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Chromosome Condensation Caused by Loss of RCC1 Function Requires the cdc25C Protein That is Located in the Cytoplasm

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We cloned the hamster *cdc25C* cDNA by using the human *cdc25C* cDNA as a probe and prepared an antibody to *Escherichia coli*-produced hamster cdc25C protein that is specific to the human cdc25C protein. The microinjected antibody inhibited a chromosome condensation induced by tsBN2 mutation, indicating that the cdc25C protein is required for an activation of $p34^{cdc2}$ kinase caused by loss of RCC1 function. The hamster cdc25C protein located in the cytoplasm, prominently in a periphery of the nuclei of cells arrested with hydroxyurea, and seemed to move into the nuclei by loss of RCC1 function. Also, we found a molecular shift of the cdc25C protein in cells showing premature chromosome condensation (PCC), in addition to normal mitotic cells. This molecular-shift appeared depending on an activation of $p34^{cdc2}$ kinase.

INTRODUCTION

The cell cycle consists of four successive intervals: G1, S, G2, and M phases. The order of these intervals is ensured by dependent relationships where the initiation of late events depends on the completion of those preceding them (Hartwell and Weinert, 1989).

In all eukaryotic cells, entry into M phase begins with an activation of $p34^{cdc2}$ kinase, which occurs on completion of DNA replication. Thus $p34^{cdc2}$ kinase is negatively regulated in the interphase by unreplicated DNA. Such a negative feedback control can be abolished either by mutations or by drug treatments (see review Enoch and Nurse, 1991), resulting in premature chromosome condensation (PCC).[1]

The tsBN2 cell line, a temperature sensitive (ts) mutant of the BHK21/13 cell line, grows normally at a permissive temperature of 33.5°C, but ceases to grow at a nonpermissive temperature of 39.5°C (Nishimoto and Basilico, 1978). Cultures of tsBN2 cells arrested with hydroxyurea (HU) show PCC by shifting a temperature to the nonpermissive one (Nishimoto *et al.*, 1978, 1981; Nishitani *et al.*, 1991). In this mutant, therefore, a negative feedback control ensuring a coupling between S and M phases is defective at the nonpermissive temperature. By the DMGT (DNA mediated gene transfer) method, the gene complementing tsBN2 mutation has been cloned and designated as *RCC1*, regulator of chromosome condensation (Kai *et al.*, 1986; Ohtsubo *et al.*, 1987). From S phase onwards, the $p34^{cdc2}$ kinase is activated upon loss of RCC1 protein (Nishitani *et al.*, 1991). Such an activation of $p34^{cdc2}$ kinase and PCC-induction are prevented by emetine and cycloheximide, inhibitors of protein synthesis. (Nishimoto *et al.*, 1981; Ajiro and Nishimoto, 1985; Nishitani *et al.*, 1991).

So far, *RCC1*-homologous genes and its ts mutants have been isolated from both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, these being *srm1/prp20* (Clark and Sprague, 1989; Aebi *et al.*, 1990) and $pim1^-$ (Matsumoto and Beach, 1991), respectively. At the nonpermissive temperature *srm1* is arrested in G1 phase restoring the mating activity to receptorless mutants and *prp20* causes the alteration of mRNA metabolism. Both have a defect in the same gene, that is, *SRM1*. Although neither *srm1* nor *prp20* showed PCC at the nonpermissive temperature, these *S. cervisiae's* mutations can be

[1] Abbreviation used: HU, hydroxyurea; PBS, phosphate-buffered saline; PCC, premature chromosome condensation; PCR, polymerase chain reaction; ts; temperature sensitive.

complemented by the human *RCC1* cDNA and vice versa (Clark *et al.*, 1991; Fleischman *et al.*, 1991; Ohtsubo *et al.*, 1991).

S. pombe's ts mutant, *pim1*$^-$, arrested with HU, prematurely enters mitosis at the nonpermissive temperature, the same as tsBN2 cells (Matsumoto and Beach, 1991). The *pim1*$^-$, however, enters mitosis even from the G1 phase arrested by the *cdc10*$^-$ mutation. Thus the *pim1*$^-$ did not show G1 arrest, which is another phenotype of tsBN2 cells.

Matsumoto and Beach (1991) found that the *pim1*$^{-1}$ mutation can be suppressed by a multicopy suppressor, the *spi1*, encoding the protein similar (81% identity) to the human TC4, a small ras-like G protein (Drivas *et al.*, 1990). Independently, Bischoff and Ponstingl who purified the RCC1 protein from HeLa cells as a protein recognized by antikinetochore autoimmune sera (Bischoff *et al.*, 1990) found that the RCC1 protein formed a complex with the 25-kDa protein, which has turned out to be the TC4 protein, and was designated as Ran, ras-related nuclear protein (Bischoff and Ponstingl, 1991b). Furthermore, they found that the RCC1 protein functions as a guanine nucleotide exchanging protein on the Ran protein (Bischoff and Ponstingl, 1991a). The amount of Ran protein is a 25-fold molar excess over RCC1 in the nuclei (Bischoff and Ponstingl, 1991b). Because RCC1 is one of the major nonhistone proteins in the chromatin (Frasch, 1991; Dasso *et al.*, 1992), these findings indicated an existence of a signal transduction pathway from the chromatin to the cytoplasm. The RCC1 protein may transfer a signal of unreplicated DNA to an activator(s) of p34^{cdc2} kinase through the Ran protein, because a loss of RCC1 function abolishes a coupling between DNA replication and mitosis (Nishitani *et al.*, 1991).

The finding that cycloheximide inhibited an activation of p34^{cdc2} kinase arising from a loss of RCC1 function is consistent with the notion that the RCC1/Ran system locates upstream in the cascade toward an activation of p34^{cdc2} kinase. Probably, in S phase, the RCC1/Ran system inhibits a synthesis of some activator(s) of p34^{cdc2} kinase until completion of DNA replication. Identification of such a protein(s) synthesized on loss of RCC1 function, therefore, will give us a clue about a role of the RCC1/Ran system in a negative feedback control of p34^{cdc2} kinase activity.

So far, both cyclin B and cdc25 proteins have been reported to be essential for an activation of p34^{cdc2} kinase (Nurse, 1990). It is quite possible, therefore, that these proteins are produced on loss of RCC1 function. We have already found that the cyclin B protein exists in tsBN2 cells arrested with HU before a temperature shift (Nishitani *et al.*, 1991).

In this report, we investigated whether the cdc25 protein exists in tsBN2 cells arrested with HU. So far, three kinds of *cdc25* genes A, B, and C have been cloned from human cell lines (Sadhu *et al.*, 1990; Galaktionov and Beach, 1991; Nagata *et al.*, 1991). Although all *cdc25* genes rescue a *S. pombe* ts mutant, *cdc25-22*, they are supposed to have a different function in the cell cycle. For instance, the cdc25C protein is considered to be involved for the initiation of mitosis (Millar *et al.*, 1991a) and the cdc25A protein is suggested to function after initiation of mitosis (Galaktionov and Beach, 1991). We chose the cdc25C as a possible cdc25 protein required for PCC induced by loss of RCC1 function.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The ts mutants of BHK21/13 cell line, the tsBN2 and tsBN7 cell lines (Nishimoto and Basilico, 1978), the BHK21/13 cell line derived from the golden hamster, the Chinese hamster cell line, V79, (Liskay, 1978) and the HeLa cell line were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. The Chinese hamster cell line, CHO-K1 (ATCC CCL 61) was cultured in the same medium containing 0.1% trypton broth. These cell lines were maintained in a humidified atmosphere containing 10% CO_2, either at 33.5°C (ts mutants) or at 37.5°C, respectively. As the nonpermissive temperature, 40.5°C was used for ts mutants.

Asynchronously growing cells were arrested at early S phase with 2.5 mM HU as described (Nishitani *et al.*, 1991). Mitotic cells were obtained by nocodazole block (0.2 μg/ml) at 37.5°C for 18 h after release from isoleucine deprivation and by gentle pipetting as described previously (Yamashita *et al.*, 1990).

Construction and Screening of the cDNA Library

A cDNA library prepared from human Raji cells was generously provided by Dr. H. Hayakawa (Hayakawa *et al.*, 1990).

The cDNA libraries of the poly-A$^+$ RNA extracted from exponentially growing BHK21/13 cells and tsBN7 cells were constructed by using the Amersham cDNA and λgt10 cDNA cloning system (Amersham, UK).

The cDNA library of BHK21/13 cells was screened with the use of the *Pst* I fragment of human *cdc25C* cDNA as a probe, and seven positive clones were obtained out of 4.5×10^5 plaques. The clone containing the longest insert of 1.9 kb was chosen for this experiment.

Expression of Hamster cdc25C in S. pombe

Expression vectors of *S. pombe*, pSM1, and pSM2 (Sadhu *et al.*, 1990) were digested with *Bam*HI and ligated with the *Not* I linker after blunting with T4 DNA polymerase, resulting in new expression vectors, pSM1BN and pSM2BN (Russell, 1989). The *Not* I fragment of the hamster *cdc25C* was inserted into the *Not* I site of pSM1BN or pSM2BN with both direct and reverse directions.

Expression and Preparation of Hamster cdc25C Protein in E. coli

Construction of the Vector. *pET3a-25/37*, the *Hinc*II-*Acc* I fragment of the hamster *cdc25C* cDNA and the *Bam*HI-digested pET3a were blunted with T4 DNA polymerase and were then ligated to each other. By this construction, the C-terminal part of hamster *cdc25C* from the 107th amino acid residue can be expressed as the T7 gene fusion-protein (p37). *pET3a-25/55*, the 5′ primer, 5′-GACTCCGAACATATGGCTACCGGA-3′, was constructed to change the second amino acid residue of the hamster cdc25C protein, Serine, to Alanine. The 3′-primer, 5′-AAACCTTGGGATCCTAGGTGA-3′, was constructed on the basis of the sequence of the 3′ terminal *Bam*HI site of hamster

cdc25C cDNA. By using both primers, the hamster *cdc25C* cDNA was amplified with polymerase chain reaction (PCR) (Randall *et al.*, 1988). The amplified fragments were digested with *Nde* I and *Bam*HI and then ligated with pET3a-25/37, which was digested with *Nde* I and *Bam*HI, to construct the plasmid containing a full length of hamster *cdc25C* cDNA (p55).

Expression and Purification. The cdc25C-expression vectors were introduced into *E. coli, BL21 (DE3) pLys S,* and were expressed according to the method of Studier *et al.* (1990). Inclusion bodies containing an expressed hamster cdc25C protein were solubilized with 8 M Urea, and were diluted stepwise by dialysis according to Kumagai and Dunphy (1991). The purity of obtained hamster cdc25C protein was >90%.

