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A family of human cdc2-related protein kinases

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The p34^{cdc2} protein kinase is known to regulate important transitions in the eukaryotic cell cycle. We have identified 10 human protein kinases based on their structural relation to p34^{cdc2}. Seven of these kinases are novel and the products of five share >50% amino acid sequence identity with p34^{cdc2}. The seven novel genes are broadly expressed in human cell lines and tissues with each displaying some cell type or tissue specificity. The *cdk3* gene, like *cdc2* and *cdk2*, can complement *cdc28* mutants of *Saccharomyces cerevisiae*, suggesting that all three of these protein kinases can play roles in the regulation of the mammalian cell cycle. The identification of a large family of cdc2-related kinases opens the possibility of combinatorial regulation of the cell cycle together with the emerging large family of cyclins.

Key words: *cdc2*-related genes/*cdk3*/cell cycle/protein kinases

Introduction

Cell division is controlled by a carefully orchestrated cycle of events. Early genetic studies in the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* demonstrated that cell division is regulated at discrete transition points (Hartwell, 1973; Nurse, 1975). The best characterized of these regulation points are the G_1-S and G_2-mitosis transitions. In the last several years there have been remarkable advances in our understanding of the regulatory events that control progression through these transition points (reviewed in Hunt, 1989; Murray and Kirschner, 1989; Draetta, 1990; Nurse, 1990; Pines and Hunter, 1990b). The most intriguing and perhaps the most important finding of this recent work has been that all eukaryotic cells use similar mechanisms to regulate progression through these transitions.

According to our current understanding of G_2-M control, the activation of a protein kinase composed of a catalytic subunit, p34^{cdc2}, and a regulatory subunit, cyclin B, is the key step in the initiation of mitosis. As the levels of p34^{cdc2} do not change appreciably during the cell cycle (Draetta and Beach, 1988), the regulation of kinase activation must be controlled by post-translational events. Cyclin levels, in contrast, oscillate in synchrony with the cell cycle; cyclins A and B are synthesized during interphase and abruptly degraded at mitosis (Evans *et al.*, 1983). The first step in the activation of p34^{cdc2} is its obligatory association with cyclin B (Draetta and Beach, 1988; Solomon *et al.*, 1990), an event that can be detected shortly after the initiation of cyclin B synthesis in late S or early G_2. Concomitant with the association of p34^{cdc2} and cyclin B, two types of p34^{cdc2} phosphorylations occur. Phosphorylation of Thr167 on *S.pombe* p34^{cdc2} (Gould *et al.*, 1991) or the corresponding Thr161 on human p34^{cdc2} (Ducommun *et al.*, 1991; Solomon *et al.* 1992) is required for activation of the kinase. Phosphorylations on Thr14 and Tyr15 (only Tyr15 in fission yeast) inhibit kinase activity (Gould and Nurse, 1989; Solomon *et al.* 1990; Krek and Nigg, 1991a,b; Norbury *et al.*, 1991). Dephosphorylation of Thr14 and Tyr15 activates the kinase at the initiation of mitosis (Norbury *et al.*, 1991). The p34$^{cdc2}-$cyclin B kinase remains active until the end of mitosis, when cyclin B is degraded and p34^{cdc2} is released as an inactive monomer (Murray *et al.*, 1989; Glotzer *et al.*, 1991). Although some modifications have recently been observed (Osmani *et al.*, 1991; Amon *et al.*, 1992; Sorger and Murray, 1992), this pattern of control has become the paradigm for cell cycle regulation and elements of this regulatory mechanism are also used to control other key cell cycle transition points.

A second well-characterized transition, which illustrates one variation in this theme of cell cycle control, is G_1-S progression in *S.cerevisiae*. This transition is controlled by a kinase complex that contains a catalytic subunit known as CDC28, which is the homologue of p34^{cdc2} in these cells. Unlike the G_2-M-controlling complex, cyclin B is not the regulatory subunit. This function is supplied by one of another group of cyclins known as CLNs (Cross, 1988; Nash *et al.*, 1988; Hadwiger *et al.*, 1989). The activation of CDC28 at the G_1-S transition requires CLN association, but it is not known whether the other regulatory mechanisms used for G_2-M are also required. Therefore in budding yeast the same CDC28 catalytic subunit is required for both the G_1-S and G_2-M transitions, but its activation at each is controlled by association with different cyclins. Similar regulatory events are also seen in *S.pombe*, where p34^{cdc2} is required at both the G_1-S and G_2-M transitions (Nurse and Bissett, 1981).

In vertebrates, a further variation of this theme appears to be used for cell cycle control. As in yeast, multiple cyclins have been identified. This is best studied in mammals, where eight different cyclins have now been discovered (Pines and Hunter, 1989, 1990a; Wang *et al.*, 1990; Koff *et al.*, 1991; Lew *et al.*, 1991; Matsushime *et al.*, 1991; Motokura *et al.*, 1991; Xiong *et al.*, 1991).

In contrast to yeast, the p34^{cdc2} kinase is not the only catalytic subunit identified in vertebrates that can interact with cyclins. While p34^{cdc2} is present and active in vertebrate cells and has been shown to be essential for the G_2-M transition (Riabowol *et al.*, 1989; Th'ng *et al.*,

1990; Fang and Newport, 1991), a second cdc2-related kinase has also been implicated in cell cycle control. The cyclin-dependent kinase 2 (*cdk2*) gene product, $p33^{cdk2}$, is a close relative of $p34^{cdc2}$ and it also binds to cyclins (Elledge and Spottswood, 1991; Ninomiya-Tsuji *et al.*, 1991; Paris *et al.*, 1991; Tsai *et al.*, 1991; Pagano *et al.*, 1992b; Rosenblatt *et al.*, 1992). Its kinase activity is temporally regulated during the cell cycle, being activated prior to or during S phase (Pagano *et al.*, 1992a; Rosenblatt *et al.*, 1992; L.-H.Tsai, unpublished results). Like *cdc2* genes from many species, human *cdk2* will complement budding yeast *cdc28* mutants (Elledge and Spottswood, 1991; Koff *et al.*, 1991; Ninomiya-Tsuji *et al.*, 1991). Depletion of $p33^{cdk2}$ from *Xenopus* oocyte lysates inhibits cell cycle progression at S phase (Fang and Newport, 1991), suggesting an essential role for this kinase in at least some vertebrate cells. In this case, a relative of $p34^{cdc2}$ appears to perform an important cell cycle role, dividing the control of different transitions in cell cycle progression.

