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Cloning of a human cDNA encoding a CDC2-related kinase by complementation of a budding yeast *cdc28* mutation

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ABSTRACT **We have cloned two different human cDNAs that can complement *cdc28* mutations of budding yeast *Saccharomyces cerevisiae*. One corresponds to a gene encoding human p34^{CDC2} kinase, and the other to a gene (*CDK2*; cell division kinase) that has not been characterized previously. The CDK2 protein is highly homologous to p34^{CDC2} kinase (65% identical) and more significantly is homologous to *Xenopus* Eg1 kinase (89% identical), suggesting that CDK2 is the human homolog of Eg1. The human *CDC2* and *CDK2* genes were both able to complement the inviability of a null allele of *S. cerevisiae CDC28*. This result indicates that the CDK2 protein has a biological activity closely related to the CDC28 and p34^{CDC2} kinases. However, *CDK2* was unable to complement *cdc2* mutants in fission yeast *Schizosaccharomyces pombe* under the condition where the human *CDC2* gene could complement them. CDK2 mRNA appeared late in G_1 or in early S phase, slightly before CDC2 mRNA, after growth stimulation in normal human fibroblast cells. These results suggest that in human cells, two different CDC2-like kinases may regulate the cell cycle at distinct stages.**

Members of the yeast CDC28/mammalian CDC2 protein kinase family are highly conserved in a wide variety of eukaryotic organisms and play key roles in the regulation of the eukaryotic cell cycle. Extensive studies of CDC28/CDC2 kinases have been directed toward elucidating their function in the initiation of mitosis. These studies have demonstrated that the human p34^{CDC2} kinase is a component of the mitosis-regulating protein kinase complex known as maturation-promoting factor or growth-associated histone H1 kinase and have identified a number of associated regulatory proteins such as cyclins and p13^{suc1} (1, 2). Beyond their central role in the regulation of mitosis, the CDC28/CDC2 kinases are also involved in the regulation of G_1/S phase transition. This G_1 role has been established through genetic analysis in budding and fission yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively (3, 4). In budding yeast, it is primarily G_1 arrest that is observed with *cdc28* mutants. Furthermore, in vertebrate cells, there is evidence that p34^{CDC2} kinase in some form is involved in the initiation of DNA replication in addition to its mitotic role (5–7). However, the mouse FT210 cell line carrying a temperature-sensitive *cdc2* mutation arrested in G_2 and showed no defect in initiation of S phase (8, 9). This result is consistent with the fact that, in mammalian cells, microinjection of antibodies to p34^{CDC2} caused cells to arrest in G_2 but had no effect on DNA replication (10). Thus, a possible G_1 role of the p34^{CDC2} kinase in mammalian cells remains uncertain.

Whereas *S. pombe cdc2* mutants arrest in both G_1 and G_2 (4), most temperature-sensitive alleles of *S. cerevisiae cdc28* arrest in G_1 at restrictive temperatures (3, 11). The G_1 bias of cell cycle arrest by *cdc28* mutations has facilitated the investigation of the G_1 function of the CDC28 kinase. In fact, G_1-specific cyclins, which activate the CDC28 kinase for its function required at the G_1/S phase transition, were first identified in *S. cerevisiae* (12–14). Therefore, G_1 cyclins perform an analogous function to that described for mitotic cyclins in the G_2/M phase transition.

By taking advantage of the G_1 bias of *S. cerevisiae cdc28* mutants, we sought to identify mammalian genes involved in regulation of G_1 as suppressors of *cdc28* mutants. We report here a human gene that we have sequenced‖ and named *CDK2* for cell division kinase that can complement *cdc28* mutations. *CDK2* encodes a protein that is highly homologous to the p34^{CDC2} (15) and the *Xenopus* Eg1 kinases (16). Furthermore, we discuss a possible role for the CDK2 protein in cell cycle control.

MATERIALS AND METHODS

Strains and Growth Media. *S. cerevisiae* mutants used were *cdc28-13* and a disruption of *CDC28* (*cdc28::LEU2*). These strains were congenic with 15Dau (*MATa ura3*Δns *ade1 his2 leu2-3, 112 trp1-1a*) (13). *S. pombe cdc2* mutants *cdc2-L7* and *cdc2-M26* have been described (4). Standard culture media for *S. cerevisiae* and *S. pombe* were used in all experiments (17, 18). Human HeLa, mouse NIH 3T3, and human WI-38 cells (ATCC CCL75, 25–28 population doubling level) were cultured in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum.

