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Genetics

Human homolog of fission yeast cdc25 mitotic inducer is predominantly expressed in G_2

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ABSTRACT **Entry into mitosis during the somatic cell cycle is regulated in response to signals that monitor the completion of DNA replication, the integrity of the nuclear genome, and, possibly, the increase in cellular mass during the cell cycle. It has been postulated that the operation of this cell cycle control involves the gradual accumulation of rate-limiting mitotic inducers, which trigger nuclear division when their cellular concentration reaches a critical level. We have cloned a human gene, which we call *CDC25*, whose product may function as a mitotic inducer. This human gene encodes a protein with a predicted molecular mass of 53,000 daltons whose C-terminal domain shares about 37% sequence identity with the fission yeast cdc25$^+$ mitotic inducer. The human *CDC25* gene rescues the defect of a fission yeast temperature-sensitive (ts) *cdc25^{ts}* mutant that is unable to initiate mitosis. In HeLa cells CDC25 mRNA levels are very low in G_1 and increase at least 4-fold as cells progress towards M phase. These data suggest that in human cells, as in fission yeast, the accumulation of CDC25 mitotic inducer during G_2 may play a key role in regulating the timing of mitosis.**

Genetic investigations of the fission yeast *Schizosaccharomyces pombe* have established that the cell cycle timing of entry into mitosis is governed by expression of the *cdc25$^+$* and *nim1$^+$* mitotic inducer genes and the inhibitor gene *wee1$^+$* (1–5). The products of these genes operate in a control network that determines cell size at mitosis and thereby coordinates growth with division. These mitotic control elements appear to act together to regulate function of the p34^{cdc2} M-phase protein kinase, whose activity is believed to be rate-limiting for the initiation of M phase (4–7). The mitotic inducer encoded by *cdc25$^+$* plays a key role in this control. In wild-type cells, *cdc25$^+$* gene function is required for entry into mitosis, p34^{cdc2} dephosphorylation, and kinase activation (2, 3, 6–9). Incremental increases in *cdc25$^+$* gene dosage cause a corresponding decrease in cell size at mitosis, showing that the level of *cdc25$^+$* expression is rate-limiting for entry into M phase in wild-type cells (3). It has been shown recently that in fission yeast the levels of cdc25$^+$ mRNA and protein increase as cells proceed through interphase, peaking at mitosis (10). These data suggest that in fission yeast the cell cycle timing of mitosis is regulated by the cyclic accumulation of the cdc25 mitotic inducer, which when accumulated to a critical level, brings about p34^{cdc2} kinase activation and the initiation of mitosis (11–13).

One of the best strategies to investigate the mitotic control in mammalian cells is to identify mammalian homologs of fission yeast mitotic control elements. One approach to this has been to rescue yeast mitotic control mutations with mammalian cDNA libraries made in expression vectors. Although this method was used to clone a human *cdc2* homolog (14), the success of this approach has so far been limited. We have used a two-stage strategy that circumvents potential problems to clone a human *cdc25* homolog. In the first stage, a *cdc25* homolog from the highly divergent budding yeast *Saccharomyces cerevisiae* was cloned by rescue of a fission yeast temperature-sensitive (ts) *cdc25^{ts}* mutation (15). The isolation of a *S. cerevisiae cdc25* homolog, named *MIH1*, established that the *cdc25* mitotic inducer gene was conserved among broadly divergent species and identified sequence similarities that were also likely to be generally conserved among eukaryotes. Here we report the second stage of this approach in which we have used the sequence similarities between the two yeast *cdc25* homologs and a *Drosophila cdc25* homolog that has been independently identified by genetic analysis (16) to design a pair of highly degenerate oligonucleotide sets that were then used to clone a human *cdc25* homolog by polymerase chain reaction (PCR) (17, 18). The product of the human *cdc25* homolog shares a conserved C-terminal domain† with the yeast and *Drosophila* cdc25 proteins. The human *cdc25* homolog rescues a fission yeast *cdc25^{ts}* mutant, indicating functional as well as structural conservation of cdc25 homologs. We also report that the human *cdc25* homolog, which we call *CDC25*, is expressed predominantly in G_2 phase in HeLa cells, indicating that periodic accumulation of the cdc25 mitotic inducer may be an evolutionarily conserved feature of the mitotic control in somatic cells.