Antibody

Polyclonal rabbit antiserum was raised against *E. coli* produced truncated hamster cdc25C protein ($p37^{cdc25C}$). The antibody was purified by the affinity column in which $p37^{cdc25C}$ was conjugated with Affi-gel 10 (Bio-Rad, Richmond, CA) at the condition, 5 mg of protein/ml of gel, according to the manufacturers manual. Purified antibodies were concentrated in the phosphate-buffered saline (PBS), pH 7.4, by using centrifugal concentrators (Centricon 10, Amicon, Danvers, MA) and were served for the needle microinjection.

Expression of Human cdc25 Proteins; A, B, and C in E. coli

Human *cdc25 A(Hu3), B(Hu2),* and *C(Hu1)* cDNAs inserted into pcD2 vector were kindly supplied from Dr. H. Okayama (Tokyo University) (Nagata *et al.*, 1991). Those cDNAs were subcloned into the following *E. coli* expression vectors.

The *cdc25A* cDNA was cut out from the vector as a *Bgl*II-*Hin*dIII fragment, ligated to a pGEX-KG vector, and expressed in *E. coli AG1* so that the human cdc25A protein, GST-cdc25AΔ168, deleted in the N-terminal side of 168 amino acids, is produced as a protein fused with GST of molecular mass, 69 kDa.

The *cdc25 B* and C cDNAs were cut out from the vector either as a *Sma* I-*Hpa* I fragment or as a *Pst* I-*Pst* I fragment, respectively. These cDNA-fragments were ligated with a pET3a vector, and expressed in *E. coli BL21 (DE3) pLysS* so that cdc25 proteins, cdc25BΔ30 and cdc25CΔ90, deleted in the N-terminal side of 30 and 90 amino acids, respectively, is produced as a protein of molecular mass, 64 and 44 kDa protein, respectively.

Two-Dimensional Polyacrylamide Gel-Electrophoresis

Total cell lysate was analyzed by two-dimensional polyacrylamide gel electrophoresis according to O'Farrell *et al.*, (1977). A sample was subjected to the nonequilibrium pH gradient electrophoresis, pH 3.5–10, at 2000 V-h. The second gel electrophoresis was performed by using 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. After electrophoresis, the gel was either stained with a Coomassie Brilliant Blue or subjected to an immunoblotting analysis with an anti-cdc25C antibody.

Microinjection

Needle microinjection of the affinity-purified anti-cdc25C antibody was carried out as described (Graessmann and Graessmann, 1976).

Asynchronously growing cells were plated at 1×10^5 cells on 18 × 18 mm glass coverslips in 35-mm dishes and were arrested at early S phase with HU.

For Analysis of PCC Induction. The antibody was injected into tsBN2 cells arrested with HU. To maintain the pH of media during the microinjection, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-NaOH pH 7.2 was added to media at a final concentration of 20 mM. As a control, a rabbit IgG was similarly injected. Injected cells were incubated at 40.5°C for 5 h and then fixed to stain cells.

For Analysis of Mitosis. BHK21/13 cells on glass coverslips arrested with HU were allowed to grow at 37.5°C in fresh media containing 10% fetal bovine serum. One hour later, the antibody was injected as described above. Injected cells were given nocodazole (final concentration, 0.4 μg/ml) at the 5th h after release from HU block, incubated for another 4 h, and then fixed to stain cells.

Cells were doubly stained with goat anti-rabbit IgG antibodies and with Hoechst 33342 as described below. Injected cells, thus, were identified by staining with an anti-rabbit IgG antibodies.

Indirect Immunofluorescence

Staining of Micro-Injected Cells. Cells grown on glass coverslips were washed once with PBS and then fixed in methanol at −20°C for 5 min. After rehydration in PBS, the cells were stained with rhodamine-conjugated goat anti-rabbit IgG antibodies (TAGO, La Jolla, CA) for 30 min at room temperature, then washed with PBS, and further incubated with 1 μg/ml of DNA-specific dye, Hoechst 33342 (Calbiochem, San Diego, CA) for 10 min. Finally cells were mounted in a solution containing 90% glycerol and 10% PBS.

Staining of the cdc25C Protein. The cdc25C protein in tsBN2 cells was stained with the method described previously (Yamashita *et al.*, 1990), with an affinity purified anti-cdc25C antibody as the primary antibody, and a Texas-Red conjugated goat anti-rabbit IgG antibody (Amersham) as the secondary antibody. DNA was visualized with 1 μg/ml of Hoechst 33258 (Calbiochem).

Photomicroscopy was performed by using Axiophot (Zeiss, Germany).

Preparation of the Cell Lysate

Cultures of BHK21/13 cells arrested with HU were treated with cytochalasin B (10 μg/ml) for 30 min and then scraped. The packed cells were washed once with Tris buffer without calcium and magnesium (TD) buffer (Sekiguchi *et al.*, 1988), once with the extraction buffer (EB) solution (20 mM HEPES-KOH pH 7.4, 50 mM KCl, 50 mM β-glycerophosphate, 15 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 10 mM $MgCl_2$, and 5 mM 1,4-dithiothreitol [DTT]), and once with the one-half diluted EB solution containing cytochalasin B (5 μg/ml) and the mixed proteinase inhibitors. Finally a cell-pellet was suspended by vortexing for 30 s in two volumes of the one-half diluted EB solution containing cytochalasin B (5 μg/ml), the mixed proteinase inhibitors, and 0.5% NP40. The cell-suspension was held for 30 min at 0°C and then centrifuged at 12 000 × *g* for 15 min. The supernatant was used as the cytoplasmic lysate. The pellet was washed twice with the one-half diluted EB solution suspended in an equal volume of the NXB solution containing 20 mM HEPES-KOH pH 7.4, 1 M NaCl, 80 mM β-glycerophosphate, 15 mM EGTA, 10 mM $MgCl_2$, 5 mM DTT, and 0.5% Triton X-100 with 30 strokes of a dounce homogenizer. The suspension was held at 0°C, for 30 min and subjected to a centrifugation of 80 000 × *g* for 30 min. The supernatant was diluted 10-fold with the EB solution and then concentrated to the original volume with Centricon-10 (Amicon) to use as the nuclear fraction.

The mixed proteinase inhibitors contain 5 μg/ml of aprotinin, antipain, leupeptin, and pepstatin, and 100 μM [p-amidinophenyl]methanesulfonyl fluoride (pAPMSF).

Histone H1 Kinase Assay

Histone H1 kinase assays were performed on immunoprecipitates made with an anti-C terminal peptide antibody of human $p34^{cdc2}$ from the cell lysate as previously described (Yamashita *et al.*, 1990).

Immunoblotting

After electrophoresis, proteins were transferred onto a nitocellulose membrane or a poly[vinyldine difluoride] (PVDF) membrane. Protein-blotted membranes were incubated at room temperature for 1 h in the PBS-blocking buffer containing 0.1% Tween 20, 5% nonfat dry milk, for 1 h in the same buffer containing an affinity purified anti-

$p37^{cdc25C}$ antibody, and then for 1 h with horseradish peroxidase-conjugated protein A. The antibody was detected with enhanced chemiluminescence (ECL) kit (Amersham).

RESULTS

Analysis of Cloned Hamster cdc25 cDNA

The cloned hamster cdc25 cDNA encodes the 47 kDa of protein consisting of 421 amino acids (Figure 1), which is highly homologous to the human *cdc25C* used as a screening probe (Figure 2) (Sadhu *et al.*, 1990; Galaktionov and Beach, 1991). However, a central part of the cloned hamster cdc25 protein, 152–185th amino acid-residues, corresponding to the region of a human cdc25C protein, 208–290th amino acid-residues, can not be aligned by each other. Using the nucleotide-sequence outside the nonaligned region (the nucleotide sequence underlined in Figure 2) as a primer of PCR reaction, the DNA fragments of 500 and 650 bp, the same length as estimated from the determined nucleotide-sequence, were obtained from a poly-A^+ RNA preparation of BHK21/13 and HeLa cells, respectively (Figure 2b, lanes 3 and 8). Using the same primer, the DNA-fragment with the same size as the BHK21/13 cell line was obtained from the cDNA library of the tsBN7 cell line, another ts cell line derived from the BHK21/13 cell line (Figure 2b, lane 4), but the DNA fragments of ~600 bp were amplified from the poly-A^+ RNA fractions of cell lines derived from Chinese hamster: CHO-K1 and V79 (Figure 2b, lanes 5 and 6). Thus the nonaligned region was not an artifact but seems to be unique to the *cdc25* gene of BHK21/13 cell line.

Except for the central part, the cloned hamster cdc25 protein was 74% identical at the amino acid level with the human cdc25C protein.

To examine whether the cloned hamster *cdc25* cDNA encodes a protein functionally related to the fission yeast *cdc25*, the cloned hamster *cdc25* cDNA was inserted into pSM1 and pSM2 plasmids, in both direct (D) and reverse (R) transcriptional orientations as described in MATERIALS AND METHODS. The pSM plasmids are *pBR322/LEU2/2-μm* chimeras containing the Simian virus 40 early promoter to direct moderate levels of expression in fission yeast (Jones *et al.*, 1988). After introduction of plasmids into a *S. pombe cdc25-22 leu-32* strain, transformants were plated on media at a permissive (26°C) or a restrictive temperature (33.5°C). In both pSM1- and pSM2 transformants, only the *cdc25-22 leu 1-32* mutants transfected with plasmids containing a hamster *cdc25* cDNA in the direct orientation were able to grow at 33.5°C (Figure 3). Thus the cloned cDNA has a function as a mitotic inducer in fission yeast.

Preparation of the Antibody to the Hamster cdc25 Protein

We prepared an antibody to the *E. coli*-produced hamster cdc25 protein ($p37^{cdc25}$), which was truncated in the N-terminal region. By using the affinity column, the antibody was purified to a single band (Figure 4a, lane 1). This antibody specifically recognized the cdc25 protein of whole-cell lysate of BHK21/13 cells (Figure 4a, lanes 3 and 4). The hamster cdc25 protein recognized by the antibody was confirmed to be a single protein by the two dimensional gel electrophoresis (Figure 4b).

The $p37^{cdc25}$ encodes the C-terminal part of cdc25 protein, which is well conserved through evolution (Galaktionov and Beach, 1991; Gautier *et al.*, 1991; Millar *et al.*, 1991b; Nagata *et al.*, 1991; Kakizuka *et al.*, 1992). To investigate what type of a human cdc25 protein was recognized by the presently prepared anti-cdc25 antibody, the antibody was blotted onto *E. coli* produced human cdc25 proteins A, B, and C, which were deleted in the N-terminal one-third of the original protein. Only the human cdc25C protein was detected by our anti-cdc25 antibody (Figure 4c). This finding is consistent with a similarity of amino acid-sequence between the cloned hamster *cdc25* and the human *cdc25C* cDNA (Figure 2a). Thus we will describe afterwards the protein encoded by the cloned hamster *cdc25* cDNA as the hamster cdc25C protein.