Plasmids and Transformation. The HeLa cDNA library used in this work was made in the vector pNV7 in which the *GAL1* promoter and *TRP5* terminator flank the cDNA cloning site. A 0.45-kilobase (kb) *Eco*RI–*Xba* I fragment from pME18s (19) containing cDNA cloning sites and a 1.75-kb *Xba* I–*Bgl* I fragment from pGT5 (20) were ligated with a 4.4-kb *Eco*RI–*Bgl* I fragment from a *URA3*-based multicopy plasmid YEplac195 (21) to yield pNV7. pNV7 consists of the origin of replication of the *S. cerevisiae* 2-μm plasmid and the *URA3* gene. The size-selected cDNA (>1.0 kb) prepared from HeLa cells was attached with *Bst*XI adapters and was inserted into the *Bst*XI sites of pNV7. Approximately 2×10^5 independent clones were obtained. pNV7-CDC2 and pNV7-CDK2 were obtained from this HeLa cDNA library by the screening in this study (see *Results*). The 0.45-kb *Eco*RI–*Xba* I fragment from pME18s was cloned into a centromeric plasmid pGT5 (20). The resulting plasmid pNV6 contains the *GAL1* promoter, the *TRP5* terminator, the cDNA cloning

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Abbreviation: CMV, cytomegalovirus.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. M68520).

sites, *CEN3, ARS1*, and *URA3*. YCpG2-CDC28 has been described previously (13). pSE271-CDC28 is a centromeric plasmid containing *TRP1* and *CDC28* and was used to provide CDC28 function in strains that carry the *cdc28*::*LEU2* disruption. The *CDC28* disruption was constructed as follows. A fragment extending from the *Xho* I site ≈200 base pairs (bp) upstream from the initiating methionine to the *Sac* I site halfway through the coding region was replaced by a *Xho* I–*Sac* I fragment containing the *LEU2* gene. Transformation of *S. cerevisiae* was carried out by the alkali cation method and by electroporation (22). For the expression of cloned human cDNAs in *S. pombe, Xho* I fragments of pNV7-CDK2 and pNV7-CDC2 were inserted downstream of human cytomegalovirus (CMV) promoter in the vector pCMVU-X (23). *Xho* I sites are located in the cDNA cloning sites of pNV7.

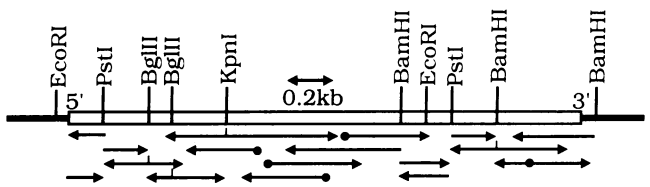
DNA Sequencing. The restriction fragments indicated in Fig. 1 *Upper* were subcloned into the vector pBluescript II SK(+) (Stratagene) and were sequenced by the dideoxy chain-termination method (24). Several synthetic oligodeoxynucleotides corresponding to internal sequences were also used as sequencing primers as indicated in Fig. 1 *Upper*.

RNA Blot Hybridization (Northern Blots). Poly(A)$^+$ RNA prepared from HeLa, NIH 3T3, and WI-38 cells (Fast Track-mRNA isolation kit, Invitrogen, San Diego) were separated by electrophoresis on agarose gels containing 6% formaldehyde, followed by blotting onto nitrocellulose filters. Entire cDNAs of *CDK2*, human *CDC2*, and mouse c-*myc* (designated *Myc*) were labeled by the random primer method to a specific activity of 2×10^9 cpm/μg. Hybridization was performed in 50% formamide containing 0.5% SDS, 5× Denhardt's solution (1× = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), 6× SSPE (1× = 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA), and 100 μg of denatured salmon sperm DNA per ml at 42°C overnight. Filters were washed in 0.2× SSC (1× = 0.15 M NaCl/0.015 M sodium citrate, pH 7) containing 0.1% SDS for 30 min at 52°C. The sizes of the mRNAs were roughly estimated by using DNA size markers and 18S and 28S ribosomal RNAs.

