A novel cyclin gene from *Drosophila* complements *CLN* function in yeast

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In the yeast *Saccharomyces cerevisiae* three G₁-S cyclins, or CLNs, have been identified that trigger the G₁-S transition of the cell cycle. The regulation of the G₁-S transition is particularly intriguing in *Drosophila* both because G₁ is added to the cell cycle developmentally and G₁-S regulators may drive the polytene cell cycle. To identify potential G₁-S regulators from *Drosophila*, a cDNA expression library was constructed in which Kc cell cDNAs were placed in a high-copy *S. cerevisiae* vector under the control of the constitutive *ADH1* promoter. Following transformation into an *S. cerevisiae* strain lacking all three CLN gene products, we identified one *Drosophila* cDNA that complemented the yeast G₁ cyclins and restored growth to near wild-type levels. The *CLNDm* gene is present as a single copy in the *Drosophila* genome and encodes a 1.2-kb mRNA. DNA sequence analysis reveals that although this gene has cyclin homology, it is a new member of the cyclin gene family. *CLNDm* mRNA expression correlates with periods of maximal cell division throughout *Drosophila* development. The transcript is most abundant in early embryos, and it is present in low levels in larvae, pupae, and adults. *Drosophila* embryos hybridized in situ to this cyclin gene show uniform expression of the message throughout the embryo, with diminishing expression as embryogenesis proceeds.

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During *Drosophila* development the cell cycle undergoes several dramatic changes (Fig. 1). The early cleavage divisions of embryogenesis are controlled by maternal components and rapidly oscillate between S phase and mitosis without detectable intervening gap (G) phases (Foe and Alberts 1983; Campos-Ortega and Hartenstein 1985). Following cellularization and blastoderm formation, at ~3 hr after fertilization, a zygotically regulated G₂ phase is added to the cell cycle (Edgar and O'Farrell 1989). This is followed by three postblastoderm mitotic divisions that occur in spatially and temporally regulated domains (Foe 1989). In at least the first two of these (cycles 14 and 15), S phase follows mitosis almost immediately, such that if a G₁ phase is present it must be <5 min in length (Edgar and O'Farrell 1990). After cycle 16, either a G₁ phase is added to the cell cycle or an existing G₁ is lengthened greatly, as evidenced both by a gap prior to S phase and by the detection of cells with a 2C DNA content (Edgar and O'Farrell 1990; Smith and Orr-Weaver 1991). During the latter half of embryogenesis, cells destined to compose larval tissues enter a modified cell cycle, the endo cell cycle, in which S phase alternates with a gap phase without mitosis, a sequence leading to polyteny (Smith and Orr-Weaver 1991). Dur-

ing the latter half of embryogenesis, only the neural cells continue in a mitotic cycle with G₁, S, G₂, and M phases.

Drosophila genes involved in regulating the entry into mitosis have been identified, and they illustrate the extreme evolutionary conservation of cell cycle control. In a number of organisms the G₂-M transition has been shown to be governed by maturation-promoting factor (MPF), a complex of the *cdc2* and cyclin proteins with serine-threonine kinase activity (for review, see Lewin 1990). *Drosophila* homologs to cyclin A and B have been cloned and, in the case of cyclin A, demonstrated genetically to be essential for mitosis (Lehner and O'Farrell 1989; Lehner and O'Farrell 1990b; Whitfield et al. 1990). The *Drosophila string* gene, a homolog of the *Schizosaccharomyces pombe cdc25* gene (a positive regulator of the *cdc2* protein kinase), is the critical regulator responsible for triggering entry into mitosis during the three postblastoderm divisions of embryogenesis (Edgar and O'Farrell 1989, 1990). A second gene capable of rescuing *S. pombe cdc25* mutants has been isolated, and it has been subsequently shown to be a *Drosophila cdc25* homolog (Jimenez et al. 1990; L. Alphey and D. Glover, pers. comm.). Two *cdc2* homologs have been identified in *Drosophila*, only one of which complements the *S. pombe* mutant (Jimenez et al. 1990; Lehner and O'Farrell 1990a). One or both of these could be targets of the two *Drosophila cdc25* genes.

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Figure 1. Regulation of the cell cycle in *Drosophila* embryogenesis. A time line for the morphogenetic events of embryogenesis is shown for the first two-thirds of embryogenesis, until cuticle deposition at 15 hr postfertilization. The types of cell cycle occurring at each stage of embryogenesis are shown schematically below. Prior to cellular blastoderm 13, rapid divisions occur in which S and M phases alternate without G phases. After cellularization, a G_2 phase is added. The parenthesis indicates that if a G_1 phase is present it is very short. From 6 hr of embryogenesis on, only the cells of the nervous system (NS) divide, by using a cell cycle with G_1 , S, G_2 , and M phases. During the latter half of embryogenesis the majority of cells are in an endo cell cycle in which S phase alternates with a G phase.

