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Novel Members of the cdc2-related Kinase Family in *Drosophila*: cdk4/6, cdk5, PFTAIRE, and PITSLRE Kinase

Karsten Sauer, Katrin Weigmann, Stephan Sigrist, and Christian F. Lehner*

Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Spemannstrasse 37–39, 72076 Tübingen, Germany

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In addition to the previously identified *Drosophila cdc2* and *cdc2c* genes, we have identified four additional *cdc2*-related genes with low stringency and polymerase chain reaction approaches. Sequence comparisons suggest that the four putative kinases represent the *Drosophila* homologues of vertebrate cdk4/6, cdk5, PCTAIRE, and PITSLRE kinases. Although the similarity between human and *Drosophila* homologues is extensive in the case of cdk5, PCTAIRE, and PITSLRE kinases (78%, 58%, and 65% identity in the kinase domain), only limited conservation is observed for *Drosophila* cdk4/6 (47% identity). However, like vertebrate cdk4 and cdk6, *Drosophila* cdk4/6 binds also to a D-type cyclin according to the results of two-hybrid experiments in yeast. Northern blot analysis indicated that the four *Drosophila* kinases are expressed throughout embryogenesis. Expression in early embryogenesis appeared to be ubiquitous according to in situ hybridization. Abundant expression already at the start of embryogenesis and long before neuron differentiation was also observed in the case of cdk5 protein, which has been described as predominantly neuron specific in mice. Sequence conservation and expression pattern, therefore, suggest that all of these kinases perform important cellular functions.

INTRODUCTION

Cyclin-dependent kinases (cdks) were identified because of their crucial roles in eukaryotic cell cycle regulation. Compared with budding and fission yeast, where the first *cdk* genes were isolated (*Saccharomyces cerevisiae CDC28* and *Schizosaccharomyces pombe cdc2*$^{+}$), higher eukaryotes appear to have a considerably more complex inventory of cdks. Most of the vertebrate kinases were originally identified in an extensive search for *cdc2*-related genes in humans (Meyerson *et al.*, 1992). Although many of these *cdc2*-related kinases were subsequently shown to associate with regulatory cyclin subunits, cyclin partners remain to be discovered in a number of cases. However, in contrast to the bonafide cdks which consist of kinase domains only and are regulated by cyclins, some cdc2-related kinases have considerable N-terminal extensions (Meyerson *et al.*, 1992; Kidd *et al.*, 1994; Xiang *et al.*, 1994). These kinases therefore might be regulated by these additional domains and not by cyclin subunits. More recently, vertebrate cdks with rather limited similarity to cdc2 and functional roles in contexts other than cell cycle control have been described (Tsai *et al.*, 1993; Roy *et al.*, 1994; Poon and Hunter, 1995; Shiekhattar *et al.*, 1995; Tassan *et al.*, 1995).

The great diversity of vertebrate cdks complicates their functional characterization. We have concentrated on an analysis of cdks and their role in cell cycle regulation in *Drosophila* where the diversity can be expected to be less complex and accessible to genetic methodology. The comparison of the families of cdc2-related kinases in vertebrates and *Drosophila* as described here reveals variable degrees of evolutionary conservation in the regulation of the different cell cycle steps.

* Corresponding author and present address: Department of Genetics, University of Bayreuth, 95440 Bayreuth, Germany.

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MATERIALS AND METHODS

Fly Strains

Stocks carrying the deficiencies *Df(2R)XTE11* and *Df(2L)l13* were obtained from Dr. Eileen Underwood (Bowling Green State University, Bowling Green, Ohio). *Df(2R)Jp4*, *Df(2R)WMG*, and *Df(2R)XTE18* were obtained from the Bloomington stock center (Bloomington, IN). All of these deficiencies are described in Underwood *et al.* (1990) and in Smith *et al.* (1993). The polymerase chain reaction (PCR) assays to test for the presence of cdk5 sequences on the deficiency chromosomes were done as described by Knoblich *et al.* (1994), except that the hybridization steps were carried out at 50°C and a cdk5-specific primer pair (5′-CTT ATT GAT TCA GTA AAA TAG TTT TAT TC-3′ and 5′-CGT CGA CAC TCA TTA AAT TTG GAA AC-3′) was used in combination with the cyclin A-specific primer pair that was used for control purposes.

PCR Screen

A pair of degenerate oligonucleotide primers was used for enzymatic amplification of cdc2-like kinases. The first primer (5′-GCA GGATCC GA(GA) AA(AG) AT(CT) GG(ACGT) GA(AG) GG(ACGT) AC(ACGT) TA-3′) was derived from the conserved motif EKIGEGTY which spans amino acids 8–15 in the *Drosophila* cdc2 kinase sequence (Lehner and O'Farrell, 1990). The second primer (5′-CCG TCTAGA (AG) (ACGT)A(CT) (CT)TC (ACGT)GG (ACGT)(GC)(ACT) (ACGT)C(GT) (AG)TA CCA-3′) was derived from the conserved motif WYR(AS)PE(IV)L which spans amino acids 168–175 in the *Drosophila* cdc2 kinase sequence. As template, we used first-strand cDNA synthesized from Schneider cell poly(A)+ RNA isolated with the help of a fast-track mRNA isolation kit (Invitrogen, San Diego, CA). One microgram of poly(A)+ RNA was reverse transcribed for 2 h at 37°C in a 50-μl reaction containing 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 30 mM KCl, 10 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 50 U of RNasin (Boehringer Mannheim, Indianapolis, IN), and 125 U of Moloney Murine Leukemia Virus reverse transcriptase (New England Biolabs, Beverly, MA). Ten microliters of this reverse transcription reaction were used for a 50-μl PCR containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 4 μM of each of the two primers, and 2.5 U of AmpliTaq polymerase (Perkin Elmer-Cetus, Norwalk, CT). Enzymatic amplification was carried out during 35 cycles (1 min at 94°C, 2 min at 55°C, 2 min at 73°C). PCR products in the expected size range of 600 bp were gel purified and digested with *Eco*RI and *Nsi*I in an attempt to eliminate the previously identified cdc2 and cdc2c fragments which are cut by these enzymes. The PCR products remaining in the 600-bp range were purified and reamplified using the same conditions as in the first PCR. The resulting PCR products in the 600-bp range were gel purified and cloned using the *Bam*HI and *Xba*I sites introduced by the PCR primers and the vector Bluescript KS+. One hundred thirty-five colonies obtained after transformation were analyzed for the presence of an insert in the 600-bp range by PCR using the original primers again. Seventy-three colonies carrying a 600-bp plasmid insert were screened for the presence of cdc2 or cdc2c sequences by PCR using nondegenerate gene-specific primers (5′-GTC ATT TCC GGC AAC AAT-3′ for cdc2c and 5′-GTT GAT AAG CAC ATG GAG-3′ for cdc2) and a T7 primer (5′-AAT ACG ACT CAC TAT AG-3′). Twenty and eight colonies, respectively, resulted in amplification of a PCR product with the size expected in the case of cdc2 and cdc2c. Plasmids from the remaining colonies were purified and classified based on the restriction fragment patterns generated by *Hae*III. The inserts from representative plasmids were sequenced, resulting in the identification of three novel cdc2-like sequences with maximal homology to either vertebrate cdk4/6, cdk5, or PITSLRE. The cdk4/6-like sequence was present in 7 colonies, the cdk5-like sequence in 27 colonies, and the PITSLRE-like sequence in 5 colonies. The plasmid inserts originating from six colonies revealed no similarity to kinase sequences.

