A mouse cdc25 homolog is differentially and developmentally expressed

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The timing and activation of the p34\(^{cde2}\) kinase in mammals is associated with dephosphorylation of phosphotyrosine and phosphothreonine residues on the p34\(^{cde2}\) kinase. For fission yeast, the timing of mitosis is regulated by cyclic accumulation of cdc25, which promotes dephosphorylation of p34\(^{cde2}\) and concomitant protein kinase activation. We report the identification and characterization of a structural and functional mouse homolog, Cdc25M2, of the cdc25 phosphatase. Cdc25M2 shows high sequence identity to the previously reported human homolog cdc25Hu2. Cdc25M2 can functionally complement for a Schizosaccharomyces pombe cdc25\(^{+}\) mutation, and when expressed in Escherichia coli and purified, Cdc25M2 is an active phosphatase. cdc25M2 mRNA shows variation in expression in different tissues in the mouse embryo and is expressed in a developmental and cell-cycle-dependent fashion. We suggest that the expression and accumulation of the cdc25 mitotic inducer may play a critical role in the regulation of mouse development.

[Key Words: cdc25M2, mouse; developmental expression; cell-cycle-dependent expression; phosphatase]

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Chromosomal replication in S phase and chromosome segregation in M phase are the two major cell cycle events that are conserved among eukaryotic cells. Central to these events is a group of regulatory gene products that control the passage through S phase and mitosis. These regulators act as intrinsic controls during cell cycle progression and respond to extracellular conditions and signals to drive the cell cycle. They include a number of protein kinases and phosphatases that were defined initially by genetic studies in budding and fission yeast [Reed 1980, Nurse and Bislett 1981] and by biochemical studies in Xenopus and sea urchin [Masui and Markert 1971; Evans et al. 1983]. Of particular interest is a universal control that regulates M-phase onset [for review, see Nurse 1990], and central to this control is the conserved p34\(^{cde2}\) protein kinase. Activation of p34\(^{cde2}\) induces M phase, and high p34\(^{cde2}\) kinase activity maintains the cell in M-phase state. A second component of this cell cycle progression control is a family of proteins called cyclins. Cyclins are regulatory components of the cdc2 family of protein kinases, and cyclin B is required for p34\(^{cde2}\) kinase activation. There are various types of cyclins [for review, see Pines and Hunter 1990, 1991; Hunter and Pines 1991; Reed 1991] that appear to have roles at different points in the cell cycle.

In fission yeast, the cell cycle timing of M-phase entry is governed by two mitotic inducer genes, cdc25\(^{+}\) and cdc1\(^{+}\)/nim1\(^{+}\) [Russell and Nurse 1986; Feilotter et al. 1991], and two mitotic inhibitor genes, wee1\(^{+}\) and mik1\(^{+}\) [Russell and Nurse 1987; Lundgren et al. 1991]. The products of these genes act together to regulate the function of p34\(^{cde2}\) for the initiation of M phase. The cdc25\(^{+}\)/nim1\(^{+}\), wee1\(^{+}\), and mik1\(^{+}\) genes encode protein kinases, whereas the cdc25\(^{+}\) gene product promotes dephosphorylation of p34\(^{cde2}\). In wild-type Schizosaccharomyces pombe cells, the cdc25\(^{+}\) mitotic inducer is required for entry into mitosis, for p34\(^{cde2}\) dephosphorylation and for p34\(^{cde2}\) protein kinase activation. It appears that the level of cdc25\(^{+}\) expression in fission yeast is rate limiting for M-phase entry in S. pombe. Incremental increases in cdc25\(^{+}\) gene dosage cause a decrease in cell size at mitosis, and the levels of cdc25\(^{+}\) mRNA and protein increase as cells approach mitosis [Russell and Nurse 1986]. For fission yeast, these data suggest that the timing of mitosis is regulated by cyclic accumulation of cdc25 and that cdc25 regulates dephosphorylation of p34\(^{cde2}\) and subsequent protein kinase activation.

The mitotic activity of fission yeast cdc25\(^{+}\) is highly conserved among eukaryotes. First, in the budding yeast Saccharomyces cerevisiae, a mitotic inducer gene called MIH1 can complement S. pombe temperature-conditional cdc25\(^{-}\) mutants [Russell et al. 1989]. Unlike S. pombe, the S. cerevisiae MIH1 gene is not essential and can be mutated without causing a significant growth defect. However, when the S. pombe wee1\(^{+}\) protein kinase is expressed in an S. cerevisiae mih1 mutant, the trans-
genic yeast strain arrests in G₂–M phase. Second, in Drosophila, a mitotic activator called string (stg) has been described that, like cdc25, encodes an essential function (Edgar and O’Farrell 1990).

Human homologs (CDC25Hs1 and CDC25Hu2) of fission yeast cdc25 have been described previously (Sadhu et al. 1990; Nagata et al. 1991). The CDC25Hs1 cDNA was identified through a polymerase chain reaction (PCR)-based strategy that amplified a region of the human gene that is conserved with the budding yeast and Drosophila cdc25 proteins, whereas CDC25Hu2 was isolated by its ability to functionally complement a S. pombe cdc25 strain. Similarly, CDC25Hs1 also complements a cdc25 mutant. Antisera raised against CDC25Hs1 and microinjected into HeLa cells result in G₂ phase arrest (Millar et al. 1991). This suggests that cdc25 may be an important regulator of the onset of mitosis in mammalian cells.

