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# Chromosome condensation induced by fostriecin does not require p34$^{cdc2}$ kinase activity and histone H1 hyperphosphorylation, but is associated with enhanced histone H2A and H3 phosphorylation

**X.W.Guo, J.P.H.Th'ng, R.A.Swank, H.J.Anderson[1], C.Tudan[1,2], E.M.Bradbury[3] and M.Roberge[1,4]**

Department of Biological Chemistry, University of California, Davis, CA 95616, USA, [1]Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada V6T 1Z3, [2]Kinetek Corporation,Vancouver, BC, Canada and [3]The Division of Life Sciences, Los Alamos National Laboratories, Los Alamos, NM 87545, USA

[4]Corresponding author

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**Chromosome condensation at mitosis correlates with the activation of p34$^{cdc2}$ kinase, the hyperphosphorylation of histone H1 and the phosphorylation of histone H3. Chromosome condensation can also be induced by treating interphase cells with the protein phosphatase 1 and 2A inhibitors okadaic acid and fostriecin. Mouse mammary tumour FT210 cells grow normally at 32°C, but at 39°C they lose p34$^{cdc2}$ kinase activity and arrest in $G_2$ because of a temperature-sensitive lesion in the *cdc2* gene. The treatment of these $G_2$-arrested FT210 cells with fostriecin or okadaic acid resulted in full chromosome condensation in the absence of p34$^{cdc2}$ kinase activity or histone H1 hyperphosphorylation. However, phosphorylation of histones H2A and H3 was strongly stimulated, partly through inhibition of histone H2A and H3 phosphatases, and cyclins A and B were degraded. The cells were unable to complete mitosis and divide. In the presence of the protein kinase inhibitor staurosporine, the addition of fostriecin did not induce histone phosphorylation and chromosome condensation. The results show that chromosome condensation can take place without either the histone H1 hyperphosphorylation or the p34$^{cdc2}$ kinase activity normally associated with mitosis, although it requires a staurosporine-sensitive protein kinase activity. The results further suggest that protein phosphatases 1 and 2A may be important in regulating chromosome condensation by restricting the level of histone phosphorylation during interphase, thereby preventing premature chromosome condensation.**

*Key words*: fostriecin/histones/mitosis/protein kinase/protein phosphatase

## Introduction

During mitosis, the cell nucleus undergoes major rearrangements required for the separation of chromosomes to the daughter cells. The most striking change is the condensation of interphase chromatin into metaphase chromosomes. Despite extensive morphological studies, the process of chromosome condensation remains an enigma at the molecular level. In *Physarum polycephalum*, chromosome condensation has been correlated with an increase in the phosphorylation of histone H1 through $G_2$ to reach a hyperphosphorylated state of 24 phosphates at metaphase (Bradbury *et al.*, 1973, 1974a,b; Mueller *et al.*, 1985). This is preceded by a 15- to 20-fold increase in activity of the growth-associated H1 kinase (Bradbury *et al.*, 1974a,b). In mammalian cells, histone H1 phosphorylation increases from two to three phosphates in early $G_2$ to five to six phosphates at mitosis (Gurley *et al.*, 1978; Davis *et al.*, 1983). Growth-associated H1 kinase is now known to be p34$^{cdc2}$ kinase (Langan *et al.*, 1989), a complex of p34$^{cdc2}$ and cyclin B, which is the major regulator of mitosis (reviewed in Nurse, 1990; Norbury and Nurse, 1992). In addition to p34$^{cdc2}$–cyclin B, purified murine p34$^{cdc2}$–cyclin A and p33$^{cdk2}$–cyclin A can phosphorylate histones H1A and H1B *in vitro* at the sites phosphorylated *in vivo* at metaphase (R.A.Swank *et al.*, manuscript in preparation).

A strong correlation exists between the phosphorylation of histone H3 and chromosome condensation. Histone H3 is not phosphorylated during interphase but becomes phosphorylated at Ser10 just prior to metaphase (Gurley *et al.*, 1978), perhaps participating in a late stage of chromosome condensation (Bradbury, 1992). This site is not phosphorylated by cyclin-dependent kinases.

The level of histone phosphorylation during the cell cycle is also determined by protein phosphatases. Four types of Ser/Thr protein phosphatases, PP1, PP2A, PP2B and PP2C, have been isolated from mammalian cells (Cohen, 1989; Cohen *et al.*, 1989). The PP2A subtype $PP2A_1$ is the major enzyme that dephosphorylates substrates for cyclin-dependent protein kinases, including histone H1 (Ferrigno *et al.*, 1993), and the treatment of mitotic cells with the protein phosphatases 1 and 2A inhibitor okadaic acid induces histone H1 dephosphorylation (Paulson *et al.*, 1994). There is also mounting evidence that protein phosphatases are involved in controlling early stages of mitosis: a PP2A subtype negatively regulates p34$^{cdc2}$ kinase in *Xenopus* egg extracts (Felix *et al.*, 1990; Lee *et al.*, 1991), and okadaic acid, calyculin A and fostriecin, all potent inhibitors of PP1 and PP2A *in vitro*, can induce premature entry into mitosis (Yamashita *et al.*, 1990; Tosuji *et al.*, 1992; Roberge *et al.*, 1994).

In this study we used a temperature-sensitive p34$^{cdc2}$ mutant cell line and protein phosphatase inhibitors to evaluate the role of histone H1 and H3 phosphorylation in chromosome condensation. We show that morphologically normal mitotic chromosome condensation can occur without p34$^{cdc2}$ kinase activity and without increased histone H1 phosphorylation, but it is accompanied by a large increase in the phosphorylation of histones H2A and H3. The cells containing these condensed chromosomes are unable to complete mitosis and divide. Our results have

important implications for current models of chromosome condensation.

## Results

### *Protein phosphatase inhibitors can induce chromosome condensation*

Entry into a mitosis-like state can be induced from the S or $G_2$ phases of the cell cycle by the protein phosphatase inhibitors okadaic acid (Yamashita *et al.*, 1990) and fostriecin (Roberge *et al.*, 1994). We have also shown that fostriecin causes little or no increase in $p34^{cdc2}$ kinase activity (Roberge *et al.*, 1994). To investigate further whether $p34^{cdc2}$ kinase activity is required for chromosome condensation induced by fostriecin or okadaic acid, we examined the response to these inhibitors in the mouse FT210 cell line which has a temperature-sensitive $p34^{cdc2}$ kinase. These cells grow normally at 32°C, but at 39°C they lose their $p34^{cdc2}$ kinase and arrest in $G_2$ (Th'ng *et al.*, 1990).