The characterization of *cdc2*-related kinases has advanced in two stages. First, *cdc2* homologs have been identified from various eukaryotic species, including humans (Lee and Nurse, 1987; Draetta *et al.*, 1987), chicken (Krek and Nigg, 1989), mouse (Cisek and Corden, 1989), *Drosophila* (Jimenez *et al.*, 1990; Lehner and O'Farrell, 1990), pea (Feiler and Jacobs, 1991), maize (Colasanti *et al.*, 1991), alfalfa (Hirt *et al.*, 1991) and *Arabidopsis* (Hirayama *et al.*, 1991). Secondly, a variety of *cdc2*-related genes have been identified from various species (Simon *et al.*, 1986; Hanks, 1987; Uesono *et al.*, 1987; Bunnell *et al.*, 1990; Lehner and O'Farrell, 1990; Matsushime *et al.*, 1990; Shuttleworth *et al.*, 1990; Irie *et al.*, 1991; Lee and Greenleaf, 1991; Lapidot-Lifson *et al.*, 1992).

We now report the cloning and characterization of a family of human *cdc2*-related kinases. Eleven kinase genes were cloned in these studies, seven of which were novel. The kinases fall into several subgroups based on sequence similarities, physical properties, functional tests and expression patterns. One of these subgroups includes *cdc2* itself, *cdk2* and *cdk3*. The properties of these kinases suggest that they will play important roles in cell cycle regulation.

Results

Isolation of multiple human cdc2-related genes

To isolate cdc2-related kinases, degenerate oligonucleotides corresponding to conserved regions of *cdc2* (Lehner and O'Farrell, 1990) were used to amplify human sequences via the polymerase chain reaction (PCR). First strand cDNAs from HeLa cervical carcinoma cells and Nalm-6 pre-B leukaemia cells were used for amplification. Sequencing of the PCR products unexpectedly revealed the presence of eight distinct clones encoding related polypeptides. Three had been previously identified; these included *cdc2* itself (Lee and Nurse, 1987), the human homolog of the *Xenopus cdk2* gene (Paris *et al.*, 1991; Tsai *et al.*, 1991) and $p58^{GTA}$ (Bunnell *et al.*, 1990). The five other genes were novel, although they were clearly related to *cdc2*. Upon screening cDNA libraries by hybridization with the PCR fragments, we identified three further clones, two novel clones and PSK-J3 (Hanks, 1987). We do not believe that we have identified all the human members of this *cdc2*-related kinase family. The PCR primers used in these experiments may not be suitable for amplification of all *cdc2*-related genes. At least one additional human *cdc2*-related kinase has been identified by PCR using distinct primers (Pines and Hunter, 1992), as well as a distinct clone from mouse (D.Morgan, personal communication). In addition, our screens may have missed *cdc2*-related genes whose transcripts are rare or are restricted to specific cell types or tissues that we did not sample.

At the 1991 Cold Spring Harbor Symposium on the Cell Cycle, a consensus was established that *cdc2*-related kinases would be named in one of two manners. The proteins that are most closely related to *cdc2* and that bind to cyclins are known as cyclin-dependent kinases and are numbered in order of their discovery. *cdc2* is the first member of this family. Since *cdk2* was originally identified by its interaction with cyclin A, it becomes the second family member. Because of its similarity to *cdc2* and *cdk2*, another of the kinases was assigned the provisional name, *cdk3*, by this *ad hoc* committee. Although the association of *cdk3* with cyclins has not yet been shown, we continue to use the temporary *cdk3* name because of this agreement within the field. Other kinases take their names from the amino acid sequences in the highly conserved region within the so-called PSTAIRE motif of *cdc2*, using the single-letter amino acid code. These names are meant to be temporary until more functional information can be determined.

Full-length open reading frames (ORFs) for the *cdc2*-related kinase genes were obtained by screening human cDNA libraries with probes derived from the PCR products or from the DNA fragment identified by cross-hybridization. Because of cell- and tissue-specific variations in mRNA abundance (see below), it was necessary to screen libraries from the following sources in order to obtain complete clones: NALM-6 cells, HeLa cells, FOCUS hepatoma cells, T84 colon carcinoma cells, adenovirus-transformed retinal cells and fetal brain tissue. Putative full-length ORFs for *cdk2* (Tsai *et al.*, 1991), *cdk3*, PSSALRE, PCTAIRE-1, PLSTIRE and KKIALRE have been isolated and sequenced. Criteria for full-length clones included: (i) the identification of an ORF yielding a sequence related to *cdc2*, (ii) the presence of a consensus translation initiation sequence (Kozak, 1986) at a putative initiator methionine codon and (iii) the identification of a stop codon in the same reading frame but upstream of the putative initiation codon. We do not believe that our PCTAIRE-2 and PCTAIRE-3 clones are full-length, because the 5′ ends of our longest clones are homologous to PCTAIRE-1 but lack a putative initiator codon and an upstream stop codon.

The predicted amino acid sequences for the new clones are shown in Figure 1 along with the *cdc2* sequence for comparison. All of these genes encode proteins that contain domains and residues characteristic of the protein kinase family (Hanks *et al.*, 1988) and we therefore consider them to be putative protein kinase genes. Search of the GenBank and EMBL databases for these genes showed that each was similar to *cdc2* and related genes, but there were no homologues identified from any other species. During the sequencing of multiple isolates of the various kinase cDNAs, we noted protein sequence variations for PCTAIRE-1 and KKIALRE (Figure 2).

All of the novel gene products are closely related to human $p34^{cdc2}$ and range from 44–67% predicted amino acid sequence identity with human $p34^{cdc2}$. The percentage of identity between these proteins is determined by comparing