There is increasing evidence from vertebrate systems that, as in fission yeast, the onset of mitosis is controlled by the p34cdc2/cyclin B complex. Once cyclin B associates with cdc2 in G₂ phase, cdc2 is phosphorylated on tyrosine-15 (Y-15) and threonine-14 (T-14), which are dephosphorylated as cells enter mitosis. These residues are phosphorylated on residue 347 of yeast cdc25 (Kumagai and Dunphy 1991; Strausfeld et al. 1991). The overall identity between the S. cerevisiae MIH1 protein and all other cdc25-like gene products is low (30%), and any of the other cdc25 homologs, Cdc25M2, shows greatest identity to the human CDC25Hu2 [91%] and least identity to the S. cerevisiae MIH1 protein [30%].

The highest region of homology within the five cdc25 proteins can be found between residues 433 and 453 and 475 and 495 in the Cdc25M2 protein. These regions contain the cdc25 shared motifs of IXDCRYPYEYXG-PKQXAVNL and KRXLXHCEFSSGPXXM. Because these motifs are so highly conserved between species (81% for Cdc25M2), they might represent the active functional region in all of these proteins. For example, the HCEFSSER motif is similar to the invariant HCXAGXR motif (Cool et al. 1989; Guan et al. 1990, 1991) surrounding the active site of various protein tyrosine phosphatases (PTPases) and is essential to cdc25 activity (Dunphy and Kumagai 1991; Strausfeld et al. 1991).

Unlike any of the other cdc25 homologs, Cdc25M2 shows substantial amino acid identity to CDC25Hu2 throughout the entire protein (Figs. 2 and 3). The overall identity between Cdc25M2 and CDC25Hs1 and Hu2 is 37% and 81%, respectively. Outside the conserved catalytic domain of the cdc25 family, Cdc25M2 is most closely related to CDC25Hu2 (76%) and shows little similarity to CDC25Hs1 (20%) (Fig. 2). It is for this reason that we have named this mouse homolog of cdc25 cdc25M2.

cdc25M2 is a functional homolog of S. pombe cdc25

To investigate whether the mouse cdc25M2 gene and S. pombe cdc25 + gene have similar roles in mitotic control, we determined whether cdc25M2 could rescue a fission yeast temperature-sensitive cdc25 + mutation. The cdc25M2 cDNA was isolated and ligated into a LEU2, μ origin-based plasmid containing the SV40 early promoter. The resulting plasmid as well as the parental plasmid were transformed into a S. pombe cdc25-22 leu1-32 strain. Transformants were isolated and examined for their ability to complement for the temperature-sensitive cdc25-22 lesion. The mouse cdc25M2 cDNA allowed cdc25-22 cells to form colonies at 35°C,
whereas the control transformants were unable to support robust growth on solid medium [Fig. 4a]. Similarly, the cdc25M2 cDNA was capable of rescuing the cdc25-22 G4 arrest when cells were shifted from 25°C to 35°C, whereas the control plasmid did not [Fig. 4b]. We found that the degree of rescue was variable among individual cells, but most cells divided at 1–1.5 times wild-type size. These data establish that Cdc25M2 is able to function as a mitotic inducer in fission yeast and suggest that Cdc25M2 is likely to function in mitotic control in mouse cells.

**Cdc25M2 has phosphatase activity that can dephosphorylate p-NPP**

Because of the structural similarity between Cdc25M2 and PTPases, we determined whether Cdc25M2 had as-

**Figure 1.** Sequence of the mouse cdc25M2 cDNA. The mouse cdc25M2 cDNA was identified in a XZAPII cDNA library by low-cDNA. The mouse cdc25M2 whereas the control transformants were unable to support robust growth on solid medium [Fig. 4a]. Similarly, the cdc25M2 cDNA was capable of rescuing the cdc25-22 G4 arrest when cells were shifted from 25°C to 35°C, whereas the control plasmid did not [Fig. 4b]. We found that the degree of rescue was variable among individual cells, but most cells divided at 1–1.5 times wild-type size. These data establish that Cdc25M2 is able to function as a mitotic inducer in fission yeast and suggest that

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Because of the structural similarity between Cdc25M2 and PTPases, we determined whether Cdc25M2 had associated phosphatase activity. We used the phosphatase...
assay described by Guan et al. (1990, 1991), which measures the colorimetric conversion of p-NPP to p-nitrophenol (p-NP). This assay has been used previously to examine further the possibility that Cdc25M2 functions as a mitotic inducer in mouse cells, we determined to measure the phosphatase activity associated with the shared region of homology [shaded box in a]. The species shown are mouse (M2), human (Hs1 and Hu2), Drosophila (D), S. pombe (P), and S. cerevisiae (C).

