The EMBO Journal vol.13 no.7 pp.1549–1556, 1994

Cdc25A is a novel phosphatase functioning early in the cell cycle

Shigeki Jinno[1,3], Kimihiko Suto[1,3], Akihisa Nagata[1,3], Makoto Igarashi[2], Yoshihide Kanaoka[1], Hiroshi Nojima[1] and Hiroto Okayama[1,2,3,4]

[1]Department of Molecular Genetics, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565 and [2]The Okayama Cell Switching Project, ERATO, JRDC, Pasteur Building, Sakyo-ku, Kyoto 606, Japan
[3]Present address: Department of Biochemistry, The University of Tokyo, Faculty of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan
[4]Corresponding author

Communicated by P.Nurse

The *cdc25*$^+$ tyrosine phosphatase is a key mitotic inducer of the fission yeast *Schizosaccharomyces pombe*, controlling the timing of the initiation of mitosis. Mammals contain at least three *cdc25*$^+$ homologues called cdc25A, cdc25B and cdc25C. In this study we investigate the biological function of cdc25A. Although very potent in rescuing the *S.pombe* cdc25 mutant, cdc25A is less structurally related to the *S.pombe* enzyme. Northern and Western blotting detection reveals that unlike cdc25B, cdc25C and cdc2, cdc25A is predominantly expressed in late G1. Moreover, immunodepletion of cdc25A in rat cells by microinjection of a specific antibody effectively blocks their cell cycle progression from G1 into the S phase, as determined by laser scanning single cell cytometry. These results indicate that cdc25A is not a mitotic regulator but a novel phosphatase that plays a crucial role in the start of the cell cycle. In view of its strong ability to activate cdc2 kinase and its specific expression in late G1, cdc2-related kinases functioning early in the cell cycle may be targets for this phosphatase.

Key words: cdc2/cdc25 phosphatase/cell cycle/G1-S progression

Introduction

The growth of eukaryotes results from their consecutive progression through four distinct phases called G1, S, G2 and M. This process, named cell cycle progression, is controlled mainly in the G1 and G2 phases. The cell's commitment to grow or differentiate is made in G1. On the other hand, the timing of the initiation of mitosis is determined in G2, ensuring the complete duplication of the cell's genetic information before cell division is allowed to take place. The molecular mechanism for the G2 control is best understood in the fission yeast *Schizosaccharomyces pombe*. The onset of mitosis is triggered by the activation of the *cdc2*$^+$ kinase complex containing a B-type cyclin called Cdc13 as a regulatory subunit (Goebl and Byers, 1988; Hagan *et al.*, 1988; Solomon *et al.*, 1988; Booker *et al.*, 1989). During the S and G2 phases, this kinase is phosphorylated at the Tyr15 residue by another kinase called *wee1*$^+$ and also by its sibling *mik1*$^+$, remaining inactivated (Russell and Nurse, 1987; Lundgren *et al.*, 1991). When cells are ready to divide, the *cdc2*$^+$ kinase is activated by dephosphorylation with a specific tyrosine phosphatase called *cdc25*$^+$ and initiates mitosis (Russell and Nurse, 1986; Gould *et al.*, 1990).

Higher eukaryotes contain a similar *cdc2*$^+$ kinase complex initially identified as a mitosis promoting factor (MPF). This kinase is controlled by a similar mechanism. During the interphase, MPF remains inactive because of phosphorylation at both the Thr14 and Tyr15 residues of cdc2 kinase (Solomon *et al.*, 1990; Krek and Nigg, 1991). Human cells contain a *wee1*$^+$ kinase counterpart, which phosphorylates the Tyr15 residue (Igarashi *et al.*, 1991; Parker and Piwnica-Worms, 1992). The identity of the enzyme phosphorylating Thr14 is unclear. When cells are ready to enter the M phase, both Tyr15 and Thr14 undergo dephosphorylation, resulting in the activation of cdc2 kinase. This dephosphorylation is catalysed by phosphatase(s) homologous to *cdc25*$^+$ (Gautier *et al.*, 1991). Thus, the principal mechanism for G2 regulation is highly conserved from yeast through to humans. However, mammals contain at least three *cdc25*$^+$ phosphatase homologues, called cdc25Hs (cdc25C), cdc25Hu2 (cdc25B) and cdc25Hu3 (cdc25A) (Sadhu *et al.*, 1990; Galaktionov and Beach, 1991; Nagata *et al.*, 1991; Okayama *et al.*, 1992). cdc25B has the ability to dephosphorylate both Tyr15 and Thr14 (Honda *et al.*, 1993), whereas cdc25C dephosphorylates cdc2 kinase yet undergoes phosphorylation and concomitant activation by cdc2 kinase, forming a positive feedback loop (Hoffmann *et al.*, 1993).

On the contrary, the mechanism regulating the G1/S transition is far less clear. In the budding yeast the *cdc2*$^+$/CDC28 kinase controls both transitions (Forsburg and Nurse, 1991). In mammalian cells, however, cdc2 kinase appears to regulate the G2/M transition only. Recently, several cdc2-related kinases, such as cdk2, cdk3, cdk4 and cdk5, have been identified from mammals by expression cloning using a CDC28 mutant of the budding yeast as a host or by cross-hybridization (Elledge and Spottswood, 1991; Ninomiya-Tsuji *et al.*, 1991; Meyerson *et al.*, 1992). Cdk2 is active during the S and G2 phases (Rosenblatt *et al.*, 1992), and immunodepletion of this protein results in inhibition of DNA synthesis (Fang and Newport, 1991). In addition, mammalian cells contain multiple G1-type cyclins called cyclin C, D and E. Cyclins D and E are associated with cdk4 and cdk2 kinases, respectively. Cdk4 is expressed in late G1 and early S (Dulic *et al.*, 1992; Koff *et al.*, 1992). Interestingly enough, these cdc2-related kinases possess the conserved Tyr phosphorylation site which is originally found in cdc2, and their activity appears to be regulated by phosphorylation and dephosphorylation at this Tyr residue (Gu *et al.*, 1992; Matsushime *et al.*, 1992).

In this report we show that cdc25A, one of the three mammalian cdc25 homologues, is a novel tyrosine phosphatase that plays a crucial role in the start of the cell cycle. We also suggest that the target for this phosphatase may be cdc2-related kinases expressed early in the cell cycle.

Results

Structural comparison of rat and human cdc25A and cdc25B

We and others previously have identified and cloned two human *cdc25*$^+$ homologues called cdc25A and cdc25B by *trans*-complementation of a *S.pombe* mutant or by PCR (Galaktionov and Beach, 1991; Nagata *et al.*, 1991; Okayama *et al.*, 1992). We concluded that cdc25B displayed properties as a mitotic inducer (Nagata *et al.*, 1991; Honda *et al.*, 1993). To facilitate analysis of the biological role of cdc25A, we decided to work with an immortal untransformed rat kidney fibroblast cell line called NRK. This cell line has a unique property of reversible transformability by a combination of growth factors, such as EGF and TGF-β.