Flow Cytometry. For flow cytometry, cells were stained with propidium iodide as described (25). Flow cytometric analysis using a FACS IV analyzer (Becton Dickinson) has been described (25).

RESULTS

Cloning of *CDK2*. We have used a HeLa cDNA library to isolate human genes capable of complementing *S. cerevisiae cdc28* mutations. The HeLa cDNA library was prepared in the *S. cerevisiae* expression vector pNV7. DNA prepared from the cDNA expression library was transformed into a *S. cerevisiae* temperature-sensitive mutant strain containing *cdc28-13* and *ura3* mutations. A total of 10^6 Ura$^+$ transformants were obtained and subsequently screened for the ability to grow at the restrictive temperature of 37°C on medium containing galactose. Thirty-eight transformants were capable of forming colonies. Segregation analysis showed that in each case cells that lost the plasmid were temperature sensitive for growth, indicating that the complementing activity was plasmid-linked. Plasmids from these transformants were transferred into *Escherichia coli*. Their cDNA inserts were compared by restriction enzyme analysis. Thirty-seven corresponded to the human *CDC2* gene encoding p34^{CDC2} kinase (15) shown previously to rescue *cdc28* mutations (26). The remaining one cDNA exhibited a restriction map different from that of the *CDC2* gene (Fig. 1 *Upper*), and was named *CDK2*. Recovered plasmids (pNV7-CDC2 and pNV7-CDK2) containing human *CDC2* and *CDK2*, respectively, were used for their expression in *S. cerevisiae*. pNV7-CDC2 and pNV7-CDK2 were both capable of complementing *cdc28-13* upon retransformation (Fig. 2).

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TCACCCTTGTCCTTGAGTGCTCTTGCTATTAACGTTATTTGTAATTTAGTTTGTA   2213
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FIG. 1. Nucleotide sequence of the CDK2 cDNA and the predicted amino acid sequence. (*Upper*) Restriction map and sequencing strategy. The open box shows the insert cDNA. The black dots show the synthesized primers. (*Lower*) Nucleotide and amino acid sequences.

Sequencing of CDK2 cDNA. The original insert cDNA of pNV7-CDK2 was sequenced. The sequencing strategy and the nucleotide and predicted amino acid sequences of CDK2 cDNA are shown in Fig. 1. The CDK2 cDNA is 2213 nucleotides long, and the longest open reading frame, starting at the first ATG codon, encodes a protein of 298 amino acids corresponding to M_r 33,931. Screening the GenBank peptide data base with this sequence revealed significant homology to members of the p34^{CDC2} protein kinase family; CDK2 shares 65% identity with human CDC2 (15) and 59% identity with *S. cerevisiae* CDC28 (27) (Fig. 3). The amino acid sequence identities include segments known to be highly conserved and characteristic of all functional p34^{CDC2} homologs, such as the Pro-Ser-Thr-Ala-Ile-Arg (PSTAIR) motif (Fig. 3). Moreover,

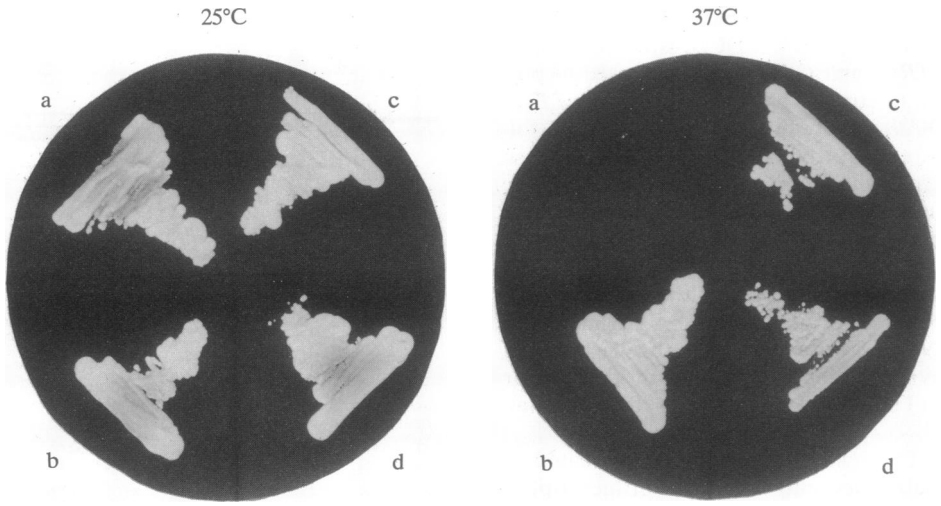

FIG. 2. Complementation of the *cdc28-13* mutation by human *CDC2* and *CDK2*. *cdc28-13* mutant cells carrying pNV7 (*a*), YCpG2-CDC28 (*b*), pNV7-CDC2 (*c*), or pNV7-CDK2 (*d*) were cultured on medium containing galactose at 25°C or 37°C for 4 days.