Synchronous populations of FT210 cells were generated by sequential incubation in isoleucine-deficient medium followed by aphidicolin treatment. The cells were released from aphidicolin block and incubated at 39°C overnight. Under these conditions, cells arrest in $G_2$ and >90% of the $p34^{cdc2}$ kinase is inactivated (Th'ng *et al.*, 1990; Hamaguchi *et al.*, 1992). Different concentrations of fostriecin were then added and the cells were maintained at 39°C for up to 2 h. Microscopic examination showed that fostriecin concentrations of ≥25 μM caused a significant increase in the mitotic index, as indicated by the presence of condensed chromosomes (Figure 1A). After 1 h incubation, 60% of cells treated with 100 μM fostriecin had condensed chromosomes. The chromosomes were highly condensed, with visible sister chromatids (Figure 2B), and were indistinguishable from those of cycling cells arrested in mitosis by colcemid (Figure 2A) or nocodazole (results not shown). Similarly, different concentrations of okadaic acid were added to FT210 cells at 39°C for up to 2 h. Over 60% of the cells treated with 1 μM okadaic acid for 2 h had condensed chromosomes (Figures 1B and 2C). These experiments show that fostriecin and okadaic acid overcome the $G_2$ block caused by the temperature-induced inactivation of $p34^{cdc2}$ in FT210 cells, and drive the chromosomes to condense fully.

We have shown previously that other mitotic events, such as nuclear lamina depolymerization and spindle pole separation, are also induced in BHK cells by fostriecin, but that the cells fail to enter anaphase (Roberge *et al.*, 1994). Okadaic acid also prevents the formation of the mitotic spindle and trilaminar kinetochores of HeLa cells (Ghosh *et al.*, 1992), disrupts the metaphase plate of pig kidney LLC-PK cells (Vandré and Wills, 1992), and does not allow the cells to complete mitosis. FT210 cells treated with fostriecin or okadaic acid also failed to complete mitosis.

### *Fostriecin-induced chromosome condensation does not require $p34^{cdc2}$ kinase activity*

Under the conditions described above, FT210 cells have little $p34^{cdc2}$ kinase activity (Th'ng *et al.*, 1990; Hamaguchi *et al.*, 1992), suggesting that chromosome condensation can occur in the absence of $p34^{cdc2}$ kinase activity. This

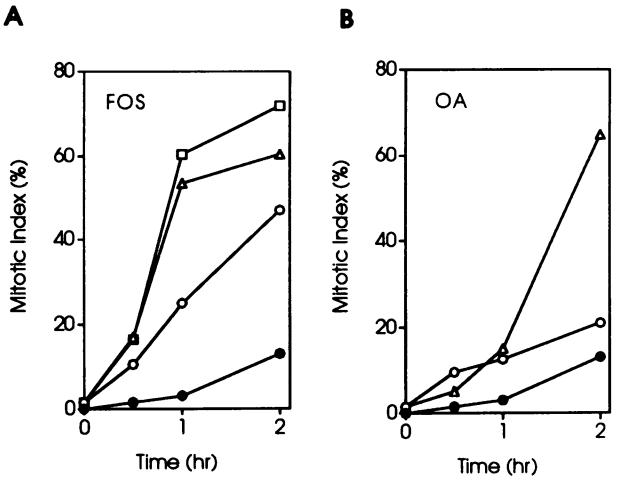


**Fig. 1.** Induction of chromosome condensation in 39°C arrested FT210 cells by fostriecin and okadaic acid. FT210 cells were incubated with different concentrations of (**A**) fostriecin (●, 0 μM; ○, 25 μM; △, 50 μM; □, 100 μM) or (**B**) okadaic acid (●, 0 μM; ○, 0.1 μM; △, 1.0 μM) for different times. The percentage of cells with condensed chromosomes (mitotic index) was determined by fluorescence microscopy.

was confirmed in the following experiment. FT210 cells arrested at $G_1$/S by aphidicolin were released and either arrested in $G_2$ by shifting the temperature to 39°C, or allowed to progress into mitosis at 32°C where they were trapped by the presence of nocodazole, an inhibitor of microtubule polymerization. Fostriecin was then added to both $G_2$- and mitosis-arrested cultures for up to 2 h. The mitotic index for nocodazole-arrested cells increased slightly in the presence of fostriecin, while the addition of fostriecin to cells arrested in $G_2$ at 39°C led to a large increase in the mitotic index (Table I). No significant increase in the mitotic index was observed when these cells were maintained at 39°C in the absence of fostriecin. The cells were then lysed, $p34^{cdc2}$ kinase was immunoprecipitated and its histone H1 kinase activity was measured (Table I). Cells arrested in mitosis with nocodazole had high levels of $p34^{cdc2}$ kinase activity, while cells blocked in $G_2$ by temperature shift had little or no $p34^{cdc2}$ kinase activity. The addition of fostriecin for up to 2 h did not result in an increase in $p34^{cdc2}$ kinase activity in either cell population. Thus, cells arrested in $G_2$ at 39°C and treated with fostriecin had a mitotic index similar to that of cells treated with nocodazole, yet their $p34^{cdc2}$ kinase activity was 50- to 100-fold lower. These experiments, as well as most of those described below using fostriecin, were also performed with okadaic acid, with the same outcome. The results demonstrate clearly that $p34^{cdc2}$ kinase activity is not required for chromosome condensation induced by fostriecin or okadaic acid.

### *$p34^{cdc2}$ kinase activity is not correlated with fostriecin-induced chromosome condensation*

Cell cycle progression is also controlled by the activity of other cyclin-dependent protein kinases (reviewed in Solomon, 1993) whose activity is not temperature-sensitive in FT210 cells. These are associated with the $G_1$/S transition. An exception is $p33^{cdk2}$ kinase which is active in S and $G_2$ (Rosenblatt *et al.*, 1992) and is regulated by phosphorylation at sites equivalent to those of $p34^{cdc2}$. We