First we isolated full-length rat cdc25A and cdc25B cDNAs and compared their structures with the human counterparts. The rat cdc25A and cdc25B cDNAs were 3.4 and 2.8 kb in size and encoded 525 and 574 amino acid proteins, respectively, whereas their human cognates are composed of 523 and 566 amino acids (Galaktionov and Beach, 1991; Nagata *et al.*, 1991). Their amino acid sequences were compared (Figure 1). Cdc25A and cdc25B retain 84 and 78% amino acid sequence identities between human and rat, respectively. In both phosphatases, the catalytic domain residing in the 160 amino acid C-terminal region is highly conserved (Galaktionov and Beach, 1991; Nagata *et al.*, 1991). However, amino acid conservation in this region is extraordinarily high in cdc25A, marking 98% identity versus 89% for cdc25B. This unusually high conservation of the cdc25A's catalytic domain between two species must be a reflection of its functional importance.

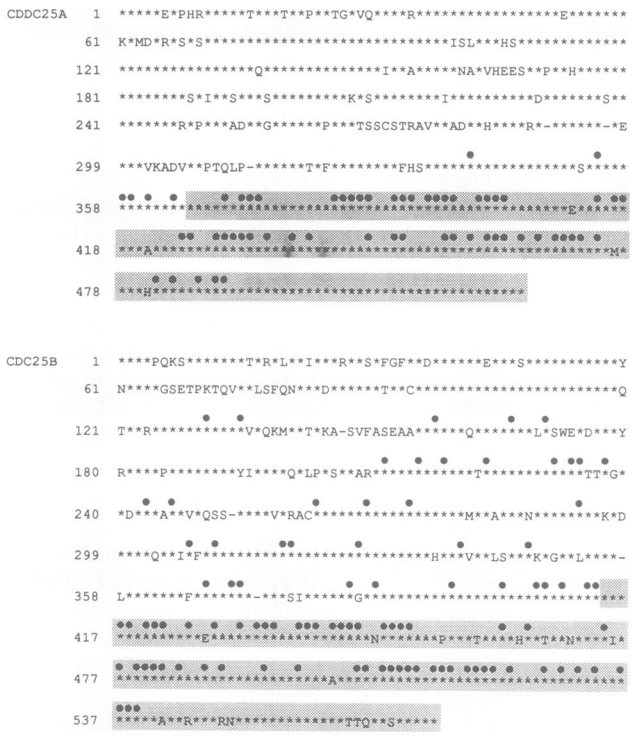

Fig. 1. The amino acid sequences of rat cdc25A and cdc25B compared with the human cognates. Asterisks denote the sequence of human enzymes. The highlighted region indicates the phosphatase domain. Filled circles on the sequences show amino acids identical to *S.pombe cdc25*$^+$. The nucleotide sequences have been deposited in the EMBL/Genbank databases under the accession numbers D6236 and D6237.

Both cdc25A and cdc25B from rat and human rescued a temperature-sensitive cdc25 mutant of *S.pombe*, but cdc25A was ~10-fold more potent than cdc25B, irrespective of the temperatures used for complementation and the source of the genes (Table I). Consistent with this, the cells rescued by cdc25A exhibited a morphology similar to that of the wild-type cells (Figure 2). Despite the ability to efficiently rescue a cdc25 mutation, cdc25A is no more closely related to the *S.pombe cdc25*$^+$ in amino acid sequence. As indicated by dots in Figure 1, cdc25B rather retains a higher similarity to the *S.pombe* enzyme throughout the entire sequence.

cdc25A mRNA is expressed early in the cell cycle

Since mammalian cells contained at least three *cdc25*$^+$ homologues and both cdc25B and cdc25C were expressed in G2 (Sadhu *et al.*, 1990; Nagata *et al.*, 1991), we next examined the cell cycle dependency of cdc25A expression in rat NRK cells. NRK cells were arrested in G1 by contact inhibition and serum deletion, and were induced to start the cell cycle by replating at a low cell density with medium containing serum and the two growth factors. Cells were harvested every 3 h and the levels of the mRNAs for cdc25A, cdc25B, cdc2 and β-actin were determined by Northern blotting. In parallel, their cell cycle progression was monitored by flow cytometry. As shown in Figure 3, 12 h after stimulation cells began to enter the S phase, and at 18–21 h most cells reached the G2 phase. cdc2 mRNA was undetectable in G1, but became visible in S and highly induced in G2. cdc25B mRNA began to be induced at 12 h, slightly behind cdc2 mRNA, and peaked in G2, in good agreement with our previous report (Nagata *et al.*, 1991). On the contrary, cdc25A mRNA started to be expressed in G1 3–6 h after stimulation and peaked at 9 h, just before the onset of the S phase. This pattern of cdc25A expression was not specific to the rat gene. As shown in Figure 4, virtually identical results were obtained with KD cells, an untransformed mortal human fibroblast cell clone. In KD

Table I. Complementation of a *S.pombe* cdc25 mutant by cdc25A and cdc25B

Gene	% Complementation		
	35°C	36°C	37°C
Rat			
cdc25A	27	26	10
cdc25B	4	3	1
Human			
cdc25A	33	27	10
cdc25B	4	3	<0.1
CAT	<0.1	<0.1	<0.1

CAT is the chloramphenicol acetyltransferase gene and was used as a negative control.

cells, however, a considerable amount of cdc25B mRNA was detectable in the quiescent state, but its biological significance is currently unclear.

cdc25A protein is predominantly expressed in late G1

To confirm the expression of cdc25A in G1, we performed immunoblot detection in synchronized NRK cells. A polyclonal antibody was generated against MYSRLKKL, the C-terminal eight amino acids, which was unique to cdc25A. This polyclonal antibody called anti-cdc25A-peptide detected rat cdc25A protein expressed in *Escherichia coli*. Its specificity was confirmed by suppression of detection with an excess amount of the peptide used for the antibody production (data not shown). When NRK cells were analysed, the antibody visualized a 65 kDa protein as a specific band which was slightly larger than the molecular weight (59 kDa) calculated from the deduced amino acid sequence.

To further confirm the specificity of the anti-cdc25A-peptide antibody, we generated another polyclonal antibody, but this time against a whole GST-fused human cdc25A protein made in *E.coli*. Immunoglobulin (Ig) G was purified from the antiserum with protein A−Sepharose and pretreated with *E.coli*-produced GST-fused human cdc25B protein to eliminate those that react with GST or the sequence common in cdc25 phosphatases. The final Ig preparation, termed anti-cdc25, did not react with GST or GST-fused cdc25B (data not shown), but efficiently detected GST-fused cdc25A by immunoblotting (Figure 5, lane e). An NRK cell extract prepared 6 h after growth stimulation was first immunoprecipitated with anti-cdc25A and then immunoblotted with anti-cdc25A-peptide following SDS−PAGE (Figure 5, lane b). As a positive control, the GST-fused rat cdc25A protein was similarly immunoprecipitated and blotted (lane d). The GST-fused cdc25A protein was used instead of the original cdc25A because IgG comigrated with cdc25A and hindered its detection. In parallel, the same amount of the 6 h NRK cell extract as for lane b was directly immunoblotted with anti-cdc25A-peptide (lane a) or anti-cdc25A (lane d). The detection of GST-fused cdc25A in lane c proved that anti-cdc25A was able to effectively recognize and precipitate rat cdc25A. When incubated with NRK cell extracts, anti-cdc25A precipitated and specifically visualized by direct blotting the same 65 kDa band as detected with anti-cdc25A-peptide (lanes b and d). Comparison of their intensities indicates that ~50% of the cdc25A protein in the cell extract was immunoprecipitated with anti-cdc25A. Thus, the two antibodies independently raised against different regions of cdc25A recognized the same 65 kDa band. From these results we concluded that the 65 kDa band was indeed the rat cdc25A protein.