higher homology was obtained with *Xenopus* Eg1 kinase (16) than with human CDC2 kinase. The amino acid sequence of the CDK2 protein is 89% identical and 95% homologous to that of the Eg1 kinase. This result suggests that CDK2 is the human homolog of Eg1.

***CDK2* Can Rescue the Deletion of *S. cerevisiae CDC28* but Does Not Complement *cdc2* Mutants in *S. pombe*.** To determine whether *CDK2* could substitute for all *CDC28* functions, we asked whether *CDK2* was able to complement a *cdc28* deletion allele (*cdc28*::*LEU2*). Human *CDK2* and *CDC2* genes were transformed into *cdc28*::*LEU2* cells carrying a plasmid copy of the wild-type *CDC28* gene (pSE271-CDC28). Transformants of this strain with pNV7-CDK2 or pNV7-CDC2 were selected, and an attempt was made to cure the cells of plasmid pSE271-CDC28 while expression of the human genes was induced. It was found that plasmid pSE271-CDC28 could be lost from cells containing plasmid pNV7-CDK2 or pNV7-CDC2 but not from cells containing the control plasmid pNV7. Therefore, *CDK2* and *CDC2* can provide all of the essential functions of the *S. cerevisiae* *CDC28* gene. In addition, *cdc28*::*LEU2* cells carrying *CDK2* or human *CDC2* responded normally to α factor (data not shown). This suggests that human CDK2 is regulated in a manner similar to CDC28 in *S. cerevisiae*.

Next, we tested whether *CDK2* could complement *S. pombe* temperature-sensitive *cdc2* mutants. Cells of *cdc2-L7 ura4-294* were transformed with pCMVU-CDC2 or pCMVU-CDK2, allowing expression of either *CDC2* or *CDK2* from the CMV promoter. Cells containing pCMVU-CDC2 were able to grow at 33°C and showed the wee phenotype at both 27°C and 33°C (data not shown), suggesting that the CMV promoter caused overexpression of $p34^{CDC2}$ in *S. pombe*. Similar results were obtained with another allele, *cdc2-M26*. On the contrary, cells containing pCMVU-CDK2 did not grow at 33°C. We confirmed expression of the CDK2 protein in *S. pombe* by Western analysis, using anti-PSTAIR antibodies (data not shown). Thus, expression of *CDK2* did not suppress *S. pombe cdc2* mutations.

Paris *et al.* (16) have reported that *Xenopus* Eg1 kinase was unable to suppress either *S. cerevisiae cdc28* or *S. pombe*