Low Stringency Screens

The coding sequence of a human cdk2 cDNA (Meyerson *et al.*, 1992) was labeled by random priming and used to probe 400,000 plaques of a genomic *Drosophila* λ DASH library (Caudy *et al.*, 1988) at reduced stringency essentially as described by McGinnis *et al.* (1984). Hybridizations were done for 72 h at 37°C in 43% deionized formamide, 5× SSC (Sambrook *et al.*, 1989), 5× Denhardt's reagent (Sambrook *et al.*, 1989), 50 mM $NaPO_4$ (pH 7.0), 0.1% SDS, and 0.25 mg/ml salmon sperm DNA. The filters were washed in 2× SSC, 0.1% SDS twice for 5 min at room temperature, and twice for 15 min at 45°C before autoradiography. Dot blot experiments confirmed that hybridization signals obtained with a *Drosophila* cdc2c cDNA were above background signals obtained with unrelated cDNAs under these conditions. Sixty-eight plaques which resulted in signals on duplicate filters were further characterized. After one or two rounds of plaque purification, we used PCR to test for the presence of cdc2, cdc2c, cdk4/6, cdk5, and PITSLRE sequences in positive plaque eluates. Plaques were eluted in 0.5 ml 10 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$. Five microliters of these eluates were digested for 1 h at 50°C in a 10-μl reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Nonidet P-40, 0.1% Tween 20, 0.03 mg/ml of proteinase K followed by an inactivation of proteinase K during 10 min at 94°C. These reactions were used in 50-μl PCR assays (see above) with primers specific for either cdc2 (5′-GCA GGA TCC TAT AAG AAC ACG TCC CC-3′ and 5′-GCA AAG CTT AGT TAA AGT CAA ACG A-3′), cdc2c (5′-GCA GGA TCC TTT AAG ACC AAG TTC CC-3′ and 5′-GCA AAG CTT CGA TCA GAC GAG CCG CGT-3′), cdk4/6 (5′-AAG CAG CTG AAT GCC AGC AAT-3′ and 5′-GGA GGT GAG CTT CAT TTC CGA-3′), cdk5 (5′-CTA AAG CTG GCT GAC TTT-3′ and 5′-TTG CAG CAG GTC GCG TCC-3′), or PITSLRE (5′-GTG GTC GGA TCC AAC ATG GAT-3′ and 5′-CTT GAT CGG CGA TCC ATA CTC-3′). PCR products were analyzed after resolution on 6% polyacrylamide gels. Several plaques were also characterized by filter hybridization under stringent conditions using random primer-labeled cdc2, cdc2c, cdk4/6, cdk5, and PITSLRE probes. The two phages with inserts hybridizing to human cdk2 under low stringency but unrelated to the previously identified cdc2-like PCR fragments were purified and found to contain overlapping inserts according to restriction digests with various enzymes. A *Pst*I fragment which showed maximal hybridization to human cdk2 was subcloned and sequenced and found to contain sequences highly similar to vertebrate PCTAIRE kinases. Of the total of 68 plaques identified by the human cdk2 probe, 7 were found to represent cdc2c clones, 37 cdk4/6 clones, 22 cdk5 clones, and 2 PFTAIRE clones.

For the identification of cdk4- and cdk6-related kinases, we screened the same genomic library using the same low stringency conditions as in the screen for the cdk2-related kinases. However, probes were generated from human cdk4 and cdk6 cDNAs (Hanks, 1987; Meyerson *et al.*, 1992; Meyerson and Harlow, 1994). Sixteen of 105 plaques were recognized by the cdk6 probe. Signals resulting under the same conditions with the cdk4 probe were very weak, but 3 of these 16 plaques were also recognized by the cdk4 probe on duplicate filters. Two of these latter plaques and two plaques recognized by the cdk6 probe only were purified and found to contain overlapping inserts according to restriction analyses. All of these phages were found to hybridize under stringent conditions to a probe derived from the cdk4/6 fragment identified in the PCR screen, and sequence analysis confirmed that these genomic phages contained identical sequences as present in the previously isolated cdk4/6 PCR fragment. Subsequent analysis of the other positive plaques recognized by the cdk6 probe revealed the presence of cdk4/6 sequences in all cases (except for two which failed to generate positive signals also when rescreened with the human cdk6 probe under low stringency).

Isolation and Sequencing of cDNA Clones

Drosophila cdk5 cDNAs were isolated from three different cDNA libraries. Three clones were isolated from a λ library derived from 3- to 12-h embryos (Poole *et al.*, 1985), 12 clones were isolated from a λ library derived from ovaries (Hay *et al.*, 1988), and 1 clone from a plasmid library derived from 0- to 4-h embryos (Brown and Kafatos, 1988). None of these cDNA clones contained sequences extending beyond the 5′ end of the largest cDNA that was completely sequenced (accession number X99511). Since the size of the cDNA insert (1.6 kb) is in agreement with the size of the cdk5 transcript detected on Northern blots, this cDNA corresponds most likely to a full-length or near full-length clone. The putative amino acid sequence encoded by the large open reading frame starting at the first initiation codon therefore presumably represents the complete protein product even though no stop codons were found upstream of the first initiation codon.

The plasmid library (Brown and Kafatos, 1988) was also used for the isolation of five PFTAIRE cDNA clones, six PITSLRE cDNA clones, and six cdk4/6 cDNA clones.

Three of the five PFTAIRE cDNA clones contained 3.5-kb inserts corresponding in size to the transcript detected on Northern blots, while two cDNAs were partial. One of the 3.5-kb cDNAs was sequenced completely (accession number X99512). Stop codons were found upstream of the first initiation codon of the largest open reading frame and a poly(A) tail at the 3′ end, suggesting that the complete coding sequence is contained within this cDNA.

Four of the six cdk4/6 cDNAs clones had inserts of similar length (1.2 kb) as the transcript detected on Northern blots. Sequence analysis (accession number X99510) did reveal a stop codon immediately upstream of the first initiation codon of the largest open reading frame present in these clones. It appears, therefore, that the complete coding sequence of the putative cdk4/6 kinase is contained within the sequenced region.

The PITSLRE cDNA clone with the largest insert (4.6 kb) contained an artifactual extension with rearranged vector sequences within the 5′ end. Therefore, we selected a cDNA clone with a 3.2-kb insert corresponding in size to the major maternal transcript detected on Northern blots for complete sequence analysis (accession number X99513). The presence of stop codons upstream of the first initiation codon of the largest open reading frame and of a poly(A) stretch in the 3′ region suggests that the complete coding sequence is contained within this cDNA. The additional four cDNA clones carried partial fragments of this sequence as suggested by PCR experiments and partial sequence analysis.