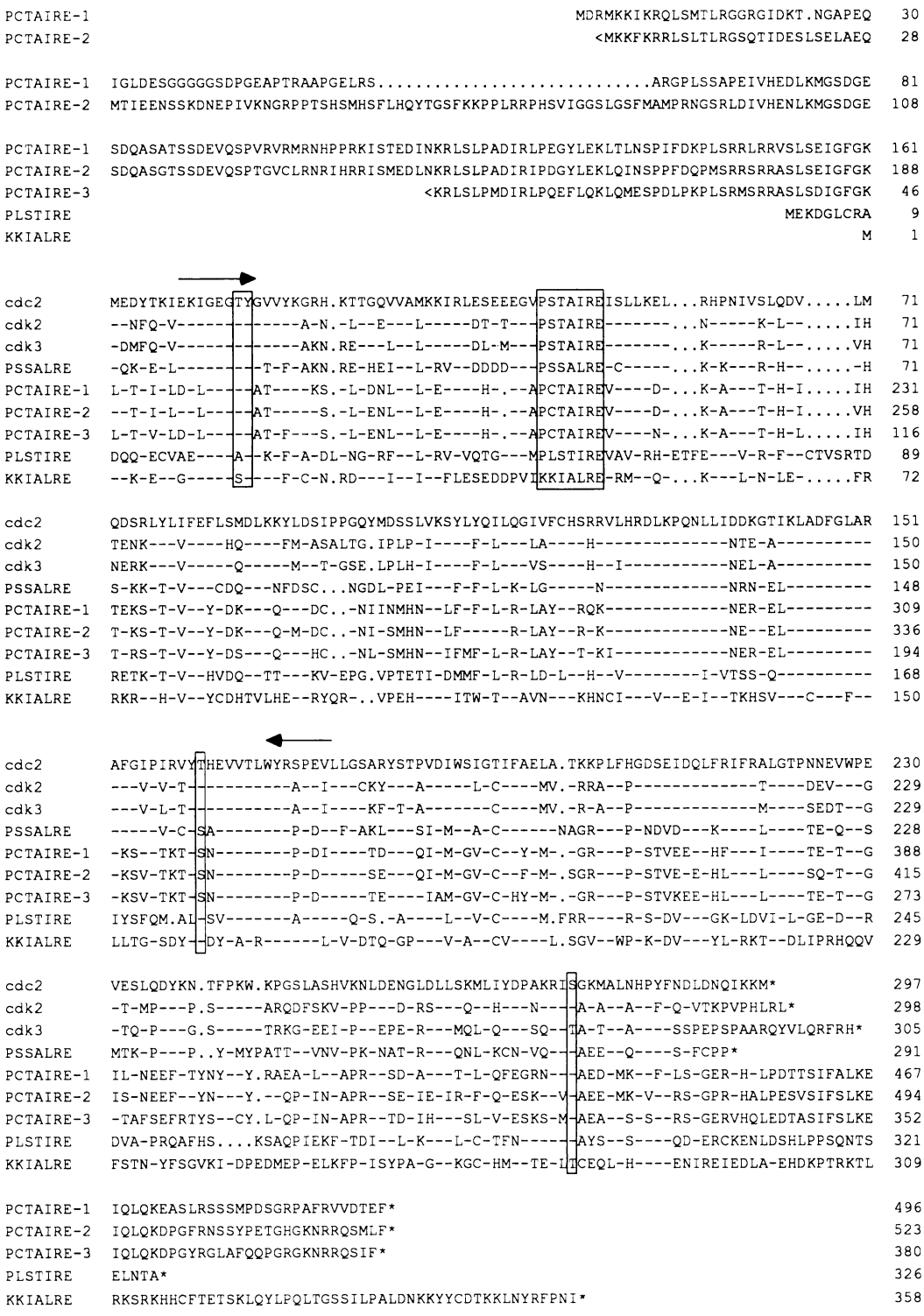

Fig. 1. Comparison of the predicted amino acid sequences of the cdc2-related kinases. Boxes indicate the region of the conserved PSTAIRE motif of cdc2 used for the kinase nomenclature, as well as the conserved phosphorylation sites of cdc2. Dashes denote residues identical to human $p34^{cdc2}$. When no residue is present, it is indicated by a dot. Arrows designate the regions used to generate PCR primers. < represents the predicted continuation of the ORF. Accession numbers for the nucleotide and amino acid sequences are as follows: cdk3, X66357; PSSALRE, X66364; PCTAIRE-1, X66363; PCTAIRE-2, X66360; PCTAIRE-3, X66362; PLSTIRE, X66365; and KKIALRE, X66358, X66359 and X66361.

regions corresponding to the full-length sequence of $p34^{cdc2}$. N- or C-terminal extensions relative to the $p34^{cdc2}$ sequence have not been used to calculate the sequence identities. The kinases fall into several subgroups based on their sequence homologies (Table I). The *cdc2*, *cdk2* and *cdk3* gene products form a subfamily. The PCTAIRE gene products form a second subfamily, while the PLSTIRE and PSK-J3 products define a third group. The KKIALRE and p58-GTA proteins are more distantly related. Two well characterized kinase families that are related to *cdc2* are the MAP and casein kinase II families. For comparison, the rat ERK-1 (Boulton *et al.*, 1990) kinase and human casein kinase

II (Meisner *et al.*, 1989) are far more distant from human p34^{cdc2}, sharing 37% identity and <30% identity with p34^{cdc2}, respectively.

The primary sequence comparisons showed that the PCTAIRE kinases contain N-terminal extensions relative to p34^{cdc2}. These extensions are 162 amino acids long in PCTAIRE-1 and at least 189 amino acids long in PCTAIRE-2; their function is unknown. No homology to any other proteins, including cyclins and suc1$^+$ homologs, was detected by search of the databases.

Phosphorylation and dephosphorylation of specific amino acid residues in p34^{cdc2} is known to regulate its kinase activity. The residues whose phosphorylation appears to be significant for vertebrate *cdc2* activity—Thr14, Tyr15, Thr161 and Ser277—are well conserved throughout the family of *cdc2*-related gene products (Gould and Nurse, 1989; Ducommun *et al.*, 1991; Gould *et al.*, 1991; Krek and Nigg, 1991a; Norbury *et al.*, 1991). The only non-conservative substitution is the replacement of Thr14 by alanine in PLSTIRE. Thus the *cdc2*-related kinases have preserved these potential regulatory sites and thereby the potential to be regulated in an analogous manner to p34^{cdc2}.

Characterization of the protein products

To characterize the human *cdc2*-related proteins, we subcloned full-length cDNAs into vectors for *in vitro* transcription and translation. We assayed the binding of the *in vitro* translated proteins to several antibodies and other reagents that are widely used to study *cdc2* function. These include antibodies to the conserved PSTAIRE motif of p34^{cdc2} (Lee and Nurse, 1987; a gift of M.Yamashita), antibodies to the C-terminal region of human p34^{cdc2} (including G6: Draetta and Beach, 1988) and antibodies to the entire *S.pombe* p34^{cdc2} protein (G8: Draetta *et al.*, 1987), as well as p13^{suc1}–agarose beads (Arion *et al.*, 1988; Dunphy *et al.*, 1988). Figure 3 shows the example of *in vitro* translation products from the *cdk3* gene. p36^{cdk3} was efficiently recognized by the anti-PSTAIRE and G8 antibodies and it bound weakly to p13 beads. All of the *cdc2*-related kinases for which we have full-length clones, except for KKIALRE, have been assayed under similar conditions. Sizes were assigned by comparison to molecular weight markers and to p34^{cdc2}. The results are summarized in Table II.