The Hamster cdc25C Protein is Essential for PCC-Induction Caused by "BN2" Mutation

To investigate a requirement of the cdc25C protein for PCC-induction caused by loss of RCC1 function, we examined whether the anti-cdc25C antibody inhibited PCC-induction of tsBN2 cells.

The anti-cdc25C antibody was injected either into the cytoplasm, or into the nuclei as described in MATERIALS AND METHODS. As a control, a rabbit IgG was injected similarly. Injected cells were incubated at 40.5°C for 5 h, and then the frequency of cells showing PCC was counted (Table 1). The frequency of cells showing PCC was reduced by injecting an anti-cdc25C antibody. Contrarily, a control rabbit IgG had no significant inhibitory effect on PCC induction, indicating that a needle injection itself is not harmful for chromosome condensation. Because chromosome condensation was similarly inhibited by both cytoplasmic and nuclear injection, an antibody was injected into the cytoplasm afterwards.

Inhibition of PCC with the anti-cdc25C antibody was further confirmed by injecting various doses of the anti-cdc25C antibody (Figure 5). To equalize a nonspecific effect of injection, in this case, a protein-concentration of an injection-buffer at each dose was adjusted to 5 mg/ml by mixing the anti-cdc25C antibody with a control rabbit IgG.

PCC induction of tsBN2 cells was significantly inhibited by injecting an anti-cdc25C antibody at a concentration of >0.1 mg/ml.

To further confirm that the effect of the anti-cdc25C antibody is specific to the cdc25C protein, we mixed

Hamster cdc25C

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 1
 gc ggc cgc ctt aca aat acc atc tct aag aca gcg gtc cgc gtc tgc gcc tct ggg
tcc tcc tca tcc ggc gga gcc ttc gct ccc gtg cag gag ttt ctt tct cca gag ccg
agt ctg cct gtg aag cga aga tag ctg tcc ccc tgc ctc ggt gaa gac tcc gaa gtc
1
ATG TCT ACC GGA CCC TTT CCA TCT TCG AGA AGA GAG GAG AGT TCT GTT TCA GCA CCC
Met Ser Thr Gly Pro Phe Pro Ser Ser Arg Arg Glu Glu Ser Ser Val Ser Ala Pro

AGT TTT AGG TTC AGT CAG AGG AAG ATG TTA AAC CTA CTT TTG GAA AGA AAC ACT TCT
Ser Phe Arg Phe Ser Gln Arg Lys Met Leu Asn Leu Leu Leu Glu Arg Asn Thr Ser

TTT ACC CAA GAT TTC CCC AGA TCT CCA GGA GAC AAG CTT CTC GAC TCG ACA AAC CTA
Phe Thr Gln Asp Phe Pro Arg Ser Pro Gly Asp Lys Leu Leu Asp Ser Thr Asn Leu

AGC ATT TTA TCT GGA GGG ACC CCA AAA CGT TGC CTT GAT CTT TCA AAT CTT AGC AAT
Ser Ile Leu Ser Gly Gly Thr Pro Lys Arg Cys Leu Asp Leu Ser Asn Leu Ser Asn

GGA GAG ATG TCT GCT TCT CCG CTT ATC ACT TCG GCT GAC TTC GAT GAC ACC GGT TCC
Gly Glu Met Ser Ala Ser Pro Leu Ile Thr Ser Ala Asp Phe Asp Asp Thr Gly Ser
                                    Hinc II
TTG GAT TCC TCA GGA CCC CAG GAT GTG CAG TTA ACT GAG AAG AAC CAT CAC CAG GAC
Leu Asp Ser Ser Gly Pro Gln Asp Val Gln Leu Thr Glu Lys Asn His His Gln Asp

CCT ATG AAA GGC ATC CCA GTT CAG CTT CTC TGT AGT ACT CCA AAC GCT TTG GAC CAT
Pro Met Lys Gly Ile Pro Val Gln Leu Leu Cys Ser Thr Pro Asn Ala Leu Asp His

AGC CAC AGA AAG AAA GAT GCA GTG CGT GGC TTA TCT GCA AAT AAA GAA AAC ATA AAC
Ser His Arg Lys Lys Asp Ala Val Arg Gly Leu Ser Ala Asn Lys Glu Asn Ile Asn
                                          Bam H1
ACC AAT CTA AAG ACA TTG CAG TGG GAG TCA CCT AGG ATC CCA AGG TTT CAA AAC ACG
Thr Asn Leu Lys Thr Leu Gln Trp Glu Ser Pro Arg Ile Pro Arg Phe Gln Asn Thr

CCT GGA GAT CCT CTG GCT TCT CCC CTT CCT TTG CTG GGA AAT GGA GTC TCG ATG GAC
Pro Gly Asp Pro Leu Ala Ser Pro Leu Pro Leu Leu Gly Asn Gly Val Ser Met Asp

ACT GAA GTG AGG TCT CTG GGG AGT CCC ATC ACT GCA GTT CCC AAG CTG AGT AAA AAT
Thr Glu Val Arg Ser Leu Gly Ser Pro Ile Thr Ala Val Pro Lys Leu Ser Lys Asn

CTA AAT CTA GAA GAC CAA GAA GAG ATT TCA GAG GAG CCA ATG GAG TTT TCC CTG GAA
Leu Asn Leu Glu Asp Gln Glu Glu Ile Ser Glu Glu Pro Met Glu Phe Ser Leu Glu

GAC CAC GAT ACC AAG GAG TGT GTG CTT CCA ACG GTG TCA GGG AAA CAC CAA GAT CTG
Asp His Asp Thr Lys Glu Cys Val Leu Pro Thr Val Ser Gly Lys His Gln Asp Leu

AAG TAC ATC ACC CCA GAC ACG GTG GCT GCT TTA CTG TCT GGA AAG TTC CAG GGT CTG
Lys Tyr Ile Thr Pro Asp Thr Val Ala Ala Leu Leu Ser Gly Lys Phe Gln Gly Leu

ATC GAG AAG TTT TAC ATC ATC GAT TGC CGC TAC CCG TAT GAG TAC CTT GGA GGA CAC
Ile Glu Lys Phe Tyr Ile Ile Asp Cys Arg Tyr Pro Tyr Glu Tyr Leu Gly Gly His

ATC CTG GGA GCC ATA AAC CTG TGC AGC CAG AAG GAA CTG CAC GAG TTC TTT CTG AAG
Ile Leu Gly Ala Ile Asn Leu Cys Ser Gln Lys Glu Leu His Glu Phe Phe Leu Lys

AAG CCT ATT GTC CCT CTG GAC ATC CAG AAA AGA GTC ATC ATT GTG TTC CTC TGT GAA
Lys Pro Ile Val Pro Leu Asp Ile Gln Lys Arg Val Ile Ile Val Phe Leu Cys Glu

TTC TCC TCA GAG AGA GGC CCC CGA ATG TGC CGT TCT CTG AGA AGG AAA GAC AGA GCT
Phe Ser Ser Glu Arg Gly Pro Arg Met Cys Arg Ser Leu Arg Glu Lys Asp Arg Ala

CTG AAC CAG TAT CCA GCA TTG TAC TAC CCA GAG CTG TAT ATC CTC AAA GGG GGC TAC
Leu Asn Gln Tyr Pro Ala Leu Tyr Tyr Pro Glu Leu Tyr Ile Leu Lys Gly Gly Tyr

AGA GAC TTC TTT CCA GAA TAT ACG GAG CTG TGC GAA CCC CAG GGC TAC TGT CCT ATG
Arg Asp Phe Phe Pro Glu Tyr Thr Glu Leu Cys Glu Pro Gln Gly Tyr Cys Pro Met

CAC CAC CAG GAC CAC CAG GCT GAG CTG CTG ATG TGG CGA AAC CAG AGC AAA GCC CAG
His His Gln Asp His Gln Ala Glu Leu Leu Met Trp Arg Asn Gln Ser Lys Ala Gln

GAA GGG GAG CGG CAG CTT TCG GAG CAG ATT GCC CTC CTG ATG AAG AAG GGT GTG AGC
Glu Gly Glu Arg Gln Leu Ser Glu Gln Ile Ala Leu Leu Met Lys Lys Gly Val Ser

CTC CCA TAG tgg gtg gtg gtg gcg cac acc ttt aac ccc agc acc cgg gag gca gag
Leu Pro Ter
      421                                        ACC 1
gta gga gga tct ctg cgt ttg aaa tca gcc tgt cta cga cac agc aag ttc cag gac
agc cag agc tac aag gaa aac atc tca gaa aaa caa aaa aca att tct gtc aag tat
gga gca gat gcc tgt tgc ctg tgt gct tga aag gtg gac cta gag gac ctg aag ctc
agt tca agg cca gcc tca gct cct gga ttc cct agc tca cta aat gga aac aaa acc
gaa agg cat agt aac cat tgc tct gga acc aag gga aag ttt cat ttg tat tct tga
act ggt gtt ggt ggt aaa tgt ttg tat tcc taa gag gtc atg gca gga aga tga tga
gtt aaa gaa caa gat aaa gtc tgt ggt gcg gcc gc
                                                1858
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Figure 1. Nucleotide sequence of cloned hamster *cdc25C* cDNA. The indicated *Hinc*II, the *Bam*HI, and the *Acc* I sites were used for subcloning the fragment of cloned cDNA to express in *E. coli*. The nucleotide sequence data reported in this figure will appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence Databases with the following accession number D10878, under Description, hamster *cdc25*.