Figure 2. Comparison of the structure and similarities between cdc25 proteins from various species. [a] Conceptual alignment of cdc25 proteins from various species. Open boxes indicate regions with little amino acid similarity, hatched boxes indicate homology outside of the conserved carboxyl terminus, and shaded boxes indicate the ~155-amino-acid region of high identity between all cdc25 proteins. [b] A comparison of the level of amino acid identity among various cdc25 proteins through the shaded region of homology [shaded box in a]. The species shown are mouse (M2), human (Hs1 and Hu2), Drosophila (D), S. pombe (P), and S. cerevisiae (C).

cdc25M2 expression is regulated temporally and developmentally

To examine further the possibility that Cdc25M2 functions as a mitotic inducer in mouse cells, we determined whether cdc25M2 mRNA is expressed periodically in the mouse cell cycle by examining cdc25M2 mRNA levels after serum addition to quiescent cells. Swiss 3T3 cells were serum starved for 72 hr to enrich for G0 cells and were then released into fresh medium containing 10% fetal bovine serum [FBS] (Stein and Stein 1989). Cell cycle progression was followed for 42 hr by measuring the phosphorylation of DNA precursors [see Materials and methods]. We observed a synchronous round of DNA synthesis as seen by a peak of [3H]thymidine incorporation [Fig. 6a]. RNA was prepared from these cells, and cdc25M2 levels were measured by Northern blot analysis [Fig. 6b]. We observed that cdc25M2 mRNA levels were low in G0 phase of the cell cycle, remained low through G1, which occurs 9–15 hr after addition of serum in these experiments), increased during S phase (which occurs 15–30 hr after serum addition), and reached a plateau ~21–24 hr after the start of this experiment. The peak cdc25M2 mRNA levels reached maximum midway through S phase and remained elevated through the completion of S phase and into G2–M. Densitometric scanning indicated that the level of cdc25M2 increased about three- to fivefold from G1 to G2 levels. Similar to CDC25Hs1 in HeLa cells, these data establish that the level of mouse cdc25M2 mRNA increases as cells progress through the cell cycle from G1 and approach mitosis. We also found that cdc25M2 mRNA levels were readily detectable in logarithmic cells [Fig. 6b], and we note that cdc25M2 mRNA levels do not drop dramatically as cells progress through the cell cycle.

To characterize further the expression of cdc25M2 mRNA, we examined its levels in various tissues from an adult rat [Fig. 7a] and at different stages during mouse development by Northern analysis [Fig. 7b]. We found that cdc25M2 mRNA was detectable in most adult tissues. The level of cdc25M2 mRNA was lowest in adrenal, kidney, liver, and muscle, was moderately expressed in brain, heart, and intestine and was expressed highest in lung and spleen. We examined the amount of cdc25M2 expression during embryonic development by Northern blotting of mRNA samples from day-10.5 to day-18.5 embryos. We found that total cdc25M2 mRNA levels fluctuate during embryonic development and that cdc25M2 mRNA peaks between day 12.5 and 13.5 and remains elevated until day 15.5. After this period, cdc25M2 mRNA levels gradually diminish. We are unable to determine by this analysis whether variation in cdc25M2 mRNA level is the result of altered transcription rates, mRNA stability, or merely variation among tissue types.

Because of the variation in mRNA levels in adult tissues and during embryonic development, we determined the spatial distribution of cdc25M2 mRNA in embryonic tissues by in situ hybridization. We observed a high and widespread distribution of cdc25M2 transcripts in differentiating tissues containing dividing cells (days 10.5–16.5). In particular, the liver shows high expression in a day-13.5 embryo [Fig. 8a,b], but at day 16.5, the signal has decreased to near-background level [Fig. 8c]. We also found high levels of cdc25M2 throughout the central nervous system in the early embryo. As cells differenti-
ate, this expression becomes restricted to regions containing rapidly proliferating cells. For example, as cells in the telencephalon (endbrain) become postmitotic, they migrate toward the periphery and eventually differentiate into the basal ganglia and cerebral cortex. We observed that cdc25M2 expression is concentrated to the ventricular surface of the telencephalon and that there is only weak labeling in the periphery (Fig. 9). There also appears to be a correlation between the order in which the brain vesicles divide and cdc25M2 expression. Cells in the mesencephalon (midbrain) divide after those in the metencephalon (afterbrain) (Kandel and Schwartz 1985). The metencephalon shows high expression of cdc25M2 mRNA in a day-10.5 embryo (not shown) but is reduced to near-background level in a day-13.5 embryo, whereas at this point the mesencephalon (midbrain) shows a strong, highly localized signal (Fig. 8).

Extracellular signals, including growth factors and morphogens, are widely used for controlling mammalian cell proliferation and development. Although the specific intracellular targets for these signals are still obscure, they must change the expression patterns of genes involved in growth control and differentiation. To understand the alteration in expression of cdc25M2 during mouse development, we determined whether cdc25M2 mRNA levels are responsive to a growth factor (serum) that induced immediately early gene expression (Treisman 1985) and to a morphogen [retinoic acid] that induces proliferation during development (Roberts and Sporn 1984). We starved Swiss 3T3 cells of serum for 72 hr, added 10% serum, and examined cdc25M2 levels immediately after the induction. In contrast to the well-characterized immediate-early response to serum seen with genes like fos (Kruijer et al. 1984), we found that cdc25M2 transcription was not induced rapidly by serum (Fig. 6).