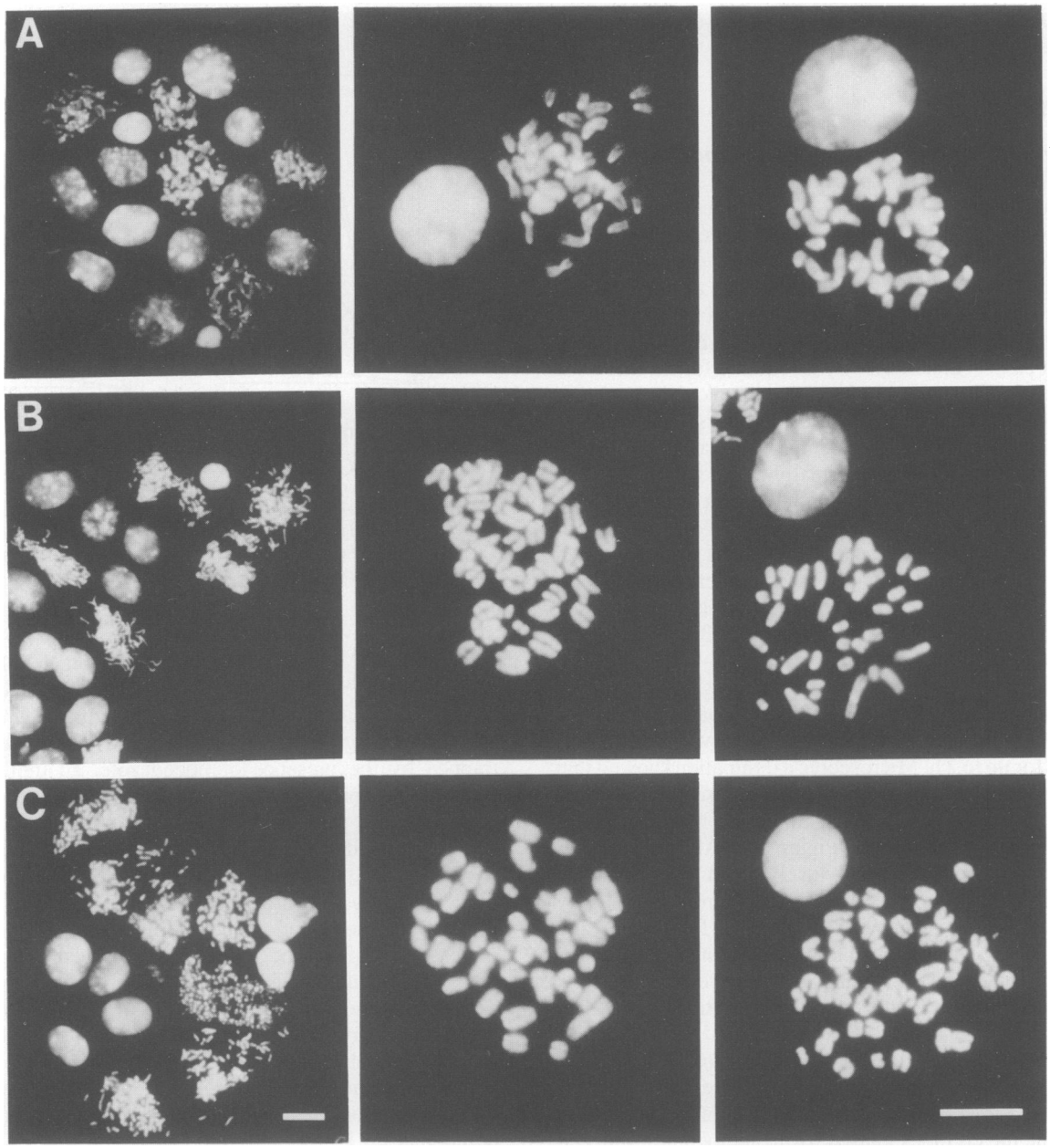


**Fig. 2.** Chromosome condensation induced by fostriecin or okadaic acid. FT210 cells grown at 32°C were arrested in mitosis using colcemid (**A**) or were arrested in $G_2$ at 39°C and then incubated for 2 h with 100 μM fostriecin (**B**) or 0.5 μM okadaic acid (**C**). The cells were harvested, fixed and stained with the DNA dye bisbenzimide, and the chromosomes were visualized by fluorescence microscopy. The column on the left shows a field containing several cells with condensed chromosomes. The other two columns show individual cells with condensed chromosomes at a higher magnification. Bar: 20 μm.

considered the possibility that fostriecin causes chromosome condensation through activating $p33^{cdk2}$ kinase. $p33^{cdk2}$ kinase was immunoprecipitated from fostriecin-treated cells as described above and its histone H1 kinase activity was determined in parallel with that of $p34^{cdc2}$ kinase. The treatment of nocodazole-arrested cells with fostriecin caused a decrease in $p33^{cdk2}$ kinase activity, as observed for $p34^{cdc2}$ kinase activity (Table I). The addition of fostriecin to cells blocked in $G_2$ at 39°C caused an initial 2-fold increase in $p33^{cdk2}$ kinase activity at 30 min, followed by a significant decrease in activity at 1 or 2 h, while the mitotic index continued to rise. This inverse correlation and the absence of increased histone phosphorylation after 30 min treatment with fostriecin (see below) suggest that chromosome condensation induced by fostriecin is not due to $p33^{cdk2}$.

### *Fostriecin causes cyclin degradation*

The results presented thus far show that fostriecin can cause chromosome condensation in cells that contain little or no $p34^{cdc2}$ kinase activity. Surprisingly, fostriecin caused a decrease in $p33^{cdk2}$ and $p34^{cdc2}$ kinase activities in nocodazole-arrested cells (Table I). Fostriecin had no effect on the activity of purified preparations of $p33^{cdk2}$–cyclin A, $p34^{cdc2}$–cyclin A or $p34^{cdc2}$–cyclin B kinases (results not shown). This suggests that the decrease in

**Table I.** Histone H1 kinase activity from fostriecin-treated FT210 cells

| Treatment[a] | Mitotic index (%) | $p34^{cdc2}$ kinase[b] activity | $p33^{cdk2}$ kinase[b] activity |
|---|---|---|---|
| NOC | 41 | 100 | 100 |
| NOC + Fos 0.5 h | 50 | 112 | 50 |
| NOC + Fos 1 h | 41 | 52 | 53 |
| NOC + Fos 2 h | 63 | 40 | 30 |
| 39°C | 2 | 1 | 75 |
| 39°C + Fos 0.5 h | 6 | 4 | 142 |
| 39°C + Fos 1 h | 31 | 2 | 32 |
| 39°C + Fos 2 h | 53 | 0 | 3 |
| STSP | 0 | 5 | 115 |
| STSP + Fos 0.5 h | 0 | 1 | 43 |
| STSP + Fos 2 h | 0 | 1 | 36 |

[a]FT210 cells were arrested in mitosis with nocodazole (NOC) or in $G_2$ either at 39°C or with staurosporine (STSP). Fostriecin (Fos) was added to these cultures for up to 2 h.
[b]The histone H1 kinase activity of $p34^{cdc2}$ and $p33^{cdk2}$ immunoprecipitated from treated cells is expressed as a percentage of the activity from nocodazole-arrested cells, which was defined as 100%.

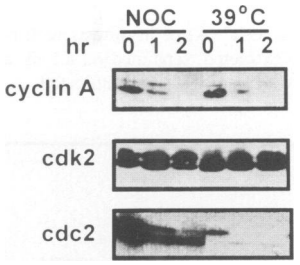


**Fig. 3.** Effect of fostriecin on cyclin A, $p33^{cdk2}$ and $p34^{cdc2}$. FT210 cells arrested in $G_2$ at 39°C or in mitosis at 32°C with nocodazole (NOC) were treated with fostriecin for 0, 1 or 2 h. Cell extracts were prepared and the proteins were run on a 9.5% polyacrylamide gel containing SDS. Cyclin A, $p33^{cdk2}$ and $p34^{cdc2}$ were visualized by immunoblotting.

$p33^{cdk2}$ and $p34^{cdc2}$ kinase activity observed during the treatment of cells with fostriecin is not due to direct inhibition by fostriecin.