Yeast Two-Hybrid Experiments

Methods, vectors, and yeast strains used for the two-hybrid screen have been described (Gyuris *et al.*, 1993; Finley and Brent, 1994, 1995). A cDNA library prepared from *Drosophila* ovary cDNA in the yeast expression vector pJG4-5 was kindly provided by J. Großhans (Max Planck Institut für Entwicklungsbiologie, Tübingen, Germany). This library was transformed into a yeast strain (EGY48) carrying the cdk4/6-coding sequence in the expression vector pEG202. One hundred six transformants were selected on plates. After scraping the colonies of transformants into medium containing galactose to induce the synthesis of the fusion proteins encoded by the library plasmids, 107 colony-forming units were replated onto galactose medium lacking leucine. One hundred Leu^+ colonies were picked and assayed for the dependence of the Leu^+ phenotype on the presence of galactose. The galactose-dependent colonies were subsequently assayed for blue color development on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The library plasmids from 25 blue galactose-dependent Leu^+ colonies were isolated and assayed for the presence of inserts using a PCR assay with primers flanking the insertion site. The resulting PCR products were classified based on the restriction fragment patterns generated by *Hae*III and *Alu*I. Colonies which resulted in maximum levels of blue color development on the X-gal plates were found to fall into two distinct classes. Sequence analysis revealed *Drosophila* cyclin D sequences (Finley *et al.*, 1996) in one of these classes. To address the specificity of the *Drosophila* cyclin D-cdk4/6 interaction, we performed interaction mating assays (Finley and Brent, 1994). For these experiments, pEG202 constructs with *Drosophila* cdc2 and cdc2c cDNAs were kindly provided by R. Finley, Jr. Moreover, using PCR strategies, we made additional pJG4-5 constructs with *Drosophila* cyclin A and B cDNAs. Interaction mating was done as described by Finley and Brent (1994).

Northern Blots and in Situ Hybridization Experiments

$Poly(A)^+$ RNA was isolated form various developmental stages using a fast-track mRNA isolation kit (Invitrogen). This RNA was resolved on formaldehyde containing 1% agarose gels and transferred to GeneScreen Plus nylon membranes (DuPont, Wilmington, DE). Northern blots were probed with antisense probes generated by in vitro transcription in the presence of [γ-^{32}P]-CTP. As templates for the in vitro transcription, we used appropriately linearized plasmids containing the cdk5, PITSLRE, and PFTAIRE cDNAs. In the case of cdk4/6, we generated labeled antisense RNA with the fragment isolated in the PCR screen. In the case of rp49, which served as a loading control, we generated a probe by random primer labeling of plasmid DNA containing a rp49 cDNA fragment (O'Connell and Rosbash, 1984). Hybridizations were performed according to standard procedures (Sambrook *et al.*, 1989).

In situ hybridization to polytene chromosomes for the determination of the genomic localization of the *cdc2*-related genes was carried out as described by Courtot *et al.* (1992), and in situ hybridization to embryos for the analysis of the mRNA distributions was carried out as described by Knoblich *et al.* (1994). Antisense RNA probes were transcribed in vitro from the same templates that were used to generate the Northern blot probes. Sense probes transcribed from the same plasmids were used for control experiments.

Immunoblotting Experiments

For the isolation of anti-cdk5 antibodies, rabbits were immunized with a C-terminal peptide (CAEAAMQHPYFTDSTSSGH) coupled to keyhole limpet hemocyanin. This C-terminal cdk5 sequence was found in the cDNA clones isolated from the ovary library and in a genomic clone. The corresponding sequence in the cDNAs from the embryonic library revealed a single nucleotide exchange resulting in SSSSGH instead of STSSGH in the predicted C-terminal cdk5 sequence. This sequence difference presumably represents a strain-specific polymorphism.

The immune sera obtained after four booster injections in 4-week intervals were affinity purified using the immunogenic peptide coupled to a SulfoLink Coupling Gel (Pierce ImmunoPure Ag/Ab Immobilization kit 2). Embryo extracts from different developmental stages were prepared and used for immunoblotting experiments as described by Sauer *et al.* (1995).

RESULTS

Two *Drosophila* genes (*cdc2* and *cdc2c*) were identified using PCR and degenerate oligonucleotides primers deduced from conserved regions of cdc2 as described previously (Lehner and O'Farrell, 1990). Since the same approach led to the identification of 10 different *cdc2*-related genes in humans (Meyerson *et al.*, 1992), we analyzed the PCR products obtained in *Drosophila* more extensively. Moreover, we complemented our PCR strategy by probing a genomic library with a human cdk2 cDNA at low stringency. In addition to

the previously described genes, we identified four *Drosophila* genes encoding putative protein kinases with high similarity to cdc2. The putative amino acid sequences of these kinases were deduced by sequencing cDNA clones. Sequence comparisons indicated that the *Drosophila* kinases are most similar to vertebrate kinases.

The first of the *Drosophila* amino acid sequences is most similar (78% identity) to vertebrate cdk5 (Hellmich *et al.*, 1992; Lew *et al.*, 1992; Meyerson *et al.*, 1992; Kobayashi *et al.*, 1993; Gervasi and Szaro, 1995). An independent identification of the same *Drosophila cdk5* gene has been described while this work was in progress (Hellmich *et al.*, 1994).

The second *Drosophila* sequence that we have identified (Figure 1) is most similar to the vertebrate PCTAIRE kinase family (55–58% identity in the kinase domain) (Meyerson *et al.*, 1992; Okuda *et al.*, 1992). These PCTAIRE kinases as well as other cdc2-related kinases which have not yet been shown to associate with cyclin subunits were named according to the amino acid sequence in a characteristic region (Meyerson *et al.*, 1992; Grana *et al.*, 1994a,b; Brambilla and Draetta, 1994; Xiang *et al.*, 1994). The *Drosophila* sequence with maximal similarity to the vertebrate PCTAIRE kinases has a single amino acid exchange in this region (PFTAIRE instead of PCTAIRE).

The third *Drosophila* sequence (Figure 2) is most similar to the human PITSLRE kinase family (65% identity in the kinase domain) (Bunnell *et al.*, 1990; Kidd *et al.*, 1994; Xiang *et al.*, 1994). Finally, the fourth *Drosophila* sequence (Figure 3) is most similar to vertebrate cdk4 and cdk6 kinases (47% identity) (Hanks, 1987; Matsushime *et al.*, 1992; Meyerson *et al.*, 1992).