To determine whether cdc25M2 levels are responsive to retinoic acid, we examined the induction of cdc25M2 after an 8-hr treatment of embryonal carcinoma P19 cells with varying concentrations of retinoic acid. As shown in Figure 10, we found that cdc25M2 mRNA was induced 5- to 10-fold by retinoic acid treatment. The time course of retinoic acid induction of cdc25M2 mRNA is similar to the retinoic acid induction seen for retinoic acid receptor β mRNA (RARβ) (Song and Siu 1989). To test whether retinoic acid induction was a primary event, we determined whether induction occurred in the presence of cycloheximide. However, similar to many highly unstable mRNAs (Graves et al. 1987; Mullner and Kühn 1988; Shyu et al. 1989), we found that the addition of cycloheximide resulted in higher steady-state cdc25M2 mRNA levels after induction with retinoic acid. This precludes determination of whether retinoic acid induction is a primary event.
Developmental expression of a mouse cdc25 homolog

Figure 4. The mouse cdc25M2 gene is a functional homolog of S. pombe cdc25. [a] S. pombe strains were transformed to Leu and grown at room temperature. Both wild-type cdc25 and cdc25 strains were transformed. Transformants were single-colony-purified on EMM medium, streaked for single colonies on YES medium, and incubated at the cdc25 nonpermissive temperature of 35°C. Sector 1 shows a vector-control Leu cdc25 transformant; sector 2 contains a cdc25 strain transformed to Leu with a cdc25M2-containing plasmid; sector 3 shows a Leu vector-alone cdc25 strain. [b] Various S. pombe strains were grown overnight in EMM liquid medium to mid-log phase. Cultures were transferred by dilution into YES media, grown for several hours at permissive temperature, and shifted to nonpermissive temperature (35°C). Arrow indicates the time point when cultures were shifted to nonpermissive temperature (35°C). (X) cdc25-22 cdc25M2; (•) cdc25-22 + vector; (D) cdc25-22.

Discussion

To understand how extracellular signal molecules such as growth factors and morphogens act in mammalian cell proliferation and development, we have isolated a mouse homolog of the Drosophila stg gene. The D. melanogaster stg gene product is a regulator of embryonic cell cycles [Edgar and O’Farrell 1990]. The stg gene product is functionally identical to the fission yeast rate-limiting mitotic inducer cdc25 and is one possible candidate for a developmental target for growth control and differentiation.

cdc25M2 is a functional homolog of the S. pombe cdc25 gene. The mouse gene is fully capable of restoring the temperatureconditional growth defect associated with the cdc25-22 temperature-sensitive mutation and allows cdc25M2 transgenic fission yeast to grow at nearly wild-type rates in broth medium, to form normal colonies on agar medium at the nonpermissive temperature, and to divide at nearly normal cell size [Fig. 4]. Complementation occurs despite the fact that cdc25M2 expression is not cell cycle regulated in S. pombe, as the protein is overexpressed from the SV40 promoter.

Cdc25M2 shows striking amino acid identity throughout its amino-terminal noncatalytic region to CDC25Hu2, whereas neither of these predicted gene...
Figure 5. Mouse Cdc25M2 can dephosphorylate p-NPP. The indicated amounts of purified recombinant GST-Cdc25M2 protein (•) or GST protein (■) were incubated with p-NPP as described in Materials and methods. The reactions were incubated for 45 min at 30°C, terminated with the addition of 0.2 M NaOH, and p-NP production was measured by absorbance at 410 nm. Under these conditions, the dephosphorylation of p-NPP is linear with respect to protein concentration and with time (not shown).

products shows any significant identity to the corresponding region of CDC25Hsl [Fig. 2]. This suggests that at least two distinct types of cdc25 homologs exist in mammals. A second and structurally distinct cdc25 homolog in P19 cells has been identified, suggesting that multiple cdc25 homologs exist within a single cell [B. Sebastian, A. Kakizuka, R.M. Evans, and T. Hunter, unpubl.].

The importance of cdc25 homologs in regulating the onset of mitosis has been established in a number of systems. The cdc25 homologs identified in S. pombe and D. melanogaster are essential [Russell and Nurse 1987;...
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Edgar and O’Farrell 1990). S. pombe cdc25+ is required for viability and cell cycle progression, and cdc25 mutants can only be maintained if they contain temperature-conditional mutations or if they contain additional mutations in the wee1 protein kinase. In Drosophila, stg mutants are unable to complete early embryonic development, and cell cycle progression is prevented after cellularization. The observation that antibodies raised to CDC25Hsl microinjected into HeLa cells cause G2 arrest suggests that cdc25 homologs might also play an important role in the regulation of mitosis in mammalian cells [Millar et al. 1991].

The products of the fission yeast cdc25+ gene and its homologs MIHI1 in budding yeast and the stg gene product in Drosophila are positive regulators of the G2 M-phase transition. In fission yeast, the mutation cdc2-F15, whose product cannot be phosphorylated on amino acid residue tyrosine 15, bypasses the requirement for cdc25 function [Gould and Nurse 1989], and a human PTPase can rescue a cdc25+ mutation [Gould et al. 1990]. These experiments support the view that cdc25 protein is required for promoting the dephosphorylation of Y-15 in S. pombe p34cdc2.

Biochemical and structural evidence support this conclusion. A number of reports have shown that cdc25 is a protein phosphatase that can dephosphorylate Y-15 of cdc2 in vitro. Addition of bacterially produced stg protein into Xenopus extracts efficiently induces the dephosphorylation of the Xenopus cdc2 protein and appears to activate the p34cdc2/cyclin B complex [Gautier et al. 1991; Kumagai and Dunphy 1991]. Similarly, purified bacterially expressed CDC25Hsl protein can dephosphorylate and activate starfish p34cdc2/cyclin B complex in vitro [Strausfeld et al. 1991]. Structural comparisons of cdc25+ from S. pombe to known PTPases identified an “HCXXXXXR” motif that is found in the vaccinia virus VHI phosphatase and is conserved among all cdc25 homologs [Guan et al. 1991; Moreno and Nurse 1991]. The cysteine in this motif is essential to VHI phosphatase activity [Guan et al. 1991]. When this cysteine is mutated, bacterially expressed cdc25 loses its phosphatase activity and its ability to activate p34cdc2/cyclin B [Dunphy and Kumagai 1991; Gautier et al. 1991].