We next examined the effect of fostriecin on the amount and state of cellular $p34^{cdc2}$, $p33^{cdk2}$, cyclin A and cyclin B using Western blot analysis. $p34^{cdc2}$ was readily detectable in cells arrested in mitosis with nocodazole at 32°C (Figure 3). The addition of fostriecin for 1 or 2 h caused a decrease in the amount of $p34^{cdc2}$. In cells arrested at 39°C, very little $p34^{cdc2}$ was present and the residual amount was mostly in the slowly migrating, highly phosphorylated state characteristic of the inactive form of the enzyme. The addition of fostriecin caused the complete disappearance of the residual $p34^{cdc2}$. In contrast, fostriecin had no effect on the amount of $p33^{cdk2}$ and did not cause any mobility shift indicative of dephosphorylation in nocodazole-arrested cells or cells arrested in $G_2$ at 39°C. Interestingly, fostriecin caused a rapid loss of cyclin A (Figure 3) and cyclin B (results not shown) in both nocodazole-arrested cells and cells arrested in $G_2$ at 39°C. Thus, the fostriecin-induced decrease in activity of the $p33^{cdk2}$ kinase is probably due to degradation of its cyclin subunit, and the decrease in $p34^{cdc2}$ activity is probably caused by the loss of both enzyme subunits.

### *Fostriecin does not stimulate histone H1 phosphorylation during chromosome condensation*

$p34^{cdc2}$ kinase is the major mitotic histone H1 kinase (Langan *et al.*, 1989). Mitotic phosphorylation of histone H1 is correlated with chromosome condensation. Because fostriecin can cause chromosome condensation in the absence of $p34^{cdc2}$ kinase activity, we next determined its effect on histone H1 phosphorylation in the cell. Nocodazole-arrested cells and cells arrested in $G_2$ at 39°C were treated with fostriecin in the presence of [$^{32}$P]inorganic phosphate. Histones were extracted, separated by electrophoresis on acid urea–polyacrylamide gels and subjected to autoradiography. In this gel system, increasingly phosphorylated histones migrate as discrete bands of decreased mobility (Langan, 1982).

The fostriecin treatment of cells arrested in $G_2$ at 39°C did not result in any increase in the level of histone H1 phosphorylation compared with untreated cells after 30 min (results not shown) or 1 or 2 h (Figure 4A); rather, a small decrease was observed (see also Figure 6A). Similarly, fostriecin-treated mitotic cells showed no increase in histone H1 phosphorylation (Figure 4B). The phosphorylation state of the slowest migrating histone H1 species remained largely unchanged while that of the fastest migrating species decreased, indicating that fostriecin affects interphase and mitotic histone H1 phosphorylation differently. These results show that chromosome condensation can be induced in $G_2$ by fostriecin in the absence of histone H1 hyperphosphorylation.

### *Phosphorylation of histones H2A and H3 is stimulated by fostriecin*

Histone H3 is normally phosphorylated only at mitosis, whereas histone H2A is phosphorylated throughout the cell cycle. Unlike histone H1 phosphorylation, the phosphorylation of histones H2A, H2A.X and H3 was enhanced greatly in FT210 cells treated with fostriecin at 39°C (Figure 4A). No unphosphorylated histone H3 was detected by Coomassie blue staining (results not shown), suggesting that all histone H3 molecules were phosphorylated. Furthermore, phosphorylated bands with lower mobility than phosphorylated histone H3 from mitotic cells were observed upon treatment of nocodazole-arrested cells with fostriecin (Figure 4B). This suggests phosphorylation at a second site, in addition to the mitotic site. 2-D gel electrophoresis confirmed this suggestion. Figure 5 (lower panel) shows the position of phosphorylated histones H1, H2A and H3 in nocodazole-arrested cells not treated with fostriecin. Three subtypes of phosphorylated histone H3 are present in these cells, and are revealed as three radioactive spots migrating identically in the second dimension. The upper panel in Figure 5 shows that upon the addition of fostriecin to nocodazole-arrested cells, histone H3 migrated slower in the first dimension. This shows that an additional phosphate was added to histone H3 in the presence of fostriecin. During treatment with fostriecin, histone H2A phosphorylation increased in intensity without any change in migration in the first dimension, suggesting an increase in the number of molecules that were phosphorylated. A similar increase in phosphorylation was also observed for histone H2A.X which comigrates with histone H3 in the second dimension,

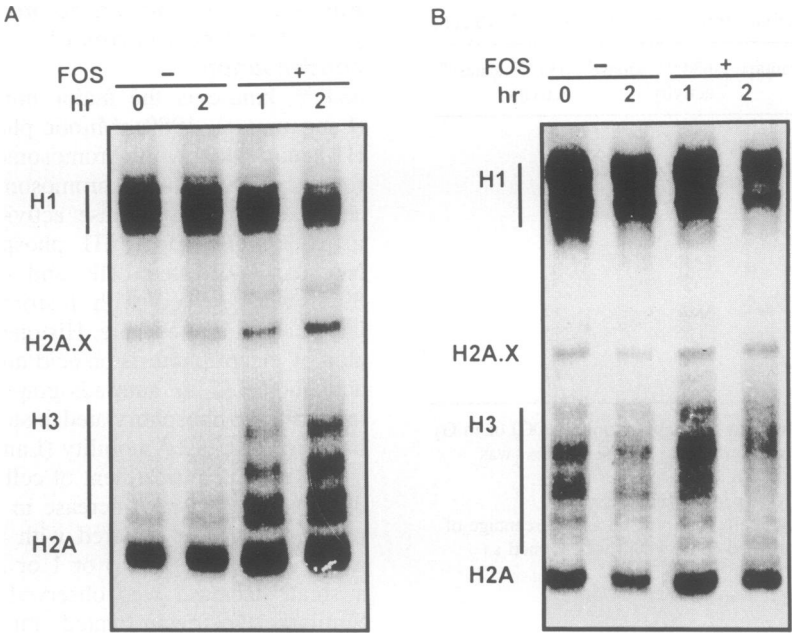


**Fig. 4.** Phosphorylation of histones in the presence of fostriecin. FT210 cells were arrested in $G_2$ at 39°C (**A**) or in mitosis with nocodazole (**B**) in the presence of [$^{32}$P]inorganic phosphate and treated with fostriecin for 0, 1 or 2 h. Histones were extracted, separated on a long acid urea–polyacrylamide gel and visualized by autoradiography. The positions of histones H1, H2A.X, H3 and H2A are indicated.

as shown by the filled arrows. This result indicates that a higher proportion of the family of histone H2A became phosphorylated, to the level of one phosphate per histone. Further, these results show that the phosphorylation of histone H1 is regulated very differently from that of histones H3 and H2A. This raises the possibility that the additional phosphorylation of histones H3 and H2A induced by fostriecin may cause chromosomes to condense in the absence of H1 hyperphosphorylation.