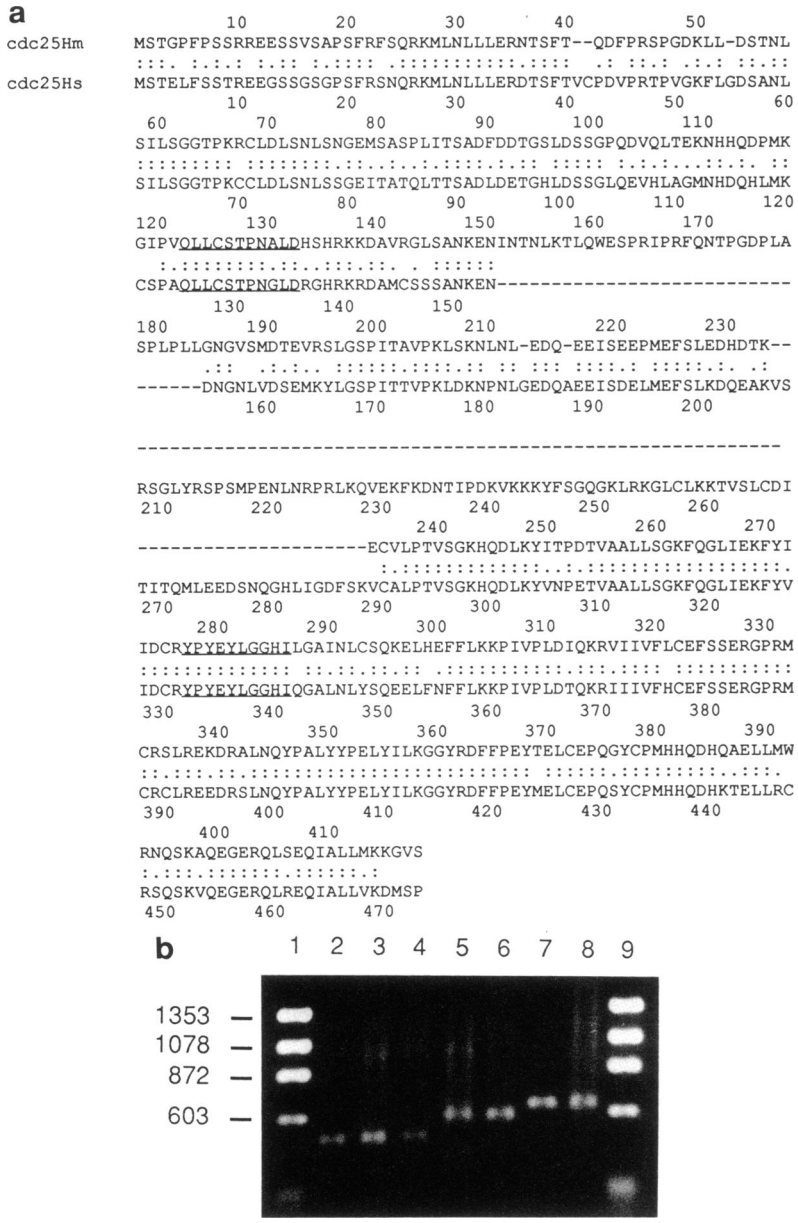

Figure 2. Characterization of amino acid sequence of the cloned hamster cdc25 protein. (a) Maximum alignment of the amino acid sequences between hamster (Hm) and human (Hs) cdc25C proteins. A deduced amino acid sequence of the cloned hamster cdc25 protein is aligned to maximize a similarity with the human cdc25C protein. Identical amino acids between two proteins are marked by double dots whereas conserved changes are marked by single dots. Classification of amino acids based on their chemical similarity is as follows: A, T, G, P, and S; D, E, N, and Q; H, K, and R; I, L, M, and V; F, Y, and W; C. The underlines indicate the positions of the nucleotide-sequences used for the PCR amplification. (b) Amplification of the region non-aligned between hamster and human cdc25C proteins by PCR. Poly-A$^+$ RNA extracted from BHK21/13, CHO, V79, and HeLa cells, and the cDNA library of BHK21/13, tsBN7, and human Raji cells were amplified with the following two primers, 5′ primer: CAGCTTCTT/CTGTAGC/TACTCCG/AAAT/CGG/CTTTGGA, and 3′ primer: GATGTGTCCTCCC/AAGA/GTACTCATAT/CGGA/GTA. Amplified DNA fragments were analyzed by an electrophoresis. Lanes 1 and 9 contain DNA-size marker (bp) of ϕX174 *Hae*III digest. Other lanes contain DNA-fragments amplified from the following: lanes 2, 4, and 7 from the cDNA libraries of BHK21/13, tsBN7 and human Raji cells, and lanes 3, 5, 6, and 8 from the poly-A$^+$ RNAs of BHK21/13, V79, CHO, and HeLa cells.

the anti-cdc25C antibody either with the *E. coli* produced hamster cdc25C protein or with the control rabbit IgG. The effect of the injected anti-cdc25C antibody was reduced depending on a dose of *E. coli* produced hamster cdc25C protein (Figure 6). On the other hand, the control rabbit IgG showed a little inhibitory effect (Figure 6, ○), compared with the *E. coli* produced cdc25C protein. Thus the anti-cdc25C antibody itself prevented an induction of chromosome condensation.

The cdc25C Protein Exists in tsBN2 Cells Arrested With Hydroxyurea

Because inhibitors of protein synthesis prevent PCC induction of tsBN2 cells, a new protein synthesis is considered to be essential for tsBN2-induced PCC (Nishimoto *et al.*, 1981). So far, both cyclin B and p34^{cdc2} protein kinase have been proved to exist in tsBN2 cells arrested with HU (Nishitani *et al.*, 1991). Because an antibody to the hamster cdc25C protein inhibited PCC-induction of tsBN2 cells, we investigated whether the hamster cdc25C protein is newly synthesized upon loss of RCC1 function.

Cultures of tsBN2 cells synchronized with isoleucine deprivation were allowed to grow at a permissive temperature of 33.5°C and then arrested with either mimosine or HU. The cell lysates were prepared from these growth-arrested cells and analyzed by immunoblotting with an anti-cdc25C antibody. The cdc25C protein ap-

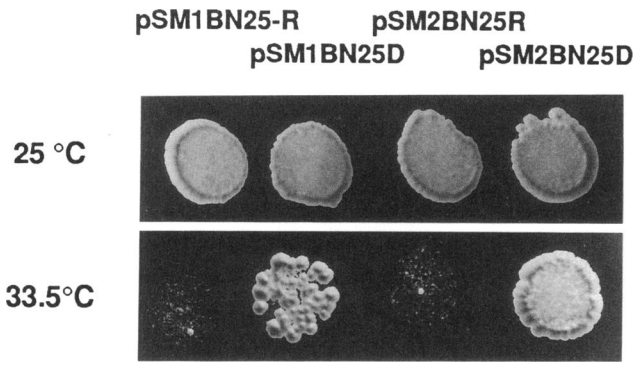

Figure 3. Rescue of *S. pombe cdc25-22* by cloned hamster *cdc25* cDNA. Cloned hamster *cdc25* cDNA expression vectors were transfected into the *S. pombe cdc25-22*. Transformants were cultured on the EMM plate (Moreno *et al.*, 1991) either at 25°C (permissive temperature) or at 33.5°C (nonpermissive temperature). Plasmids pSM1BN25R and pSM2BN25R contain cloned hamster *cdc25* cDNA in the reverse direction, and the plasmids, pSM1BN25D and pSM2BN25D, contain the cloned hamster *cdc25* cDNA in the right direction.

peared in cells treated with mimosine (Figure 7a, Mino), which arrests the cell cycle in late G1 phase (Lalande, 1990), and accumulated in cells arrested with HU (Figure 7a, HU). Thus in tsBN2 cells arrested with HU, the cdc25C protein already existed at the time of a temperature shift, indicating that it is not a protein synthesized upon loss of RCC1 function.

Modification of the cdc25C Protein Occurs after an Activation of p34^{cdc2} Kinase

After release from HU block, an abundance of cdc25C protein did not alter at both temperatures, 33.5°C and 40.5°C. At 40.5°C, nonpermissive temperature, as found previously (Nishitani *et al.*, 1991), an activation of p34^{cdc2} kinase was observed at 2 h after a temperature shift. Interestingly, we found a higher molecular form of the cdc25C protein after incubation for 4 h at 40.5°C (Figure 7a, 40.5°C). In the presence of cycloheximide where no activation of p34^{cdc2} kinase occurs, however, no molecular shift was observed (Figure 7a, 40.5°C + CHX). Thus the molecular shift of cdc25C protein depended on an activation of p34^{cdc2} kinase.

Molecular shift of the cdc25C protein was also observed in normal mitotic cells (Figure 7b). By gentle pipetting, cultures of BHK21/13 cells arrested with nocodazole were separated into the floating cells (92% of cells in mitosis) (M0) and the remaining adherent cells (temporarily designated as G2) (Morla *et al.*, 1989). An immunoblotting of whole-cell lysate revealed that most cdc25C proteins took on a higher molecular form in mitotic cells arrested with nocodazole (M0), but not in nonmitotic cells (G2). Metaphase arrested cells were washed and replated in fresh media. The inhibitory effect of nocodazole was readily reversible, and within 60 min, cells had completed mitosis and returned to the interphase (Figure 7b, mitotic index). At different times after removal of nocodazole, samples were taken for immunoblotting with an anti-cdc25C antibody. After release from nocodazole arrest, the higher molecular form of cdc25C disappeared, and the previous lower form appeared. An abundance of cdc25C protein did not alter during an entrance into the interphase. These findings indicated that a molecular shift of the hamster cdc25C protein observed in tsBN2 cells showing PCC reflected a normal modification of the hamster cdc25C protein, which transiently occurred in M phase.

Localization of the cdc25C Protein in tsBN2 Cells

The subcellular localization of the cdc25C protein was examined by immunofluorescence staining of synchronized and exponentially growing tsBN2 cells.

In tsBN2 cells synchronized with isoleucine deprivation, a few staining spots were observed in the nuclei by anti-cdc25C antibodies (Figure 8a, G1). In tsBN2 cells arrested with HU (early S phase), however, the anti-cdc25C antibody gave rise to a punctuate staining throughout the cytoplasm and a prominent staining in the periphery of nuclei (Figure 8a, early S). The cytoplasmic localization of the cdc25C protein was not specific to cells arrested with HU. In tsBN2 cells growing exponentially at 33.5°C, the cytoplasm was prominently stained with anti-cdc25C antibodies (Figure 8c).

After incubation at 40.5°C, the nuclei were strongly stained with anti-cdc25C antibodies (Figure 8a, 40.5°C, 1 h). It seems likely that the cdc25C protein gathered on the surface of nuclei was dumped into the nuclei by a temperature shift, that is, by loss of RCC1 function. In the presence of cycloheximide, no staining of nuclei occurred (Figure 8b, +CHX). Thus the remarkable staining of nuclei depended on a new protein synthesis.

In cells showing PCC, the whole cytoplasm was stained with the anti-cdc25C antibody (Figure 8a, 40.5°C, 2 hr) indicating that the cdc25C protein was dispersed into the cytoplasm due to a breakdown of nuclear membranes.

At an equivalent concentration of antibodies, no signal was observed with the secondary antibody alone (Figure 8c).

In vitro Activation of Nuclear p34^{cdc2} Kinase by E. coli Produced cdc25C Protein

Subcellular localization of the cdc25C protein in tsBN2 cells suggested that the cdc25C protein moved into the nuclei upon loss of RCC1 function, to activate p34^{cdc2} kinases. Consistently with this finding, the nuclear p34^{cdc2} kinase was activated *in vitro*, depending on externally added hamster cdc25C proteins.