As expected, anti-PSTAIRE antibodies precipitated the p34^{cdc2}, p33^{cdk2} and p36^{cdk3} proteins containing the complete PSTAIRE motif. These antibodies also recognized the PSSALRE and PCTAIRE-1 kinases that lack canonical PSTAIRE sequences. The only antibody we have tested that was specific for p34^{cdc2} was the anti-carboxy-terminal peptide antibody (Draetta and Beach, 1988). All other reagents that we tested bound to more than one kinase. Thus, only experiments using the anti-p34^{cdc2}-C-terminal peptide antibodies will give results that can be convincingly ascribed to p34^{cdc2}, while the use of other reagents is complicated by cross-reaction with additional *cdc2*-related kinases.

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PCTAIRE-1   478  SSSMPDSGRPAFRVVDTEF*                    496
PCTAIRE-1A  478  SSSMPEPCSPAHLSPAACPNTPPLACQPTHWPVC*    510

KKIALRE     142  KLCDFGFARLL.................TGPSDYYTDY  162
KKIALRE-A   142  KLCDFGFARLLILIPAFRCCLFSMFHHWLAGPSDYYTDY  180
KKIALRE-B   142  KLCDFGFARLLKTGFHHIAQAGLQLLGRSKPPTSASRSAGITSLDRVT  179
                 TIQTTWLPGGTAPLSCWWGTRSTAPRWMFGQLAVSLLSCCQECLCGQENR  229
                 MWISCI*                                  235
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Fig. 2. Alternative coding sequences in PCTAIRE-1 and KKIALRE genes. The sequence of PCTAIRE-1 and KKIALRE shown in Figure 1 is denoted by bold type, while the alternate sequences are shown in normal type. One of six PCTAIRE-1 cDNA clones sequenced at the C-terminus contained a 149 bp deletion between a heptanucleotide repeat, TGCCTGA, giving rise to an alternate C-terminal sequence. Three independent clones of KKIALRE contained different coding sequences downstream from amino acid residue 152. Two contained insertions relative to the sequence shown in Figure 1. One insertion of 54 bp maintained the reading frame while the other insertion of 95 bp shifted the reading frame and terminated 83 amino acids downstream. These insertions do not appear to contain consensus splice donor and acceptor sites.

Expression of the cdc2-related genes

The presence of multiple human *cdc2*-related kinases led us to wonder whether each of these genes was required in all cell types, or whether some might have cell- or tissue-specific functions and show correspondingly restricted patterns of mRNA expression. Because we had isolated a number of family members by cross-hybridization at high stringency, we first needed to generate specific probes for each mRNA. Potential probes were tested on DNA slot blots of all the related clones to establish their specificity and sensitivity (data not shown). The probes are described in more detail in Materials and methods. RNA blot analysis was performed on poly(A)$^+$mRNA from 293 (adenovirus-transformed embryonic kidney cell), ML-1 (myeloid leukemia), U118MG (glioblastoma), MCF-7 (mammary carcinoma) and NGP (neuroblastoma) cells. Representative lanes from these blots

Table I. Predicted amino acid sequence identity among human cdc2-related kinases[a]

	cdc2	cdk2	cdk3	PSSALRE	PCTAIRE-1	PCTAIRE-2	PCTAIRE-3	PLSTIRE	PSK-J3	p58-GTA	KKIALRE
cdc2	–	65	66	57	52	55	51	47	44	42	43
cdk2		–	76	60	54	52	53	50	48	44	40
cdk3			–	62	54	53	51	48	48	45	42
PSSALRE				–	58	57	55	47	45	42	43
PCTAIRE-1					–	83	79	43	42	42	35
PCTAIRE-2						–	80	41	42	41	36
PCTAIRE-3							–	45	44	39	36
PLSTIRE								–	71	40	39
PSK-J3									–	41	34
P58-GTA										–	32
KKIALRE											–

[a]Percentage amino acid sequence identity calculated for region corresponding to p34^{cdc2} amino acid sequence. Triangles denote sequence subfamilies.

are shown in Figure 4 to illustrate the sizes of the mRNAs and the specificities of the probes. *cdc2*, PCTAIRE-1, and PLSTIRE show obvious multiple transcripts. On other gels it is clear that PCTAIRE-2 can be resolved into a doublet (for example, see Figure 5, brain RNA). Longer exposures of KKIALRE also showed two major transcripts. The sizes of each transcript are shown in Figure 4.

The levels of the various transcripts from different human sources are given in Figure 5. The *cdk2* and PSSALRE transcripts were expressed at uniformly high levels, as judged by relative signal strength on the RNA blots normalized by signal intensity of these probes on DNA slot blots. The *cdc2*, PCTAIRE-1, PCTAIRE-2, PCTAIRE-3, and PLSTIRE transcripts vary in abundance across the different cell lines, with each gene showing a distinct pattern. *cdk3* also appears to be ubiquitously expressed, although the levels of *cdk3* mRNA detected were low compared with the other *cdc2*-related kinase genes, and were highly variable. An unexpected finding was that the PLSTIRE mRNA was

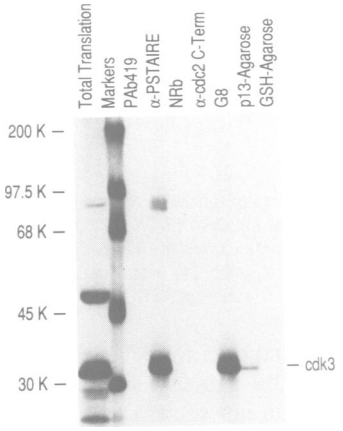

Fig. 3. Characteristics of *in vitro* translated cdk3 protein. The molecular weight of cdk3 protein synthesized *in vitro* by translation in a rabbit reticulocyte lysate (lane 1) is estimated to be 36 kDa by comparison to protein marker standards (lane 2). cdk3 protein was immunoprecipitated with a control monoclonal antibody, PAb419 (lane 3), mouse monoclonal antibody to the PSTAIRE conserved motif of $p34^{cdc2}$ (lane 4), normal rabbit serum (lane 5), polyclonal rabbit antibody to the human $p34^{cdc2}$ C-terminal peptide (lane 6) and polyclonal rabbit antibody to the entire *S.pombe* $p34^{cdc2}$ protein (G8, lane 7). cdk3 protein was also tested for binding to $p13^{suc1}$–agarose beads (lane 8), with glutathione–agarose beads as control (lane 9). The 80 kDa band seen in the total and anti-PSTAIRE lanes is of unknown origin.

Table II. Properties of cdc2-related proteins

Protein	Apparent molecular weight (kDa)	anti-PSTAIRE[a]	G8[b]	anti-cdc2 C-terminal[c]	p13 beads[d]
cdc2	34	+	+	+	+++
cdk2	33	+	+	−	++
cdk3	36	+	+	−	+
PSSALRE	31	+	+	−	−
PCTAIRE-1	55	+	−	−	−
PLSTIRE	40	−	−	−	−

[a]Mouse monoclonal anti-peptide antibody to conserved PSTAIRE region of cdc2.
[b]Rabbit polyclonal antibody to *S.pombe* $p34^{cdc2}$ protein.
[c]Rabbit polyclonal anti-peptide antibody to C-terminus of human cdc2.
[d]Bacterially-expressed $p13^{suc1}$ coupled to agarose beads.