Regulators of the G_1 -S transition and the entry into S phase have not been identified in *Drosophila*. The *cdc2* gene has been shown in the yeasts *S. pombe* and *Saccharomyces cerevisiae* to regulate both the G_1 -S and G_2 -M transitions [Nurse and Bissett 1981; Piggott et al. 1982; Reed and Wittenberg 1990]. Therefore, it is likely that at least one of the two *Drosophila cdc2* homologs will function in controlling entry into S phase, but this remains to be established. The regulation of the G_1 -S transition is a particularly interesting problem in *Drosophila*, as a G_1 phase is added to the cell cycle, or greatly lengthened, during embryogenesis in response to developmental signals. The identification of *Drosophila* G_1 -S regulators will facilitate understanding how this alteration of G_1 occurs. In addition, because the endo cell cycle (S-G) is independent of G_2 -M *string* control, it is of interest to determine whether this novel cell cycle is also independent of G_1 -S regulators [Smith and Orr-Weaver 1991].

In *S. cerevisiae* the *CDC28* gene, the homolog of *cdc2*, controls the G_1 -S transition by interacting with three redundant G_1 -specific cyclins, *CLN1*, *CLN2*, and *CLN3* [Richardson et al. 1989]. The CLN cyclins act to regulate the G_1 -S transition because strains mutated for all three CLN genes arrest in G_1 . Furthermore, dominant, gain-of-function alleles of *CLN3* result in precocious entry into S phase [Cross 1988; Nash et al. 1988]. The levels of the CLN1 and CLN2 transcripts and proteins vary with the cell cycle and are maximal in G_1 . Although the CLN3 transcript does not change with the cell cycle, presumably the activity of the CLN3 protein is modulated [Wittenberg et al. 1990]. Thus, the levels or activity of the CLN proteins appear to trigger the G_1 -S transition, most likely by the association of these proteins with the CDC28 protein to form an active kinase. The CLN2 protein has been shown to associate with the CDC28 protein in an active kinase complex [Wittenberg et al. 1990].

The recent isolation of four G_2 -M cyclins from *S. cerevisiae* [Ghiara et al. 1991; Surana et al. 1991] implies that although *CDC28* is responsible for both the G_1 -S and G_2 -M transitions, its specificity is dictated by associated cyclin proteins.

The isolation of G_1 -S regulators is important for the investigation of cell cycle regulation in *Drosophila*. We chose to isolate *Drosophila* homologs to the CLN genes by functional complementation because this has the stringent criterion that the complementing genes are capable of conducting the same biological function. Nevertheless, the possibility remains that the properties exhibited by a *Drosophila* gene in the yeast cell may not accurately reflect its true function in the fly. Transformation of an *S. cerevisiae* strain mutated for all three CLN genes with a cDNA expression library yielded a novel *Drosophila* cyclin gene that complements the CLN function. This cyclin gene is expressed maximally during developmental periods when cell division is occurring.

Results

Isolation of a functional CLN homolog from *Drosophila*

Many cell cycle regulators are evolutionarily conserved and can complement the same function in heterologous species. For example, the human *cdc2* gene complements *cdc2*⁻ *S. pombe* mutants [Lee and Nurse 1987]. We used such an approach to search for possible G_1 cyclins in *Drosophila* by screening for *Drosophila* cDNAs capable of complementing *cln*⁻ deficiencies in *S. cerevisiae*. During the *Drosophila* life cycle, cells that utilize a cycle with both G_1 and G_2 phases make up only a small percentage of the total cell population. We therefore constructed the cDNA library using mRNA obtained from actively dividing *Drosophila* Kc cells. Kc cells are an embryonically derived cell line but utilize a cell cycle that contains both a G_1 and a G_2 phase [Echalier 1976], thereby increasing the chances of isolating G_1 regulatory factors.

We screened for rescue of growth of an *S. cerevisiae* strain mutated for the CLN genes by transforming the strain with the *Drosophila* Kc cell cDNA library that had been constructed in a yeast expression vector (Fig. 2). Loss of all three *S. cerevisiae* CLN genes is lethal; therefore, the viability of the host strain [884-21B[pFC204]] lacking all three CLN genes (*cln1*, *cln2*, *cln3*) was sustained by the presence of the CLN3 gene on a *URA3*-marked *CEN* plasmid. After transformation of this strain with the cDNA library, the *URA3*-marked CLN3 plasmid was removed by the plasmid shuffle technique using 5-fluoro-orotic acid (5-FOA) selection [Boeke et al. 1987], and the colonies that retained the ability to grow without the CLN3 gene were selected. Cells lacking the CLN3 plasmid should grow only if they contain a *Drosophila* gene capable of complementing the G_1 -S defect of the *cln* triple mutant. To compensate for the possibility that the *Drosophila* gene would complement the yeast

Figure 2. Complementation strategy for cloning *Drosophila* CLN homologs. The *Drosophila* Kc cell cDNAs were cloned into an expression vector in the *Bst*XI site downstream of the yeast *ADH1* promoter to overexpress the cDNA (Becker et al. 1991). This vector is maintained at high copy in yeast by the 2μ origin of replication and also contains *ARG4* as a selectable marker. This cDNA library was then transformed into a triply deleted *cln*[−] strain, in which growth was rescued by the presence of *CLN3* on a *URA3*-marked plasmid. Independent cDNA transformants (2×10^5) were selected on medium lacking arginine. These transformants were replica plated onto medium containing 5-FOA to select for loss of the *URA3*-marked plasmid. Any colonies that grew on 5-FOA were assumed to grow because the *CLN3* gene was replaced by a functional *Drosophila* homolog. These positive growers were then analyzed by Southern blot to verify loss of the *CLN3* gene.