### *Histone H2A and H3-specific phosphatases are inhibited by fostriecin*

The overall phosphorylation level of proteins in cells is in a dynamic equilibrium determined by the opposing activities of kinases and phosphatases. In the presence of fostriecin, histone H1 phosphorylation remained unchanged or decreased, but histone H2A and histone H3 phosphorylation increased compared with untreated cells. This increased phosphorylation could result from an increase in kinase activity or a decrease in phosphatase activity, or both. Fostriecin is an inhibitor of PP1 and PP2A *in vitro* (Roberge *et al.*, 1994) and is expected to inhibit these protein phosphatases in cells. In addition, since the activity of some kinases is positively regulated by phosphorylation, the inhibition of a protein phosphatase may cause the activation of a protein kinase. To distinguish between these possibilities, pulse and chase experiments were performed.

The lower level of [$^{32}$P]phosphate incorporation in histone H1 caused by fostriecin in FT210 cells at 39°C (Figures 4A and 6A) is compatible with an inhibition of histone H1 kinase activity or a stimulation of histone H1 phosphatase activity, but it is not compatible with the inhibition of histone H1 phosphatase activity. Since we have shown above that p33$^{cdk2}$ and p34$^{cdc2}$ kinase activity decreased during treatment with fostriecin, we conclude

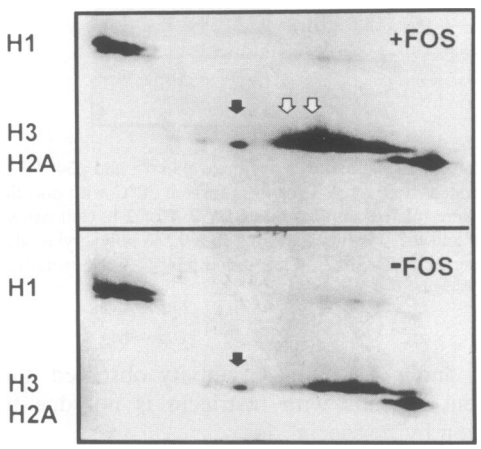


**Fig. 5.** 2-D gel electrophoresis of phosphorylated histones. FT210 cells were arrested in mitosis with nocodazole in the presence of [$^{32}$P]inorganic phosphate and treated with or without fostriecin for 2 h. Histones were extracted, separated on a long acid urea–polyacrylamide gel in the first dimension and by SDS–PAGE in the second dimension, and visualized by autoradiography. The open arrows indicate histone H3 containing more than one phosphate per molecule; the filled arrows indicate histone H2A.X. The positions of histones H1, H3 and H2A in the second dimension are also indicated.

from these experiments that fostriecin acts by causing a decrease in histone H1 kinase activity, rather than by inhibiting histone H1 phosphatase activity.

The addition of fostriecin resulted in an increase in the phosphorylation level of histones H2A and H3, through either activating histone H2A and H3 kinases or inactivating histone H2A and H3 phosphatases, or both. FT210 cells arrested in $G_2$ at 39°C were labeled with [$^{32}$P]inorganic phosphate for 18 h; [$^{32}$P]phosphate was washed out and incubation was continued in normal medium for up to 3 h with or without fostriecin (Figure 6B). Untreated cells

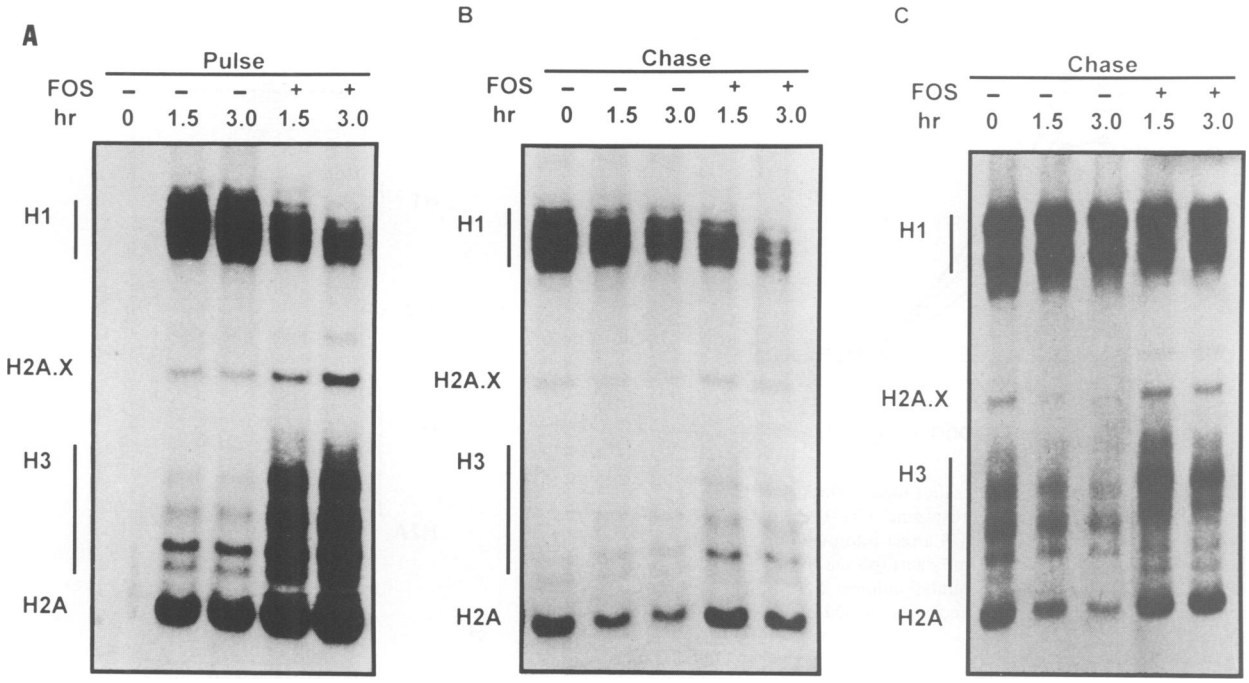


**Fig. 6.** Histone phosphorylation in the presence of fostriecin during pulse and chase labeling. In the pulse experiment (**A**), FT210 cells were arrested in $G_2$ at 39°C and then treated with [$^{32}$P]inorganic phosphate for 1.5 or 3.0 h in the absence or presence of fostriecin. In the chase experiment (**B**), FT210 cells were arrested in $G_2$ at 39°C in the presence of [$^{32}$P]inorganic phosphate for 18 h to label histones. At 0 h, the cells were transferred to medium without [$^{32}$P]inorganic phosphate and incubated in the absence or presence of fostriecin for 1.5 and 3.0 h. In a second chase experiment (**C**), FT210 cells were arrested in mitosis with nocodazole in the presence of [$^{32}$P]inorganic phosphate for 18 h. At 0 h, the cells were transferred to medium without [$^{32}$P]inorganic phosphate and incubated in the absence or presence of fostriecin for 1.5 and 3.0 h. Histones were extracted, separated on long acid urea–polyacrylamide gels and detected by autoradiography. The positions of histones H1, H2A.X, H3 and H2A are indicated.

showed a steady decrease in $^{32}$P incorporated into histones H2A and H3 over 3 h, indicating that histone H2A and H3 phosphatases were active in those cells. Conversely, this decrease in $^{32}$P incorporation was not only stopped by fostriecin treatment, but a slight increase was observed. This is consistent with both an inhibition of the histone H2A and H3 phosphatases and an activation of histone H2A and H3 kinases.