To determine the cell cycle-dependent production of the cdc25A protein, NRK cells were similarly arrested in G1 and induced to start growth by replating at a low density with medium containing serum, EGF and TGF-β, as for the Northern analysis. But this time, cell cycle progression was monitored for 27 h. In this particular experiment, NRK cells entered the G2 phase 18−21 h after stimulation, and most of the cells returned to G1 by 24 h (Figure 5). As an internal control, the cdc2 protein was quantitated. In accordance with the level of the mRNA, the cdc2 protein started to appear at 12 h (early S phase), increased significantly at 18 h (S to G2 phase), and peaked at 21−24 h (G2 to M and G1). The G1-arrested NRK cells contained a band detectable by the anti-cdc2 mAb, but whether it was cdc2 itself or an antigenically-related protein was unclear. In contrast, the cdc25A protein began to appear at 3 h (slightly ahead of the mRNA), peaked at 6 h and decreased in early S phase. At 24 h when the cells re-entered the G1 phase, the production of the cdc25A protein was up again. Thus, the cdc25A protein was produced most in G1 and least in G2. These results indicate that, unlike cdc25B and cdc25C, cdc25A is not a mitotic regulator, but is a novel phosphatase which may function early in the cell cycle.

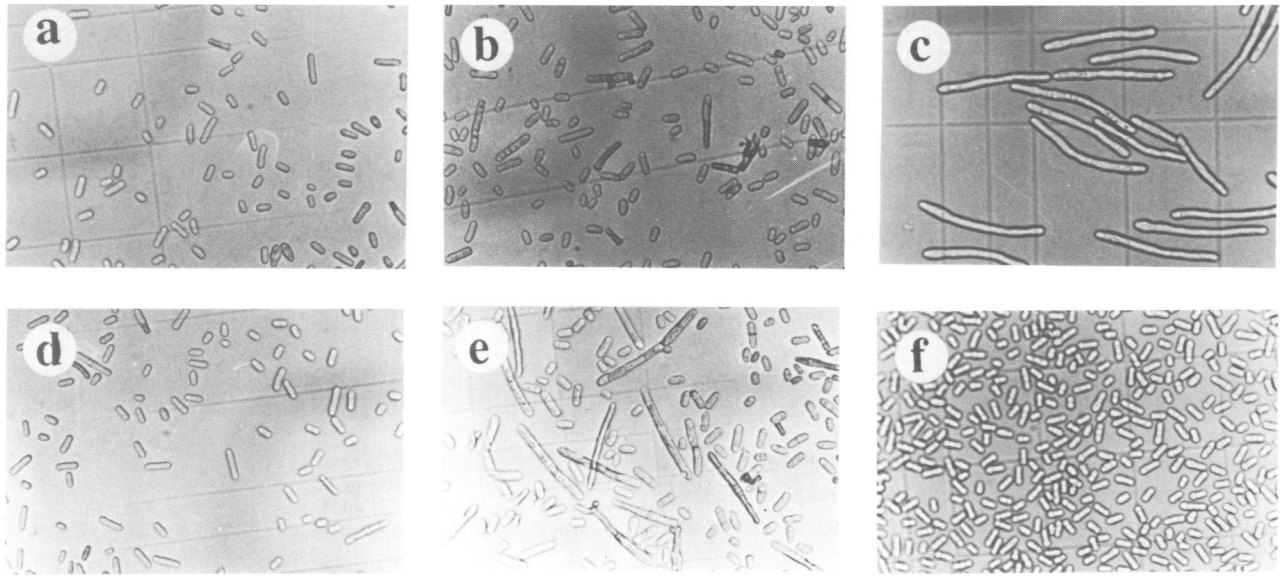

Fig. 2. The morphology of a temperature-sensitive *S.pombe* cdc25 mutant rescued by rat or human cdc25A or cdc25B. The *S.pombe* strain *cdc25-22 leu1-32 h*$^-$ was transfected with the pcD2 vector containing rat cdc25A cDNA (**a**), rat cdc25B cDNA (**b**), the chloramphenicol acetyltransferase gene as a negative control (**c**), human cdc25A cDNA (**d**) or human cdc25B cDNA (**e**). Cells were then spread on minimal medium agar plates and incubated at 23°C for 24 h and then at 34°C for 5 days. (**f**) Wild-type strain grown under identical conditions.

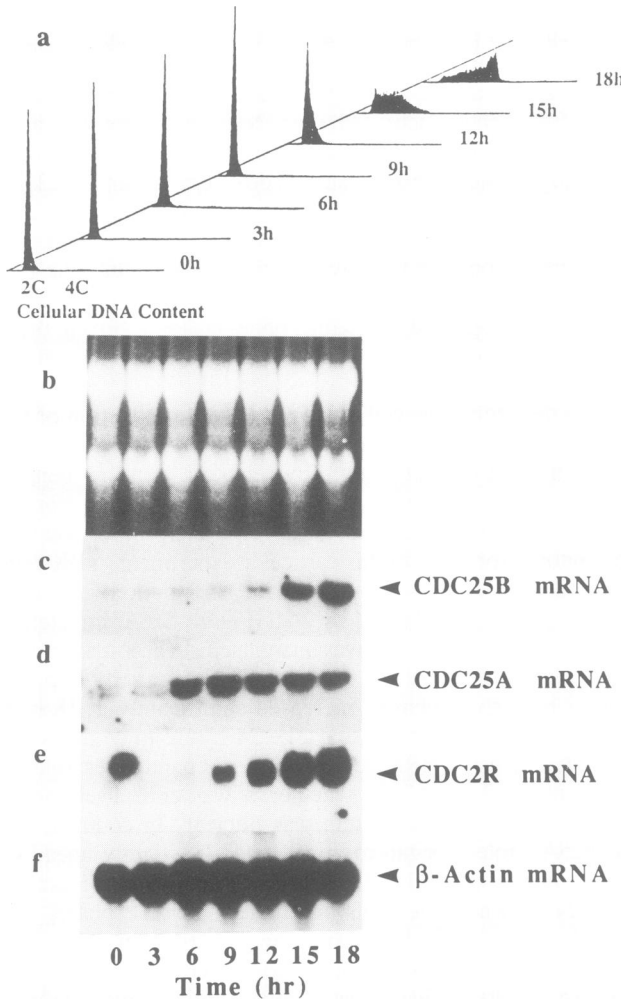

Fig. 3. Cell cycle-dependent expression of cdc25A mRNA in NRK cells. NRK cells were arrested by contact inhibition and serum depletion, and stimulated to grow by replating in DMEM containing 5% FCS, EGF and TGF-β (see Materials and methods for details). Cell cycle patterns were analysed by flow cytometry (**a**). Cells were harvested at the indicated time, and total RNA (**b**) was prepared and analysed by Northern blot hybridization with rat cdc25B cDNA (**c**), rat cdc25A cDNA (**d**) and rat cdc2 cDNA (**e**) as probes. As an internal control, rat β-actin mRNA (**f**) was quantitated.