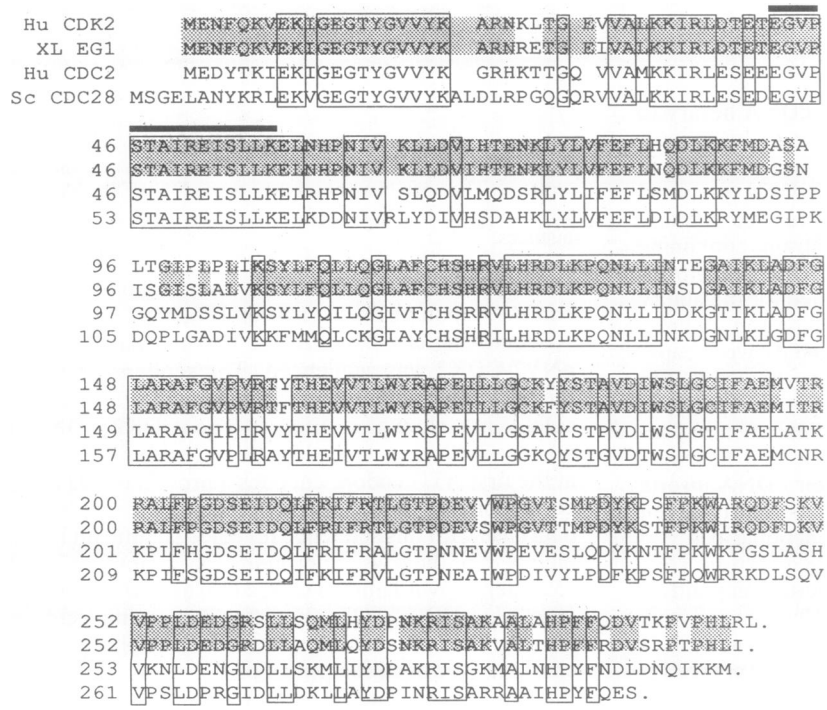

FIG. 3. Comparison of amino acid sequences of CDK2 with *Xenopus* Eg1, human CDC2, and *S. cerevisiae* CDC28. Abbreviations and source of data: Hu CDK2, human CDK2; XL EG1, *X. laevis* Eg1 (16); Hu CDC2, human CDC2 (15); Sc CDC28, *S. cerevisiae* CDC28 (27). The black bar designates the PSTAIR region.

cdc2 mutations. As shown above, the human Egl homolog (CDK2) was able to suppress *cdc28* mutations. One possible explanation of this discrepancy is that expression levels of the *Xenopus* and human Egl are critical for suppression of *cdc28*. To test this possibility, we constructed a single copy plasmid (pNV6) expressing *CDC2* or *CDK2* from the *GAL1* promoter. pNV6-CDC2 and pNV6-CDK2 also rescued the growth defect caused by *cdc28*::*LEU2*. However, *cdc28*::*LEU2* cells with pNV6-CDK2 grew more slowly than those with pNV6-CDC2 or pNV7-CDK2 and were noticeably larger with an elongated shape reminiscent of the disruption of *CLB2* encoding one of the *S. cerevisiae* G_2-specific cyclins (28). The cell cycle distribution of these cells was examined by flow cytometric analysis (Fig. 4). Compared with *cdc28*::*LEU2* cells carrying pNV7-CDC2, those with pNV7-CDK2 had a higher proportion of cells with fully replicated DNA. The phenotype was even more extreme with the single-copy plasmid pNV6-CDK2. This result suggests that cell-cycle progression was delayed in the post-S-phase interval when cells were dependent on CDK2. In addition, high levels of CDK2 expression are necessary for good suppression of *cdc28*. These results suggest that CDK2 is poor at performing mitotic functions. It is possible that CDK2 is incapable of performing mitotic functions in *S. pombe*, accounting for its inability to suppress *cdc2* mutations. An alternative explanation for the failure of Paris *et al.* (16) to observe suppression of a temperature-sensitive *cdc28* mutation by the *Xenopus* Egl gene is that the kinase encoded by this gene might itself be temperature sensitive, as a result of the relatively low growth temperature of frogs compared with mammals.

Northern Analysis. To analyze the expression of the *CDK2* gene in mammalian cells, poly(A)$^+$ RNA was prepared from HeLa and mouse NIH 3T3 cells. Both RNAs were blotted and probed with human *CDK2* and *CDC2* genes. The *CDK2* probe detected a major transcript of ≈2.3 kb in HeLa cells (Fig. 5*A*, lane 1). The transcripts detected by the human *CDC2* probe were 2.0 kb and 1.6 kb long (Fig. 5*A*, lane 3) as reported previously (29). The human *CDK2* probe detected a transcript (≈2.4 kb) in mouse, while under the same conditions human *CDC2* did not cross-hybridize with the mouse homolog of *CDC2* (Fig. 5*A*, lanes 2–4). This likely reflects a higher degree of conservation of *CDK2* than *CDC2* between human and mouse.