CLN function only poorly, the library was cloned into an overproducing vector. This vector, pBL15, utilizes the strong and constitutive yeast *ADH1* promoter, and it contains a high-copy 2μ origin as well as the selectable marker *ARG4*.

The Kc cell library was transformed into 884-21B[pFC204], and 2×10^5 Arg⁺ transformants were obtained. These transformants were replica plated onto 5-FOA to select for loss of the *URA3*-marked *CLN3* plasmid. We obtained 265 Ura[−] colonies. To ensure that these colonies had lost the pFC204 plasmid, total DNA prepared from each of these strains was analyzed by Southern blots. Only one of these colonies had lost the *CLN3*-containing plasmid. The rest appeared to have mutated or deleted the *URA3* marker.

DNA from the single positive isolate, temporarily designated *CLNDm* until assigned to a cyclin class, was electroporated into *Escherichia coli* to recover the cDNA-containing plasmid. The transformants contained only one plasmid, with an insert size of ~1.1 kb. The purified *CLNDm* plasmid was retested for ability to complement *CLN* function by transforming strain 884-21B[pW16]. This strain, isogenic with 884-21B, contains a plasmid with the yeast *CLN3* gene under the control of the *GAL1* promoter, so the strain is able to grow on galactose but not on medium containing glucose. However, the *CLNDm* transformants of this strain grew quite well on medium containing glucose. Thus, under two different selection criteria, the *CLNDm* gene complemented the *cln*[−] deficiency.

To verify that the cDNA insert was derived from *Drosophila*, we probed a Southern blot of genomic *Drosophila* DNA with the *CLNDm* insert. Five different restriction digests of *Drosophila* DNA hybridized to *CLNDm* in patterns indicative of a single-copy gene, as all but one digest yielded a single band by Southern analysis. Moreover, the *CLNDm* DNA hybridized to polytene bands 88D,E on larval salivary gland squashes (data not shown). These results show that the DNA is derived from *Drosophila* and that the gene is present in only a single copy in the *Drosophila* genome.

Rescue of the *cln*[−] phenotype in yeast

The growth and morphology of the yeast strain rescued by the *CLNDm* gene was examined to determine how well the *Drosophila* gene complemented the mutant yeast phenotype. The rescued strain showed seemingly wild-type growth as evidenced by the colony size on YPD plates (Fig. 3) or synthetic complete medium (data not shown). When examined under the microscope, however, the cell shapes were often aberrant, with many large cells having long projections and squared or elongated cell morphology. In addition, chains of incompletely separated cells were observed. This morphology may be the result of the overproduction of the *Drosophila* *CLN* homolog, as the same aberrant morphology was seen in the isogenic strain containing the *CLN3* gene overproduced by the galactose promoter. Because the growth rate of the *CLNDm* transformants was near wild type and the altered morphology may be a nonspecific stress response, we did not pursue this issue further.

Strain 884-21B containing *CLNDm* was tested for its ability to respond to α-factor in a halo assay. The *CLNDm*-containing strain displayed the same, or slightly greater, sensitivity to α-factor as a pFC204 (*CLN3*) transformant of the same strain (data not shown). Therefore *CLNDm* not only complements the *cln* growth defect but is also responsive to the mating pheromone signal for cell cycle arrest.

DNA sequence of *CLNDm*

The *CLNDm* gene was sequenced to determine whether it was homologous to previously identified genes, espe-

Figure 3. Comparison of the growth rate of a transformant with *CLNDm* with a transformant of *CLN3*. The growth rates of strain 884-21B, triply mutated for the *cln* genes, are compared when transformed with *CLNDm* in the high-copy expression vector pBL15 (left) versus the starting strain containing *CLN3* on the *CEN* vector pFC204 (right). Growth was for 3 days at 30°C on YPD plates.

cially cyclin genes. The gene sequence was not found in the GenBank data base. The DNA sequence of 1084 bp (Fig. 4) encodes a putative open reading frame of 267 amino acids with a consensus translation start sequence 5' to the first AUG (Cavener 1987), and a putative polyadenylation signal 3' to the coding sequence (Fitzgerald and Shenk 1981). A poly(A) tail of 16 bp is present in the cDNA clone. The open reading frame encodes a polypeptide of ~31 kD, with an estimated pI of 5.95.

Comparison of *CLNDm* with previously identified cyclins, using either the PIMA or IALIGN protein homology alignment programs (Dayhoff et al. 1983; Devereux et al. 1984; Smith and Smith 1990), indicated that *CLNDm*, though clearly unique, has homology to cyclins. Both programs identified the same region of the *CLNDm* gene as homologous to the other cyclins, the "cyclin box." This box consists of ~100 amino acids located between amino acids 39 and 138 in the *CLNDm* protein. The regions aligned between *CLNDm*, human cyclin D (Xiong et al. 1991), mouse *cyl3* (one of several mouse cyclin D's recently identified; Matsushime et al. 1991), and *CLN1* by the IALIGN program are shown in Figure 5. The region of *CLNDm* that appears most conserved is the LLAPTCILLASK stretch. However, *CLNDm* is quite diverged from other members of the cyclin family. The cyclin box of *CLNDm* is ~20% identical to other cyclins and 45% similar if conservative amino acid changes are considered.