The extensive similarity to vertebrate sequences observed in the case of the first three *Drosophila* sequences strongly suggests that the corresponding kinases perform homologous functions. However, the similarity to vertebrate homologues was clearly less extensive in the case of the cdk4/6-like *Drosophila* kinase. To identify potential *Drosophila* genes with more extensive similarity to vertebrate cdk4 and cdk6, we probed a genomic library with human cdk4 and cdk6 cDNAs at low stringency. All of the 14 clones identified after screening several equivalents of the *Drosophila* genome were found to correspond to the previously identified putative *cdk4/6* gene. Since vertebrate cdk4 and cdk6 are known to bind specifically to D-type cyclins (Matsushime *et al.*, 1992; Bates *et al.*, 1994; Meyerson and Harlow, 1994), we determined whether the putative *Drosophila* cdk4/6 homologue can bind to D-type cyclins as well. We performed a two-hybrid interaction screen and found that several of the most strongly interacting clones identified in an ovarian cDNA library encoded the same *Drosophila* D-type cyclin that was described while this manuscript was in preparation (Finley *et al.*, 1996). The specificity of the *Drosophila* cyclin D-cdk4/6 interaction was further addressed by interaction mating experiments (Finley and Brent, 1994). *Drosophila* cyclin D was found to interact strongly with cdk4/6 and cdc2c and to a lesser extent with cdc2 according to these interaction matings (Figure 4). An analogous behavior, i.e., the preferred binding to cdk4, cdk6, and cdk2, has also been observed with vertebrate D-type cyclins (Xiong *et al.*, 1992; Bates *et al.*, 1994; Meyerson *et al.*, 1994). Although the mating assay revealed an interaction of *Drosophila* cdk4/6 and cyclin D, we could not

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  1 MATTTTTTTT TGATNAKDGV TMREKKGGAL QKLKKRLSHS FGRLTISRED GDESTHHHHH
    *

 61 HHHHRGGHGG HHNHGNKVPY NGYNSEEYLD RLEPNGNIPA DKTNCRYGDW RSTDSTDHHD

121 RVQRQLSVSS DSKLLDEDIR EEMKYHSHVV MRPKKPPRPK SEVFLNKQET HPRRKRFSAF
                 * *  ***                    *               **   *

181 GGDSPFGKQE AYVKLEPLGE GSYATVYKGF SKLTYQRVAL KEIRLQEEEG APFTAIREAS
    *    *** *  * **  *** * **** **  ****   *** *****  *** ** ***** *

241 LLKELKHSNI VTLHDIVHTR ETLTFVFEYC DTDLSQYMEK HPGGLDHRNV RLFLFQLLRG
    *** *** ** *****  **    ** ****  * ** **            **   *  * ***

301 LSYCHKRRVL HRDVKPQNLL ISDCGELKLA DFGLARAKSV PSHTYSHEVV TLWYRPPDVL
    * ***    * *** ****** *   ****** *********  *  *** *** ******** *

361 LGSTEYSTSL DMWGVGCIFV EMVTGMPTFP GIRDTYDQLD KIFKLLGTPT EDTWPGVTHF
    ***  * *    *******   **  * * ** *       *   **  ****     ***

421 PGYKPHKLGF YRPRKLGHNF PRLYDIIEGE TIANGFLQLN PEQRLGADDA LQHPYFAQLP
               *    *     ***     *        *       *  *  *   *  *  *

481 KKLYELPDET SIFTVEGVQL YTEPNRQNK
         *     ***     **
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Figure 1. Putative amino acid sequence of *Drosophila* PFTAIRE kinase, a homologue of vertebrate PCTAIRE kinases. The amino acid sequence deduced from a 3.5-kb PFTAIRE cDNA is shown. Stars below the sequence indicate positions identical in *Drosophila* PFTAIRE, human PCTAIRE 1 to 3 (Meyerson *et al.*, 1992), and mouse PCTAIRE 1 and 3 (Okuda *et al.*, 1992). The similarity between the *Drosophila* and vertebrate sequences is maximal within the kinase domain (186–509), but extends into the N-terminal region flanking the kinase domain, in particular when amino acid similarity in addition to identity is taken into account.

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  1 MVNSSGSEDG QLRSPNDVHY HSRGEEDEHE GDADALYIQP PQASRESGSG PRREKKKHSR
    *

 61 ERRRHKERED VGGAALALER DHRYDYRSRE EHYHHHQRER SSNAAAAYAK HHLGHAYHYP

121 QPPQQQQQPL PPAPSYAAHH YHHHQHLSGA RAAPREYHSY PSGYHSGSRH GDYPMEEPTR

181 RSSKYAESKD AESLEQDLRS RLLKKRHNYV KDYETEENYE HRAERSDRRE GGRKERERTV

241 RSTHKQNRHD RVIELLDSPE QEHHHQHQHK SHRSKWREEV EVSRRKVPED LELLARREKL

301 LAAERESRQR KQTAREELEA RRELLRERNE HSDALSPTTV AASVTAGLNI HVKRKSKPDN

361 YEKEIKLKKR REDDIEVIRD DDDEESEESD SNEEVPEQDS EGSATESGSE DSYASKKKSK

421 IKSKSQLEDD DEDLPLPDSP LSVGELYKSP KQRQRSRSVS SKSSSQSSRS SRSRSRSRSQ
           *      *  * *               *

481 SSLEDEVDRQ DAGADASPSS STRSEERGMT QEQPEEKPEE KLKEKQKSLE EQIPCDDKGI
                      * * *   *         *   * *  **

541 PLPNYYPGVQ GCRSVEEFQC LNRIEEGTYG VVYRAKDKRT NEIVALKRLK MEKEKEGFPI
     ** * *  * ***** **** ********** ******** *  ********* **** *****

601 TSLREINTLL KGQHPNIVTV REIVVGSNMD KIFIVMDYVE HDLKSLMETM KNRKQSFFPG
    ******** * * ******** ********** ** *** *** ********** *   * * **

661 EVKCLTQQLL RAVAHLHDNW ILHRDLKTSN LLLSHKGILK VGDFGLAREY GSPIKKYTSL
    *** *  *** * * ****** ********** ***** **** ********** *** * **

721 VVTLWYRAPE LLLCSPVYST PIDVWSVGCI FAEFLQMLPL FPGKSEIDEL NRIFKELGTP
    *** ****** ***    ***   * ****** * * *   ** *** ****   *  ** ****

781 NEKIWPGYTE LPAVKNMLSQ NSQFTEYPVS QLRKHFQEKT SEMGLSLLQG LLTYDPKQRL
     ******* * ** ** *       *   *    *** *     *  *  *     *** *  *

841 SADAALKHGF FKELPLPIDP SMFPTWPAKS ELGARKAQAS SPKPPSGGSQ FKQLGRDEPI
    **   ***   * * ****** ********** *   *      ** ** **     *** *

901 IVGPGNKLSS GIITGNKKSH GAGGSSASTG FVLNAGITQR QLAMGPGFSL KF
           *                      ** * *     *    * ****** **
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Figure 2. Putative amino acid sequence of *Drosophila* PITSLRE kinase. The amino acid sequence deduced from a 3.2-kb PITSLRE cDNA, which was isolated from an early embryonic cDNA library and thus presumably corresponds to the major maternally derived transcript (see Figure 5A, transcript 1) is shown. Stars below the sequence indicate positions identical in *Drosophila* PITSLRE and in human PITSLREa1, PITSLREa2-1, PITSLREa2-2, PITSLREa2-3, PITSLREa2-4, PISLREb1, PITSLREb2-1, and PITSLREb2-2 (Kidd *et al.*, 1994; Xiang *et al.*, 1994). The complex family of human PITSLRE kinases is encoded by a complex locus composed of three genes giving raise to multiple protein products encoded by differentially spliced transcripts (Kidd *et al.*, 1994; Xiang *et al.*, 1994). The amino acid sequence differences among the various human kinases are essentially restricted to the region N-terminal of the kinase domain (560–952 in *Drosophila* PFTAIRE kinase). The N-terminal regions in many of these different kinases are characterized by highly basic or highly acidic stretches. Similar stretches are also found in the *Drosophila* PITSLRE sequence (underlined).

observe an interaction of cdk4/6 with the *Drosophila* cyclins A and B, whereas these cyclins interacted with cdc2 kinase as expected (Figure 4). Our observations, therefore, strongly argue that we have identified a cdk4/6 homologue in *Drosophila*.