We believe that Cdc25M2 also acts as a protein phosphatase. A purified bacterially expressed GST-Cdc25M2 fusion protein dephosphorylates p-NPP [Fig. 5]. Given the evidence discussed above, a possible in vivo substrate for Cdc25M2 is p34cdc2/cyclin B. Vertebrate p34cdc2/cyclin B activity is inhibited by phosphorylation on T-14 as well as Y-15 [Krek and Nigg 1991; Norbury et al. 1991]. This raises the possibility that Cdc25M2 is perhaps a “dual-specificity” phosphatase that dephosphorylates both Y-15 and T-14; alternatively, two different phosphatases may be required—one for each residue.

Whereas expression of cdc25 is rate limiting for mitosis in S. pombe and varies throughout the cell cycle [Russell and Nurse 1987; Moreno et al. 1990], a similar relationship has not been established for mammalian cells. CDC25Hsl protein levels appear to be relatively constant throughout the cell cycle [Millar et al. 1991]. Despite the variation in CDC25Hsl mRNA [Sadhu et al. 1990], CDC25Hu2 mRNA varies only slightly [Nagata et al. 1991]. Upon stimulating Swiss 3T3 cells to

Figure 7. CDC25M2 expression varies during embryonic development and between tissues. [a] Five micrograms of poly[A] + RNA from various adult rat tissues was analyzed by Northern blotting. The probe was CDC25M2 cDNA. (b) Ten micrograms of poly[A] + RNA from various stage mouse embryos was examined by Northern blotting.

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enter the cell cycle from $G_0$, we found that $cdc25M2$ mRNA levels are first detected during late $G_1$ to early $S$ phase and are elevated three- to fivefold above early $G_1$ levels, but not fall later in the cycle.

In *Drosophila*, increasing levels of regulation act upon mitosis as an embryo develops. The first 13 rapid cycles in the fly syncytial embryo rely on maternal gene products (for review, see Glover 1991). These 13 divisions...
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Figure 9. cdc25M2 expression in the central nervous system of a mouse embryo. [a] A coronal section of the head of an E12.5 mouse embryo. (Ne) Neuroepithelium; (Tl) telencephalon; (R) retina; (Na) nasal cavity; (Fm) facial mesenchyme; (E) eye. [b] Dark-field autoradiograph showing expression of cdc25M2 in the embryonic section in a.

lack the cdc25-dependent feedback regulation that monitors completion of S phase. Such regulation is introduced in cycle 14 with the beginning of an apparent G2 phase and a coordinated network of mitotic control that is under the overall control of stg. After the onset of cellularization in the fourteenth division cycle, fly somatic cells undergo mitotic divisions that are coordinated with the process of gastrulation and morphogenesis. In wild-type embryos, short-lived zygotic stg transcripts precede and induce the spatiotemporal onset of mitosis.

We anticipated that the universal mitotic control defined by developmental studies in *D. melanogaster* may hold true in mouse development. To test the notion that high levels of cdc25M2 might correlate with proliferating cells, we analyzed the levels of cdc25M2 mRNA in the developing mouse. The cdc25M2 gene shows a temporal and spatial pattern of expression during embryonic development. cdc25M2 mRNA is most abundant during days 13.5–16.5 in development; after day 15.5, this level declines rapidly. In situ hybridizations of cdc25M2 antisense RNA show that the cdc25M2 gene is widely expressed in differentiating tissues containing dividing cells. In particular, there appears to be a correlation between cdc25M2 expression and the presumed region and timing of mitosis in cells in the liver and the central nervous system. cdc25M2 expression in the liver is found in day-13.5 embryos, during which rapid cell division occurs, and has decreased in the liver in a day-16.5 embryo, at which point proliferation in liver cells has presumably dropped sharply (Fig. 8). A high level of cdc25M2 transcript was detected in regions in the nervous system containing rapidly dividing cells such as the neuroepithelium (Fig. 9) and proliferative zone (Fig. 8b), whereas strong signals were not observed in the periphery of the neural tube, which contains postmitotic, differentiated cells (Fujita 1964). Finally, the timing of cdc25M2 expression in the brain vesicles such as the telencephalon, mesencephalon, and metencephalon is consistent with the order in which the cells in these regions divide. The differential localization of cdc25M2 in the mouse embryo suggests that elucidation of the developmental role of each type of cdc25 protein within an organism might be informative.