Using the IALIGN program, we quantitatively compared the *CLNDm* gene with seven cyclin genes: human cyclin D and mouse *cyl3*; the *CLN1*, *CLN2*, and *CLN3* genes from *S. cerevisiae* (Nash et al. 1988; Hadwiger et al. 1989); and the *Drosophila* G₂-M cyclins A and B (Leh-

ner and O'Farrell 1990b; Whitfield et al. 1990). Human cyclin D was most homologous to *CLNDm*, although the homology with mouse *cyl3* was also significant with alignment scores of 5.08 and 3.63, respectively. An alignment score between 3 and 4 is considered significant in cases where evidence of a common biological function is present. Scores of 4 to 5 are significant if no independent information regarding protein function is available (Dayhoff et al. 1983). The alignment score is a measure of the number of standard deviation units between the score

AGAATTGGATAGCTAACGAAATATTGGCCGACCTAATCAGCTAGGCCTACTAGTGA	60
CGAATGGCGGCAATTTTGGCAGAGTTCGCAGCTCGCAGCAGTGGATTGGACAAACC	120
M A G N F W Q S S H S Q Q W I L D K P	19
CGATCTGCTCCGGGAGCGTCAGCAGATTGTTGGCCCTTAACGAGGATGAATACCAAAA	180
D L L R E R Q H D L L A L N E D E Y Q K	39
GGTGTATTATTTCTTCGCAATGTGATCCAGGTGCTCGCGAGCAGCTAAAGCTGAGGCA	240
V F I F F A N V I Q V L G E Q L K L R Q	59
ACAGGTGATCGCCACGGCGACGGTTTACTTCAAGAGATTCTATGCGAGGAACCTCCCTGAA	300
Q V I A T A T V Y F K R F Y A R N S L K	79
GAACATCGATCTCTGCTGCTGGCGCCACTTGCATCTTGTGCGCTCGAAGGTGGAGGA	360
N I D P L L L A P T C I L L A S K V E E	99
GTCGGTGTCAATTTCAATTCAGACTGATCTCGATCTGCCAGTCCGCCATTAAGACAAA	420
F G V I S N S R L I S I C Q S A I K T K	119
GTTTCAGTATGCGTACGCTCAGGAGTTCCCTACCGCACCAATCAGATCCTGGAGTCCGA	480
F S Y A Y A Q E F P Y R T N H I L E C E	139
GTTTACCTGCTGGAGAACCTGGATTGCTGCTGATGTCTACAGCCGTACAGACCGCT	540
F Y L L E N L D C C L I V Y Q P Y R P L	159
GCTGCAGCTGGTTCAGGACATGGGCCAGGAGSACAGTTGCTACCCCTGAGCTGGCGTAT	600
L Q L V Q D M G Q E D Q L L T L S W R I	179
CGTAACGATTCCTCGCGCACCGATGTCTGCCTGCTCTCTCTCTTACAGATCGCTAT	660
V N D S L R T D V C L L Y P P Y Q I A I	199
AGCCTGCCTTCAATTCGCTGCGTATCTGCTGCAAAAGGACGCGACAGCAGTGGTTCGC	720
A C L Q I A C V I L Q K D A T K Q W F A	219
AGAATTGAATGTTGATTAGACAAGGTCCAGGAGATCGTCCGCCCATTTGTAATCTGTA	780
E L N V D L D K V Q E I V R A I V N L Y	239
CGAGTTGTGGAAGGACTGGAAGGAAAGGACGAGATTCAGATGCTGCTTCCAAGATTCC	840
E L W K D W K E K D E I Q M L L S K I P	259
GAAACGAAACCCCGCTCAGCGTTAGAAGAGCTTCATAATCATTATCATTAGCTGGT	900
K P K P P P Q R	279
ATTGCCCAATTGTGTGTTGTACTCATATTACCAATGATTTATGCCAATTAAACGAAAG	960
TTTAAGCTAGGTGACTAAGCAATCAAAATGTCATTACAAATTATTTGCTTTTATAAT	1020
AATTATTCTAAATTAATACATAATTGCTTAAGATTACATGCGGTAAAAA	1080
AAAA	

Figure 4. Nucleotide sequence and predicted amino acid sequence of the *Drosophila* *CLNDm* gene. The nucleotide sequence of the *Drosophila* *CLNDm* cDNA (1084 bp) has a putative open reading frame of 267 amino acids. The predicted amino acid sequence is shown in single-letter code below the nucleotide sequence. The putative initiating ATG is underlined, along with flanking bases matching consensus sequences for translational start sites in *Drosophila*. A putative polyadenylation signal is also underlined.

A.

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CLNDm      39  QKVFIFANVIQ--VLG--EQLKLRQQVIATATVYFKRFYARNSLKNIDPLLLAPTCLLASKVEEFGVISNSRLISICQSAIKTKFSYAYAQEFPYRTNHILECE
                |  **  *  |  *  |  **  *  |  |  |  *  **  |  ||**||*  *|||*  |  **  *  |  ***|**  *
Cyclin D    51  -EVLPSMRKIVATWMLVCEEQKCEEVFFLAMNYLDRFLSLEPVKKSRLQLLGATCMFVASKMKETIPLTAEKLCIYTDGSIKPELLQMEILLVNLKWKW----

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B.