It has been reported that CDC2 mRNA is induced in late G_1 or early in S phase in T cells and fibroblasts when resting cells are stimulated by mitogens (6, 29). We examined the expression of *CDK2* and *CDC2* in normal human cells after they were arrested in G_0 by serum starvation and then stimulated by readdition of serum. In this experiment, cells began DNA synthesis after 12–18 hr (Fig. 5*C*), and mitotic cells were observed at 24 hr. The expression of CDK2, CDC2, and Myc mRNAs was evaluated by Northern blotting after serum stimulation (Fig. 5*B*). The Myc mRNA was induced through G_0 and G_1 and then decreased as reported (30). Before serum stimulation, CDC2 mRNA was undetectable, became first detectable after 12 hr, and was significantly increased after 18–24 hr. The accumulation of CDK2 mRNA was similar but not identical to that of CDC2 mRNA. A basal level of CDK2 mRNA was detected in the cells arrested in G_0, followed by an increase at 12–24 hr (Fig. 5*B*).

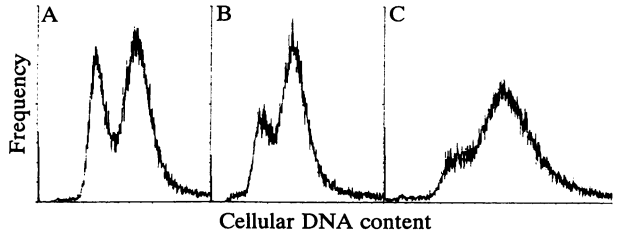

FIG. 4. Flow cytometric analysis of cells dependent on human CDC2 or CDK2. *cdc28*::*LEU2* cells carrying pNV7-CDC2 (*A*), pNV7-CDK2 (*B*), or pNV6-CDK2 (*C*) were cultured in medium containing galactose at 30°C. Cultures were stained with propidium iodide and analyzed for nuclear DNA content by using a FACS analyzer. The left peak (or shoulder) corresponds to cells in G_1, and the right peak corresponds to cells in G_2 and/or M phase. The increased fluorescence in *C* is due to the greater contribution of mitochondrial DNA to the fluorescent signal in larger cells.

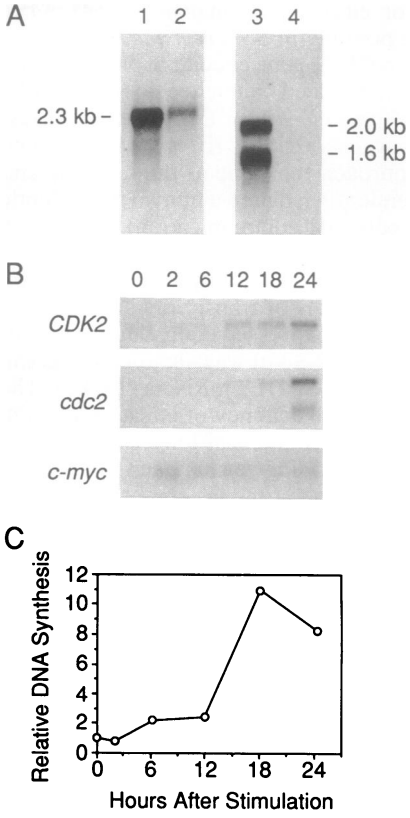

FIG. 5. Northern analysis of CDK2 and CDC2 mRNAs in mammalian cells. (*A*) Poly(A)$^+$ RNA was prepared from growing HeLa (lanes 1 and 3) and NIH 3T3 cells (lanes 2 and 4). Each lane was loaded with 5 μg of poly(A)$^+$ RNA. The probes used were CDK2 cDNA (lanes 1 and 2) and human CDC2 cDNA (lanes 3 and 4). (*B*) Poly(A)$^+$ RNA was prepared from WI-38 cells at resting stage (lane 0) and at 2, 6, 12, 18, and 24 hr after serum stimulation. Each lane was loaded with 5 μg of poly(A)$^+$ RNA, and cDNAs corresponding to *CDK2*, human *CDC2*, and mouse *Myc* were used for probes. (*C*) DNA synthesis was measured by incorporation of [^{3}H]thymidine [40-min pulse at 5 μCi (185 kBq)/ml] into an acid-insoluble fraction and normalization to that at zero time.

DISCUSSION

Lee and Nurse (15) isolated a human *CDC2* gene by direct complementation of *S. pombe cdc2* mutants with a human cDNA library. Encouraged by their success, we have developed a procedure to isolate mammalian genes in *S. cerevisiae* using biological screening. Our approach has been to use a library prepared by inserting mammalian cDNAs into a *S. cerevisiae* expression vector proximal to the *GAL1* promoter. In the present work, we cloned the human CDK2 cDNA by screening a cDNA library for complementation of an *S. cerevisiae cdc28* mutation. In addition, using a biological screen, we recently succeeded in isolation of human cDNAs that affect the signal transduction pathway mediated by *S. cerevisiae* mating pheromone (S.N. and K.M., unpublished data). Thus, this system is likely to be a generally useful

approach for cloning mammalian cDNAs when functional screens are possible in *S. cerevisiae*.