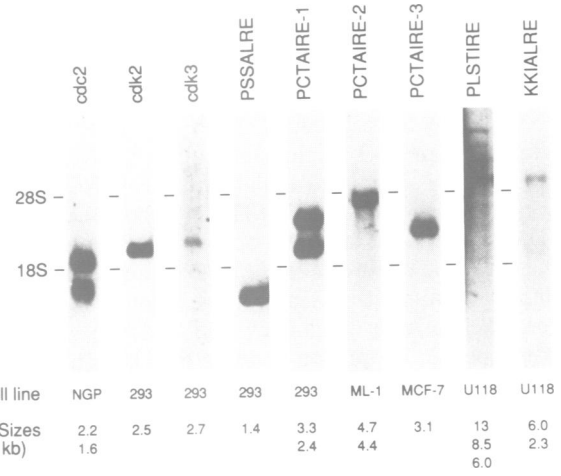

Fig. 4. mRNA sizes of *cdc2*-related transcripts. Representative lanes from Northern blots of human cell line poly(A)$^+$RNA are shown. Dashes indicate the position of the 28S and 18S ribosomal RNA subunits for each lane. Sizes were determined by comparison to these ribosomal RNA markers, with 28S and 18S RNAs considered to be 4.7 kb and 1.9 kb.

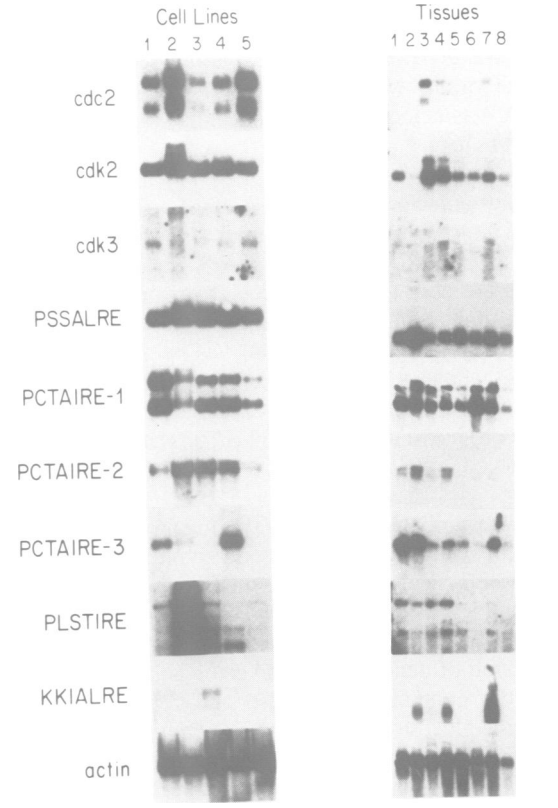

Fig. 5. Northern blot analysis of poly(A)$^+$RNA from various human sources. **(Left panel)** Human cell lines (7.5 μg per lane); 293 (lane 1), ML-1 (lane 2), U118 (lane 3), MCF-7 (lane 4) and NGP (lane 5). **(Right panel)** Normal human tissues (2 μg per lane); heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7) and pancreas (lane 8). Exposure times were (times for cell lines/times for tissue blots): cdc2, 8 h/11 h; cdk2, 3 h/3 h; cdk3, 3 days/3 days; PSSALRE, 13 h/11 h; PCTAIRE-1, 3.5 h/5.5 h; PCTAIRE-2, 6 days/6 days (oligo probe); PCTAIRE-3, 6 days/6 days (oligo probe); PLSTIRE, 1 day/1 day; KKIALRE, 40 h/40 h; and actin, 1 h/1 h. A 700 bp band (not shown) was hybridized with the *cdc2* and PSSALRE probes in the placenta, muscle and kidney samples. This band was not seen in any other samples. In addition, faint bands of 4.4 kb and 3.9 kb (not shown), most abundant in ML-1 cells, hybridized with the *cdc2* and *cdk3* probes.

expressed at extremely high levels in ML-1 cells relative to the other cell lines. We have confirmed this finding by using independent non-overlapping probes on independent blots. We do not yet know whether the protein levels of PLSTIRE are correspondingly high, nor whether this is a characteristic of other myeloid leukemias. The closely related PSK-J3 gene is expressed at highest levels in NGP cells and lowest levels in ML-1 cells (data not shown).

The differential expression of several of the *cdc2*-related genes in tissue culture cells led us to examine their expression in normal human tissues: heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (Figure 5). Tissue-specific expression patterns for chicken *cdc2* have been previously described (Krek and Nigg, 1989); we were surprised to find that mRNA levels of all the human kinases showed tissue-specific variations. One such pattern that may reflect a functional difference was seen by comparing the expression levels of the *cdk2* and PSSALRE genes. These genes were abundantly and equally expressed in the tissue culture cells, yet showed divergent patterns in different tissues. The *cdk2* gene, like *cdc2*, was expressed at highest levels in placenta, which is a rapidly dividing tissue. However, the corresponding RNAs were almost absent in adult brain, which contains few dividing cells. The PSSALRE gene, in contrast, was expressed at highest levels in adult brain; while a significant amount of PSSALRE mRNA was detected in placenta, this was the lowest level of all tissues.

Complementation of cdc28 mutants of S.cerevisiae

One of the critical questions about a family of kinases with close structural resemblance to $p34^{cdc2}$ is whether these kinases are able to regulate cell cycle progression. Functional complementation of budding yeast *cdc28* mutants (or of fission yeast $cdc2^-$ mutants) has been used to identify kinases that play a *cdc2*-like role in controlling the vertebrate cell cycle. As mentioned above, both human *cdc2* and *cdk2* have been shown to complement *cdc28* mutants (Elledge and Spottswood, 1991; Koff *et al.*, 1991; Ninomiya-Tsuji *et al.*, 1991).

To determine whether any of the human *cdc2*-related genes could complement temperature-sensitive *cdc28* mutants of *S.cerevisiae*, each full-length ORF was subcloned into a multicopy yeast expression vector containing a *GAL1* promoter at the cloning site and a *URA3* selectable marker. Expression of genes from this vector is induced by growth on media containing galactose and repressed on media containing glucose. Transformation with plasmids expressing genes that complement thermosensitive *cdc28* mutants should restore growth at the restrictive temperature of 37° C on galactose plates but not on glucose plates. Yeast strains bearing the *cdc28*-1N allele, which arrests predominantly at the G_2-M transition (Piggott *et al.*, 1982; Surana *et al.*, 1991) and the *cdc28*-4 allele, which arrests predominantly at START in G_1 (Reed, 1980), were transformed with constructs expressing *cdc2*-related genes.