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CLNDm      39  QKVFIFANVIQVLGEQLKLRQQVIATATVYFKRFYARNSLKNIDPLLLAPTCLLASKVEEFGVISNSRLISICQSAIKTKFSYAYAQEFPYRTNHILECE
                |  *  **  ***  |  |  *  **  *  |  |  |  *  *  *  *  |  ||**  *  *|||*  |  **  *  |  *  |  *  *  *  *  |
M-Cyl3     59  RKMLAYW--MLEVCEEQ--RCEEDVFPLAMNYLDRFLSCVPTRKAQLQLGTVCLLASKLRETTPLTIEKLCIYTDQAVAPWQLREWEVLVLGKLGKLDLAHV

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C.

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CLNDm      39  QKVFIFANVIQVLGEQLKLRQQVIATATVYFK--RFYARNSLKNI---DPLLLAPTCLLASKV--EEFGVISNSRLIS---ICQSAIKTKFSYAYAQEFPY--RTNHILECE
                **  |  ||  *  |  *  ***  |  |||  |  |  *  **  |  *  *  *  *  *  ||*  |  |  |  |  |  |  |  |  |  |  *  *  *  *  *  *  *
CLN1       67  MNPNQTREAIVTFL--YQLSVMTRV--SMGIFHFSVRFYDRYCSKRVVLKDAQKLVVGTCLWLAAKTGGCNHIINNVS IPTGGFRFYGPNNRARIPLRLSELVHYCGGSDLFDES

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Figure 5. Amino acid sequence comparison of *CLNDm* with two cyclin D's and *CLN1*. The alignments obtained from the IALIGN program are indicated. In pairwise comparisons of *CLNDm* with human cyclin D (A), mouse cyl3 (B), or *CLN1* (C), the same region of *CLNDm* was identified by the computer. The numbers indicate the amino acid position within each protein sequence. Identical residues are designated by vertical bars; conserved amino acids are shown by asterisks (*). The following groups of amino acids were classified as conserved: N, D, E, Q; K, R, S, T, A, G, P; F, W, Y; I, V, L, M (Dayhoff et al. 1983).

obtained by comparing the real sequences versus the mean of scores for random permutations of those sequences. Thus, an alignment score of 5.08 means that the probability of obtaining the similarities by chance is 1.5×10^{-7} , and a score of 3.63 has a probability 1.3×10^{-4} . Of those compared, the only other cyclin that showed homology to *CLNDm* was *CLN1* (Fig. 5); however, its alignment score is not significant. *CLNDm* is very different from the other cyclins tested.

CLNDm is predicted to encode the lowest molecular mass protein in the cyclin family (31 kD), although the predicted cyclin D protein is small as well (34 kD; Xiong et al. 1991). *CLNDm* also parallels cyclin D and *CLN1* in having its cyclin box located in the amino-terminal region of the protein. In contrast with the *CLN1* protein, the predicted *CLNDm* protein does not appear to contain a PEST region, a region associated with proteins with short intracellular half-lives (Rogers et al. 1986).

While this manuscript was in preparation we learned that Pierre Leopold and Patrick O'Farrell had isolated a *Drosophila* cyclin-like gene by using a similar complementation strategy. Their gene appears to be the same as ours, as evidenced by the same cytological location. They have designated their *CLN* complementer as cyclin C, as a result of its high degree of homology with the human cyclin C gene isolated by Reed and co-workers (Leopold and O'Farrell 1991).

Expression of *CLNDm* during *Drosophila* development

We wanted to examine when in development the *Drosophila* *CLN* gene was expressed transcriptionally to determine whether its expression was enhanced at times when cell division was occurring or during developmental periods when most cells were in the endo cell cycle. The cell cycle changes that occur during embryogenesis have been described (Fig. 1). During the larval and pupal stages, the majority of cells are in the endo cell cycle, and mitotic divisions occur only in imaginal or neural tis-

sues. In the adult, few cells undergo division; mitosis is largely confined to the gonads. Because both the type of cell cycle and the extent of cell division vary during *Drosophila* development, a developmental profile of *CLNDm* expression would be suggestive of possible functions of the gene.

Northern blots of Kc cell mRNA and mRNA isolated at different times of development were probed with *CLNDm* DNA (Fig. 6A,B). The probe hybridized to a single transcript of ~1.2 kb in Kc cells and at all developmental time points examined. There was no evidence for the presence of alternatively spliced or polyadenylated messages (Fig. 6A,B).

The developmental profile showed that the *CLNDm* transcript is most abundant during early embryogenesis (Fig. 6B). The level of *CLNDm* mRNA [as standardized to actin-5C levels] present in ovary is almost as high as that in early embryos, indicating that the transcript is most likely maternally synthesized and deposited in the developing egg. No significant increase was apparent in transcript levels in 2- to 5-hr embryos, following zygotic induction of mRNA transcription. The *CLNDm* transcript level drops significantly (~10- to 20-fold) at 8–24 hr of embryogenesis, correlating with the cessation of mitotic division in the majority of embryonic cells, and the mRNA remains low in second- and third-instar larvae and adults (except females, where its presence can be attributed to oogenesis). There is a possible increase in transcript levels during pupation. The level of *CLNDm* message in Kc cells is twofold higher than in 8- to 24-hr embryos (Fig. 6A).