Cytoplasmic and nuclear lysates were prepared from BHK21/13 cells arrested with HU as described in MATERIALS AND METHODS. The cdc25C protein was

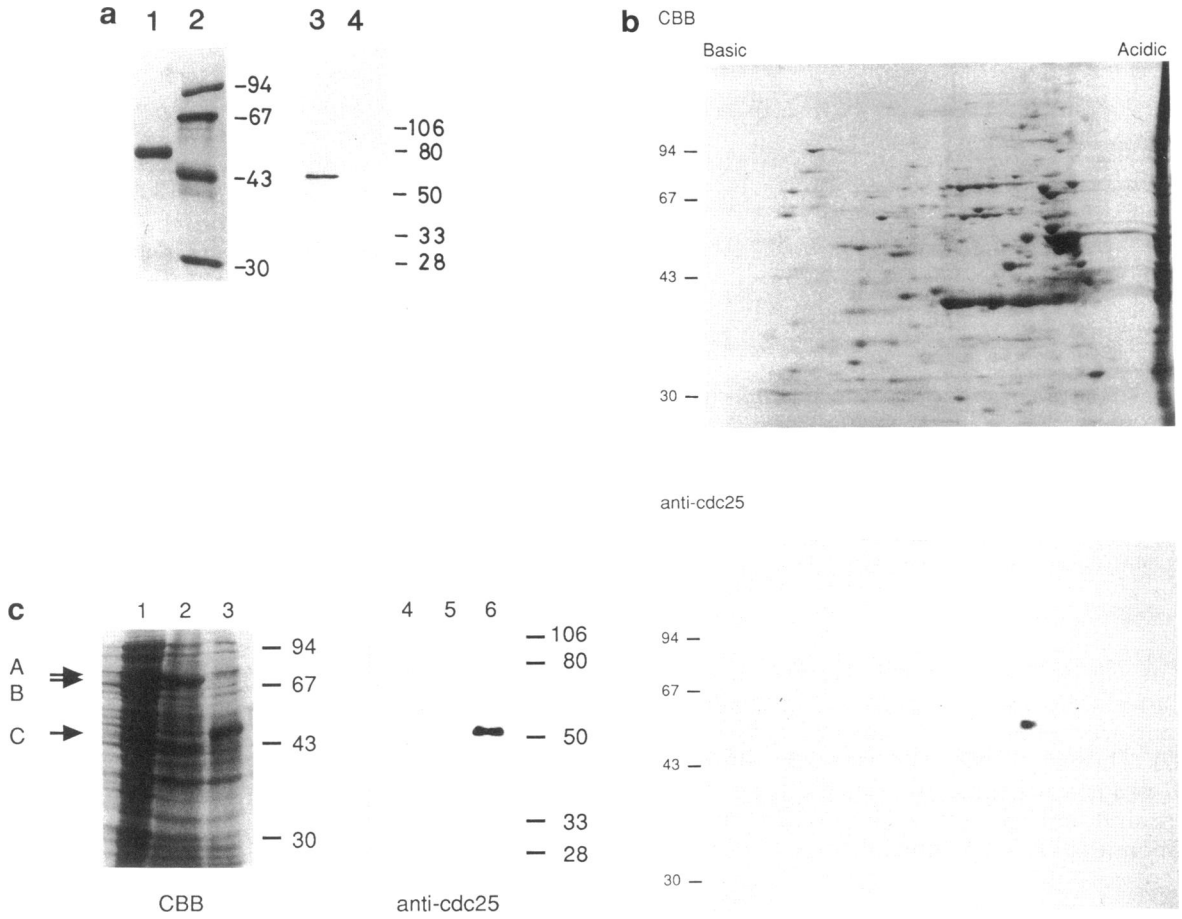

Figure 4. Characterization of the anti-cdc25 antibody. (a) lanes 1 and 2. The purified anti-cdc25 analyzed by 10% SDS-polyacrylamide gel electrophoresis stained with Coomassie Brilliant Blue R250 and the sized marker coelectrophoresed (kDa). Lanes 3 and 4. The whole-cell lysate of BHK21/13 cells arrested with HU was electrophoresed in 10% SDS-polyacrylamide gel, and analyzed by immunoblotting with the purified antibody to the *E. coli* produced cloned hamster cdc25 protein in the absence (Lane 3) or the presence (Lane 4) of *E. coli*-produced cdc25 proteins as a competitor. (b) Two dimensional analysis of the cell lysate. Total cellular proteins extracted from 1×10^6 BHK21/13 cells arrested with HU were subjected to two dimensional gel-electrophoresis as described in MATERIALS AND METHODS. CBB stained with Coomassie Brilliant Blue R250 and anti-cdc25 immunoblotted with the purified anti-cdc25C antibody. Only one spot was observed even after overexposing. (c) The anti-cdc25C antibody recognizes the human cdc25C protein. Homogenates of *E. coli* cells expressing human cdc25 proteins as described in MATERIALS AND METHODS were electrophoresed and analyzed by immunoblotting with the hamster anti-cdc25 antibody. CBB, lanes 1 to 3, stained with Coomassie Brilliant Blue R250, and anti-cdc25, lanes 4 to 6, immunoblotted with the anti-cdc25C antibody. Lanes 1 and 4, GST-cdc25AΔ168; lanes 2 and 5, cdc25BΔ30; lanes 3 and 6, cdc25CΔ90. Arrows indicate positions of *E. coli* produced human cdc25 A, B, and C proteins.

detected in the cytoplasmic, but not the nuclear, fraction by the immunoblotting analysis (Figure 9a). On the contrary, the $p34^{cdc2}$ proteins were identified in both cytoplasmic and nuclear fractions.

After incubation at 33.5°C for 60 min, the $p34^{cdc2}$ kinase of the cytoplasmic fraction was spontaneously activated without an addition of *E. coli* produced cdc25C protein. The maximum activity increased a little if any by an addition of purified *E. coli* producecd cdc25C protein. On the contrary, the $p34^{cdc2}$ kinase of the nuclear lysate was activated depending on an addition of *E. coli* produced cdc25C protein (Figure 9b).

When H1 kinase activity increased, an abundance of a high molecular form of $p34^{cdc2}$ protein decreased in both nuclear and cytoplasmic lysates (Figure 9c) indicating that $p34^{cdc2}$ protein was dephosphorylated. Additionally the activation of nuclear $p34^{cdc2}$ kinase with *E. coli*-produced cdc25C proteins was inhibited by vanadate, an inhibitor of tyrosine phosphatase (Tonks *et al.*, 1988; Morla *et al.*, 1989) (Figure 9d). The *E. coli*-produced hamster cdc25C protein, thus, activated the $p34^{cdc2}$ kinase through tyrosine-dephosphorylation as reported previously (Dunphy and Kumagai, 1991; Galaktionov and Beach, 1991; Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Millar *et al.*, 1991b). As reported previously (Dunphy and Newport, 1989), such an *in vitro* activation of nuclear $p34^{cdc2}$ kinase required ATP (Figure 9d, lanes 3 and 4). Consistently with the pre-

Table 1. Anti-cdc25C antibody inhibits tsBN2-induced PCC

Injected samples	Frequency of PCC (%)			
	Exp I	R[a]	Exp II	R[a]
Into cytoplasm				
IgG	32 (39)	100	26 (40)	100
Anti-cdc25C	3.8 (40)	12	4.1 (35)	18
Into nuclei				
IgG	34 (35)	100	20 (31)	100
Anti-cdc25C	1.7 (35)	5.0	3.6 (23)	24

Anti-cdc25C antibodies (1 mg/ml) were injected into either the cytoplasm or the nuclei of tsBN2 cells arrested with HU. Injected cells were incubated at 40.5°C for 5 h, and then fixed to calculate a frequency of cells showing PCC as described in MATERIALS AND METHODS. As a control, a nonspecific rabbit IgG (1 mg/ml) was injected, The numbers in parentheses show the frequency of uninjected cells showing PCC on the same glass coverslips as the injected cells.

[a] R, the frequency of cells showing PCC was normalized based on that of uninjected cells as follows. In the case of Exp I, Cytoplasm: (3.8/40)/(32/39) × 100 = 12%.

vious report that the C-terminal part of cdc25 protein is essential for tyrosine dephosphorylation of $p34^{cdc2}$ protein (Millar *et al.*, 1991b), both full-sized and truncated cdc25C proteins showed a similar activity (Figure 9d).

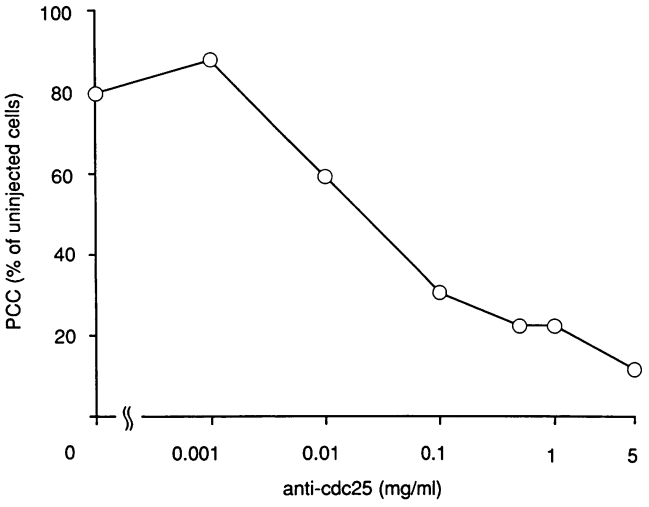

Figure 5. Dose-dependent inhibition of tsBN2-induced PCC by the anti-cdc25C antibody. tsBN2 cells growing on glass coverslips were arrested with HU, and were given a microinjection of the purified anti-cdc25C antibody at the indicated dose. In each injection, the nonspecific control rabbit IgG was mixed to adjust a final protein-concentration of the injected solution to 5 mg/ml. Within 1 h after injection, injected cells were shifted to 40.5°C. After incubation for 5 h, they were fixed and processed to calculate the frequency of cells showing PCC as described in MATERIALS AND METHODS. At each dose, >100 cells were injected. Vertical value indicates a frequency (%) of injected cells showing PCC. At zero point, the control rabbit IgG alone was injected at the concentration of 5 mg/ml.