Plasmids expressing the *S.cerevisiae CDC28*, human *cdc2* (not shown), human *cdk2* and human *cdk3* genes were able to restore growth of *cdc28*-1N and *cdc28*-4 mutants at 37° C on galactose plates (Figure 6 and data not shown). Plasmids expressing PSSALRE, PCTAIRE-1, PCTAIRE-2, or PLSTIRE were not able to restore growth of *cdc28*-1N or *cdc28*-4 mutants at 37° C (Figure 6 and data not shown).

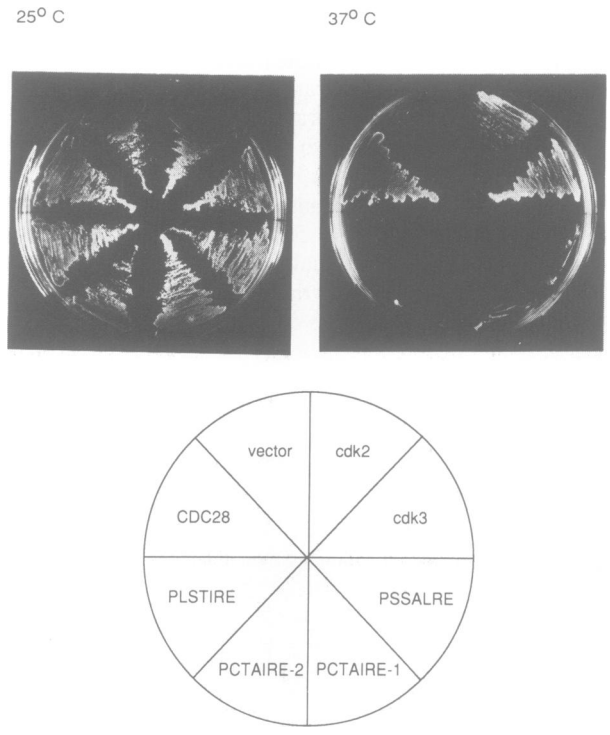

Fig. 6. Complementation of *cdc28-1N* temperature-sensitive yeast mutants by cdc2-related kinases. *cdc28-1N* mutants of *S.cerevisiae*, transformed with plasmids expressing cdc2-related kinase genes, were streaked onto plates containing 2% raffinose and 2% galactose and grown for 60 h at 25° C (left) or 37° C (right).

When *cdc2*, *cdk2* and *cdk3* were cloned in the reverse orientation to transcribe the antisense strand, they failed to rescue *cdc28* mutants (data not shown).

The failure of *cdc2*-related genes other than *cdc2*, *cdk2* and *cdk3* to complement *cdc28* mutants could be due to lack of expression at the RNA or protein level, or to mutations introduced by PCR, as well as being due to the intrinsic properties of these genes. mRNA expression of the appropriate gene could be detected by RNA blot analysis when *cdc28*-1N mutants containing plasmids expressing each of the *cdc2*-related genes were cultured in media containing galactose (data not shown), however, similar experiments for protein expression will require specific antibodies. We did not sequence each clone and cannot exclude PCR-induced mutations. In addition, failure of the other *cdc2*-related genes to rescue *cdc28* mutants must be interpreted with some caution in light of the observation that complementation of *cdc28* mutants by *cdc2*-related genes may be dependent on strain-to-strain variations (Elledge and Spottswood, 1991).

To confirm that introduction of the *cdk2* or *cdk3* gene, rather than selection of a spontaneous revertant, was responsible for the rescue, multiple colonies growing at 37° C were streaked onto non-selective plates and then replica-plated onto plates lacking uracil. 100% of colonies that had lost the plasmid, as assayed by failure to grow on these plates, did not grow at 37° C on rich media; 100% of colonies that retained the plasmid could still grow at 37° C. In summary, *cdk3*, like *cdc2* and *cdk2*, can complement *cdc28* temperature-sensitive mutants of *S.cerevisiae*. The degree of complementation cannot be assessed by these experiments; *cdk3* was expressed from the strong *GAL1* promoter in a

high copy number plasmid and the mutants rescued were point mutants. Nevertheless, the ability to rescue *cdc28* mutants suggests that the *cdc2*, *cdk2* and *cdk3* genes share significant functional similarities.

Discussion

The isolation of a large family of *cdc2*-related kinases poses a series of important questions. First, which of these kinases play a role in control of the cell cycle? Secondly, if they are involved in cell cycle control, how does their regulation differ from *cdc2*? Finally, does their identification in multicellular organisms reflect roles in regulatory pathways unique to these organisms?

Which kinases are cell cycle regulators?

The complementation of *S.cerevisiae cdc28* mutants by *cdc2*, *cdk2* and *cdk3* suggests that these kinases can interact effectively with yeast cyclins and respond correctly to the essential regulatory signals in these yeast. This strongly suggests that the cdks can play similar roles in human cell cycle control. The question of whether other members of this family of kinases regulate cell cycle progression is unresolved at present. CDC28 functions at several points in the yeast cell cycle, and it would be premature to exclude a cell cycle role for *cdc2*-related kinases based on their failure to complement. There remains a distinct possibility that the non-complementing kinases may contribute to cell cycle control in specialized cells or circumstances. For now, however, there is no evidence that any of the human *cdc2*-related kinases other than *cdc2*, *cdk2*, and *cdk3* are involved in cell cycle regulation.

It seems likely that *cdc2* is essential for the G_2-M transition in vertebrate cells. In rat fibroblasts, microinjection of antibodies to $p34^{cdc2}$ blocks entry into mitosis (Riabowol *et al.*, 1989). Mouse cells with temperature-sensitive *cdc2* mutants are also arrested at the G_2-M transition at the restrictive temperature (Th'ng *et al.*, 1990) and immunodepletion of $p34^{cdc2}$ from *Xenopus* oocyte lysates prevents entry into mitosis (Fang and Newport, 1991). For *cdk2*, the observation that depletion of $p33^{cdk2}$ blocks DNA synthesis in *Xenopus* oocyte lysates (Fang and Newport, 1991) suggests a role in controlling the G_1-S transition. The role of *cdk3* is still unknown.