We analyzed the expression of the cdc2-related *Drosophila* kinases during development by Northern blot experiments. The most complex profile of transcripts was detected with the *Drosophila* PITSLRE probe (Figure 5A). At least five different transcripts (3.6, 4.4, 4.9, 5.8, and 7.1 kb) were detected on Northern blots. Total transcript levels correlated well with mitotic proliferation during embryogenesis. Maximum levels were found in early embryos (Figure 5A, lanes 1 and 2) where proliferation is exponential and extremely rapid. Minimal expression was detected in late embryos (Figure 5A, lane 4) where cell proliferation occurs only to a very limited extent in few tissues. The 3.3-kb PITSLRE clone that we have sequenced (Figure 2) was isolated from a 0- to 4-h embryo cDNA library and therefore corresponds presumably to the 3.6-kb transcript which is the major maternally derived transcript during these stages. The transcript patterns revealed by probes derived from the other cdc2-related *Drosophila* genes were simple. Single transcripts (a 1.6-kb cdk5 transcript, a 3.4-kb PFTAIRE transcript, and a 1.2-kb cdk4/6 transcript) and limited variations in abundance at different developmental stages were observed (Figure 5, C–E).

To determine the spatial pattern of expression during embryogenesis, we performed in situ hybridization experiments with antisense RNA probes. Sense RNA probes were used for control experiments. In all cases, maternal transcripts were found to be distributed throughout the early embryos. Also during subsequent embryogenesis, we observed a low level of

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  1 MSYVRQLKRQ KMSQAKKFGD GDPFNYQELN IIGEGAYGTV YRARDVITGN IVALKKVRIS
    *              *           *      ** **** *   ***   *   **** **

 61 LNENGVPMST LREISLLKQL NASNHANIVK LYEVCQFLER DGQLLILLAF EHVEQDLSDL
        * * **  **   *  *     * * *  *  **      *      * * **  ***

121 IDRLPKSGMS PPTIQRLSRE LLTGVDFLHS HRIIHRDLKP QNLLVSSQGH LKIADFGLAK
     *  *  *     **        * * ****      ******  * ** * *   * ******

181 TYGSEMKLTS VVVTLWYRAP EVLLAQPYNS TVDIWSAACI IFEMFNRRAL FPGTSEKNQL
     *   * **  ********** ****   *    ** **  **   *** *  * * * *   **

241 DRIFELTGRP TEQQWPQTIS VALEHFPQRH PKRPKDFCPH LCKYADDLLN KMLSYDLHLR
      *    * *  *  **          *                       **    *      *

301 PSALACLEHD YFQQEPL
     **   * *  *
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Figure 3. Putative amino acid sequence of *Drosophila* cdk4/6 kinase. The amino acid deduced from a 1.1-kb cdk4/6 cDNA is shown. Stars below the sequence indicate positions identical in *Drosophila* cdk4/6 and human cdk4 and cdk6 kinase (Matsushime *et al.*, 1992; Meyerson *et al.*, 1992; Bates *et al.*, 1994; Meyerson and Harlow, 1994).

signal throughout the embryo. In addition to these ubiquitous signals, we observed somewhat stronger signals in the CNS of late embryos after germ band retraction with the cdk5 probe and the PITSLRE probe. With the cdk4/6 probe, we observed somewhat stronger signals in internal tissues of germ band-retracted embryos in a pattern correlated with endoreduplication in these tissues (Smith and Orr-Weaver, 1991). The most extensive modulation of signal intensity above the general basal signal was observed with the PFTAIRE probe (Figure 6). With this probe, we observed transient strong signals in a number of cell types. These strong signals were apparent predominantly in cells actively rearranging cell-to-cell contacts: in the anterior and posterior midgut cells during gastrulation (Figure 6A), in tracheal precursors during invagination (Figure 6C), most likely in myoblasts during fusion, and in discrete cells of the CNS (Figure 6D) that might be migrating glial cells.

The presence of high levels of cdk5 transcripts throughout the *Drosophila* embryo was unexpected, since murine cdk5 protein and, in particular, cdk5 kinase activity have been described to be limited to postmitotic neurons (Tsai *et al.*, 1993). With the help of an antibody raised against a C-terminal peptide of *Drosophila* cdk5, we were able to detect constant levels of cdk5 protein by immunoblotting (Figure 7A), in contrast to cdc2 and cdc2c kinase where lower protein levels were observed late in embryogenesis (Figure 7, B and C). The immunoblot result demonstrates that *Drosophila* cdk5 is present already in early embryos long before the onset of neuron differentiation. We have also tried to assay for the presence of histone H1 kinase activity in immunoprecipitates obtained with the anti-C-terminal antibodies. Histone H1 is known to be a substrate for vertebrate cdk5 isolated from brain (Tsai *et al.*, 1993). Nevertheless, we were unable to detect histone H1 phosphorylation above background. We do not know whether cdk5 kinase has very low activity or whether our antibody is unable to precipitate active complexes.

For future genetic analysis, we determined the chromosomal localization of the *cdc2*-related genes. In situ hybridization to polytene chromosomes localized the *cdk4/6* gene to 53C (Figure 8A), the *PFTAIRE* gene to 63E (Figure 8B), the *PITSLRE* gene to the chromosomal region 77E (Figure 8C), and the *cdk5* gene in agreement with results reported by Hellmich *et al.* (1994) to 52A. The *cdk5* gene, therefore, is located in a region of the *Drosophila* genome that is required for DNA replication during embryogenesis (Smith *et al.*, 1993). The

Figure 4. Characterization of the cdk4/6 interactions by two-hybrid experiments in yeast. Strains containing plasmids that allow expression of *Drosophila* cdc2 (cdc2), cdc2c (cdc2c), and cdk4/6 (cdk4/6) fusions to a DNA-binding domain were mated with strains containing plasmids that allow expression of *Drosophila* cyclins D (D), B (B), and A (A) fusions to a transcriptional activation domain (Finley and Brent, 1994). Strains containing the corresponding vectors without inserts (V) were included for control. Interactions were analyzed on X-gal containing indicator plates containing galactose, which results in the expression of the cyclin fusion proteins. Interaction of cyclin and cdk fusion proteins results in blue color development on these plates, which leads to darkening of the yeast patches in the black and white representation. No blue color development was observed on indicator plates with glucose instead of galactose, which represses the expression of the cyclin fusion proteins. Strains with the cyclin A plasmids grew very slowly even in the absence of galactose and growth after mating with the strain containing the cdc2c plasmid failed completely.