A similar set of experiments was performed with cells arrested in mitosis at 32°C with nocodazole, which have high levels of histone H1 and H3 phosphorylation (Figure 6C). Untreated controls showed a steady decrease in incorporated [$^{32}$P]phosphate during the chase, showing that phosphatases remain active when histones are maximally phosphorylated. In the presence of fostriecin, $^{32}$P-labeling of the faster migrating forms of histone H1 decreased more rapidly than that of the slower migrating forms, compared with controls. This indicates a more rapid decrease in the interphase level of histone H1 phosphorylation than in the mitotic level of phosphorylation. Since the kinases responsible for H1 phosphorylation were inactivated in the presence of fostriecin (Table I and Figure 3), the different effects of fostriecin on interphase and mitotic phosphorylation of histone H1 suggest that fostriecin inhibits mitotic histone H1 phosphatase activity but not interphase histone H1 phosphatase activity.

A chase in the presence of fostriecin resulted in a slight increase in $^{32}$P incorporation into histones H2A and H3 and a shift to lower mobility of histone H3 (Figure 6C). These results suggest that fostriecin inhibits histone H3 and H2A phosphatases in mitotic cells.

### *Chromosome condensation induced by fostriecin requires the activity of staurosporine-sensitive protein kinases*

To determine whether the induction of chromosome condensation by fostriecin requires protein phosphorylation, we examined the effect of staurosporine on condensation. Staurosporine has been shown recently to inhibit the mitotic histone H1 kinase activity and H3 kinase activity (Th'ng *et al.*, 1994) and to cause $G_2$ arrest.

FT210 cells were presynchronized and then released at 32°C into growth medium containing different concentrations of staurosporine, together with nocodazole to capture cells that enter mitosis. Staurosporine prevents entry into mitosis in a concentration-dependent manner (Figure 7). The effect was half-maximal at 50 ng/ml staurosporine; 100 ng/ml was sufficient to block entry into mitosis completely. The addition of up to 100 μM fostriecin for up to 4 h to cells blocked in $G_2$ with staurosporine did not overcome $G_2$ arrest. Okadaic acid at a concentration of 1 μM was also unable to overcome this effect.

The addition of fostriecin to cells arrested in $G_2$ with 150 ng/ml staurosporine caused no increase in histone H1 or H3 phosphorylation, but did cause an increase in the phosphorylation of H2A and H2A.X (Figure 8A). This result shows that distinct histone kinases phosphorylate H2A and H3 and that increased histone H2A phosphorylation is not sufficient to induce chromosome condensation, supporting the proposal that histone H3 phosphorylation is required for chromosome condensation. The treatment of staurosporine-arrested cells with fostriecin did not result in any changes in the amount or electrophoretic mobility

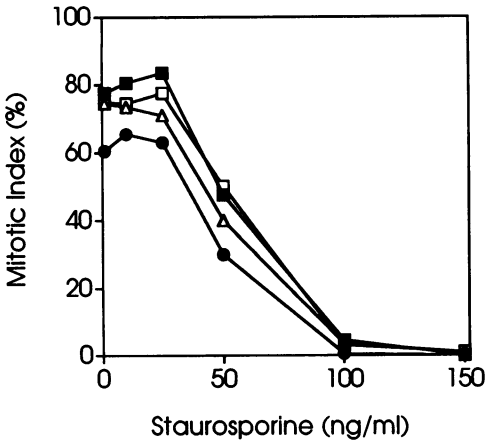


**Fig. 7.** Fostriecin and okadaic acid cannot induce chromosome condensation in the presence of staurosporine. FT210 cells were released from aphidicolin-induced $G_1$/S arrest into medium containing 50 ng/ml nocodazole and 0–150 ng/ml staurosporine for 18 h, and the mitotic index was determined (●). Parallel cultures were further incubated for 4 h with 100 μM fostriecin (△), 1 μM okadaic acid (□), or neither (■).

of cyclin A, $p34^{cdc2}$ or $p33^{cdk2}$ (Figure 8B). The $p34^{cdc2}$ kinase activity in staurosporine-arrested FT210 cells was very low and was not affected significantly by the presence of fostriecin (Table I). On the other hand, $p33^{cdk2}$ kinase activity in staurosporine-arrested cells was comparable with that in mitotic cells, and the addition of fostriecin led to a loss of activity (Table I).

Th'ng *et al.* (1994) have shown that the addition of staurosporine to mitotic cells causes histone dephosphorylation and chromosome decondensation. Cells were arrested at 39°C and induced to condense their chromosomes by the addition of fostriecin for 1 or 2 h. Staurosporine was then added for up to an additional 4 h and chromosome decondensation was not observed (results not shown). The simplest interpretation of this result is that in the presence of fostriecin, staurosporine does not cause any histone dephosphorylation or chromosome decondensation because fostriecin inhibits histone phosphatases. Together, the results show that chromosome condensation induced by fostriecin requires protein phosphorylation, and that both protein kinases and phosphatases are important for regulating chromosome condensation.

## Discussion

Using a temperature-sensitive $p34^{cdc2}$ mutant cell line and protein phosphatase inhibitors that can induce chromosome condensation, we have been able to uncouple the process of chromosome condensation from the activity of $p34^{cdc2}$ kinase and H1 phosphorylation. These results have important implications for our understanding of the mechanisms governing entry into mitosis and chromosome condensation.

$p34^{cdc2}$ kinase is highly conserved in eukaryotes and is essential for entry into mitosis in yeast (Nurse and Bisset, 1981; Reed and Wittenberg, 1990) and higher eukaryotes (Th'ng *et al.*, 1990; van den Heuvel and Harlow, 1993). How can we reconcile this with our finding that chromosome condensation, a major mitotic event, can occur in