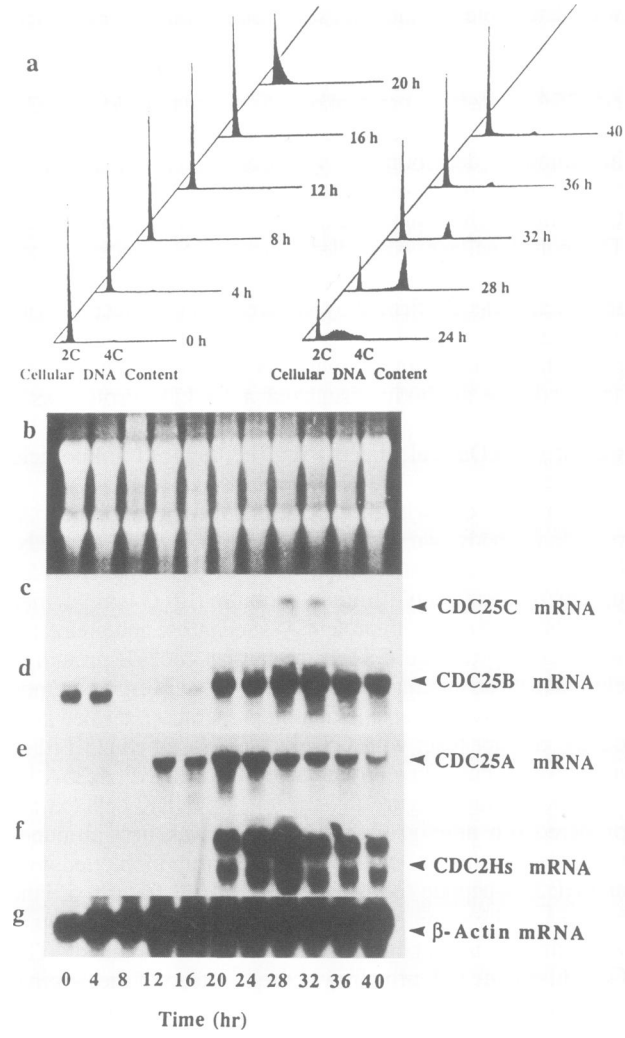

Fig. 4. Cell cycle-dependent expression of cdc25A mRNA in human cells. KD cells were analysed as described in Figure 3. The cell cycle progression was monitored by flow cytometry (**a**). Total RNA (**b**) was prepared and analysed by Northern blot hybridization with ^{32}P-labelled human cdc25C (**c**), cdc25B (**d**), cdc25A (**e**) and cdc2Hs (**f**) as probes. As an internal control, human β-actin mRNA was quantitated (**g**).

cdc25A is required for the start of the cell cycle

To identify the biological role of cdc25A, the anti-cdc25A antibody was microinjected into NRK cells and their cell cycle progression was examined. NRK cells were sparsely plated on culture dishes, arrested in G1 by serum depletion and induced to start the cell cycle by the addition of 5% FCS, EGF and TGF-β. One hour later, an anti-cdc25A or a control Ig solution containing 1% fluorescein isothiocyanate (FITC)-dextran was microinjected into the cells. FITC-dextran was used to identify injected cells. Twenty-four hours later, the number of injected cells and those that were entering, or were already in, the M phase (rounded and dividing cells) was counted (see Figure 7). Under these growth stimulation conditions, which did not include the replating step, the start of the cell cycle was slightly slower and less synchronized than that in Figure 6, and none of the cells entered the M phase before this time. As summarized in Table II, in three separate experiments ~20% of the cells injected with the control Ig were entering, or had already entered, the M phase. On the contrary, only <3% of the cells injected with

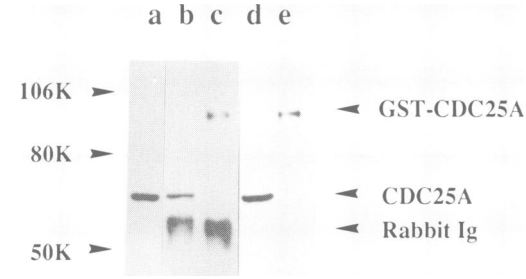

Fig. 5. Immunoprecipitation and immunoblot detection of rat cdc25A by two independently-raised antibodies. An NRK cell extract 6 h after growth factor addition (lane b) and an *E.coli* lysate containing GST-fused cdc25A protein (lane c) were immunoprecipitated with the anti-cdc25A antibody, electrophoresed and immunoblotted with the anti-cdc25A-peptide antibody. The same amount of the 6 h NRK cell extract was electrophoresed and immunoblotted with anti-cdc25A-peptide (lane a) or anti-cdc25A (lane d) without prior immunoprecipitation. An *E.coli* lysate containing GST-fused cdc25A protein was also electrophoresed and directly blotted with anti-cdc25A (lane e).

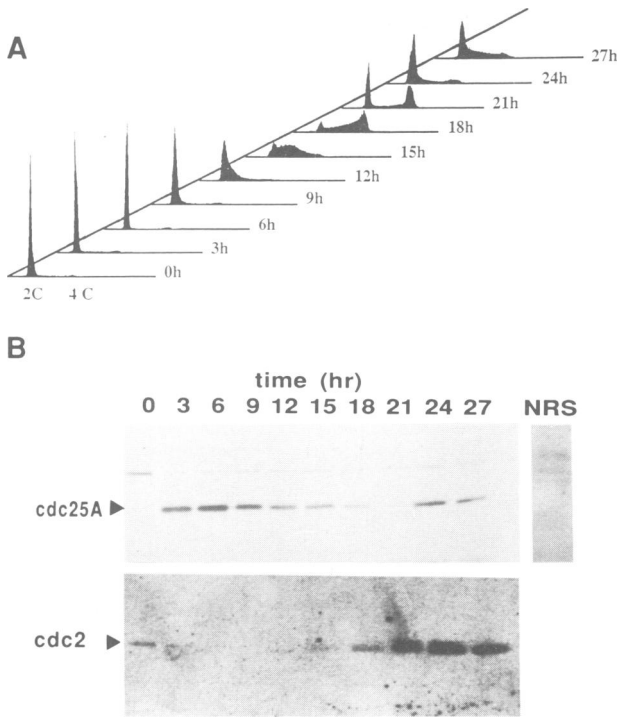

Fig. 6. Cell cycle-dependent expression of cdc25A and cdc2 proteins in NRK cells. NRK cells were arrested in G1 and stimulated to grow as described in Figure 3. Cell cycle patterns at each time point were determined by flow cytometry (**A**). 20 μg cell extracts were electrophoresed on a 10% SDS−polyacrylamide gel, and the cdc25A and cdc2 proteins were detected by Western blot using the anti-cdc25A-peptide antibody or the anti-cdc2 mAb (**B**). In lane NRS, 20 μg of the 6 h cell extract was electrophoresed and detected with normal rabbit serum following blotting.

the anti-cdc25A antibody was in the M phase. The block in the cell cycle progression by anti-cdc25A was also confirmed by almost no increase in the cell number after a 28−30 h incubation.