MATERIALS AND METHODS

Cloning Human CDC25 cDNA. The 40-mer 5′ degenerate oligonucleotide set had the sequence: 5′-ATCTCGAGATCGATNATNGAYTGYMGNTWYGARTAYGART-3′, where R = A + G, Y = T + C, M = A + C, S = G = C, and W = A + T. The underlined sequence contains *Xho* I and *Cla* I restriction enzyme sites, and the 3′ proximal 28 nucleotides correspond to the peptide sequence Ile-Ile-Asp-Cys-Arg-(Phe or Tyr)-Glu-Tyr-Glu-(Phe or Tyr). This oligonucleotide is 16,384-fold degenerate. The 44-mer 3′ degenerate oligonucleotide set had the sequence: 5′-ATCTCGAGYTTRTANCCNCCRTSNARNANRTANAYNTCNGGRTA-3′. The underlined sequence contains an *Xho* I site, and the reverse complement of the 3′ proximal 36 nucleotides correspond to the peptide sequence (Tyr-Pro-(Glu or Asp)-(Val or Ile)-Tyr-(Ile or Leu)-Leu-(His or Asp)-Gly-Gly-Tyr-Lys. This oligonucleotide set is 16,777,216-fold degenerate. The 0.1-ml PCR reaction mixture consisted of 50 mM KCl; 10 mM Tris (pH 8.3); 1.5 mM $MgCl_2$; 0.01% gelatin; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 0.025 unit of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin–Elmer/Cetus), 10

Abbreviations: PCR, polymerase chain reaction; ts, temperature-sensitive.
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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M34065).

μg of each oligonucleotide set, and 5 μg of a HeLa cDNA library made in vector pCD (19) by Steve Hanks (Salk Institute) and kindly provided by Steve Gould (University of California, San Diego). Forty cycles of 94°C for 1 min, 45°C for 2 min, and 72°C for 3 min were performed in a DNA thermal cycler (Perkin–Elmer/Cetus). The reaction products were digested with *Cla* I and *Xho* I restriction enzymes and separated on a 1.5% agarose gel; the 280-nucleotide product was cloned into the *Cla* I/*Xho* I-digested vector pBluescript SK (pBSK) (Stratagene). Three clones were sequenced. They all were at least 99% identical. The HeLa *CDC25* PCR clone was used as a probe to obtain cDNA clones from a HeLa (D98/AH-2) cDNA library in vector Lambda ZAP II (Stratagene). pBSK phagemids were derived by *in vivo* excision using the manufacturer's protocol. The longest clone, pBSK1, was sequenced completely.

Expression of Human CDC25 cDNA in Fission Yeast. The complete CDC25 cDNA from pBSK1 was isolated by digestion with *Bam*HI and *Xho* I (the *Bam*HI site is at the 3′ end of the gene) and then ligated into pSM1 and pSM2 digested with *Bam*HI and *Xho* I. The pSM plasmids are pBR322/LEU2/2-μm chimeras containing the simian virus 40 early promoter upstream of a polylinker (20, 21). The pSM2 clone containing CDC25 cDNA in correct transcription orientation was called pSM25H, and the pSM1 derivative containing CDC25 cDNA in the opposite orientation was called pSM25H-rev. Both plasmids were transformed into a *Sc. pombe cdc25-22 leu1-32 ura4-D18* strain. Transformants were isolated, grown to midlogarithmic phase in EMM +leucine liquid medium (22) at 25°C, collected by centrifugation, and resuspended in YES medium (22). These cultures were incubated at 25°C for 4 hr and then incubated for a further 6 hr at 35°C. Cell number was monitored with a Coulter Counter model ZM. Cells were stained with Calcofluor (fluorescent brightener 28, Sigma F-6259).