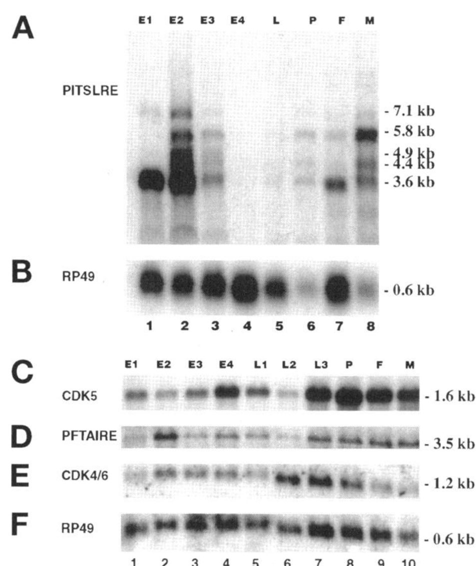

Figure 5. Expression of *Drosophila* cdk4/6, cdk5, PITSLRE, and PFTAIRE. poly(A)$^+$ RNA isolated from different developmental stages was analyzed on Northern blots with probes derived from *Drosophila* PITSLRE (A), cdk5 (C), PFTAIRE (D), cdk4/6 (E), or from the *rp49* gene (B and F) which encodes a ribosomal protein (O'Connell and Rosbash, 1984) and was used to control for equal loading. The poly(A)+ RNA resolved for the blot shown in A and B was isolated from 0- to 2-h embryos (lane 1, E1), 2- to 6-h embryos (lane 2, E2), 6- to 14-h embryos (lane 3, E3), 14- to 22-h embryos (lane 4, E4) larvae (lane 5, L), pupae (lane 6, P), adult females (lane 7, F), and adult males (lane 8, M). The poly(A)$^+$ RNA resolved for the blot shown in C–F was isolated from 0- to 2.5-h embryos (lane 1, E1), 2.5- to 8.5-h embryos (lane 2, E2), 8.5- to 16-h embryos (lane 3, E3), 16- to 22-h embryos (lane 4, E4), first instar larvae (lane 5, L1), second instar larvae (lane 6, L2), third instar larvae (lane 7, L3), pupae (lane 8, P), adult females (lane 9, F), or adult males (lane 10, M). Only the relevant parts of the filters which were probed sequentially with the different probes are shown. Transcript sizes are indicated on the right side.

ubiquitous distribution of *cdk5* observed during embryogenesis and its extensive evolutionary conservation appeared to be consistent with a functional involvement in DNA replication. However, by analyzing chromosomes carrying various deficiencies in the cdk5 region (Figure 8D and see MATERIALS AND METHODS for details), we found that the *cdk5* gene is not deleted by the two deficiencies (*Df(2R)XTE11* and *Df(2R)l13)*, which result in the embryonic DNA replication defect. Moreover, cdk5 is deleted by *Df(2R)WMG* which is not associated with an embryonic DNA replication phenotype. Therefore, we can conclude that the embryonic DNA replication defect described by Smith *et al.* (1993) is not caused by a loss of cdk5 function.

DISCUSSION

The identification and functional characterization of cdc2 kinase, first in yeast and subsequently in a wide

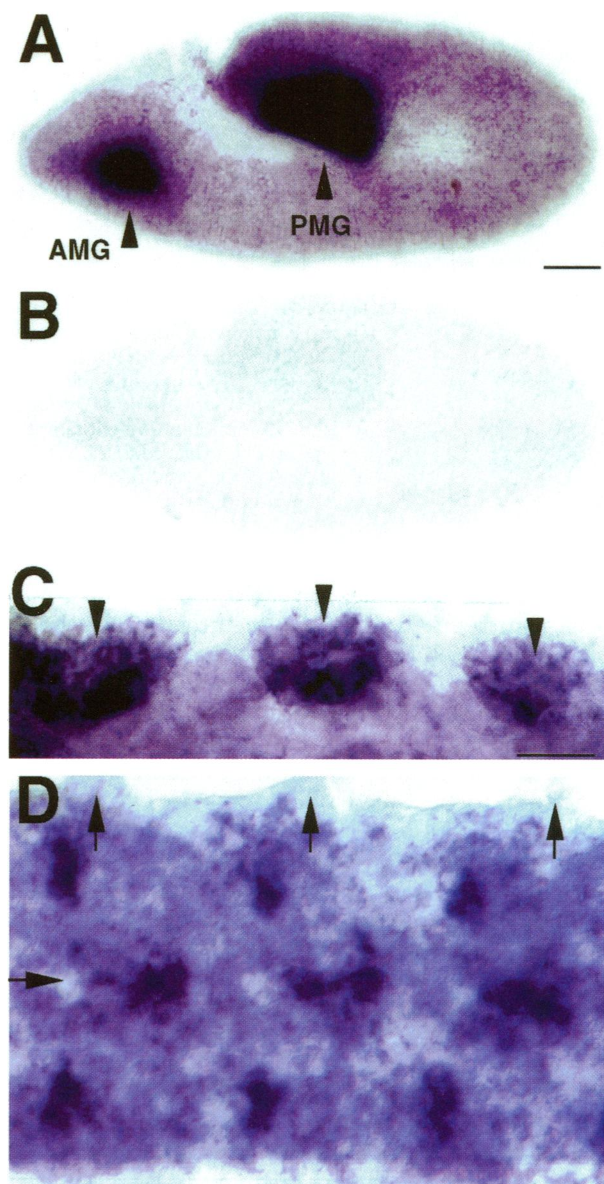

Figure 6. Spatial distribution of PFTAIRE transcripts during embryogenesis. The distribution of PFTAIRE transcripts during embryogenesis was analyzed by whole-mount in situ hybridization. Control experiments with a sense RNA probe (B) demonstrated the specificity of the signals obtained with the antisense RNA probe (A, C, and D). PFTAIRE transcripts were found to be present ubiquitously at low levels. In addition to this low level expression, we observed a dynamic pattern of maximal expression in the region of the anterior midgut (AMG) and posterior midgut (PMG) during stage 9 (A), in the invaginating tracheal precursors (arrowheads) during stage 11 (C), and in cells of the central nervous during stage 14 (D). Embryos at the same developmental stage are shown in A and B. High magnification views of optical sections show the tracheal invaginations (C) or the CNS (D) from three abdominal segments. The horizontal arrow in D indicates the ventral midline and the vertical arrows the segmental nerves. Bars in A and C correspond to 50 μm and 25 μm, respectively.