Thus, the abundance of *CLNDm* transcript parallels the developmental times at which maximal cell division is occurring, implicating *CLNDm* in the regulation of the cell cycle in *Drosophila*. In contrast, *CLNDm* does not appear to be induced transcriptionally either when G_1 is added to the cell cycle (after 5 hr of embryogenesis) or when the transition to the endo cell cycle occurs at 9 hr of embryogenesis (Fig. 1).

Figure 6. Northern analysis of *Drosophila* *CLNDm* mRNA in Kc cells and during development. Northern blots of poly(A)⁺ RNA were hybridized to the *Drosophila* *CLNDm* cDNA. As a control for the amount of mRNA loaded, the same blots were probed with actin-5C DNA (Fryberg et al. 1983) (bottom). The developmental stages shown are a composite of two different Northern blots in which lanes labeled ovary through third-instar are from one blot, and lanes labeled pupae and adult are from another. (A) Molecular weight standards (bases); Kc cell mRNA, mRNA from 8- to 24-hr staged embryos. (B) Poly(A)⁺ RNA from ovary, 0- to 2-, 2- to 5-, 5- to 8-, and 8- to 24-hr staged embryos, second-instar larvae, third-instar larvae, pupae, adult female, and adult male.

Localization of the *CLNDm* transcript in embryos

The developmental Northern blot suggested that the transcript was expressed in dividing cells and not in polytene tissues. To examine whether the transcript was localized to cells undergoing cell division we performed digoxigenin whole-mount in situ hybridization to wild-type embryos at different developmental stages using the *CLNDm* DNA as a probe (Fig. 7). The level of *CLNDm* transcript is high in preblastoderm embryos and diminishes progressively during embryogenesis (Fig. 7A,B). Although the staining is faint by germ-band extension, the staining is more intense than in control embryos without probe or those probed with vector sequences (Fig. 7B,C; data not shown). We cannot be certain of the exact developmental point at which the transcript can no longer be detected above background.

No tissue-specific localization of the *CLNDm* transcript was detected from preblastoderm to cuticle deposition, the latest developmental time point that we could analyze using this technique (Fig. 7A,B; data not shown). The *CLNDm* message levels were not detectably higher

either in mitotically dividing cells or in polytene cells. This is in contrast to the *string* gene, the *Drosophila* homolog of *S. pombe cdc25*, which labels specific post-blastoderm mitotic domains (Fig. 7D) (Edgar and O'Farrell 1989). Thus, it appears that *CLNDm* is not preferentially transcribed in mitotic cells, but because the message is present in low levels it is possible that we failed to detect some specific labeling. The fact that the gene does not appear to be expressed at higher levels in dividing cells does not preclude a direct role by *CLNDm* in the *Drosophila* cell cycle. Neither *Drosophila* cyclins A or B nor the *cdc2* gene are regulated transcriptionally during the cell cycle (Jimenez et al. 1990; Lehner and O'Farrell 1990a,b; Whitfield et al. 1990).

Discussion

We have identified a novel cyclin gene from *Drosophila* that complements the *CLN* function in *S. cerevisiae*. Yeast strains triply mutated for the *CLN* genes grow at near wild-type levels in the presence of *CLNDm*. Per-

Figure 7. Distribution of *CLNDm* transcript during embryogenesis. Whole-mount embryos were hybridized in situ with the *CLNDm* or *string* cDNA, which had been labeled by incorporation of digoxigenin dUTP by random priming. Hybridization was detected by an alkaline phosphatase histochemical reaction. (A) Preblastoderm embryo hybridized to *CLNDm*; (B) Germ-band-extended embryo hybridized to *CLNDm*; (C) preblastoderm embryo hybridized without probe and stained; (D) germ-band-extended embryo hybridized with *string*. All embryos are oriented with anterior to the left and dorsal up. Bar, 50 μ m.

haps unexpectedly, cells bearing *CLNDm* respond to α -factor, implying that the pheromone signal transduction pathway is able to regulate *CLNDm*. Whereas *CLNDm* contains a cyclin box and is clearly a member of the cyclin family, the sequence of *CLNDm* is quite divergent from the other cyclins and represents a new branch on the cyclin family tree. Of the published cyclin genes, *CLNDm* is closest to cyclin D. However, this gene has been independently isolated and assigned to a new class of cyclins, cyclin C (Leopold and O'Farrell 1991). *CLNDm* is the same gene as *Drosophila* cyclin C.

When expressed in high copy in yeast, *CLNDm* can regulate the G_1 -S transition. Complementation is a stringent criterion of gene function, which strongly implies that *CLNDm* may regulate the cell cycle in *Drosophila*. Although it is possible that *CLNDm* may control the G_1 -S transition in *Drosophila*, this conclusion must be regarded as tentative for two reasons. The precedent exists for genes from one species to complement yeast mutations, yet normally have a different function, as is the case with mammalian *ras* genes. Whereas RAS proteins control adenylate cyclase in *S. cerevisiae*, they do not do so in mammalian cells (Hall 1990). Furthermore, both *S. pombe* and human cyclin B, which normally regulate G_2 -M, have been reported to rescue yeast *cln* mutants (cited in Xiong et al. 1991). Second, although members of the cyclin family share the cyclin box, individual members are highly diverged and show only 20% homology within this region. Because the conservation is so limited it is possible that the cyclin box represents a structural motif, and it may be diagnostic solely of a kinase interaction domain rather than a cell cycle function. When initially identified, the homeo box was thought to be diagnostic of a gene involved in developmental determination. As more examples of homeo box-containing proteins have been identified, it has become clear that this domain represents a DNA-binding motif that is not restricted to developmental genes.