Mammalian cell proliferation and development are responsive to diverse extracellular signals. We found that cdc25M2 mRNA levels were clearly inducible by retinoic acid treatment in P19 cells and that cdc25M2 mRNA could be stabilized by cycloheximide treatment. This suggests that cdc25M2 mRNA is unstable and might be prone to regulation by cotranslational processing in a fashion similar to the regulation of early response genes like *fos* and *myc* or by cell cycle-controlled instability as seen for histone mRNA or for tubulin. In addition, retinoic acid induction of cdc25M2 expression suggests that cdc25M2 gene is one potential intracellular target for growth control and differentiation. The precise mechanism of cdc25M2 induction will require the isolation of its promoter element. Furthermore, it is unclear whether the degradation of cdc25M2 mRNA is translation dependent like tubulin mRNA (Pachter et al. 1987; Gay et al. 1989) or cell cycle controlled like histone mRNA (Gallwitz 1975; DeLisle et al. 1983; Heintz et al. 1983). However, these results indicate that a universal mitotic control element can act either as a direct or indirect target for extracellular developmental signals.
heximide without retinoic acid (lanes 1,5) with 10⁻⁶ M retinoic acid (lanes 2,4) or 10⁻⁷ M (lanes 3,6) of retinoic acid for 8 hr. Paired samples of 15 μg of total RNA (lanes 2,3,5) and 5 μg of 5,6-PDCA were prepared from P19 cells treated with 10 μg/ml of cycloheximide without retinoic acid (lanes 1,2) and with 10⁻⁶ M retinoic acid (lanes 3,4) or 5 x 10⁻⁷ M (lanes 5,6) of retinoic acid for 8 hr. Paired samples of 15 μg of total RNA (lanes 1,3,5) and 5 μg of poly[A⁺] RNA (lanes 2,4,6) each treatment were analyzed by Northern blotting using CDC25M2 cDNA as a probe. (b) The filter above was rehybridized with a cDNA whose expression was described in Kakizuka et al. 1991). The library was constructed in λZAPII (Stratagene). A portion of the Drosophila stg gene encoding the conserved catalytic domain of the cdc25 homologs was isolated (Fig. 1) and ligated into Smal-Ncol-digested pGEX-KG (Pharmacia-LKB) was cloned adjacent to the 5' end of cdc25M2. This plasmid and a control vector lacking the cdc25M2 cDNA were transformed into a leu1 cdc25-22 strain (kindly provided by P. Nurse and K. Gould), and Leu⁺ transformants were selected at 24°C on EMM media (Moreno et al. 1991). S. pombe transformation followed standard procedures. Transformants were grown at room temperature to mid-log phase in EMM media, and the cultures were split into YES media (Moreno et al. 1991) and shifted to the cdc25° nonpermissive temperature (35°C). Growth of the culture was followed by microscopic examination of Calcofluor-stained cells (Russell and Nurse 1987) and by cell number determination with a Coulter counter as described (Russell and Nurse 1987; Sadhu et al. 1990).

Expression and purification of a GST–CDC25M2 fusion protein and detection of phosphatase activity

A 1-kb DraI–Ncol fragment was isolated from the cloned cDNA of cdc25M2 (Fig. 1) and ligated into Smal–Ncol-digested pGEX-KG (Guan and Dixon 1991) to produce pGEX-K288. E. coli strain BL21 containing plasmid pLYSs was transformed with pGEX-K288, and cells containing both plasmids were grown in 800 ml of LB broth at 37°C to mid-log phase and treated with 0.6 mM IPTG for 2 hr at 25°C. Cells were harvested, washed in TD (150 mM NaCl, 10 mM Tris–HCl at pH 7.5), resuspended in TD containing both 1% Tween 20 and 1% Triton X-100, and the mixture was sonicated for 30 sec. The extract was centrifuged twice at 2500 rpm for 10 min in a Beckman J6B centrifuge, and the clarified supernatant was collected. Five hundred microliters of 50% (vol/vol) of Sepharose (Pharmacia), coupled with glutathione, was added to the extract, and the slurry was incubated for 2-3 hr at 4°C. The beads were collected by centrifugation for 10 sec at 1000 rpm and washed extensively in 500 mM NaCl, 10 mM DT, and 10 mM HEPES (pH 8.0). Protein was eluted by incubating the beads for 3-4 hr at 4°C in 100 mM Tris–HCl (pH 8.0), 10 mM DT, and 5 mM glutathione. GST from pGEX-KG was purified in an identical manner. Protein concentration was determined by gel electrophoresis.
To assay the activity of the purified GST–Cdc25M2 fusion protein, we measured dephosphorylation of p-NPP using the conditions described by Guan et al. (1990, 1991). Reaction mixtures (200 μl) contained 50 mm imidazole [pH 7.4], 0.5% β-mercaptoethanol, and 10 mm p-NPP, and either purified GST–Cdc25M2 fusion protein or GST, and the reactions were incubated at 30°C for 45 min. Reactions were terminated by adding 800 μl of 2 M NaOH. Absorbance at 410 nm was measured.

**Analysis of cdc25M2 mRNA levels**

cdc25M2 mRNA levels were analyzed by Northern blots using a randomly primed cdc25M2 cDNA insert as a probe. Total and poly(A)+ RNA was separated by electrophoresis in a 1% agarose gel containing 0.66 M formaldehyde, transferred to a Nyttran filter (Schleicher & Schuell), and hybridized with cdc25M2 cDNA. Hybridization was carried out at 42°C in a buffer containing 50% formamide, 5× SSC, 4× Denhardt’s solution, 0.1% SDS, 100 μg/ml of denatured salmon sperm DNA, 200 μg/ml of yeast RNA, and 5 ng/ml of 32P-labeled probe [5 × 108 cpm/ml]. The filters were washed with 0.5× SSC and 0.1% SDS at 50°C and exposed to film with an intensifying screen. Total RNA from adult male rat embryos for RNA preparation and Northern analysis.