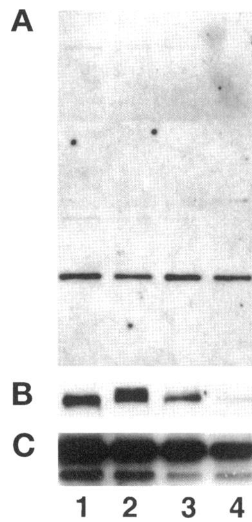

Figure 7. *Drosophila* cdk5 protein levels are constant during embryogenesis. Total extracts from 0- to 2-h (lane 1), 2- to 6-h (lane 2), 6- to 14-h (lane 3), and 14- to 22-h embryos (lane 4) were analyzed by immunoblotting with antibodies against *Drosophila* cdk5 (A), cdc2 (B), or cdc2c (C). Only the relevant regions are shown in B and C.

range of eukaryotes, has revealed a universal control mechanism regulating the G_2-M transition of the cell cycle (Nurse, 1990). This cell cycle transition is usually not influenced by extracellular conditions in most cell types. In contrast, progression through the G_1 phase is profoundly influenced by extracellular conditions in many cells. Since adaptation to external conditions is considered to be the driving force in evolution, G_1 regulation can be expected to be less conserved than the G2-M regulation. Our characterization of the cdc2-related kinase family in *Drosophila* combined with the results obtained in other species provides insights into the molecular conservation in the regulation of different cell cycle steps and is consistent with this idea.

The family of cdc2-related kinases in humans includes, in order of decreasing similarity to cdc2 (cdk1), the kinases named cdk2, cdk3, cdk5, PCTAIRE, PITSLRE, cdk7, cdk6, cdk4, PISSLRE, KKIALRE, PITALRE, and cdk8 (Meyerson *et al.*, 1992; Brambilla and Draetta, 1994; Grana *et al.*, 1994a,b; Levedakou *et al.*, 1994; Xiang *et al.*, 1994; Tassan *et al.*, 1995; Yen *et al.*, 1995), with the last four most distantly related members being clearly out of reach of the search strategies (PCR Experiments and Low Stringency Screens) that we have applied. Our strategies have identified *Drosophila* homologues for most of the human cdc2 family members and the extent of similarity is summarized in Figure 9. A *Drosophila* cdk7 as well as its partner cyclin H have been identified with alternative approaches and will be described elsewhere (Sigrist, Lehner, and Léopold, unpublished observation; Léopold, unpublished observation). Cdk8 and its partner cyclin C have also been identified in both *Drosophila* and humans (Lahue *et al.*, 1991; Leopold and O'Farrell, 1991; Lew *et al.*, 1991; Tassan *et al.*, 1995; Leclerc *et al.*, 1996). Although the conservation between *Drosophila* and human cyclin/cdk complexes is maximal in the case of

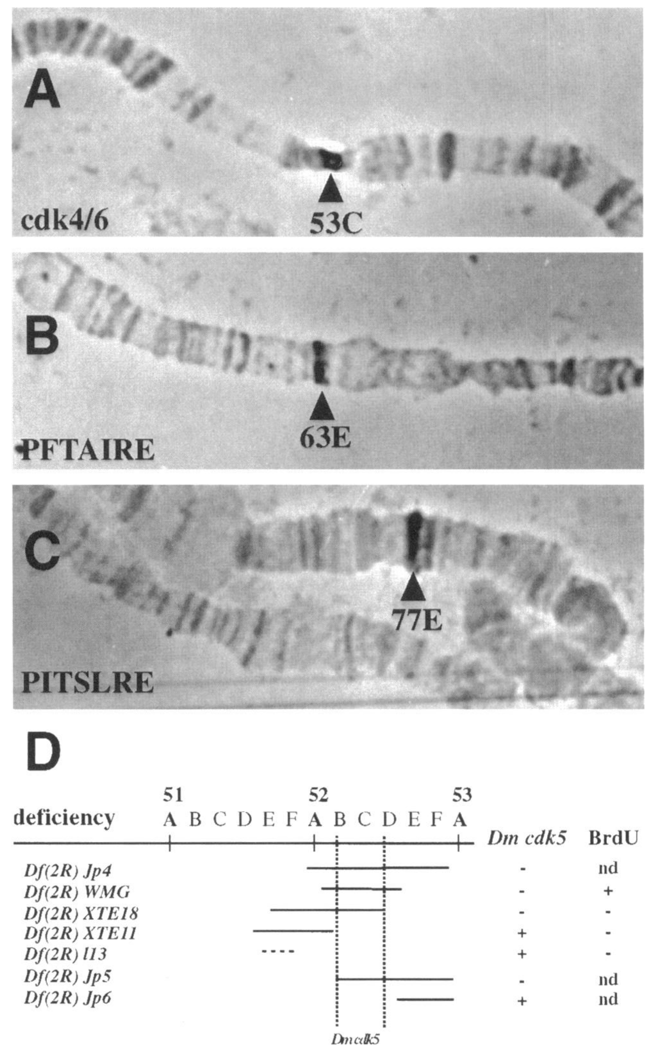

Figure 8. Chromosomal localization of *Drosophila* PFTAIRE, PITSLRE, cdk4/6, and cdk5. (A–C) Probes derived from *Drosophila* cdk4/6 (A), PFTAIRE (B), or PITSLRE (C) were used for in situ hybridization experiments with polytene chromosomes. The resulting chromosomal localization is indicated by arrowheads. (D) The presence (+) or absence (−) of cdk5 on various chromosomes carrying deficiencies in the chromosomal interval 52 as revealed by PCR experiments is listed (see column *Dm cdk5*) on the right of the scheme in which deleted regions are indicated by black lines. *Df(2R)l13* is not visible cytologically, and the deleted regions, as determined genetically, are indicated by - - -. The data in the case of *Df(2R)Jp5* and *Df(2R)Jp6* are from Hellmich *et al.* (1994). According to the description of the deficiency breakpoints (see MATERIALS AND METHODS), therefore, the *Dm cdk5* gene must be located between 52A13-14 and 52C9-D11. A locus required for progression through the S phase was identified within a similar chromosomal region by analyzing bromodeoxyuridine (BrdUrd) incorporation in late homozygous-deficient embryos (Smith *et al.*, 1993). Deficiencies which result in a failure of bromodeoxyuridine incorporation are indicated in the column BrdUrd by −, deficiencies which allow BrdUrd incorporation by +, and deficiencies that have not been analyzed by nd. The fact that *Df(2R)WMG* deletes *Dm cdk5* and yet does not interfere with BrdUrd incorporation in late embryos indicates that the BrdUrd incorporation defect described by Smith *et al.* (1993) is not caused by a loss of cdk5.