If the human cdks are required for specific cell cycle transitions, their kinase activities should vary in a cell cycle-dependent manner. For human *cdc2* and *cdk2* this is already clear. Human $p33^{cdk2}$ is activated before the onset of S phase and its activity decreases by the G_2-M transition (Pagano *et al.*, 1992a; Rosenblatt *et al.*, 1992; L.-H.Tsai, unpublished results); there is also evidence for a second peak of activity during G_2 (Rosenblatt *et al.*, 1992). The peak of $p34^{cdc2}$ kinase activity is later in the cell cycle, at the G_2-M transition (Draetta and Beach, 1988; Pines and Hunter, 1989). Similar studies of *cdk3* will be possible after the production of specific antibodies.

Are the novel kinases regulated similarly to cdc2?

The three levels of control that are known to regulate the timing of $p34^{cdc2}$ kinase activity are cyclin association, phosphorylation/dephosphorylation of critical residues and cyclin degradation. Of these, only cyclin association has been demonstrated for any of the new kinases. $p33^{cdk2}$ associates with both cyclin A and cyclin E (Tsai *et al.*, 1991; Faha *et al.*, 1992; Pagano *et al.*, 1992b; Rosenblatt *et al.*, 1992; E.Lees and L.-H.Tsai, unpublished observations; S.Reed, personal communication). Since $p34^{cdc2}$ also interacts with cyclin A, the same cyclin can interact with more than one kinase. Previous work had shown that $p34^{cdc2}$ could interact with both cyclin A and cyclin B. These data suggest that combinatorial regulation will be important for cell cycle control.

The identification of novel *cdc2*-related protein kinases expands the diversity of potential cyclin−kinase combinations. Clearly, *cdc2*, *cdk2* and probably *cdk3* are catalytic partners for cyclins. Other kinases of this family may partner cyclins as well, based on their sequence homologies and small size. Combinatorial regulation would permit control of the cell cycle either at a large number of transition points or at a small number of control points in response to many different signals. Alternatively, the multiple cyclin−kinase complexes might provide redundancy. Combinatorial regulation may not be limited to cyclin interactions, as $p34^{cdc2}$ from *S.pombe* can interact with other cell cycle regulatory proteins, such as the *cdc25* phosphatases and $p13^{suc1}$ (for a review see Nurse, 1990). Multiple human homologs of these regulatory proteins have been described recently (Richardson *et al.*, 1990; Sadhu *et al.*, 1990; Galaktionov and Beach, 1991).

Multicellular versus unicellular controls

In budding and fission yeast, both the G_1-S and the G_2-M transitions are controlled by the same cdc2/CDC28 kinase. However, in humans, *cdc2*, *cdk2* and probably *cdk3* all appear to participate in cell cycle regulation. The identification of the other *cdc2*-related kinases is not unique to humans, as homologs have now been described in several other species. PCTAIRE genes have been found in mouse (J.Downing, personal communication), *Xenopus* (R.Poon and T.Hunt, personal communication) and *Caenorhabditis elegans* (S.van den Heuvel, unpublished results), while a PSSALRE homologue has been isolated from *Drosophila* (K.Sauer, E.M.Illgen, and C.F.Lehner, personal communication), suggesting that these kinases function in diverse metazoans. The presence of a multigene family of *cdc2*-related kinases suggests that they may have separate and distinct functions in the control of cell growth, divison, differentiation and/or development. Coordination of the activities of these *cdc2*-related kinases may be essential to control the varied and distinct cell cycles and signalling pathways found within metazoan organisms. If true, this may explain the apparent absence of these kinases in yeast where intercellular communication is less prevalent.

Materials and methods

cDNA cloning

First strand cDNA was prepared from total cytoplasmic RNA made from HeLa cervical carcinoma cells or Nalm-6 pre-B leukemic cells. Amplification of these cDNA templates by PCR (Mullis *et al.*, 1986; Saiki *et al.*, 1986) was carried out using degenerate oligonucleotides derived from two highly conserved regions of *cdc2* proteins, the EKIGEGTY motif for the 5′ primer and the WYRSPEV motif for the 3′ primer. Another set of PCR amplifications was carried out using degenerate primers derived from the EGVPSTAI (5′) and DLKPQNL (3′) regions. PCR was carried out either for 30 cycles of 1 min at 94° C, 1 min at 55° C and 2 min at 72° C, or for five cycles of low stringency amplification (1 min at 94° C, 1 min at 37° C and 2 min at 72° C) followed by 30 cycles at higher stringency

(1 min at 94° C, 1 min at 50° C and 2 min at 72° C). PCR products were gel-purified and subcloned into pBSK vectors (Stratagene) for DNA sequencing. DNA sequences were determined using double-stranded plasmid DNA by the dideoxy sequencing method (Sanger *et al.*, 1977). Sequence analysis was performed using the University of Wisconsin Genetics Computer Group programs (Devereux *et al.*, 1984). Of the ~150 cloned PCR products sequenced, 69 contained inserts representing *cdc2*-related protein kinase genes. These included 28 *cdc2* clones, 13 PSSALRE clones, 13 PCTAIRE-2 cones, four *cdk2* clones, four PCTAIRE-1 clones, four PLSTIRE clones, two p58-GTA clones and one KKIALRE clone.

Three human cDNA libraries, Nalm-6 (in λZAP II), fetal brain (Stratagene, in ZAP II) and HeLa (in λgt11), were screened for full-length cDNA clones using PCR fragments of the *cdc2*-related genes. Each PCR fragment was labelled with [α-^{32}P]dCTP by the random primer method (Feinberg and Vogelstein, 1983). Hybridizations were performed at 42° C in 45% formamide, 5 × SSC (750 mM NaCl, 85 mM Na citrate pH 7.0), 5 × Denhardt's solution (0.1% Ficoll, 0.1% polyvinyl pyrollidone, 0.1% bovine serum albumin), 50 mM Na phosphate, pH 6.5, 5% dextran sulfate, 0.3% sodium dodecyl sulfate (SDS) and 100 μg/ml sonicated salmon sperm DNA. The washing conditions were 2 × SSC−0.1% SDS for 20 min at room temperature; 2 × SSC−0.1% SDS for 20 min at 65° C; 0.5 × SSC−0.1% SDS for 2 × 20 min at 65° C; 0.1 × SSC (15 mM NaCl, 1.7 mM Na citrate pH 7.0)−0.1% SDS at 65° C for 2 × 20 min. At least two million plaques were screened for each PCR probe.