To identify the point at which anti-cdc25A blocked the cell cycle, we quantitated the DNA content of the injected cells by propidium iodide (PI) staining followed by laser scanning single cell cytometry. Cells were prepared, injected and incubated similarly, but this time for 22 h, to determine the DNA content of the cells before they enter the M phase. Injected cells were identified by the presence of FITC-dextran, marked and fixed with ethanol followed by RNase A treatment and PI staining. The marked cells were scanned by an argon laser scanning microscope, the intensity of fluorescent light emitted from DNA-bound PI was digitized in 256 grades and the total PI fluorescence intensity in each nucleus (relative DNA content) was calculated by the IBAS image analysis system. To validate this new method for cell cycle analysis, the DNA contents of NRK cells in both log and stationary phases were determined (Figure 8A and B, respectively). As shown in this figure, the distribution pattern of DNA content in these cells was very similar to those generally obtained by flow cytometry (Vindelov *et al.*, 1983). To avoid confusion, anti-cdc25A and control Ig were injected into cells grown in separate dishes. Consequently, it had to be confirmed that cells in two separate dishes had progressed in the cell cycle to the same extent after 22 h incubation. Therefore, ~500 uninjected cells near injected cells in each dish were analysed. As shown in Figure 8C and D, uninjected cells in both dishes had similar cell cycle patterns; 55% of the cells were in S or G2 and 45% stayed in G1. The cells injected with control Ig entered the cell cycle to the same extent as uninjected cells (54% in S or G2 and 46% in G1; Figure 8D), indicating that neither injection of an irrelevant antibody nor mechanical damage to cells by a needle influenced cell cycle progression. On the contrary, the injection of anti-cdc25A effectively blocked the cells from starting the cell cycle; 76% of the cells remained in G1 and few reached G2 (Figure 8E). These data strongly indicate that cdc25A is required for cells to enter and/or progress in the S phase.

Discussion

Unlike yeast (Russell and Nurse, 1986), mammals contain at least three distinct *cdc25*$^+$ homologues, all of which possess a tyrosine phosphatase activity and the ability to rescue a *S.pombe* cdc25 mutant (Sadhu *et al.*, 1990; Galaktionov and Beach, 1991; Nagata *et al.*, 1991; Strausfeld *et al.*, 1991). However, they greatly differ in their ability to act as *cdc25*$^+$ phosphatase. As we reported previously (Nagata *et al.*, 1991), cdc25B (cdc25Hu2) is more active than cdc25C (cdc25Hs) in both complementation assay and mitosis induction assay (Enoch and Nurse, 1990). cdc25A is 10 times more potent than cdc25B, irrespective of whether they are human or rat genes (Table I). Despite its strong ability to act as a mitotic inducer in the fission yeast, the biological role of cdc25A is unlikely to be as a mitotic regulator.

As we have shown in this paper, the expression of cdc25A mRNA began in early G1, peaked late in G1 and decreased in G2, in both human and rat cells. The level of the cdc25A protein behaved similarly. Consistent with its expression pattern, immunodepletion of cdc25A by microinjection of a specific antibody effectively blocked cells to start the cell cycle, and most of the cells remained in G1 with few reaching G2. These results lead us to conclude that cdc25A is not a mitotic regulator, but is a crucial factor that is required for cells to enter or to progress in the S phase. Thus, despite its strong ability to activate cdc2 kinase, cdc25A sharply differs in biological function from the other cdc25s (Sadhu *et al.*, 1990; Nagata *et al.*, 1991). Consequently, the target for this phosphatase is unlikely to be cdc2 kinase. As we have shown, neither mRNA nor protein of cdc2 is detectable in late G1, the time when cdc25A protein is produced at maximum levels.

Fission as well as budding yeast utilizes cdc2/cdc28 kinase as a key component for cell cycle progression in both G1 and G2 (Forsburg and Nurse, 1991). In contrast, higher eukaryotes appear to employ cdc2-related kinase(s), instead of cdc2, for G1 regulation (Th'ng *et al.*, 1990). Among the cdc2-related kinases identified to date, those called cdk require cyclin for their activity and are highly related to cdc2 kinase. These include cdk2, cdk3, cdk4 and cdk5 (Elledge and Spottswood, 1991; Lew *et al.*, 1991; Matsushime *et al.*, 1991, 1992; Ninomiya-Tsuji *et al.*, 1991; Xiong *et al.*, 1991; Koff *et al.*, 1991; Meyerson *et al.*, 1992). Cdk2 is expressed at highest levels during the S and G2 phases (Rosenblatt *et al.*, 1992), whereas the *cdk4* gene product accumulates during the G1 phase and declines in the S phase in macrophage (Matsushime *et al.*, 1992). Cdk2 kinase is associated with cyclin E and is active in the G1 phase of