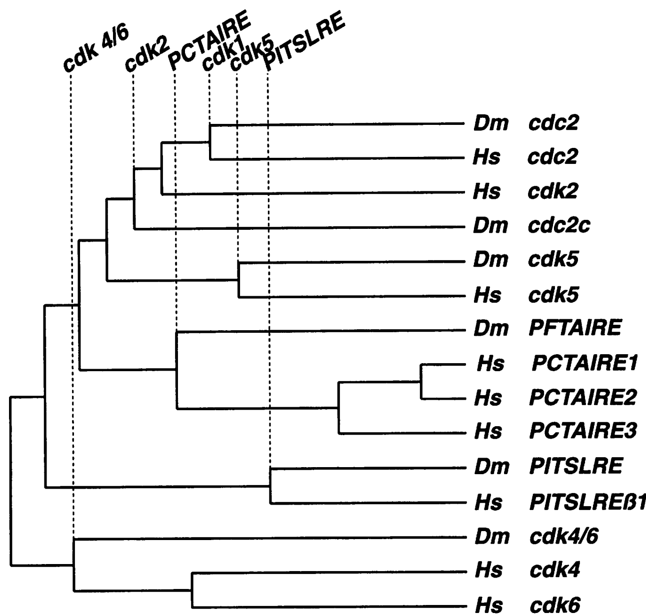

Figure 9. The structural similarity of cdc2-related kinases in *Drosophila* and humans. The putative full-length protein sequences of the known human and *Drosophila* kinases with high similarity to cdc2 were compared using CLUSTAL of the PCGene DNA analysis software package. The horizontal distance from the right end of the lines in the dendrogam is inversely correlated to the similarity score. The extent of sequence conservation observed for the different cdc2-related kinases is indicated by - - - and is minimal in the case of cdk4/6 and maximal in the case of PITSLRE (see top).

cyclin C/cdk 8, it is evident that cyclin C/cdk8 has minimal similarity to other known cyclin/cdk complexes.

Extensive conservation in higher eukaryotes is also observed in the case of cdk5 where conservation is clearly more extensive than in the case of cdc2 (cdk1). Although most cdks have been implicated in cell cycle regulation, murine cdk5 has been proposed to function exclusively in postmitotic neurons (Tsai *et al.*, 1993). Our results indicate that the *Drosophila* cdk5 protein is present in all cells, at least in early embryos. This ubiquitous presence in combination with the extensive conservation suggests that cdk5 may not only function in postmitotic neurons but could have a more general cellular function in proliferating cells as well. Consistent with this idea, cdk5 is found in all vertebrate cell lines tested (Tsai *et al.*, 1993). However, a cdk5 homologue has not been described in yeast so far.

Homologues from yeast to plants and humans have only been described in the case of cdc2 (cdk1). However, in both budding and fission yeast, cdk1 appears to provide all of the functions that are provided by several specialized cdks in higher eukaryotes. Although cdk1 is clearly involved in the regulation of the G_2-M transition in all eukaryotes, it has been implicated in the control of progression through the G_1 phase and entry into the S phase only in yeast. The function provided by yeast cdk1 early in the cell cycle during G_1 and G_1-S transition is thought to be provided by cdk2, cdk4, and cdk6 in vertebrates. These latter kinases are clearly less well conserved in higher eukaryotes than cdk1.

Based on primary sequence comparison alone, it is not possible to identify the *Drosophila cdc2c* gene unambiguously as the homologue of vertebrate cdk2. The cdc2c sequence is only marginally more similar to vertebrate cdk2 than to vertebrate cdc2. However, biochemical and genetic analyses have shown that *Drosophila* cdc2c, like vertebrate cdk2, binds to cyclin E and regulates the G_1-S transition (Knoblich *et al.*, 1994; Sauer *et al.*, 1995). In our extensive screens, we have failed to identify a *Drosophila* gene that has more extensive similarity to vertebrate cdk2 than cdc2c. Based on all of these observations, cdc2c appears to be the *Drosophila* cdk2 homologue. Potential cdk2-like kinases have been identified in a number of plant, nematode, and lower eukaryote species. However, none of these kinases can be classified as a cdk2 homologue without additional functional analysis.

cdk4 and cdk6 which associate with D-type cyclins in vertebrates apparently show the lowest conservation among the cdc2-related kinases. The only *Drosophila* gene that we have identified in our low stringency screens with human cdk4 and cdk6 probes shows even less similarity to the vertebrate counterparts than what is observed in the case of *Drosophila* cdk2. Nevertheless, we consider this *Drosophila* kinase to be a cdk4/6 homologue because we find that it can associate with a *Drosophila* D-type cyclin. Obviously, this interaction which we have observed in yeast two-hybrid experiments remains to be demonstrated in vivo as well. D-type cyclin-dependent kinases are thought to inhibit the growth-inhibitory action of the retinoblastoma tumor suppressor protein by phosphorylating this protein substrate late in G_1 and thereby causing the release of E2F/DP transcription factor activity. The identification of cyclin D/cdk4/6 (Finley *et al.*, 1996 and this study), a retinoblastoma family member (Du *et al.*, 1996) and E2F/DP (Dynlacht *et al.*, 1994; Ohtani and Nevins, 1994; Hao *et al.*, 1995) in *Drosophila* indicates that this regulatory pathway has been conserved in higher eukaryotes. It might exist in plants as well, since putative D-type cyclins have been described in several plant species (Dahl *et al.*, 1995; Soni *et al.*, 1995).

Therefore, the comparison of cell cycle regulators from yeast, *Drosophila*, and vertebrates indicates that the conservation is extensive only in the case of mitotic regulators. Although it is attractive to speculate that higher eukaryote cdk2, in association with cyclin E and cyclin A, fulfills the same function in S phase regulation as budding yeast cdk1 in association with

Clb5 and 6, it is clear that these yeast cyclins are more closely related to the other B-type cyclins of budding yeast (Clb1-4) than to higher eukaryotes cyclins A and E. It remains to be analyzed whether the S phase regulators of lower and higher eukaryotes (cdk1/Clb 5/6 and cdk2/cyclins A/E) act on the same substrates. Moreover, although higher eukaryote D-type cyclins display clear functional analogies with budding yeast Cln cyclins, the present evidence indicates that they are almost certainly not homologous. Although both D-type and Cln cyclins control progression through the G_1 phase in response to extracellular conditions (nutrients, pheromones in budding yeast, growth factors in higher eukaryotes), they have minimal structural similarity and function with structurally different cdk partners in apparently unrelated pathways.

Apart from cdks, we have also identified *Drosophila* homologues of cdc2-related kinases which have not been shown to associate with cyclins. The *Drosophila* PFTAIRE and PITSLRE kinases appear to be homologues of the vertebrate PCTAIRE and PITSLRE kinase families, respectively. Three different genes have been described in both of these human kinase families, and alternative splicing in the case of the *PITSLRE* genes potentiates the product complexity dramatically (Meyerson *et al.*, 1992; Okuda *et al.*, 1992; Kidd *et al.*, 1994; Xiang *et al.*, 1994). The functional significance of these kinases is poorly understood. Some of the PITSLRE kinases have been implicated in apoptosis (Lahti *et al.*, 1995). The identification of homologues in *Drosophila* (and by the *Caenorhabditis elegans* sequencing project in the case of PITSLRE, accession number U21317), where the complexity of these families appears to be greatly reduced should allow a future genetic characterization of these strongly conserved kinases.

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