Incorporation of [3H]thymidine into DNA was measured as described (Stein and Stein 1989) but briefly involved washing the blot with 0.5× SSC and 0.1% SDS at 50°C and exposed to film with an intensifying screen. Total RNA from adult male rat embryos was isolated as described previously (Chomczynski and Sacchi 1987).

To examine cdc25M2 mRNA levels during cell cycle progression, the methods described by Lee et al. (1988) were used. Briefly, Swiss 3T3 cells were maintained in DMEM with 5% FBS for 72 hr. At this time we estimate that >90% of the cells were in G0. Fresh medium [DMEM] containing 10% FBS was added to the serum-depleted cells, and samples were taken at various times after addition. Progression through the cell cycle was monitored by labeling cells for 30 min with 10 μCi of [3H]thymidine (83 Ci/mmol, Amersham) prior to harvesting. Incorporation of [3H]thymidine into DNA was measured as described (Stein and Stein 1989) but briefly involved washing the media from cells, lysing the cells, precipitating DNA with cold trichloroacetic acid [TCA], and measuring incorporation of [3H] into TCA-precipitated DNA. Parallel samples of cells were harvested for RNA preparation and Northern analysis.

Retinoic acid induction experiments were performed as follows: Monolayer cultures of P19 cells were maintained in αMEM with 10% FBS and treated by 10 μg/ml of retinoic acid (Sigma) for 8 hr. The effect of retinoic acid was monitored by Northern blot using the RARp cDNA as a probe (Song and Siu 1989). Developmental expression of a mouse cdc25 homolog.

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**Note added in proof**

Galaktionov and Beach (1991, Cell 67: 1181–1194) reported the third member of human cdc25.

**References**

Bettler, B., J. Boulter, I. Hermans-Borgmeyer, A. O’Shea-Greenfield, E.S. Deneris, C. Moll, U. Borgmeyer, M. Hollmann, and S. Heinemann. 1990. Cloning of a novel glutamate receptor subunit, GluR5: Expression in the nervous system during development. Neuron 5: 583–595.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation using acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156–159.

Cool, D.E., N.K. Tonks, H. Charbonneau, R.A. Walsh, E.H. Fischer, and E.G. Krebs. 1989. cDNA isolated from a human T-cell library encodes a member of the protein-tyrosine-phosphatase family. Proc. Natl. Acad. Sci. 86: 5257–5261.

DeLisle, A.J., R.A. Graves, W.F. Marzluff, and L.F. Johnson. 1983. Regulation of histone mRNA production and stability in serum-stimulated mouse 3T6 fibroblasts. Mol. Cell. Biol. 3: 1920–1929.

Devereux, J., P. Haebeli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12: 387–395.

Dupphy, W.G. and A. Kumagai. 1991. The cdc25 protein contains an intrinsic phosphatase activity. Cell 67: 189–196.

Edgar, B.A. and P.H. O’Farrell. 1990. The three postblastoderm cell cycles of Drosophila embryogenesis are regulated in G2 by string. Cell 62: 469–480.

Evans, T., E.T. Rosenthal, J. Youngblom, D. Distel, and T. Hunt. 1983. Cyclin: A protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell 33: 389–396.

Feilotted, H., P. Nurse, and P.G. Young. 1991. Genetic and molecular analysis of cdc1/nim1 in Schizosaccharomyces pombe. Genetics 127: 309–318.

Fujita, S. 1964. Analysis of neuron differentiation in the central nervous system by tritiated thymidine autoradiography. J. Comp. Neurol. 122: 311–327.

Gallwitz, D. 1975. Kinetics of inactivation of histone mRNA in the cytoplasm after inhibition of DNA replication in synchronised HeLa cells. Nature 257: 247–248.

Gautier, J., M.J. Solomon, R.N. Booher, J.F. Bazan, and M.W. Kirschner. 1991. cdc25 is a specific tyrosine phosphatase that directly activates p34^cdc2. Cell 67: 197–211.

Gay, D.A., S.S. Sisodia, and D.W. Cleveland. 1989. Autoregulation of β-tubulin mRNA stability is linked to translation elongation. Proc. Natl. Acad. Sci. 86: 5763–5767.

Glover, D.M. 1991. Mitosis in the Drosophila embryo—In and out of control. Trends Genet. 7: 125–132.

Gould, R.L. and P. Nurse. 1989. Tyrosine phosphorylation of the fusion yeast cdc2+ protein kinase regulates entry into mitosis. Nature 342: 39–45.

Gould, R.L., S. Moreno, N.K. Tonks, and P. Nurse. 1990. Com-

**GENES & DEVELOPMENT** 589
Krek, W. and E.A. Nigg. 1991. Mutations of p34<sup>cdk2</sup> phosphorylate DNA sequences governing alternative mRNA production of rat kinogen genes. J. Biol. Chem. 263: 3884–3892.

Kakizuka, A., W.H. Miller, Jr., K. Umesono, R.P. Warrell Jr., S.R. Guan, K. and J.E. Dixon. 1991. Cyclin-dependent kinases: A new cell cycle mechanism controlled by a novel putative transcription factor. PML. Cell 66: 663–674.

Kandel, E.R. and J.H. Schwartz. 1985. Principles of neural science, 2nd ed. Elsevier, New York.