The human *CDK2* gene encodes a protein that shares a high degree of amino acid sequence identity with the human $p34^{CDC2}$ (65%) and *Xenopus* Eg1 protein kinases (89%). This homology suggests that CDK2 is a human homolog of Eg1. Using an approach the same as ours, Ellege and coworkers have independently isolated a human gene identical to *CDK2* (S. Ellege, personal communication). In our screening system, the frequency of isolation of CDK2 cDNA from a HeLa cDNA library was much lower than that of CDC2. Yet analysis of the levels of transcripts showed that CDK2 and CDC2 cDNAs are expressed equivalently. However, the size of CDK2 mRNA (2.3 kb) was significantly greater than that of the shorter of the CDC2 mRNAs (1.6 kb). Therefore, it is likely that the low frequency of isolation of CDK2 cDNA is due to poor representation of longer cDNAs in our library.

In fission and budding yeasts, one CDC28/CDC2 kinase is required at both the G_1 and G_2 control points (3, 4). At each stage of the cell cycle, the kinase is thought to be regulated by different types of cyclin. In fact, in *S. cerevisiae* two distinct classes of cyclin have been identified: G_1-specific cyclins that activate the CDC28 kinase required for its G_1/S function (14) and G_2-specific cyclins that activate the same kinase for its mitotic function (28, 31). Thus, two forms of kinase are generated by association of a common catalytic subunit, CDC28, with G_1- or G_2-specific cyclins. It is likely that the same holds true for *S. pombe*, where the CDC2 kinase has been shown to have both G_1 and G_2 functions (4). In mammalian cells, $p34^{CDC2}$ is known to play a critical role for the G_2/M transition (10, 32, 33). Recently, however, it has been shown that some form of $p34^{CDC2}$ is likely to be involved in the initiation of DNA synthesis (5–7). Could the same $p34^{CDC2}$ kinase also act at the G_1/S transition point in mammalian cells as in the case for yeasts? The observation that a mouse temperature-sensitive *cdc2* mutation conferred arrest in G_2 and did not affect the G_1/S phase transition (8, 9) suggests that a different CDC2-related kinase assumes the G_1/S function. Consistent with this possibility, injection of antibodies directed against $p34^{CDC2}$ into mammalian cells resulted in inhibition of mitosis but had no effect on DNA replication (10). Therefore, as hypothesized by Murray and Kirschner (1), it is possible that two different CDC2-related kinases are involved in the regulation of different cell cycle transitions. CDK2 is very similar in structure to $p34^{CDC2}$ and can provide both G_1/S phase and G_2/M phase functions of CDC28 in *S. cerevisiae* as can human $p34^{CDC2}$. However, *CDK2* cannot rescue the cell cycle defect of *S. pombe cdc2* mutants arrested primarily in the G_2/M phase, whereas human *CDC2* can. Furthermore, when the level of expression of CDK2 was reduced, *S. cerevisiae cdc28::LEU2* cells experienced a cell cycle delay, most likely at the G_2/M phase boundary. One can infer from these results that CDK2 is less efficient at providing G_2/M functions than human $p34^{CDC2}$ in yeast. This suggests that CDK2 might play a role distinct from that of $p34^{CDC2}$; for example, CDK2 might have a G_1 function mediated by association with mammalian G_1-specific cyclins. The earlier appearance of CDK2 mRNA than of CDC2 mRNA in mitogen-stimulated cells is consistent with this idea. Paris *et al.* (16) have discussed the possibility that *Xenopus* Eg1 is involved in the G_1/S phase transition in embryonic cell divisions. It is noteworthy that *Xenopus* Eg1 kinase does not form complexes with mitotic cyclins but binds to $p13^{suc1}$, consistent with its being closely related to CDC2 kinase (34). It will be important to determine the nature of the cyclins that interact with CDK2 and to examine whether CDK2 has a G_1 role in human cells.

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