Analysis of Human CDC25 mRNA Levels. Cell synchrony and RNA blot hybridization (Northern) analysis were performed as described (23). The CDC25 probe was made with

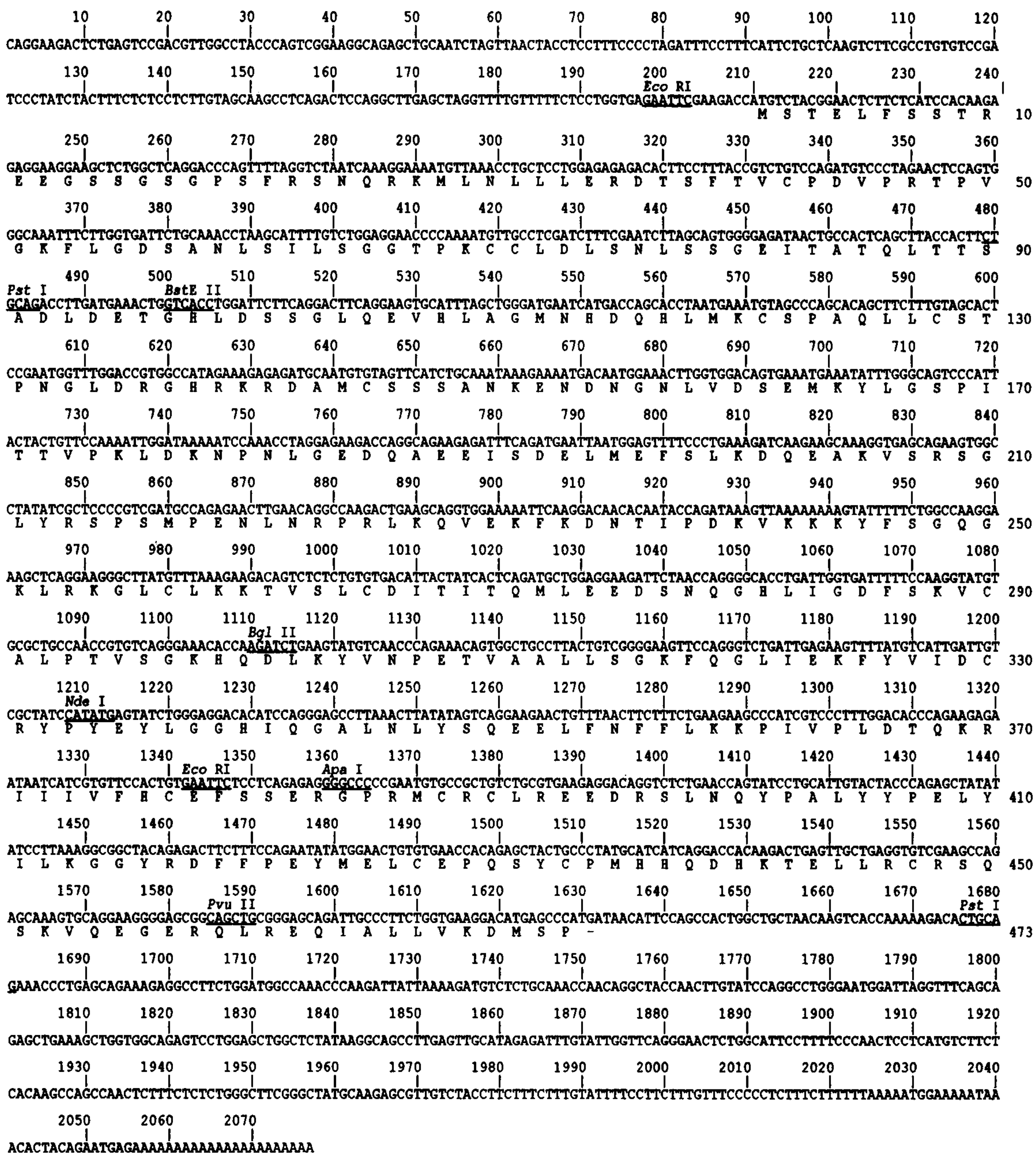

FIG. 1. Sequence of human CDC25 cDNA clone. Key restriction enzyme sites are noted.

the full-length cDNA. The actin probe is as described (24). Autoradiographs were quantified with an LKB Ultroscan XL laser densitometer.