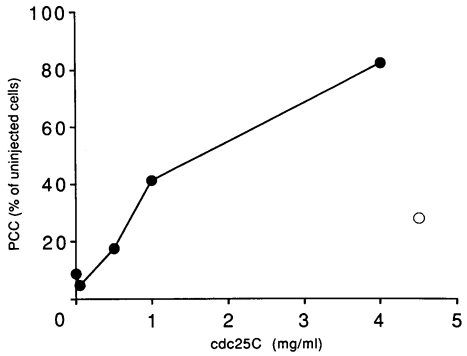

Figure 6. Effect of the anti-cdc25C antibody was blocked by *E. coli* produced cdc25C proteins. Injection buffer containing an anti-cdc25C antibody (0.5 mg/ml; final concentration) and an indicated dose of *E. coli* produced $p55^{cdc25C}$ protein (●) or control rabbit IgG (○) was injected into tsBN2 cells arrested with HU. Injected cells were incubated at 40.5°C for 5 h and fixed to calculate the frequency of cells showing PCC as described above. The vertical value indicates a frequency (%) of injected cells showing PCC.

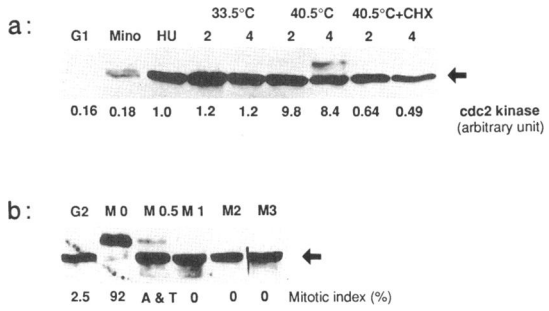

Figure 7. Appearances of the hamster cdc25C protein during a progression of the cell cycle. (a) G1 and S phase. Cultures of tsBN2 cells synchronized with isoleucine deprivation (G1) were allowed to grow in normal media, either in the presence of mimosine (150 μM) (Mino), or in the presence of hydroxyurea (HU) for 12 h. After release from HU arrest, cultures were incubated at 33.5°C (33.5°C) or at 40.5°C (40.5°C) with (+CHX) or without cycloheximide, for 2 h (2) or 4 h (4). Ten μg of whole-cell lysate prepared from indicated cultures, was electrophoresed in 10% SDS-polyacrylamide gel and analyzed by immunoblotting with the purified anti-cdc25C antibody. The activity of histone H1 kinase (cdc2 kinase) estimated as described in MATERIALS AND METHODS was shown as the ratio of radioactivity incorporated into the histone H1 either at the indicated times, or at indicated culture-conditions, relative to the value of the culture arrested with HU (HU). (b) Mitosis. Mitotic cells were collected after gentle pipetting from cultures of BHK21/13 cells treated with nocodazole as described in MATERIALS AND METHODS and then released to grow in normal media. At the indicated time (0, 0.5, 1, 2, and 3 h), cells were collected and whole-cell lysate were analyzed by immunoblotting with the anti-cdc25C antibody. Cells attached to the dish after gentle pipetting were considered to be nonmitotic cells (designated as G2) and used as the control of the interphase. For comparison, the mitotic index of each cell preparation is shown. A&T indicate the anaphase and the telophase. Arrows indicate a position of the cdc25C protein.

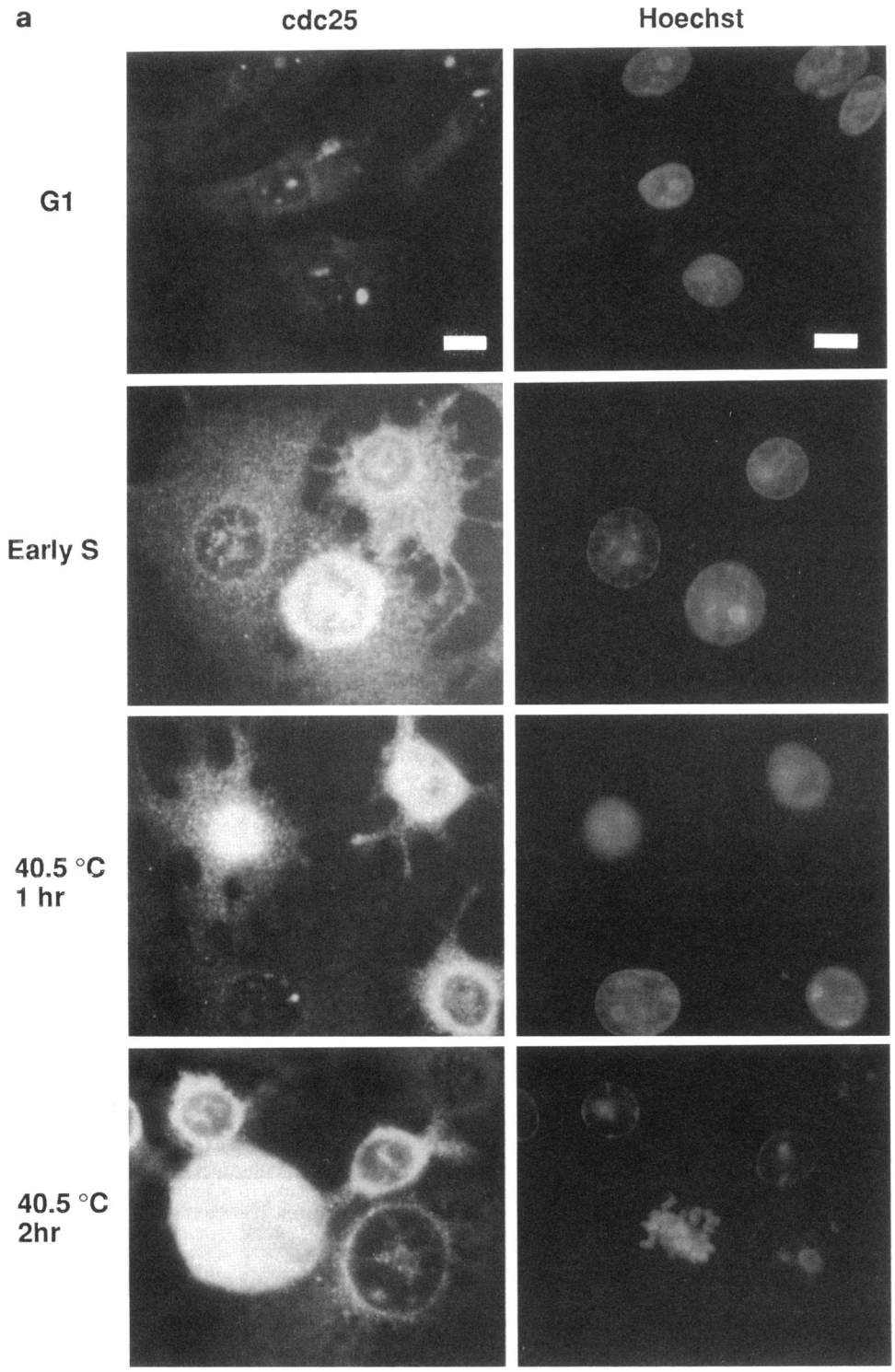

Figure 8. Localization of the hamster cdc25C protein in tsBN2 cells. (a) In synchronized cultures. Cultures of tsBN2 cells growing on coverslips were synchronized with isoleucine deprivation (G1), and then allowed to grow in normal media containing HU (early S). HU arrested cells were shifted up to 40.5°C and incubated for indicated time (h). At the indicated time, cultures were fixed for 30 sec in acetone at −20°C and stained with anti-cdc25C antibodies (1 μg/ml) and then with Texas-Red conjugated anti-rabbit IgG. Bar, 2.5 μm. (b) Effect of cycloheximide. tsBN2 cells were synchronized at early S phase as described above (early S), and then incubated at 40.5°C either with or without cycloheximide for 3 h (CHX, + or −). After incubation, cultures were processed for staining with the anti-cdc25C antibody as described in (a). (c) In exponentially growing cultures. Exponentially growing cultures of tsBN2 cells were stained with the anti-cdc25C antibody as described in (a). As a control, tsBN2 cells were stained with Texas-Red conjugated anti-rabbit IgG alone as described in (a).

Anti-cdc25C Antibodies Partly Inhibited both Normal Mitosis and Caffeine-Induced PCC

Three human *cdc25* genes, A, B, and C, (Galaktionov and Beach, 1991) have been cloned so far. It was suggested that these *cdc25* genes might have different functions, since the antibody to the human cdc25A protein arrests the cell cycle in the middle of mitosis (Galaktionov and Beach, 1991), whereas the anti-

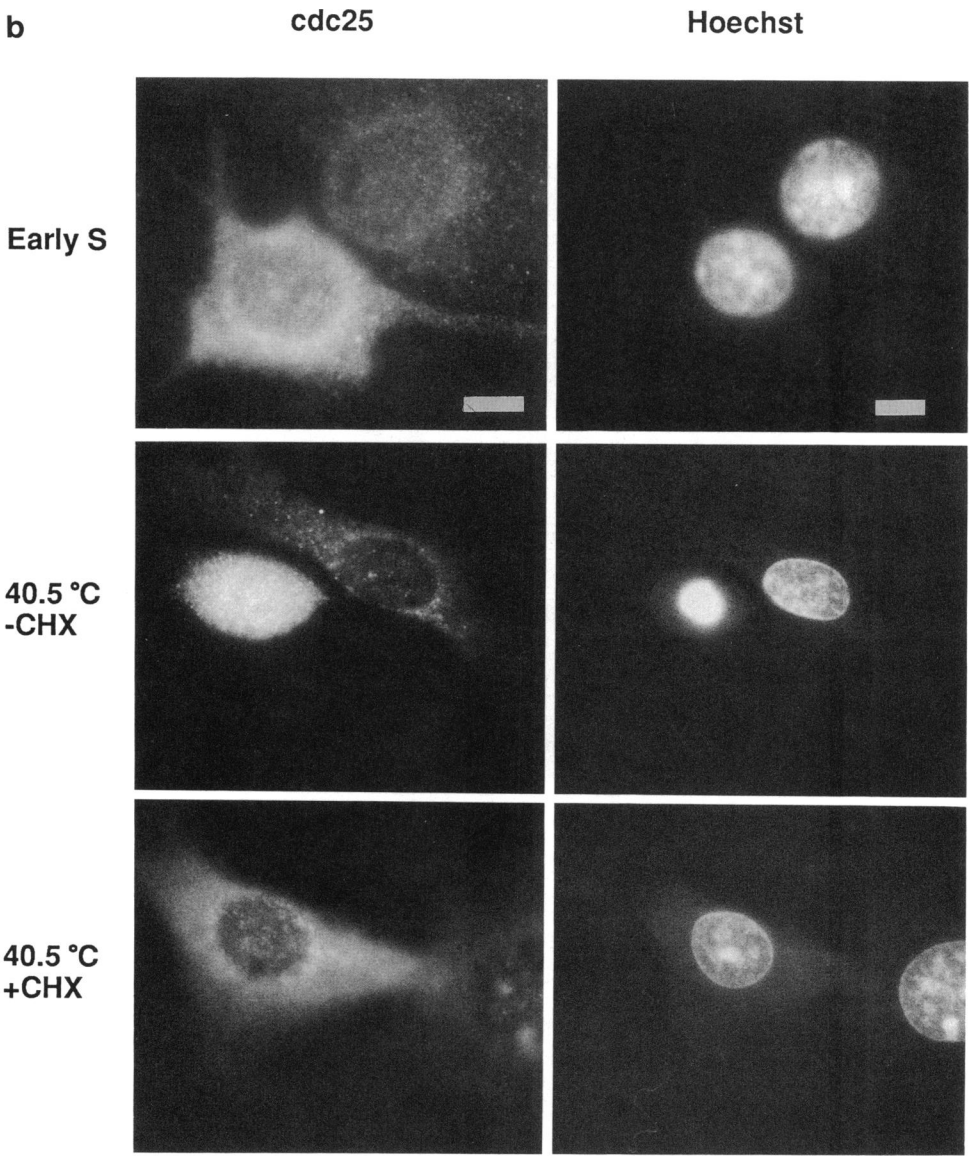

Figure 8. (Continued)