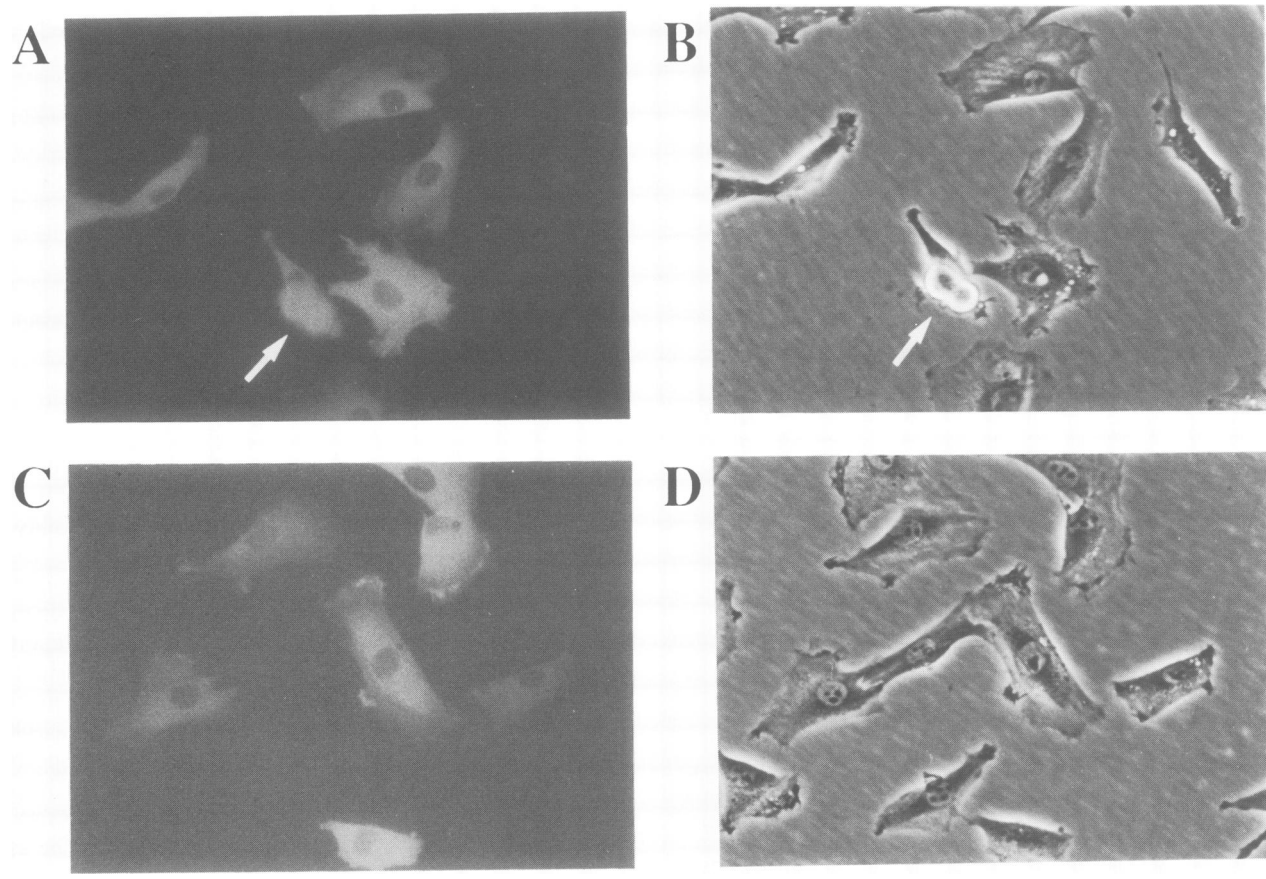

Fig. 7. Effect of anti-cdc25A injection on cell growth. NRK cells were arrested in G1 phase by serum depletion and stimulated by serum and the growth factors. Cells were then microinjected with anti-cdc25A antibody or control Ig. 24 h later, cells were photographed under a blue excitation or a phase contrast (see also Table II for quantitation). (**A–D**) The morphology of cells microinjected with control Ig (A and B) or anti-cdc25A (C and D). (A and C) FITC-labelled (antibody microinjected) cells. (B and D) Phase contrast images of A and C, respectively. The white arrow indicates a dividing cell.

HeLa cells (Dulic *et al.*, 1992; Koff *et al.*, 1992). On the other hand, cdk4 kinase is associated with cyclin D and phosphorylates the retinoblastoma gene product (Matsushime *et al.*, 1992). Interestingly, these cdk kinases contain the conserved Thr and Tyr phosphorylation sites initially found in cdc2 kinase, and the activity of these kinases appears to be regulated by a similar mechanism. Indeed, it has been shown that cdk2 kinase is phosphorylated at Thr14 and Tyr15 *in vivo*, and that its dephosphorylation activates the kinase (Gu *et al.*, 1992; Sebastian *et al.*, 1993). These data suggest that early cell cycle checkpoints are regulated by the cdc2-related kinases, which may in turn be regulated by some cdc25-related phosphatases. Our data that cdc25A phosphatase functions in G1 yet has an ability to activate cdc2 kinase, suggest that this phosphatase may be a putative regulator of the cdk kinases. The unusually high conservation of the cdc25A's catalytic domain between primate and rodent may be indicative of its general importance in mammalian cell cycle control.

Our results are inconsistent with the earlier report by Galaktionov and Beach (1991) that cdc25A is activated by cyclin B *in vitro*. Although the reason for the discrepancy is unclear, it is quite clear that the cdc25A protein is produced mainly in G1, the phase when cyclin B is produced the least. On the other hand, their results on antibody injection are similar to ours, but as we have shown, immunodepletion of cdc25A blocked cells to start DNA synthesis.

Table II. The effect of anti-cdc25 injection on cell cycle progression

Antibody	Cells injected	Cells in M phase	%
Control Ig			
Exp. 1	29	5	17.2
Exp. 2	65	13	20.0
Exp. 3	31	6	19.4
Anti-cdc25A			
Exp. 1	74	2	2.7
Exp. 2	37	0	<2.7
Exp. 3	121	3	2.5

Rounded and dividing cells were taken as those in M phase (see Figure 7).

Materials and methods

Cells and chemicals

KD (human lip skin primary fibroblast cell) and NRK-49F (normal rat kidney cell line) were obtained from the American Type Culture Collection. KD cells were maintained in 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Commonwealth Serum Lab., Australia), 100 U/ml penicillin G and 100 μg/ml streptomycin. NRK cells were maintained in 5% CO_2 at 35°C in the same medium but supplemented with 5% FCS. Crude human platelet-derived TGF-β was prepared as described (Assoian *et al.*, 1983). EGF was obtained from King Fermentation, Japan.

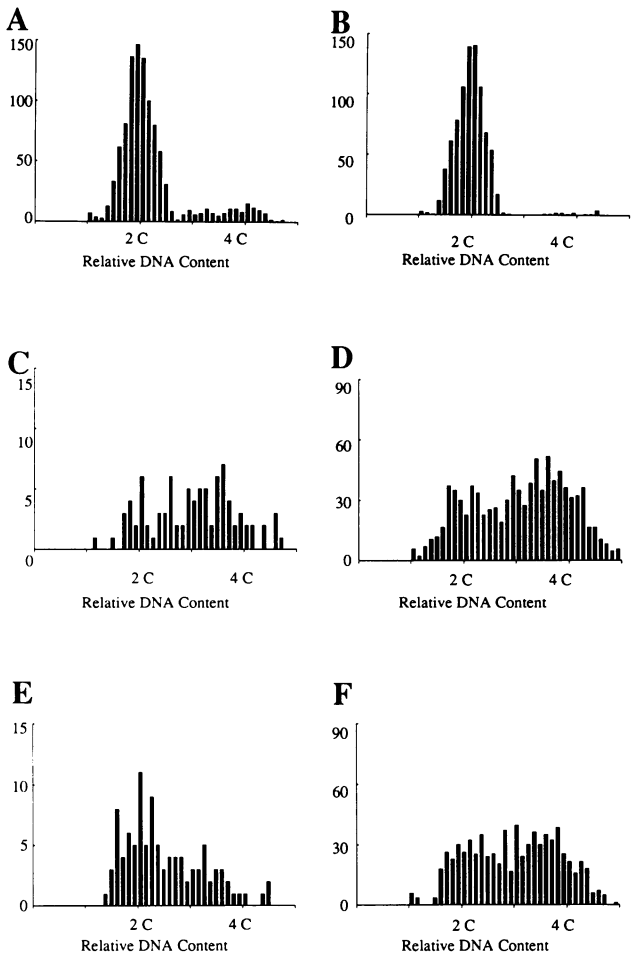

Fig. 8. Cell cycle analysis of anti-cdc25A-injected cells. NRK cells were injected with anti-cdc25A antibody or control Ig. (**A**) Log phase cells; (**B**) G1-arrested cells; (**C**) control Ig-injected cells; (**D**) uninjected cells near control Ig-injected cells; (**E**) anti-cdc25A-injected cells; (**F**) uninjected cells near anti-cdc25A-injected cells.