RESULTS

Isolation of Human CDC25 cDNA Clone. Protein sequence comparison of the known cdc25 homologs (15, 16) revealed a region of ≈35% identity extending for ≈150 amino acids in the C-terminal portions of the proteins. We designed a pair of degenerate oligonucleotides corresponding to two of the most conserved regions for use in PCR (17, 18). The oligonucleotide sets were highly degenerate: the 5′ set was 16,384-fold degenerate over 28 nucleotides, and the 3′ set was >16,000,000-fold degenerate over 36 nucleotides (see *Materials and Methods*). These oligonucleotide sets amplified the expected 280-nucleotide fragment in PCR reactions with *Sc. pombe cdc25*$^+$ or *S. cerevisiae MIH1* genes as templates. Amplification from a HeLa cell cDNA library produced a predominant 280-nucleotide-long DNA product. DNA sequence analysis of this fragment revealed an open reading frame encoding a polypeptide having about 40% identity to the yeast cdc25 homologs. A similar degree of sequence identity has been reported with the *Drosophila* cdc25 homolog (16).

The 280-nucleotide DNA fragment was used as a probe to isolate cDNA clones from a HeLa cell cDNA library. The longest cDNA contained a 1.4-kilobase open reading frame that potentially encodes a 473-amino acid protein with a predicted molecular mass of 53 kDa (Fig. 1). This protein shares a conserved C-terminal region with the other known cdc25 homologs (Fig. 2). Most of the homology is located in the region that starts at amino acid position 290 and extends for 183 residues to the C terminus. This region of the human protein is 37% identical to the C-terminal region of fission yeast *cdc25*$^+$ gene product and 28% identical to the equivalent C-terminal region of the cdc25 homolog encoded by the budding yeast *MIH1* gene. The human and *Drosophila* cdc25 protein homologs are 49% identical in this region. The N-terminal halves of the four cdc25$^+$ homologs share little or no sequence similarity.

Human *CDC25* Gene Rescues Fission Yeast *cdc25*^{ts} Mutation. To investigate whether the fission yeast *cdc25*$^+$ gene and its human homolog have similar roles in the mitotic control, we determined if the human gene could rescue a fission yeast *cdc25*^{ts} mutation that confers a ts defect in mitotic initiation. The human *CDC25* gene was placed under the control of the simian virus 40 early promoter, which directs moderate levels of expression in fission yeast (20). Plasmid pSM25H contained CDC25 cDNA in the correct orientation for expression. A plasmid containing CDC25 cDNA in the reverse orientation, pSM25H-rev, was used as a control. These plasmids were transformed into a *cdc25-22 leu1-32* ts mutant. Cell number was monitored as logarithmic-phase transformant cultures were shifted from 25°C to 35°C. Plasmid pSM25H rescued the *cdc25-22* G_2 arrest, whereas pSM25H-rev did not (Fig. 3*A*). The *cdc25-22 leu1-32* mutant transformed with pSM25H was able to grow and to divide at 35°C until reaching stationary phase. The degree of rescue was variable among individual cells (probably a result of variation in plasmid copy number), but most of the cells divided at 1–2 times wild-type size (Fig. 3*B*). Plasmid pSM25H also was able to rescue a mutant in which the *CDC25* gene had been deleted (data not shown). These data establish that the human homolog of *cdc25*$^+$ is able to function as a mitotic inducer in fission yeast and suggests that *CDC25* is likely to function in the mitotic control in human cells.

Human *CDC25* Gene Is Expressed Predominantly in G_2. To further explore the possibility that human *CDC25* functions as a mitotic inducer gene in human cells, we next determined if CDC25 mRNA is expressed periodically in the HeLa cell cycle. HeLa cells grown in suspension culture were separated on the basis of size by centrifugal elutriation (see *Materials and Methods*). Cell cycle profiles of the fractions were monitored by flow-cytometry analysis of propidium iodide-stained cells to measure nuclear DNA content. The samples were processed for total RNA, and Northern blots of the samples were probed with *CDC25* and β-actin gene probes. The first fractions eluted, containing the smallest cells, were highly enriched in cells that were in G_1 phase (Fig. 4*A*). The human CDC25 mRNA levels were very low in these cells and increased as the fraction of G_2+M cells in the samples increased. The level of a β-actin mRNA remained constant through the cell cycle. Laser densitometry of the autoradiograph indicated that the level of CDC25 mRNA relative to β-actin mRNA increased about 4-fold from the first to the last fraction.

