chalkley, R.** (1969). High resolution acrylamide gel electrophoresis of histones. *Arch. Biochem. Biophys.* **130**, 337-346.
- Paulson, J. R.** (1980). Sulfhydryl reagents prevent dephosphorylation and proteolysis of histones in isolated HeLa metaphase chromosomes. *Eur. J. Biochem.* **111**, 189-197.
- Paulson, J. R.** (1982). Isolation of chromosome clusters from metaphase-arrested HeLa cells. *Chromosoma* **85**, 571-581.
- Paulson, J. R. and Taylor, S. S.** (1982). Phosphorylation of histones 1 and 3 and nonhistone high mobility group 14 by an endogenous kinase in HeLa metaphase chromosomes. *J. Biol. Chem.* **257**, 6064-6072.
- Paulson, J. R., Mesner, P. W., Delrow, J. J., Mahmoud, N. N. and Ciesielski, W. A.** (1992). Rapid analysis of mitotic histone H1 phosphorylation by cationic disc electrophoresis at neutral pH in minigels. *Anal. Biochem.* **203**, 227-234.
- Paulson, J. R., Ciesielski, W. A., Schram, B. R. and Mesner, P. W.** (1994). Okadaic acid induces dephosphorylation of histone H1 in metaphase-arrested HeLa cells. *J. Cell Sci.* **107**, 267-273.
- Rimington, G., Dalby, B. and Glover, D. M.** (1994). Expression of N-terminally truncated cyclin B in the *Drosophila* larval brain leads to mitotic delay at late anaphase. *J. Cell Sci.* **107**, 2729-2738.
- Shenolikar, S.** (1994). Protein serine/threonine phosphatases: new avenues for cell regulation. *Annu. Rev. Cell Biol.* **10**, 55-86.
- Sola, M. M., Langan, T. and Cohen, P.** (1991). p34<sup>cdc2</sup> phosphorylation sites in histone H1 are dephosphorylated by protein phosphatase 2A<sub>1</sub>. *Biochim. Biophys. Acta* **1094**, 211-216.
- Sontag, E., Numbhakdi-Craig, V., Bloom, G. S. and Mumby, M. C.** (1995). A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. *J. Cell Biol.* **128**, 1131-1144.
- Stewart, A. A., Ingebritsen, T. S. and Cohen, P.** (1983). The protein phosphatases involved in cellular regulation: 5. Purification and properties of a Ca<sup>2+</sup>/calmodulin-dependent protein phosphatase (2B) from rabbit skeletal muscle. *Eur. J. Biochem.* **132**, 289-295.
- Taagepera, S., Campbell, M. S. and Gorbsky, G. J.** (1995). Cell-cycle-regulated localization of tyrosine and threonine phosphoepitopes at the kinetochores of mitotic chromosomes. *Exp. Cell Res.* **221**, 249-260.
- Th'ng, J. P. H., Guo, X.-W., Swank, R. A., Crissman, H. A. and Bradbury, E. M.** (1994). Inhibition of histone phosphorylation by staurosporine leads to chromosome decondensation. *J. Biol. Chem.* **269**, 9568-9573.
- Turowski, P., Fernandez, A., Favre, B., Lamb, N. J. C. and Hemmings, B. A.** (1995). Differential methylation and altered conformation of cytoplasmic and nuclear forms of protein phosphatase 2A during cell cycle progression. *J. Cell Biol.* **129**, 397-410.
- Vandre, D. D. and Wills, V. L.** (1992). Inhibition of mitosis by okadaic acid: Possible involvement of a protein phosphatase 2A in the transition from metaphase to anaphase. *J. Cell Sci.* **101**, 79-92.
- Vesely, J., Havlicek, L., Strnad, M., Blow, J. J., Donella-Deana, A., Pinna, L., Letham, D. S., Kato, J., Detivaud, L., Leclerc, S. and Meijer, L.** (1994). Inhibition of cyclin-dependent kinases by purine analogues. *Eur. J. Biochem.* **224**, 771-786.
- Yamano, H., Ishii, K. and Yanagida, M.** (1994). Phosphorylation of dis2 protein phosphatase at the C-terminal cdc2 consensus and its potential role in cell cycle regulation. *EMBO J.* **13**, 5310-5318.

(Received 19 January 1996 - Accepted 19 March 1996)