Krek, W. and E.A. Nigg. 1991. Mutations of p34<sup>cdk2</sup> phosphorylation sites induce premature mitotic events in HeLa cell cycle. Mol. Cell. Biol. 3: 539–550.

Hunter, T. and J. Pines. 1991. Cyclins and cancer. Cell 66: 1071–1074.

Kakizuka, A., N. Kitamura, and S. Nakanishi. 1988. Localization of DNA sequences governing alternative mRNA production of rat kinogen genes. J. Biol. Chem. 263: 3884–3892.

Kakizuka, A., W.H. Miller, Jr., K. Umesono, R.P. Warrell Jr., S.R. Frankel, V.V.S. Murty, E. Dmitrovsky, and R.M. Evans. 1991. Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RARα with a novel putative kinase encoded by vaccinia virus. Nature 350: 359–362.

Heintz, N., H.L. Sive, and R.G. Roeder. 1983. Regulation of rat kinogen gene expression. Cell 35: 359–370.

Lee, M.G. and P. Nurse. 1987. Complementation used to clone a human homologue of the fission yeast cell cycle control protein cdc2. Nature 327: 31–35.

Lee, M.G., C.J. Norbury, N.K. Spurr, and P. Nurse. 1988. Regulation of expression and phosphorylation of a possible mammalian cell-cycle control protein. Nature 333: 676–679.

Lundgren, K., N. Walworth, R. Booher, M. Dembski, M. Kirscher, and D. Beach. 1991. mk1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdk2. Cell 64: 903–914.

Masui, Y. and C.L. Markert. 1971. A stem-loop in the 3′ untranslated region mediates iron-dependent regulation of transferrin receptor mRNA stability in the cytoplasm. Cell 53: 815–825.

Nagata, A., M. Igarashi, S. Jinno, K. Suto, and H. Okayama. 1991. An Additional homolog of the fission yeast cdc25+ gene occurs in humans and is highly expressed in some cancer cells. New Biol. 3: 959–968.

Norbury, C., J. Blow, and P. Nurse. 1991. Regulatory phosphorylation of the p34<sup>cdk2</sup> protein kinase in vertebrates. EMBO J. 10: 3321–3329.

Nurse, P. 1990. Universal control mechanism regulating onset of G1-phase. Nature 344: 503–508.

Nurse, P. and Y. Bissell. 1981. Gene required in G<sub>1</sub> for commitment to cell cycle and in G<sub>2</sub> for control of mitosis in fission yeast. Nature 242: 558–560.

Pachter, J.S., T.J. Yen, and D.W. Cleveland. 1987. Auto-regulation of tubulin expression is achieved through specific degradation of polysomal tubulin mRNAs. Cell 51: 283–292.

Pines, J. and T. Hunter. 1990. p54<sup>cdk2</sup>: The S and M kinase? New Biol. 2: 389–401.

——. 1991. Cyclin-dependent kinases: A new cell cycle motif? Trends Cell Biol. 1: 117–121.

Reed, S.I. 1980. The selection of <i>S. cerevisiae</i> mutants defective in the start event of cell division. Genetics 95: 561–577.

——. 1991. G1-specific cyclins: In search of an S-phase-promoting factor. Trends Genet. 7: 95–99.

Robertson, A.B. and M.B. Sporn. 1984. Cellular biology and biochemistry of the retinoids. In <i>The retinoids</i> (ed. M.B. Sporn, A.B. Roberts, and D.S. Goodman), vol. 2, pp. 209–286. Academic Press, San Diego, CA.

Russell, P. and P. Nurse. 1986. cdc25+ functions as an inducer in the mitotic control of fission yeast. Cell 45: 145–153.

——. 1987. Negative regulation of mitosis by wee1′, a gene encoding a protein kinase homolog. Cell 49: 559–567.

Russell, P., S. Moreno, and S.I. Reed. 1989. Conservation of mitotic controls in fission and budding yeasts. Cell 57: 295–303.

Sadhu, K., S.I. Reed, H. Richardson, and P. Russell. 1990. Human homolog of fission yeast cdc25 mitotic inducer is predominately expressed in G<sub>1</sub>. Proc. Natl. Acad. Sci. 87: 5139–5143.

Sambrook, J., E. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York.

Shyu, A.-B., M.E. Greenberg, and J.G. Belasco. 1989. The c-fos transcript is targeted for rapid decay by two distinct mRNA degradation pathways. Genes & Dev. 3: 60–72.

Song, S. and C.-H. Szi. 1989. Retinoic acid regulation of the expression of retinoic acid receptors in wild-type and mutant embryonal carcinoma cells. FBS Lett. 256: 51–54.

Stein, G.S. and J.L. Stein. 1989. Cell synchronization. In <i>Cell growth and division: A practical approach</i> (ed. R. Baserga), pp. 133–137. IRL Press, Oxford/New York/Tokyo.

Straussfeld, U., J.C. Labbé, D. Fesquet, J.C. Cavadore, A. Picard, K. Sadhu, P. Russell, and M. Doree. 1991. Dephosphorylation and activation of a p34<sup>cdk2</sup> cyclin B complex in vitro by human CDC25 protein. Nature 351: 242–245.

Treisman, R. 1985. Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5′ element and c-fos 3′ sequences. Cell 42: 889–902.
A mouse cdc25 homolog is differentially and developmentally expressed.

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