body to the cdc25C protein prohibits the initiation of mitosis (Millar *et al.*, 1991a). The hamster *cdc25* used in this experiment corresponds to the human *cdc25C* gene. We, therefore, investigated whether our anti-cdc25C antibody inhibits the initiation of normal mitosis. We also examined an effect of the anti-cdc25C antibody on the caffeine-induced PCC of BHK21/13 cells arrested with HU (Schlegel and Pardee, 1986).

Asynchronously growing BHK21/13 and tsBN2 cells were synchronized at early S phase with HU as described (Nishitani *et al.*, 1991). A set of cultures (tsBN2 and BHK21/13 cells) were given a microinjection of the anti-cdc25C antibody and incubated at 33.5°C for 5 h in the presence of caffeine and HU. The frequency of cells showing PCC was then determined as described in MATERIALS AND METHODS. Another set of BHK21/13 cells was released to grow in fresh media containing 10% fetal bovine serum for 1 h at 37.5°C and were then injected with the anti-cdc25C antibody. The injected cells were treated with nocodazole for 4 h and then the frequency of mitotic cells was determined as described in MATERIALS AND METHODS. We confirmed by BrdU incorporation that injected cells traversed the S phase. In Tables 2 and 3, representative results are shown. Compared with the case of PCC caused by "tsBN2" mutation, our anti-cdc25C antibody did not completely inhibit both normal mitosis and caffeine-induced PCC.

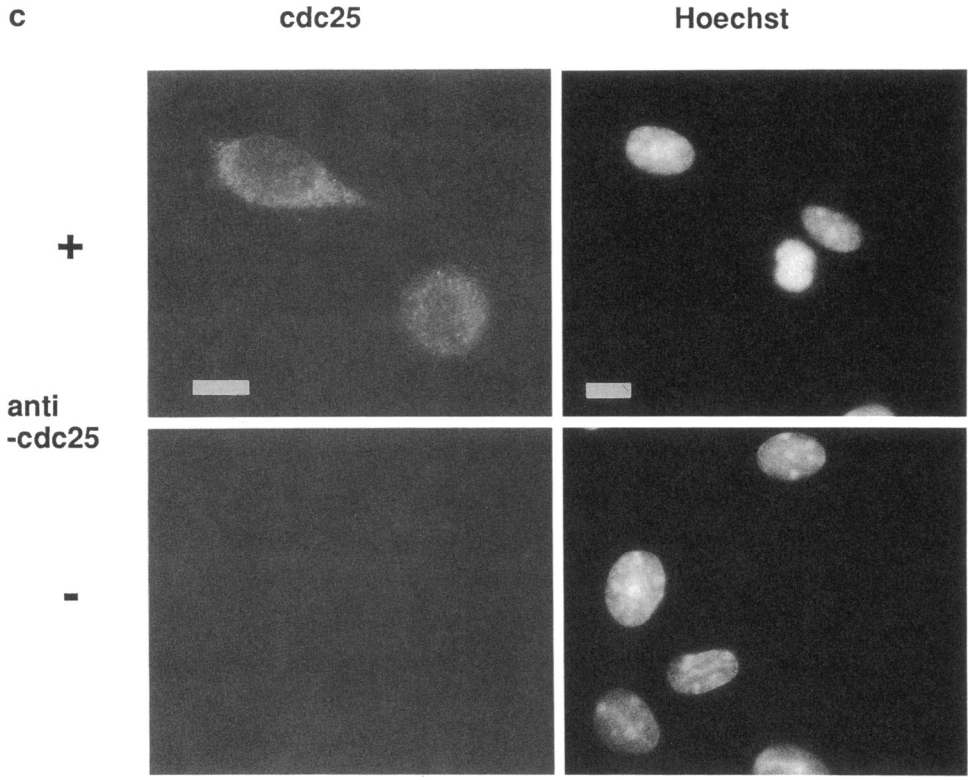

Figure 8. (Continued)

DISCUSSION

The finding that protein-synthesis inhibitors prevent both PCC-induction and an activation of $p34^{cdc2}$ kinase caused by tsBN2-mutation (Nishimoto *et al.*, 1981; Ajiro and Nishimoto, 1985; Nishitani *et al.*, 1991) indicates that the protein(s) synthesized by loss of RCC1 function is essential for an activation of $p34^{cdc2}$ kinase. So far, two proteins, cyclin B and cdc25 have been known to be required for an activation of $p34^{cdc2}$ kinase (Nurse, 1990). In this paper, we examined a requirement of the cdc25C protein for PCC-induction of tsBN2 cells. To do this, we have cloned the hamster *cdc25C* cDNA and prepared an antibody to the *E. coli* produced hamster cdc25C protein.

It is surprising that the presently prepared antibody recognized only a human cdc25C protein, since the C-terminal part of cdc25 proteins essential for an tyrosine phosphatase-activity of cdc25 protein, is well conserved through evolution (Galaktionov and Beach, 1991; Gautier, *et al.*, 1991; Millar *et al.*, 1991b; Nagata *et al.*, 1991; Kakizuka *et al.*, 1992). We further confirmed by two dimensional gel-electrophoresis of a whole-cell lysate, that the anti-cdc25C antibody recognized only a single protein (M.W. 55 kDa) of BHK21/13 cells, whose molecular mass corresponds to a protein encoded by the cloned hamster cdc25 cDNA. Because the cell lysate was prepared from BHK21/13 cells arrested at early S phase, the phosphorylated form of cdc25C protein reported by Kumagai and Dunphy (1992) should not yet appear.

Our anti-cdc25C antibodies inhibited PCC-induction of tsBN2 cells. The effect of the antibody is specific to the cdc25C protein, indicating that the cdc25C protein is essential for PCC-induction caused by tsBN2 mutation. This finding suggested that the cdc25C protein might be a newly synthesized protein upon loss of RCC1 function, which was not the case. The cdc25C protein was produced at late G1 phase in cultures released from serum starvation (G0), and its abundance did not change during a progression from mitosis to the next G1 phase, suggesting that it is present throughout the cell cycle in growing cells. Actually, exponentially growing tsBN2 cells were stained with the anti-cdc25C antibody. This finding is consistent with the previous report that in HeLa cells the cdc25C protein exists throughout the cell cycle (Millar *et al.*, 1991a).

The finding that the cdc25C protein exists in tsBN2 cells arrested with HU indicates that cells have some feed back systems to regulate an activity of cdc25C protein for an activation of $p34^{cdc2}$ kinase. For instance, until completion of DNA replication, either an activity of the cdc25C protein may be suppressed by some fac-