We wished to verify these findings using an independent method of cell synchronization. A population of HeLa cells arrested in S phase was prepared by using a double thymidine block (23). Upon removal of thymidine, the majority of the cells proceeded through S phase and entered G_2 in a syn-

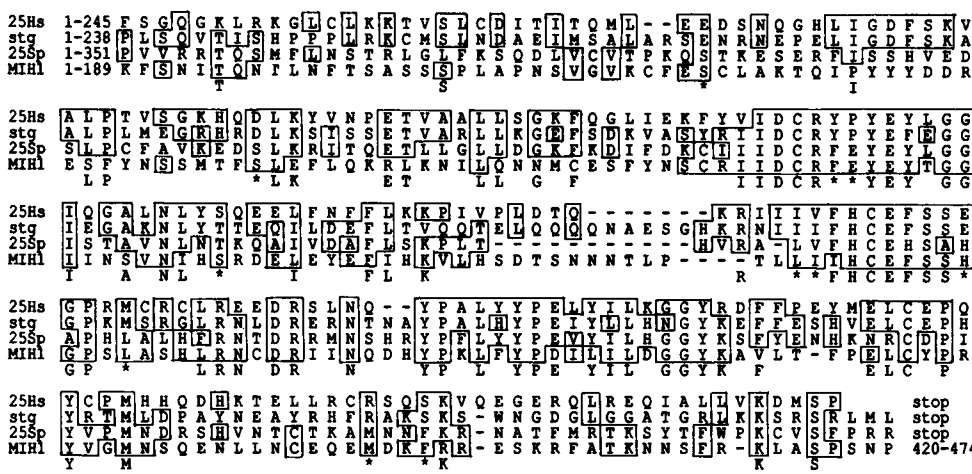

FIG. 2. Sequence comparisons among cdc25 homologs. The C-terminal regions of cdc25 homologs from HeLa (25Hs), *Drosophila* (stg), *Sc. pombe* (25Sp), and *S. cerevisiae* (MIH1) are compared. Identical matches are boxed. Positions that are identical among three or more of the gene products are indicated with the conserved residue below the MIH1 sequence. Positions at which there are two pairs of conserved residues are indicated with an asterisk. In this C-terminal region, the percentage identity between the product of the human gene and stg, 25Sp, and MIH1 is 44%, 29%, and 22%, respectively.