Expression cloning

Trans-complementation cloning of rat cdc25A was performed as described previously (Okazaki *et al.*, 1990). The temperature-sensitive lethal mutant strain *cdc25-22 leu1-32 h*$^-$ of *S.pombe* was used as a cloning host. A normal rat kidney cell line (NRK-49F) cDNA library was constructed in the pcD2 vector (Chen and Okayama, 1987) and transfected into the mutant yeast together with the pAL19 transducing vector. Cells were spread on minimum medium agar plates and incubated at 23°C for 24 h and then at 34°C for 4–5 days to select complemented colonies. Small molecular weight DNAs were prepared from the growing colonies (Rothstein, 1985) and transfected into the *E.coli* strain HB101 to recover cDNA clones (Inoue *et al.*, 1990).

Colony hybridization

A size-selected NRK cDNA sublibrary (>3.0 kb cDNA) was screened under a low stringency hybridization condition. The *Bgl*II–*Nde*I fragment (0.66 kb) of the human cdc25A cDNA and the *Nae*I–*Sac*I fragment (0.71 kb) of the human cdc25B cDNA were used as the 5′ region probes to isolate full-length clones for rat cdc25A and cdc25B cDNAs.

Complementation assay

The *S.pombe* strain *cdc25-22 leu1-32 h*$^-$ was transfected with the pcD2 vector containing the human cdc25A cDNA, the human cdc25B (cdc25Hu2) cDNA, the rat cdc25A cDNA, the rat cdc25B cDNA or the chloramphenicol acetyltransferase gene. Cells were then spread on minimal medium and incubated at 23, 34, 35 and 36°C for 5 days. The number of colonies was then counted. The percentage of complementation was calculated by dividing the number of colonies grown at the non-permissive temperatures by the number of colonies grown at 23°C.

Cell cycle analysis

NRK cells grown to confluence were incubated in DMEM containing 0.05% FCS for 48 h. Cells were stimulated to growth by replating at a density of 2 × 10^5 cells/10 cm dish) in DMEM containing 5% FCS. KD cells were arrested in G1 by incubating in DMEM with 0.2% FCS for 48 h. Cells were then growth-stimulated by replating at a density of 2 × 10^5 cells/10 cm dish in DMEM containing 10% FCS. Cells were harvested after incubation for the indicated time.

PI-labelled nuclei were prepared from synchronized cells using CycleTest (Becton-Deckinson, CA). 2 × 10^4 cells were analysed with a fluorescence-activated cell analyser as described previously (Vindelov *et al.*, 1983). The percentage of cell population in each phase was calculated with the SOBR model in the CELFIT program written for the FACScan cell analyser (Becton-Deckinson, CA).

Northern blot analysis

Total RNA was prepared from cultured cells by the modified guanidine thiocyanate method (Okayama *et al.*, 1987). Northern blot analysis was carried out as described (Maniatis *et al.*, 1989). 20 μg total RNA were loaded in each lane, electrophoresed on a 1% agarose gel containing 0.66 M formaldehyde, transferred to a Biodyne™ Transfer membrane (Paul Ultrafine Filtration Corp., Glen Cove, NY) and hybridized with each probe labelled with ^{32}P using a Multi Primer Labeling Kit (Amersham Corp.). Hybridized filters were washed in 0.2 × SSC at 60°C. The probes used are the 0.7 kb *Bgl*II–*Nde*I fragment of human cdc25A; the 1.7 kb *Nae*I–*Nae*I fragment of human cdc25B ; the 1.2 kb *Eco*RI–*Eco*RI fragment of human cdc25C; the 0.6 kb *Not*I–*Bgl*II fragment of human cdc2; the 1 kb *Xho*I–*Xho*I fragment of rat cdc25A; the 1.3 kb *Not*I–*Eco*RI fragment of rat cdc25B; and the 1.1 kb *Mro*I–*Eco*105I fragment of rat cdc2.

Antibody production

The anti-cdc25A-peptide rabbit antiserum was produced against the C-terminal decapeptide of rat cdc25A, MYSRLKKL, coupled to keyhole limpet hemocyanin (Maniatis *et al.*, 1989). The anti-cdc2 mAb that recognizes the KIRLESE sequence was obtained from MBL. The anti-cdc25A rabbit antibody was generated against the whole GST-fused human cdc25A protein produced in *E.coli* with the pGEXT2 vector (Pharmacia) containing the *Nco*I–*Sma*I fragment of the human cdc25A cDNA. The anti-cdc25A antibody was purified with protein A–Sepharose, and those that react with GST or the conserved regions of cdc25 phosphatases were eliminated by treatment with GST-fused human cdc25B protein. A GST-fused rat cdc25A protein was produced in *E.coli* from the pGEXT2 vector containing the *Nco*I–*Bam*HI fragment of the rat cdc25A cDNA, and was purified.

Immunoprecipitation

NRK cells (5 × 10^5) were lysed in 500 μl lysis buffer (10 mM Tris–HCl, pH 7.8, 1% NP-40, 0.15 M NaCl, 1 mM EDTA and 10 mg/ml aprotinin). 200 μg cell extracts were incubated with 2 μg anti-cdc25A and then incubated with 20 μl protein A–Sepharose (Yamanashi *et al.*, 1992). Immunoprecipitates were resuspended in 100 μl SDS sample buffer, and 10 μl each (20 μg as the original cell extracts) was used for Western blot analysis.

Western blot analysis

20 μg cell extracts were electrophoresed on 10% SDS–PAGE and transferred to a Hybond-ECL filter (Amersham Corp.) using the semi-dry electroblotting apparatus. The ECL filter was blocked by soaking in 5% non-fat dried milk in 10 mM Tris–HCl (pH 7.2) containing 150 mM NaCl and 0.05% Tween 20 for 1 h, and incubated with anti-cdc25A-peptide, anti-cdc25A or the anti-cdc2 mAb. The filters were then treated with horseradish peroxidase-conjugated anti-rabbit or mouse Ig (diluted 1:500) (Amersham Corp.), and signals were detected using the ECL system (Amersham Corp.).

Microinjection and laser scanning single cell cytometry

NRK cells were seeded on 3.5 cm dishes at a concentration of 10^4 cells/dish and were arrested in G1 by incubating in DMEM containing 0.05% serum for 48 h. Cells were then stimulated to start the cell cycle with medium containing 5% FCS, EGF and TGF-β. 1 h later, cells were microinjected with a phosphate-buffered saline-dialysed antibody solution (0.3 mg/ml), containing 1% FITC-dextran (mol. wt = 145 000) by using the AIS injection system (Zeiss, Germany), and were incubated for 22 or 24 h. Successfully-injected cells were identified by detecting FITC-dextran, fixed with 100% ethanol, treated with 0.1 mg DNase-free RNase A per ml, and stained with 4.16 mg PI per ml. Stained cells were then scanned with an argon laser microscope (Zeiss, Germany). The following parameters were used for the laser scanning: contrast, 394; brightness, 9208; zooming, 1; frame,

512 × 512 dots; scanning time, 8 s/frame; objective lens, 20× Plan-Neofluar lens. The fluorescence intensity for each dot was digitized in 256 grades and stored as an image data file on an optical disk. The contour of each nucleus was extracted and the total PI fluorescence intensity of each nucleus (relative DNA content) was calculated with the IBAS digital image analysis system (Zeiss, Germany).