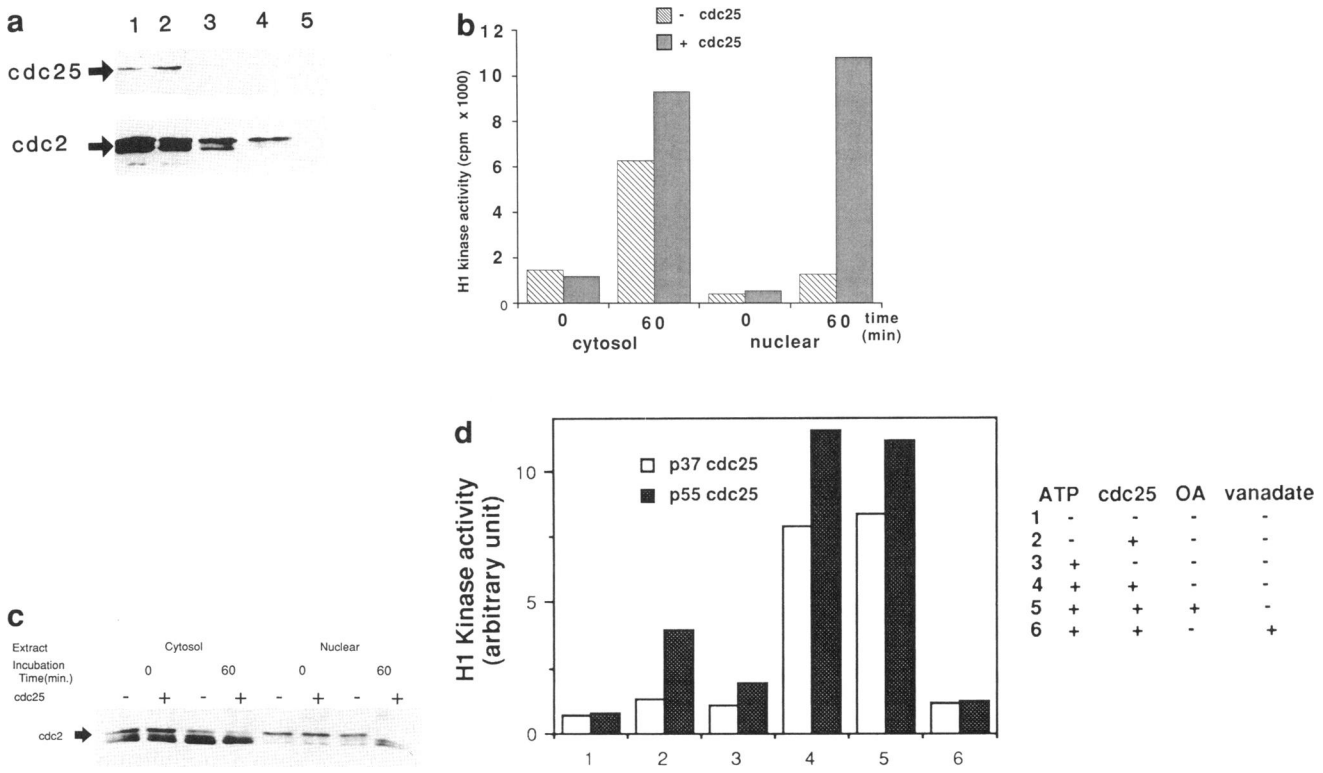

Figure 9. In vitro activation of $p34^{cdc2}$ kinase by the *E. coli* produced cdc25C protein. (a) Subcellular localization of cdc25C protein. BHK21/13 cells synchronized at early S were collected by scrapers, and fractionated into the cytoplasm and the nuclei as described in MATERIALS AND METHODS. The nuclei were washed, treated with 0.5 M NaCl and then centrifuged to fractionate into the supernatant (nuclear extract) and the pellet. After SDS-gel electrophoresis, immunoblotting analysis was performed with the anti-cdc25C and the anti-cdc2 antibodies. Lane 1, whole-cell lysate. Lane 2, cytoplasmic lysate. Lane 3, whole-nuclear preparation. Lanes 4 and 5, the nuclear extract and the pellet after treatment with 0.5 M NaCl, respectively. (b) Activation of the cytoplasmic and the nuclear $p34^{cdc2}$ kinase by *E. coli* produced cdc25C protein. Fifty μl of the cytoplasmic (5.7 mg/ml) and the nuclear extract (1 mg/ml) were incubated for 60 min, with or without 100 μg/ml (final concentration) of the purified *E. coli*-produced cdc25C protein. The activity of the $p34^{cdc2}$ kinase was estimated as described in MATERIALS AND METHODS. (c) Reduction of apparent molecular mass of $p34^{cdc2}$ kinase by its activation. Under the same condition as described in (b), both cytoplasmic (cytosol) and nuclear lysates were incubated and subjected to immunoblotting analysis with the anti-cdc2 antibody. (d) Activation of the $p34^{cdc2}$ kinase under various conditions. Fifty milliliters of the nuclear lysate (1 mg/ml) was incubated with 200 μg/ml of the purified *E. coli* produced cdc25C proteins; either the truncated (p37) or the full-sized (p55) form, under the indicated conditions as shown on the right side, for 60 min at 33.5°C. After incubation, the $p34^{cdc2}$ kinase was immunoprecipitated by the anti-cdc2 antibody, and its activity was estimated as described in MATERIALS AND METHODS. The ratio of the CPM incorporated into the histone H1 by $p34^{cdc2}$ kinase after incubation, based on the value at the beginning of incubation (time zero), is shown. The number of lanes corresponds to the number of a reaction conditions indicated on the right side. To the nuclear lysate, the indicated materials were added at the following final concentration: ATP, 1 mM; cdc25, 200 μg/ml; OA (okadaic acid), 1 μM; and vanadate, 1 mM. As a source of cdc25 proteins, either the truncated form, p37 (open column) or the full sized one, p55 (filled column) was used as indicated.

tor(s), which will be inactivated by the protein synthesized upon loss of RCC1 function, or the production of an activator for the cdc25C protein may be prevented. Another possibility is that an activity of the cdc25C protein is regulated by subcellular localization. For example, if cdc25C proteins are located in the cytoplasm, as in the case of tsBN2 cells, the nuclear $p34^{cdc2}$ kinase cannot be activated without nuclear translocation of cdc25C proteins. In *Xenopus* egg extract containing nuclei, Kumagai and Dunphy (1991) found that wheat germ agglutinin (WGA), a lectin which blocks nuclear transport by binding to glycoproteins in the nuclear pore (Finlay *et al.*, 1987) inhibits the tyrosine dephosphorylation of $p34^{cdc2}$ kinase.

Immunofluorescence figures of tsBN2 cells arrested with HU indicated that the cdc25C protein was transferred into nuclei by a temperature shift, that is, loss of RCC1 function. Consistent with this finding, nuclear $p34^{cdc2}$ kinases were activated, in vitro, depending on an addition of the purified *E. coli* produced cdc25C protein. These findings, together with the previous finding that a normal traffic through the nuclear pore is essential for the activation of $p34^{cdc2}$ kinase by tyrosine dephosphorylation (Kumagai and Dunphy, 1991), indicate that

Table 2. Effect of the anti-cdc25C antibody on the caffein-induced PCC

Injected samples	Frequency of PCC (%)					
	Exp I	R[a]	Exp II	R[a]	Exp III	R[a]
tsBN2 cells treated with caffeine						
IgG	43 (55)	100	35 (47)	100	37 (39)	100
Anti-cdc25C	22 (57)	50	19 (51)	50	22 (43)	54
BHK21 cells treated with caffeine						
IgG	56 (52)	100				
Anti-cdc25C	29 (65)	42				

The anti-cdc25C antibody (1 mg/ml) was injected into the cytoplasm of tsBN2 and BHK21/13 cells arrested with HU. Injected cells were incubated for 5 h in the presence of caffeine (10 mM) and HU at 33.5°C and then fixed to calculate the frequency of cells showing PCC as described in MATERIALS AND METHODS. The number in parentheses indicates the frequency of uninjected cells showing PCC on the same glass coverslips as the injected cells.

[a] R value was estimated as explained in Table 1.

the cdc25C protein located in the cytoplasm was transferred into the nuclei, upon loss of RCC1 function, to activate the nuclear $p34^{cdc2}$ kinase. Probably, the protein(s) synthesized by loss of RCC1 function is required for a nuclear transfer of cdc25C protein because no transfer of cdc25C protein occurred in the presence of cycloheximide. In this context, it is notable that the recent finding that some proteins including the heat shock protein, HSP70 are required for nuclear transfer of proteins (Adam *et al.*, 1989; Shi and Thomas, 1992; Stochaj and Silver, 1992). A cell-cycle specific nuclear transfer of protein has been reported for cyclin B in HeLa cells (Pines and Hunter, 1991), which was transferred into the nuclei in G2 phase.

In the same in vitro experiment of $p34^{cdc2}$ kinase-activation, the cytoplasmic $p34^{cdc2}$ kinase was spontaneously activated. This result indicated that the cytoplasmic $p34^{cdc2}$ kinase potentially could be activated even in the interphase. Cells, therefore, must possess some regulatory system for the cytoplasmic $p34^{cdc2}$ kinase, in addition to the nuclear system, which may prevent a direct contact between $p34^{cdc2}$ kinase and its activators, such as cdc25 proteins and other phosphatases, until completion of DNA replication.

In HeLa cells, the cdc25C has been reported to be present in the nuclei (Millar *et al.*, 1991a). The discrepancy concerning the subcellular localization of cdc25C protein between the HeLa and the BHK21/13 cell lines may reflect the differences of cell cycle-regulation between them. For example, caffeine causes PCC in BHK21/13 cells but not in HeLa cells, although both cell lines were arrested with HU (Steinmann *et al.*, 1991). At early S phase, the cyclin B is present in BHK 21/13 cells (Nishitani *et al.*, 1991) but not in HeLa cells (Pines and Hunter, 1989). Thus in the case of HeLa cells, coexisting cdc25C proteins can not activate $p34^{cdc2}$ kinase until cyclin B accumulated upon completion of DNA replication. In this case, cyclin B may function as a stimulator of cdc25 protein (Galaktionov and Beach, 1991).

As reported in *Xenopus* oocytes (Kumagai and Dunphy, 1992), we observed a molecular shift of the cdc25C protein in tsBN2 cells showing PCC and in normal mitotic cells of BHK21/13 cells. Such a modification probably reflects a regulatory system of the cdc25C protein as for an activation of $p34^{cdc2}$ kinase (Kumagai and Dunphy, 1992). But it is not essential for an activation of $p34^{cdc2}$ kinase, because a higher molecular form of cdc25C protein appeared after an activation of $p34^{cdc2}$ kinase. Furthermore, such a high molecular form of the cdc25C protein did not appear in the presence of cycloheximide where no activation of $p34^{cdc2}$ kinase oc-

Table 3. Effect of the anti-cdc25C antibody on the initiation of mitosis

Injected samples	Frequency of mitotic cells (%)			
	Exp I	R[a]	Exp II	R[a]
IgG	23 (22)	100	46 (49)	100
Anti-cdc25C	8.0 (39)	21	17 (39)	46

Cultures of BHK21/13 cells arrested with HU as described in MATERIALS AND METHODS were allowed to grow in fresh media containing 10% fetal calf serum at 37.5°C and then 1 h later, given a microinjection of the purified anti-cdc25C (1 mg/ml) antibody into the cytoplasm. As a control, a nonspecific rabbit IgG (1 mg/ml) was similarly injected. At the 5th h after release from HU-block, cells were given nocodazole (0.4 μg/ml) and were incubated for another 4 h. Cells were then fixed and processed to calculate the mitotic index as described in MATERIALS AND METHODS. The numbers in parentheses show the mitotic index of uninjected cells on the same glass coverslips as the injected cells.

[a] R value was estimated as explained in Table 1. More than 80% of injected cells were labeled with BrdU.

curred (Nishitani *et al.*, 1991). Thus the molecular shift of cdc25C protein depends on an activation of $p34^{cdc2}$ kinase.

The molecular shift of cdc25C protein was transient. It returned to the normal molecular mass before entering the next cell cycle. Such a behavior of a higher molecule of the cdc25C protein suggests a mitotic specific phosphorylation of cdc25C protein, like MPM-2 antigens, mitotic specific antigen (Davis *et al.*, 1983). In *Xenopus* oocyte, such a molecular shift was reported to be caused by phosphorylation (Kumagai and Dunphy, 1992).

The *pim1*$^-$, a temperature sensitive mutation of *S. pombe*'s gene corresponding to human *RCC1*, induces premature activation of $p34^{cdc2}$ kinase without the function of *cdc25* gene. It remains to be answered why the *cdc25* gene is not required in the case of *pim1*$^-$, because $p34^{cdc2}$ kinases, which were phosphorylated at the time of temperature shift, must be dephosphorylated for its activation. One possibility is that the *pim1* might not be a functional homologue of the *RCC1* gene. For example, PCC was induced even in G1 phase by *pim1*$^-$ (Matsumoto and Beach, 1991) but not by tsBN2 mutation (Nishitani *et al.*, 1991).

Three kinds of *cdc25* genes have been isolated from humans (Galaktionov and Beach, 1991). All complement a ts mutant of *S. pombe*, *cdc25-22*. The finding that our anti-cdc25C antibodies poorly inhibited both the initiation of normal mitosis and the caffeine-induced PCC may indicate a redundancy of the cdc25 function for an activation of $p34^{cdc2}$ kinase. In the case of PCC induction caused by the tsBN2 mutation, we concluded that the cdc25C protein was a principal activator of the $p34^{cdc2}$ kinase.

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