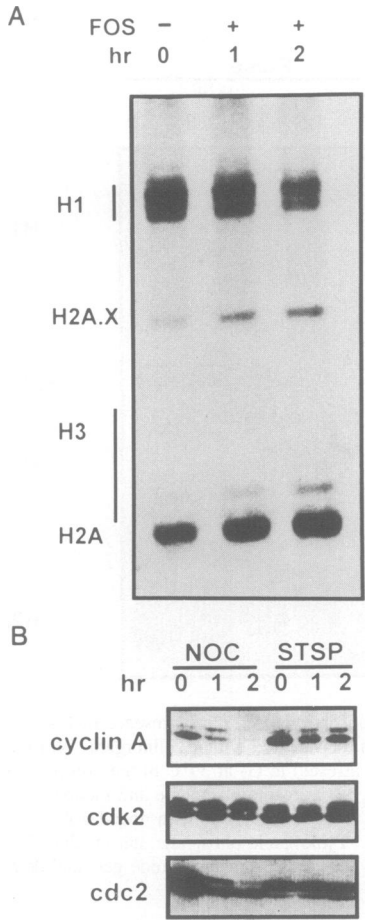


**Fig. 8.** Phosphorylation of histones in the presence of both staurosporine and fostriecin. FT210 cells were arrested in $G_2$ with 150 ng/ml staurosporine in the presence of [$^{32}$P]inorganic phosphate for 18 h. 100 μM fostriecin were then added for 1 or 2 h. Histones were extracted, separated on a long acid urea–polyacrylamide gel and visualized by autoradiography (**A**). The positions of histones H1, H2A.X, H3 and H2A are indicated. In (**B**), total proteins from parallel samples were separated by SDS–PAGE. Cyclin A, $p34^{cdc2}$ and $p33^{cdk2}$ were detected by immunoblotting (STSP). For comparison, nocodazole-arrested cells were treated with fostriecin and analyzed in the same way (NOC).

cells that do not have $p34^{cdc2}$ kinase activity? Several explanations can be put forward. One possibility is that fostriecin causes the untimely activation of a different cyclin-dependent kinase, whose function at least partly overlaps with that of $p34^{cdc2}$ kinase and which can induce chromosome condensation. Several mammalian protein kinases have been described recently that share extensive amino acid sequence identity with $p34^{cdc2}$ (Elledge and Spottswood, 1991; Ninomiya-Tsuji *et al.*, 1991; Tsai *et al.*, 1991; Lapidot-Lifson *et al.*, 1992; Meyerson *et al.*, 1992; Xiong *et al.*, 1992). One of these, $p33^{cdk2}$, is activated earlier in the cell cycle than $p34^{cdc2}$, during S and $G_2$ (Rosenblatt *et al.*, 1992). However, $p33^{cdk2}$ kinase activity was not correlated with chromosome condensation induced by fostriecin. Moreover, we showed that cyclin A and cyclin B are degraded concomitantly with chromosome condensation during fostriecin treatment. Thus, an involvement of $p33^{cdk2}$ or other kinases regulated by cyclin A or cyclin B seems unlikely. A second possibility is suggested by the fact that fostriecin and okadaic acid are potent

protein phosphatase 1 and 2A inhibitors (Cohen *et al.*, 1990; Roberge *et al.*, 1994): cyclin-independent protein kinases that can induce chromosome condensation may be active during interphase, but might not induce condensation because of the antagonizing action of protein phosphatases. A third possibility is that fostriecin and okadaic acid activate a mitotic kinase downstream of $p34^{cdc2}$ kinase in the cascade of events that induce mitosis. O'Connell *et al.* (1994) have shown recently that the expression of a stable form of NIMA kinase can induce premature chromatin condensation without a requirement for $p34^{cdc2}$ kinase. Since NIMA is required for entry into mitosis in *Aspergillus nidulans*, the authors proposed a model in which NIMA kinase is involved in chromatin condensation while $p34^{cdc2}$ kinase is required for other aspects of mitosis. Although no functional homologs have yet been described in organisms other than *A.nidulans*, it is tempting to speculate that the protein kinase required for chromosome condensation induced by fostriecin might be a NIMA kinase. However, unlike chromosome condensation induced by fostriecin, chromatin condensation induced by the expression of NIMA in HeLa cells did not resemble that of cells entering mitosis. Rather, NIMA caused the condensation of chromatin into a single mass without distinguishable chromosomes, or into condensed fragmented structures characteristic of apoptosis. Clearly, it is important to identify the protein kinase(s) required for chromosome condensation.

Fostriecin also had striking effects on the phosphorylation of histones. Unlike normal chromosome condensation, chromosome condensation induced by fostriecin in FT210 cells depleted of $p34^{cdc2}$ kinase was not accompanied by an increase in histone H1 phosphorylation even at 30 min when $p33^{cdk2}$ activity was increased slightly. This indicates that chromosome condensation was not due to transient histone H1 hyperphosphorylation caused by the transient increase in $p33^{cdk2}$ activity. This negative correlation strengthens the view that $p34^{cdc2}$ kinase is the enzyme that phosphorylates histone H1 at mitosis *in vivo*. Thus, although chromosome condensation is accompanied by histone H1 hyperphosphorylation during normal mitosis, as well as during premature chromosome condensation induced by cell fusion (Hanks *et al.*, 1983) or by temperature shift of tsBN2 cells (Ajiro *et al.*, 1983), this is not an absolute requirement.

Fostriecin induced the phosphorylation of almost 100% of H3 molecules at one or two sites. The phosphorylation of histone H3 is correlated with chromosome condensation in normal mitosis: no phosphorylation is seen during interphase, and all histone H3 molecules become monophosphorylated at mitosis (Gurley *et al.*, 1978). This raises the interesting possibility that an increase in histone H3 phosphorylation could normally cause chromosome condensation. Histone H2A was also significantly more phosphorylated in the presence of fostriecin. Histone H2A is phosphorylated throughout the cell cycle (Gurley *et al.*, 1978). The protein kinases that phosphorylate histones H3 and H2A in response to fostriecin are not known yet. Several kinases phosphorylate histone H3 *in vitro*, including a cAMP-dependent kinase (Glass and Krebs, 1979; Paulson and Taylor, 1982; Taylor, 1982), a little characterized chromatin-bound protein kinase (Shoemaker and Chalkley, 1980) and $pp90^{rsk}$ (Chen *et al.*, 1992). Histone H2A can be phosphorylated *in vitro* by a cAMP-dependent protein kinase (Martinage *et al.*, 1980), a cGMP-dependent kinase (Hashimoto *et al.*, 1976; Glass and Krebs, 1979) and protein kinase C (Takeuchi *et al.*, 1992). We are currently identifying the sites on histones H2A and H3 that are phosphorylated in response to fostriecin to identify the kinases involved.