Acknowledgements

We thank MBL for the gift of the anti-cdc2 mAb and for help with the production of the anti-GST-cdc25A polyclonal antibody. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and grants from the Asahi Glass Foundation, the Ryoichi Naito Foundation for Medical Research, the Kato Memorial Foundation, the Seijinbyo Igaku Kenkyu Shinkou Zaidan and the Osaka Cancer Society.

References

Assoian,R.K., Komoriya,A., Meyers,C.A., Miller,D.M. and Sporn,M.B. (1983) *J. Biol. Chem.*, **258**, 7155–7160.
Booker,R.N., Alfa,C.E., Hyams,J.S. and Beach,D.H. (1989) *Cell*, **58**, 485–497.
Chen,C. and Okayama,H. (1987) *Mol. Cell. Biol.*, **7**, 2745–2752.
Dulic,V., Lees,E. and Reed,S.I. (1992) *Science*, **257**, 1958–1961.
Elledge,S.J. and Spottswood,M.R. (1991) *EMBO J.*, **10**, 2653–2659.
Enoch,T. and Nurse,P. (1990) *Cell*, **60**, 665–673.
Fang,F. and Newport,J.W. (1991) *Cell*, **66**, 731–742.
Forsburg,S.L. and Nurse,P. (1991) *Annu. Rev. Cell Biol.*, **7**, 227–256.
Galaktionov,K. and Beach,D. (1991) *Cell*, **57**, 1181–1194.
Gautier,J., Solomon,M.J., Booker,R.N., Bazan,J.F. and Kirschner,M.W. (1991) *Cell*, **67**, 197–211.
Goebl,M. and Byers,M. (1988) *Cell*, **54**, 739–740.
Gould,K.L., Moreno,S., Tonks,N.K. and Nurse,P. (1990) *Science*, **250**, 1573–1576.
Gu,Y., Rosenblatt,J. and Morgan,D.O. (1992) *EMBO J.*, **11**, 3995–4005.
Hagan,I., Hayles,J. and Nurse,P. (1988) *J. Cell Sci.*, **91**, 587–595.
Hoffmann,I., Clarke,P.R., Marcote,M.J., Karsenti,E. and Draetta,G. (1993) *EMBO J.*, **12**, 53–63.
Honda,R., Ohba,Y., Nagata,A., Okayama,H. and Yasuda,H. (1993) *FEBS Lett.*, **318**, 331–334.
Igarashi,M., Nagata,A., Jinno,S., Suto,K. and Okayama,H. (1991) *Nature*, **353**, 80–83.
Inoue,H., Nojima,H. and Okayama,H. (1990) *Gene*, **96**, 23–28.
Koff,A., Cross,F., Fisher,A., Schumacher,J., Leguellec,K., Philippe,M. and Roberts,J.M. (1991) *Cell*, **66**, 1217–1228.
Koff,A. *et al.* (1992) *Science*, **257**, 1689–1694.
Krek,W. and Nigg,E.A. (1991) *EMBO J.*, **10**, 305–316.
Lew,D.J., Dulic,V. and Reed,S.I. (1991) *Cell*, **66**, 1197–1206.
Lundgren,K., Walworth,N., Booker,R., Dembski,M., Kirschner,M. and Beach,D. (1991) *Cell*, **64**, 1111–1122.
Maniatis,T., Fritsch,E.T. and Sambrook,J. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Matsushime,H., Roussel,M.F., Ashmun,R.A. and Sherr,C.J. (1991) *Cell*, **65**, 701–713.
Matsushime,H., Ewen,M.E., Storm,D.K., Kato,J., Hanks,S.K., Roussel,M.F. and Sherr,C.J. (1992) *Cell*, **71**, 323–334.
Meyerson,M., Enders,G.H., Wu,C.-L., Su,L.-K., Gorka,C., Nelson,C., Harlow,E. and Tsai,L.-H. (1992) *EMBO J.*, **11**, 2909–2917.
Nagata,A., Igarashi,M., Jinno,S., Suto,K. and Okayama,H. (1991) *New Biol.*, **3**, 959–968.
Ninomiya-Tsuji,J., Nomoto,S., Yasuda,H., Reed,S.I. and Matsumoto,K. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 9006–9010.
Okayama,H., Kawaichi,M., Brownstein,M., Lee,F., Yokota,T. and Arai,K. (1987) *Methods Enzymol.*, **154**, 3–28.
Okayama,H., Nagata,A., Igarashi,M., Suto,K. and Jinno,S. (1992) *Proceedings of the 22nd International Symposium of the Princess Takamatsu Cancer Research Fund, Tokyo (1991)*. Japan Scientific Society Press, pp. 231–238.
Okazaki,K., Okazaki,N., Kume,K., Jinno,S., Tanaka,K. and Okayama,H. (1990) *Nucleic Acids Res.*, **18**, 6485–6489.
Parker,L.L. and Piwnica-Worms,H. (1992) *Science*, **257**, 1955–1957.
Rosenblatt,J., Gu,Y. and Morgan,D.O. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 2824–2828.
Rothstein,R. (1985) In Glover,D.M. (ed.), *DNA Cloning: A Practical Approach*. Vol. 2, IRL Press, Oxford, pp. 45–65.
Russell,P. and Nurse,P. (1986) *Cell*, **45**, 145–153.
Russell,P. and Nurse,P. (1987) *Cell*, **49**, 559–567.
Sadhu,K., Reed,S., Richardson,H. and Russell,P. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 5139–5143.
Sebastian,B., Kakizuka,A. and Hunter,T. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 3521–3524.
Solomon,M., Booker,R., Kirschner,M. and Beach,D. (1988) *Cell*, **54**, 738–739.
Solomon,M.J., Glotzer,M., Lee,T.H., Philippe,M. and Kirschner,M.W. (1990) *Cell*, **63**, 1013–1024.
Strausfeld,U., Labbe,J.C., Fesquet,D., Cavadore,J.C., Picard,A., Sadhu,K., Russell,P. and Doree,M. (1991) *Nature*, **351**, 242–245.
Th'ng,J.P.H., Wright,P.S., Hamaguchi,J., Lee,M.G., Norbury,C.J., Nurse,P. and Bradbury,E.M. (1990) *Cell*, **63**, 313–324.
Vindelov,L.L., Christensen,I.J. and Nissen,N.I. (1983) *Cytometry*, **3**, 323–327.
Xiong,Y., Connolly,T., Futcher,B. and Beach,D. (1991) *Cell*, **65**, 691–699.
Yamanashi,Y., Fukui,Y., Wongsasant,B., Kinoshita,Y., Ichimori,Y., Toyoshima,K. and Yamamoto,T. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 1118–1122.

Received on January 11, 1993; revised on December 27, 1993