The original clones of *cdk2*, PSSALRE, PCTAIRE-1, PCTAIRE-2, PLSTIRE, p58-GTA, KKIALRE and *cdc2*-Hs were obtained from HeLa first strand cDNA by PCR. *cdk3*, PCTAIRE-3 and PSK-J3 clones were originally obtained by cross-hybridization upon screening the Nalm-6 cDNA library with the PCTAIRE-1 PCR fragment. The Nalm-6 cDNA library provided clones of *cdk2* (2.2 kb) and PLSTIRE (3.0 kb) containing full-length ORFs and a partial clone of PCTAIRE-3 (3.0 kb). The human fetal brain library yielded clones of *cdk3* (3.3 kb), PSSALRE (1.4 kb) and PCTAIRE-1 (3.3 kb) containing full-length ORFs and a partial clone of PCTAIRE-2 (3.8 kb). Full-length cDNA clones of KKIALRE (1.7 kb) were obtained from a HeLa cDNA library after the above libraries were found to be negative.

DNA sequences of the cDNA clones were initially analysed using 5′ and 3′ primers, and subsequently using either specific internal primers or nested sets of deletions generated by the exonuclease III/S1 nuclease digestion method (Henikoff, 1984). All sequences are deposited in GenBank.

In vitro transcription, translation and binding experiments

The *cdc2*, *cdk2*, *cdk3*, PLSTIRE and PCTAIRE-1 ORFs were cloned into a modified pBSK vector containing the human β-globin promoter and an added *Nco*I restriction site for *in vitro* transcription with T7 RNA polymerase (Promega). The PSSALRE cDNA was transcribed *in vitro* with T3 RNA polymerase. The [^{35}S]methionine labelled proteins were synthesized *in vitro* using rabbit reticulocyte lysate (Promega). Each labelled protein was tested for binding with a mouse monoclonal antibody to a 16 amino acid peptide derived from the PSTAIRE region of p34^{cdc2} (gift of M.Yamashita), an affinity-purified polyclonal rabbit antibody to the C-terminal peptide of human p34^{cdc2} (LTI/BRL-Gibco), an affinity-purified polyclonal rabbit antibody to *S.pombe* p34^{cdc2} (G8: gift of D.Beach) and p13−agarose beads (Oncogene Science). Negative controls were normal rabbit serum, the PAb 416 monoclonal antibody to SV40 large T antigen (Harlow *et al.* 1981) and glutathione−agarose beads (Sigma). Immunoprecipitations were carried out as described (Harlow *et al.*, 1985).

RNA blot analysis

Total cellular RNA was isolated by the method of Chomczynski and Sacchi (1987). Poly(A)$^+$RNA was prepared by oligo-dT cellulose chromatography (Aviv and Leder, 1972). RNA was fractionated by electrophoresis on a 1.2% formaldehyde agarose gel, and transferred to Hybond-N$^+$ membranes (Amersham). Each cell line blot contains 7.5 μg of poly (A)$^+$ RNA derived from the following human cell lines: 293 (adenovirus-transformed kidney epithelial cell), ML-1 (pre-myeloid leukemia), U118 (glioblastoma), MCF-7 (mammary carcinoma) and NGP (neuroblastoma). The human tissue blot (Clontech Laboratories, Inc.) contains 2 μg per lane of poly (A)$^+$ RNA from heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas.

For most of the clones, random primed DNA fragments labelled with [^{32}P]dCTP were used as probes for Northern hybridization. For *cdk2*, PSSALRE, PLSTIRE and KKIALRE, these fragments were generated by PCR using the cloning primers. The *cdc2* probe was a *Kpn*I−*Bgl*II restriction fragment (nucleotides 41−508). The *cdk3* probe was a PCR fragment (nucleotides 630−915). The PCTAIRE-1 probe was a *Kpn*I−*Bam*HI fragment (nucleotides 977−1355). In addition, complementary oligonucleotides labelled with DNA polymerase I large fragment (Ullrich *et al.*, 1984) were used as highly specific probes for several clones. These oligonucleotides were nucleotides 403−441 of PCTAIRE-1 and the corresponding regions of PCTAIRE-2 and PCTAIRE-3 and nucleotides 317−357 of PLSTIRE. The *cdk3* probing was confirmed with a *Nco*I−*Eco*RV restriction fragment (nucleotides −1−554) and the PLSTIRE probing was confirmed with an *Xmn*I−*Pvu*II restriction fragment (nucleotides 634−1083). A human β-actin probe (Clontech Laboratories, Inc.) was used as a control for the level of RNA in each lane. Hybridizations were carried out for 24−48 h at 42°C in 4 × SSC, 50% formamide, 10 × Denhardt's solution, 50 mM Na phosphate, pH 6.5, 1% SDS and 0.5 mg sonicated salmon sperm DNA per ml. Filters hybridized with probes derived from DNA fragments were washed as described above. The filters hybridized with oligonucleotide probes were washed twice at room temperature with 2 × SSC−0.1% SDS and at 65°C with 1 × SSC−0.1% SDS once for 20 min and once for 15 min.

Complementation of yeast cdc28 mutants

The coding regions of the *cdk2*, *cdk3*, PSSALRE and PLSTIRE genes were PCR amplified and cloned into the *Bam*HI site of pMR438 (gift of M.Tyers; Colasanti *et al.*, 1991). The entire coding regions of PCTAIRE-1 and PCTAIRE-2 clones, beginning with the initial methionine residue of the N-terminal extension, were excised with *Eco*RI and cloned into pDAD2 (gift of J.Celenza, Whitehead Institute). Each of these vectors contains a yeast 2μ origin of replication and a *URA3* selectable marker; a *GAL1* promoter is upstream of the inserted genes. Plasmids with genes inserted in both orientations were isolated and used to transform yeast. Controls included plasmid alone, *CDC28* expressed in pDAD2 (gift of J.Celenza) and *cdc2*Zma expressed in pMR438 (gift of J.Colasanti).

The following strains of *S.cerevisiae* were used for transformation experiments: K699 (*MAT***a** *GAL cdc28-1N ade-1*-1 *can1*-100 *his3*-11 *leu2 trp1*-1 *ura3*; gift of B.Futcher) and 10083-5C (*MAT***a** *GAL cdc28*-4 *gcn4 ura3*; gift of J.Celenza and G.Fink). The *cdc28-1N* mutation was initially described by Piggott *et al.* (1982) and the *cdc28*-4 mutation by Reed (1980).

Plasmids were transformed into yeast by electroporation or lithium acetate methods; transformants were selected on minimal media lacking uracil and containing 2% glucose. Transformants were then patched overnight onto plates lacking uracil and containing 2% raffinose to relieve repression of the *GAL1* promoter, before streaking onto quadruplicate plates containing either 2% glucose or 2% galactose−2% raffinose and grown at 25°C or 37°C.

Yeast RNA was isolated by resuspending cells in 0.5 M NaCl, 0.2 M Tris pH 7.6, 10 mM EDTA and 1% SDS, adding acid-washed glass beads and an equal volume of phenol−chloroform and vortexing for 2 min. The aqueous phase was re-extracted and then ethanol precipitated. The RNA blot analysis was performed as described above.

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