Despite these cautionary notes, we think it most likely that *CLNDm* is involved directly in regulating the cell cycle in *Drosophila*. The expression of *CLNDm* during development closely parallels the extent of cell division. Developmental Northern analysis showed that the *CLNDm* mRNA is present maximally during the early stages of embryogenesis when the cleavage and postblastoderm divisions occur. The low levels of *CLNDm* transcript during larval, pupal, and adult male stages correlate with the relatively infrequent cell division occurring at these times. In addition to its expression pattern correlating with a role of *CLNDm* in the cell cycle, of the published cyclin genes *CLNDm* is most homologous to the cyclin D class. Mouse cyclin D has been shown to be regulated by colony-stimulating factor, and the timing of its expression suggests that this cyclin may be involved in S-phase commitment (Matsushime et al. 1991).

At present, we cannot distinguish between a role of *CLNDm* in G_1 -S versus G_2 -M regulation. In situ hybridizations to embryos showed that the transcript was distributed uniformly throughout the embryo, showing neither a preponderance in G_1 or G_2 cells nor an increased

abundance in cells in a dividing cell cycle. Although it would have been informative, it is not surprising that the transcripts for this cyclin gene do not fluctuate with the cell cycle. Cyclin A and B message levels are constant during the cell cycle in *Drosophila* (Lehner and O'Farrell 1989, 1990b), and so far only the *string* gene has been demonstrated to be transcriptionally regulated in the *Drosophila* cell cycle (Edgar and O'Farrell 1989). The fact that the *CLNDm* mRNA is present at low levels during larval stages when many tissues are in the endo cell cycle implies that *CLNDm* is not involved in the endo cell cycle. Nevertheless, *CLNDm* may play a role if it is subject to post-transcriptional regulation. Alternatively, another as yet unidentified cyclin may regulate the endo cell cycle. Several mouse cyclin D's with different tissue specificities have been identified (Matsushime et al. 1991), raising the possibility that a family of cyclins with developmental specificity may exist in *Drosophila*.

Analysis of the *CLNDm* protein levels during the mitotic and endo cell cycles will provide further insights into the function of this gene. Such an analysis will be facilitated greatly by the spatial and temporal map of the mitotic divisions and polytene S phases that has been defined for *Drosophila* embryogenesis (Foe 1989; Smith and Orr-Weaver 1991). The accumulation and disappearance of the cyclin A and B proteins during the *Drosophila* cell cycle have been suggestive of their roles in mitosis (Lehner and O'Farrell 1990b; Whitfield et al. 1990). Characterization of the *CLNDm* protein may also clarify whether this gene functions in the endo cell cycle or is restricted to regulating the early embryonic and, possibly, germ-line divisions. Ultimately, the isolation of mutations within the *CLNDm* gene, taking advantage of the ability to apply a genetic approach in *Drosophila*, will permit us to elucidate fully the contribution of this novel cyclin gene to the cell cycle.

Materials and methods

Strains

S. cerevisiae strain 884-21B[pW16], *MATa arg4-Bcl ura3- Δ ns leu2-3,113 trp1-1^a ade1 cln1::TRP1 cln2::LEU2 cln3 Δ* ; pW16, *URA3 CEN4 ARS1 GAL-CLN3 Amp^r cole1* was obtained from Fred Cross (Rockefeller University) (Richardson et al. 1989). The *arg4*, *ura3*, and *leu2* mutations are nonreverting. The pW16 plasmid in this strain was replaced with pFC204, *URA3 CEN4 ARS1 CLN3 Amp^r cole1* (obtained from Fred Cross) to generate a strain that transformed efficiently. Strain 884-21B[pW16] will not grow on glucose because the complementing *CLN3* gene is under the control of the galactose promoter. Transformants of pFC204 were selected by their ability to grow on glucose. After serially streaking onto YDP several times, colonies that had lost the pW16 plasmid were identified by Southern analysis. *E. coli* HB101 (Sambrook et al. 1989) and XL1 blue (Stratagene) were described previously.

Expression vector construction

The *S. cerevisiae* expression vector pBL15 contains the cDNA-cloning site downstream from the constitutive *ADH1* promoter and upstream of the *ADH1* termination sequence. Plasmid pBL15 was derived from plasmid pDB15 (Becker et al. 1991) by replacing the *URA3* marker with the *ARG4* marker by digesting

with *Nde*I and *Stu*I to remove the majority of the *URA3* sequence, and blunt-end-ligating a 3.3-kb *Pst*I fragment containing the entire *ARG4*-coding region into the vector ends.

cDNA library construction

Drosophila Kc cell mRNA was kindly provided by Paul Kaufman (Whitehead Institute). Full-length cDNA was prepared by using the cDNA synthesis kit from BRL but substituting SuperScript RNase H⁻ reverse transcriptase (BRL) for the reverse transcriptase supplied with the kit. Reverse transcriptase reactions were performed at 42°C to improve processivity. The double-stranded cDNA was blunt end ligated to self-incompatible *Bst*XI monomer linkers (Invitrogen), which prevents more than one linker from being added per cDNA molecule end. The cDNA was then ligated into pBL15 vector DNA that had been linearized with *Bst*XI. This cDNA library was transformed into *E. coli* HB101, and 1×10^6 independent transformants were obtained. The CsCl₂-purified *Drosophila* cDNA obtained from this transformation was used to transform *S. cerevisiae* for the CLN complementation screen.