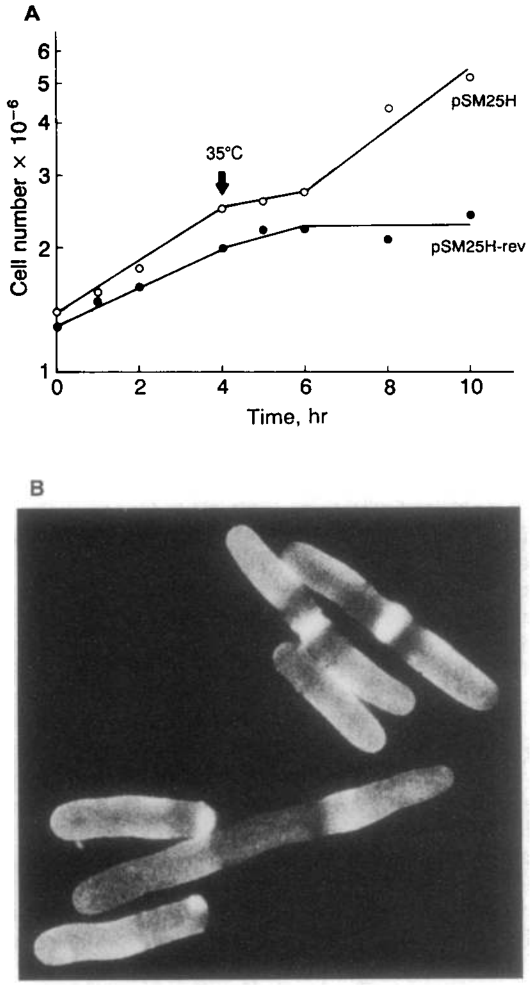

FIG. 3. Human *CDC25* gene rescues fission yeast *cdc25ᵗˢ* mutant. A *cdc25-22 leu1-32 ura4-D18* strain, containing pSM25H or pSM25H-rev, growing in YES medium at 25°C, was shifted to 35°C at the 4-hr time point. (*A*) Growth curve. (*B*) Cells containing pSM25H grown for 5 hr at 35°C and then stained with Calcofluor, which binds to the cell plate.

chronous fashion (Fig. 4*B*). Northern blot analysis showed that human CDC25 mRNA levels rose as cells progressed from S into G_2 phase, followed by a decrease as cells proceeded further into G_1.

These data establish that the level of human CDC25 mRNA increases as HeLa cells progress into G_2 phase and approach mitosis. At the present time we don't know if the periodic increase in CDC25 mRNA level is due to increased transcription or increased stability of the mRNA.

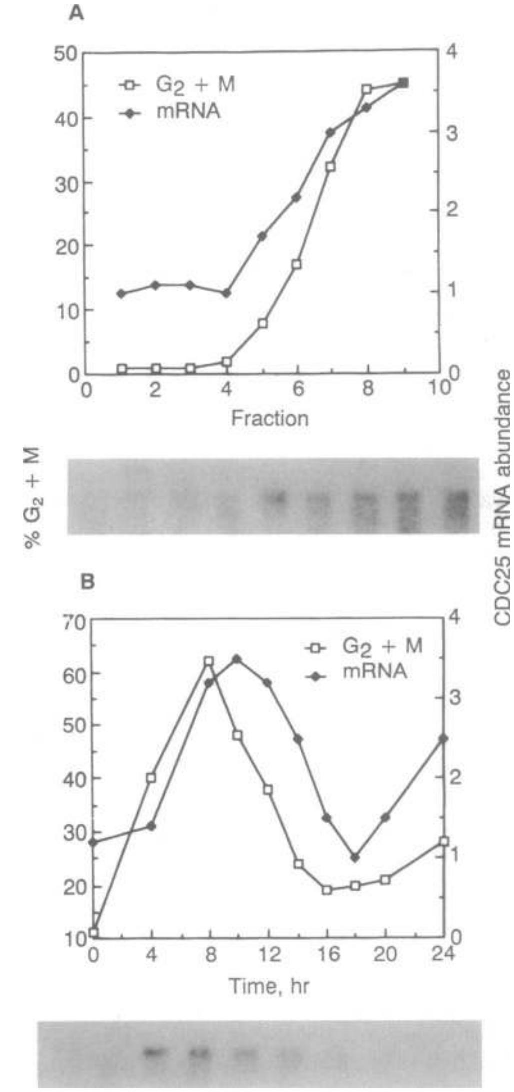

FIG. 4. Human *CDC25* is expressed late in the HeLa cell cycle. (*A*) Cells in a HeLa suspension culture were separated on the basis of size by elutriation centrifugation. Aliquots from each sample were either stained with propidium iodide for fluorescence-activated cell sorting or were processed for total RNA and subsequent Northern analysis. (*Upper*) Fraction of cells in each sample that had a 4N DNA content (N = one haploid gene content) and so therefore must be in G_2 or M phase, together with the relative level of CDC25 mRNA normalized to β-actin mRNA. (*Lower*) Northern blot probed with human *CDC25*. This blot was then washed and rehybridized with a β-actin gene probe (data not shown). (*B*) Cell cycle progression in a HeLa suspension culture was arrested in S phase with a double thymidine block (23). At the 0-hr time point, thymidine was washed out. Aliquots at each time point were processed as described above. (*Upper*) Fraction of cells in each sample that had a 4N DNA content (N = one haploid gene content), together with the relative level of CDC25 mRNA normalized to β-actin mRNA. (*Lower*) Northern blot probed with human *CDC25*. This blot was then washed and rehybridized with a β-actin gene probe (data not shown).