The increased histone H2A and H3 phosphorylation observed during chromosome condensation induced by fostriecin is strongly suggestive of a role in condensation but does not prove it. How might histone phosphorylation cause chromosome condensation? All the histones have a globular domain and one or two flexible basic N- or C-terminal domains. The nucleosome consists of DNA coiled twice around the globular domains of the core histones $(H3_2H4_2)(H2AH2B)_2$, with histone H1 sealing off the two turns of DNA (Bradbury, 1992). The flexible domains are not required to generate the nucleosome. Instead, they probably interact with the DNA linking the nucleosomes. This has been demonstrated for the C-terminal flexible domain of histone H2A in chromatin (Usachenko *et al.*, 1994). The histone tails also have the potential to interact with each other or with other proteins. It is significant that phosphorylation occurs only in these flexible domains (reviewed in Bradbury, 1992) and has been shown to alter their interactions with DNA and with proteins. Phosphorylation of histone H1 tails weakens their interaction with DNA (Hill *et al.*, 1991), perhaps promoting chromosome condensation by allowing the binding of non-histone proteins to the vacated sites (Marion *et al.*, 1985; Lennox and Cohen, 1988). Phosphorylation also promotes interactions between histone H1 and non-histone proteins such as nucleolin (Kharrat *et al.*, 1991), and may modulate interactions between histones themselves, such as H1 and H2A (Boulika *et al.*, 1980), and H3 and H1 (Mazen *et al.*, 1987; Shibata and Ajiro, 1993). Such protein−protein interactions could also cause chromosome condensation. Histone H2A phosphorylation has been shown to be necessary for the regular spacing of nucleosomes assembled in cell-free extracts (Kleinschmidt and Steinbeisser, 1991) and could perhaps facilitate chromosome condensation by establishing regularly ordered chromatin.

It is possible that a major factor in chromosome condensation is simply the degree to which the positive charges of histone tails are neutralized, either to offset electrostatic repulsion between histones or to modulate the charge balance between histones and DNA in the region of the linker DNA. Then, the overall degree of histone tail phosphorylation may be more important for chromosome condensation than the particular identity of the phosphorylated histone. Our observation that the increase in histone phosphorylation during fostriecin-induced chromosome condensation is comparable with the increase observed during a normal $G_2$/M transition is compatible with this proposal.

Our results showing that protein kinase activity is required for chromosome condensation induced by protein phosphatase inhibitors strongly suggest that chromosome condensation is controlled by an interplay of kinases and phosphatases. The inhibition of histone H2A and H3 phosphatases by fostriecin indicates a more significant role for protein phosphatases in controlling chromosome

condensation than has been recognized previously. We suggest that an important function of protein phosphatases during interphase is to negatively regulate histone phosphorylation and prevent premature condensation of chromatin.

## Materials and methods

### *Cell synchronization and drug treatment*

FT210 cells were routinely maintained at 32°C at a density of ~$5\times10^5$ cells/ml in RPMI-1640 supplemented with 10% calf serum (Th'ng *et al.*, 1990). The cells were synchronized by incubation in isoleucine-deficient RPMI-1640 medium supplemented with 10% heat-inactivated, dialyzed calf serum for 15 h, followed by incubation in regular medium containing 2.5 μg/ml aphidicolin for 9 h to arrest the cells at the $G_1$/S boundary. For $G_2$ arrest, the cells were released from $G_1$/S arrest into regular medium at 39°C or in the presence of 100 ng/ml staurosporine at 32°C for 18 h. For mitotic arrest, cells were released from $G_1$/S arrest into regular medium containing 50 ng/ml nocodazole for 18 h at 32°C. Fostriecin (NSC-339638, lot 94528) was obtained from NCI and was added to cells from a fresh stock solution in PBS. Okadaic acid was obtained from Gibco as a 0.5 mM solution in 10% dimethyl sulfoxide (DMSO) and was maintained at −20°C. Staurosporine was obtained from LC Laboratories and stored in DMSO at 0.1 μg/ml at −20°C. The effect of these inhibitors on protein phosphorylation was determined by labeling with [$^{32}$P]inorganic phosphate as described by Th'ng *et al.* (1994).

### *Fluorescence microscopy*

After drug treatment, the cells were harvested by centrifugation, swelled in hypotonic medium (75 mM KCl), fixed with methanol:acetic acid (3:1), spotted onto microscope slides and stained with bisbenzimide (Roberge *et al.*, 1994). Cells were photographed on Kodak Tmax 400 film using a Zeiss Axiophot microscope.

### *Protein analysis and protein kinase assays*

Western blotting and protein kinase assays were as described in Guo *et al.* (1994) with minor modifications. Briefly, 2.5–$5.0\times10^6$ cells were harvested and lysed on ice for 30 min with vigorous pipetting in lysis buffer (50 mM Tris−HCl, pH 7.4, 300 mM NaCl, 5 mM $MgCl_2$, 0.5% Triton X-100, 50 U/ml micrococcal nuclease, 50 μg/ml aprotonin, 50 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM okadaic acid and 1 mM sodium orthovanadate). Cell debris was then removed by centrifugation at 10 000 *g* for 5 min. For Western blot analysis, samples were boiled for 5 min in lysis buffer supplemented with 1% SDS, 10% β-mercaptoethanol and trace amounts of bromophenol blue. 50 μg cellular protein were loaded per lane on 9.5% polyacrylamide−SDS gels. After electrophoresis, the proteins were transferred to polyvinylidine difluoride (PVDF) immobilon membrane (Millipore) using a Bio-Rad semi-dry blotter. The membrane was incubated with blocking buffer (BB; 25 mM Tris−HCl, pH 7.4, 150 mM NaCl) supplemented with 3% non-fat dried milk for 30 min at room temperature. The membrane was then incubated in BB supplemented with 3% dried milk, 0.05% Tween 20 and 350 mM NaCl for 1–2 h at room temperature with 1/2000 dilution of antiserum to C-terminal peptides of $p33^{cdk2}$ and $p34^{cdc2}$, to cyclin A (a gift from T.Hunter and J.Pines) or to cyclin B (GNS1, Santa Cruz Biotech.). The membrane was then washed three times with BB containing 0.05% Triton X-100 and 1% dried milk, and then incubated for 1 h with HRP-conjugated anti-rabbit or anti-mouse antibody followed by three washes and detection by enhanced chemiluminescence (Amersham).

For protein kinase assays, cell lysates containing 0.5–1.0 mg protein were immunoprecipitated and assayed for the presence of protein kinase activity as in Guo *et al.* (1994). Briefly, the lysates were pre-cleared by incubation with 0.5 μl preimmune serum and 25 μl of a 50% slurry of protein A−Sepharose 6MB on ice for 30 min with rotation. After centrifugation to remove the protein A−Sepharose 6MB, 1 μl of antiserum and 50 μl of a 50% slurry of protein A−Sepharose 6MB were added to the supernatant and incubated on ice for 2 h with rotation. The beads were then washed five times with 0.5 ml lysis buffer. To assay histone H1 kinase activity, the beads were washed once with H1 kinase buffer (50 mM Tris−HCl, pH 7.4, 2 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl, 0.05 mM ATP). To start the reaction, 30 μl of H1 kinase buffer supplemented with 1 mM sodium orthovanadate, 1 μM okadaic acid, 0.625 mg/ml steer thymus histone H1 (a gift from R.D.Cole) and 0.25 mCi/ml [γ-$^{32}$P]ATP (1000 Ci/mmol) were added to the immunoprecipitates and incubated at 30°C for 10 min. The reaction was stopped by adding an equal volume of 2× SDS sample buffer and boiling for 10 min. 5–10 μl were loaded per lane of a 13% polyacrylamide gel containing SDS. The level of phosphorylation was determined using a Bio-Rad Molecular Imager.

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