CLN complementation screen

S. cerevisiae 884-21B[pFC204] was transformed with the *Drosophila* Kc cell cDNA library by using the lithium acetate method (Schiestl and Gietz 1989), and Arg⁺ transformants were selected at 30°C on plates lacking arginine. Transformants were then replica plated onto synthetic complete medium containing 5-FOA; only cells that are Ura⁻ will survive on 5-FOA (Boeke et al. 1987). Because the *CLN3* gene is contained on the *URA3*-marked plasmid, theoretically only those cells that lose the *URA3* plasmid and retain a functional *Drosophila* CLN homolog will survive. Colonies that grew on the 5-FOA plates were tested for the loss of the *CLN3* plasmid by examining Southern blots of total yeast DNA from each colony (Hoffman and Winston 1987). Southern blots were probed with a restriction fragment from yeast *CLN3* to ensure that the plasmid had been lost and that the transformant therefore contained a *Drosophila* CLN gene, as opposed to a mutated *URA3* gene on the *CLN3* plasmid.

Total DNA from the positive colony that did not contain the yeast *CLN3* gene was electroporated into *E. coli* XL1 blue, and the plasmid DNA was purified and examined as single-colony isolates. The purified DNA was then retransformed into *S. cerevisiae* 884-21B[pW16] to determine whether the purified plasmid DNA would still complement CLN function. Although 884-21B[pW16] was unable to grow on glucose, transformants bearing the cDNA plasmid grew on glucose.

Sensitivity to α -factor was measured by using the halo assay as described previously (Elion et al. 1990). Seven microliters of a 1.5, 0.5, and 0.167 mM solution of α -factor in methanol was applied to filter disks placed on a lawn of the strain to be tested, and the plates were incubated for 18–24 hr at 24°C.

DNA sequence analysis

The *Drosophila* cDNA, designated *CLNDm*, was subcloned into the Bluescript (KS⁺) vector (Stratagene) by excising the cDNA insert with *Hind*III, and ligating the isolated insert into the *Hind*III site of the Bluescript vector. The cDNA insert was sequenced as double-stranded DNA by the dideoxy method (Sanger et al. 1977) using the Sequenase kit (U.S. Biochemical). Initially, sequencing was done with the Universal primer (U.S. Biochemical) and the Reverse primer (New England Biolabs). The sequence obtained was used to synthesize additional oligo-

nucleotide primers (Research Genetics). The entire sequence was determined for both DNA strands, employing 10 primers. It was verified that the entire cDNA sequence had been subcloned into the Bluescript vector by sequencing the amino terminus of the original yeast clone and by the presence of the poly(A) tail.

Northern and Southern blots

The developmental Northern blots were kindly provided by Charlotte Wang and Doug Barker (Whitehead Institute), and the Kc cell Northern blot was a gift of Amanda Hannaford (Whitehead Institute). Southern blots were performed as described previously (Church and Gilbert 1984) and were hybridized with either nick-translated or random-primed probes in aqueous solution at 65°C. Northern blots were hybridized to probes labeled by random priming in 50% formamide at 42°C as described previously (Orr-Weaver and Spradling 1986). For quantification of mRNA levels, X-ray film was preflashed and the intensity of exposure was quantitated on a Zeineh Soft Laser Scanning Densitometer (Biomed Instruments).

In situ hybridization

In situ hybridizations to polytene chromosomes and embryos were done with a wild-type Canton S stock. Polytene chromosome squashes and hybridizations were performed as described previously (Engels et al. 1986), with the modification of hybridization overnight at 30°C to increase detection. In situ hybridizations to embryos were performed essentially as described (Tautz and Pfeifle 1989), with the modification that the initial paraformaldehyde fixation was performed by using 2 ml of 4% paraformaldehyde/0.2 ml DMSO/8 ml heptane. The digoxigenin-labeled probe was made as described in the Genius Kit (Boehringer Mannheim), with the modifications that the hexanucleotide mix was used at double the recommended concentration and the ethanol-precipitated probe was resuspended in 100 μ l of hybridization buffer. For digoxigenin labeling of *CLNDm* the cDNA insert was isolated and digested with *Alu*I prior to labeling. The 1.4-kb *Eco*RI restriction fragment of the *string* cDNA was isolated, digested with *Alu*I, and labeled (Edgar and O'Farrell 1989). As a control for background hybridization, Bluescript vector was labeled and used as a probe. Ten microliters of probe were used per hybridization. Salivary gland in situ hybridizations were examined under phase on a Zeiss Axiophot microscope with a Plan-Neofluar 40 $\times$ objective. Embryos were examined by using Nomarski optics and a Plan-Neofluar 25 $\times$ objective.

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Note added in proof

The human cyclin C gene referred to as unpublished has been published by D. Lew, V. Dulić, and S. Reed. 1991. *Cell* **66**: 1197–1206.

Sequence data described in this paper have been submitted to EMBL/GenBank data libraries under accession number X62948.

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