DISCUSSION

Strategy for Cloning Human Homologs of Yeast Genes. The strategy used for identifying the human *cdc25* homolog utilized two powerful gene cloning techniques that allowed us to divide a difficult cloning task into two more simple steps. We first cloned the budding yeast *cdc25* homolog by rescue of a fission yeast *cdc25ᵗˢ* mutation (15). With the sequences of the homologous genes from both yeasts, we were then able to design degenerate oligonucleotide primers that were used to clone the human *cdc25* homolog by PCR. This approach should be broadly applicable to the cloning of many types of higher eukaryotic homologs of genes first identified in yeast. In spite of the extreme divergence between the two yeasts, it is frequently possible to clone gene homologs from genomic DNA libraries of budding yeast by rescue of fission yeast mutants. In practice, the cloning of homologous genes from both yeasts by cross-species rescue of mutations often may provide the most efficient route to the identification of sequences that are likely to be generally conserved among all eukaryotes. Having identified sequences conserved between the two yeasts, highly degenerate oligonucleotide sets can be made that should have a high probability of hybridizing to divergent homologs in PCR amplifications. Indeed, the PCR primers described here have been used to clone *cdc25* homologs from mouse, *Xenopus*, *Dictyostelium*, and other species (P.R., unpublished data). We have used the same approach to clone homologs of fission yeast *suc1*$^{+}$ and budding yeast *CKS1* genes, which encode small proteins that interact with the p34^{cdc2}/CDC28 mitotic kinase (25).

Roles of the Putative CDC25 Mitotic Inducer. We have presented evidence showing that the human *CDC25* gene shares a number of structural and functional features with *cdc25* homologs identified in the yeasts and *Drosophila*. Human *CDC25* encodes a protein of predicted molecular mass of 53 kDa, which is very close to the predicted molecular mass of 54 kDa for the *Drosophila* and budding yeast cdc25 homologs. The fission yeast cdc25 protein has a somewhat larger predicted molecular mass of 67 kDa. The C-terminal half of the human CDC25 protein ranges in identity from about 25% to 45% relative to the C-terminal regions of the other cdc25 homologs. No primary sequence homology is apparent between any of the N-terminal halves of the cdc25 homologs. This suggests that sequences essential and possibly sufficient for function are located in the C-terminal halves of the cdc25 homologs. This suggestion is supported by the observation that expression of a truncated form of the *MIH1* gene product, lacking the N-terminal quarter of the protein, complemented the product of a fission yeast *cdc25*^{ts} mutation (15).

Expression of human *CDC25* in fission yeast rescued a *cdc25*^{ts} mutation, showing that the product of the human *cdc25* homolog is capable of functioning as a mitotic inducer in fission yeast. Although we do not yet have any direct evidence establishing a mitotic function of *CDC25* in human cells, the rescue of a fission yeast *cdc25*^{ts} mutation by *CDC25*, together with data demonstrating mitotic inducer functions for cdc25 homologs in the budding yeast and *Drosophila* (15, 16), strongly suggest that CDC25 is likely to function as an inducer in the mitotic control of human cells.

The proposed mitotic function of human *CDC25* is further supported by the observation that CDC25 mRNA is predominantly expressed late in the cell cycle. A similar pattern of *cdc25* gene expression has been seen in the mitotic cell cycles of fission yeast and *Drosophila* (10, 16). In fission yeast it has been shown that appearance of the *cdc25*$^{+}$ gene product, a phosphoprotein migrating with the apparent molecular mass of 80 kDa, oscillates during the cell cycle, increasing during G_2 and peaking at mitosis. In fission yeast it has been shown that the level of *cdc25*$^{+}$ expression is rate-limiting for the initiation of mitosis, and it has been proposed that the cyclic accumulation of p80^{cdc25} during interphase drives the mitotic cycle in fission yeast (3, 10). The apparent similarities of gene function and pattern of expression of the cdc25 homologs suggest the possibility that the mitotic cycle of some types of human cells may be driven by the rate of accumulation of *CDC25* gene product during G_2.

Although genetic evidence from fission yeast indicates that cdc25 protein, p80^{cdc25}, functions as a mitotic inducer by promoting activation of the p34^{cdc2} protein kinase (3, 4), the biochemical mechanism by which this occurs remains unknown. However, new biochemical evidence suggests that p80^{cdc25} may be involved in regulating the p34^{cdc2} kinase activity by modifying the phosphorylation state of p34^{cdc2}. Upon shift of a *cdc25*^{ts} mutant from the permissive to restrictive temperature, cells arrest in late G_2 with inactive p34^{cdc2} kinase (6, 7). At the arrest point, p34^{cdc2} is maximally phosphorylated on tyrosine and threonine residues (9). Upon shift of *cdc25*^{ts} cells down to the permissive temperature, p34^{cdc2} rapidly becomes dephosphorylated and activated as a kinase. These observations, together with mutagenesis studies showing that alteration of the p34^{cdc2} tyrosine phosphorylation site enhances its activity as a mitotic inducer, suggest that p34^{cdc2} is activated by dephosphorylation (9). We propose that cdc25 mitotic inducer promotes the dephosphorylation of p34^{cdc2}, either by activating phosphatases or inhibiting kinases.

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1. Nurse, P. (1975) *Nature (London)* **256**, 547–551.
2. Fantes, P. (1979) *Nature (London)* **279**, 428–430.
3. Russell, P. & Nurse, P. (1986) *Cell* **45**, 145–153.
4. Russell, P. & Nurse, P. (1987) *Cell* **49**, 559–567.
5. Russell, P. & Nurse, P. (1987) *Cell* **49**, 569–576.
6. Moreno, S., Hayles, J. & Nurse, P. (1989) *Cell* **58**, 361–372.
7. Booher, R. N., Alfa, C. A., Hyams, J. S. & Beach, D. H. (1989) *Cell* **58**, 485–497.
8. Hagan, I. M. & Hyams, J. S. (1988) *J. Cell Sci.* **89**, 343–357.
9. Gould, K. L. & Nurse, P. (1989) *Nature (London)* **342**, 39–45.
10. Moreno, S., Nurse, P. & Russell, P. (1990) *Nature (London)* **344**, 549–552.
11. Murray, A. W. & Kirschner, M. W. (1989) *Science* **246**, 614–621.
12. Hartwell, L. H. & Weinert, T. A. (1989) *Science* **246**, 629–634.
13. O'Farrell, P. H., Edgar, B. A., Lakich, D. & Lehner, C. F. (1989) *Science* **246**, 635–640.
14. Lee, M. G. & Nurse, P. (1987) *Nature (London)* **327**, 31–35.
15. Russell, P., Moreno, S. & Reed, S. I. (1989) *Cell* **57**, 295–303.
16. Edgar, B. A. & O'Farrell, P. H. (1989) *Cell* **57**, 177–187.
17. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239**, 487–491.
18. Lee, C. C., Wu, X., Gibbs, R. A., Cook, R. G., Muzny, D. M. & Caskey, C. T. (1988) *Science* **239**, 1288–1291.
19. Okayama, H. & Berg, P. (1983) *Mol. Cell. Biol.* **3**, 280–289.
20. Jones, R., Moreno, S., Nurse, P. & Jones, N. (1988) *Cell* **53**, 659–667.
21. Russell, P. (1989) in *Molecular Biology of the Fission Yeast*, eds. Nasim, A., Young, P. & Johnson, B. F. (Academic, San Diego), pp. 244–271.
22. Moreno, S., Klar, A. & Nurse, P. (1990) *Methods Enzymol.*, in press.
23. McGowan, C. H., Russell, P. & Reed, S. I. (1990) *Mol. Cell. Biol.*, in press.
24. Ponte, P., Ng, S.-Y., Engel, J., Gunning, P. & Kedes, L. (1984) *Nucleic Acids Res.* **12**, 1687–1696.
25. Richardson, H. E., Stueland, C. S., Thomas, J., Russell, P. & Reed, S. I. (1990) *Genes Dev.*, in press.