A Screen for Modifiers of *Cyclin E* Function in *Drosophila melanogaster* Identifies *Cdk2* Mutations, Revealing the Insignificance of Putative Phosphorylation Sites in Cdk2

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ABSTRACT

In higher eukaryotes, cyclin E is thought to control the progression from G1 into S phase of the cell cycle by associating as a regulatory subunit with cdk2. To identify genes interacting with cyclin E, we have screened in *Drosophila melanogaster* for mutations that act as dominant modifiers of an eye phenotype caused by a *Sevenless-CycE* transgene that directs ectopic *Cyclin E* expression in postmitotic cells of eye imaginal disc and causes a rough eye phenotype in adult flies. The majority of the EMS-induced mutations that we have identified fall into four complementation groups corresponding to the genes *split ends*, *dacapo*, *dE2F1*, and *Cdk2(Cdc2c)*. The *Cdk2* mutations in combination with mutant *Cdk2* transgenes have allowed us to address the regulatory significance of potential phosphorylation sites in Cdk2 (Thr 18 and Tyr 19). The corresponding sites in the closely related Cdk1 (Thr 14 and Tyr 15) are of crucial importance for regulation of the G2/M transition by myt1 and wee1 kinases and cdc25 phosphatases. In contrast, our results demonstrate that the equivalent sites in Cdk2 play no essential role.

ENTRY into or exit from the cell division cycle occurs in general during the G1 phase in eukaryotes. The regulation of progression through the G1 phase, therefore, is crucial for the control of cell proliferation. Progression through the G1 phase is governed by G1 cyclins in association with cyclin-dependent protein kinases (cdks). The understanding of the precise roles of these G1 cyclin/cdk complexes is most advanced in yeast where efficient genetic methodology is available. However, metazoans have a distinct set of G1 cyclin/cdk complexes. The genetic characterization of these higher eukaryote regulators is feasible in *Drosophila melanogaster*. Moreover, genetic screens for mutations modifying the effects of another mutation have recently been used with great success in this species for the dissection of various signal transduction pathways. Here, we describe a genetic screen designed to identify components involved in the function of cyclin E, one of the higher eukaryote G1 cyclins.

Mammalian cyclin E was originally identified because of its ability to complement the loss of G1 cyclin function in yeast cells (Lew *et al.* 1991). Cyclin E was found to bind to cdk2, which was originally designated as *Cdc2c* in Drosophila (Lehner and O'Farrell 1990). Protein kinase activity of cyclin E/cdk2 complexes is maximal in mammalian cells during late G1 and early S phase (Dulic *et al.* 1992; Koff *et al.* 1992), and the phenotypic characterization of *Cyclin E* (*CycE*) mutations in Drosophila has demonstrated that Cyclin E is required for progression into S phase (Knoblich *et al.* 1994).

The physiological substrates that need to be phosphorylated by cyclin E/cdk2 for progression into S phase are not yet known very well. The protein product of the retinoblastoma tumor suppressor gene (pRB) appears to be an important physiological target of cyclin E/cdk2 (for a review see Mittnacht 1998). A family of pRB-related genes (pRB, p107, and p130) is present in mammals (Weinberg 1995) and a pRB-related gene (*RBF*) has been described in Drosophila as well (Du *et al.* 1996; Du and Dyson 1999). All RB family proteins bind to E2F/DP heterodimers (for review see Dyson 1998; Helin 1998). In mammals, the complexity of E2F and DP genes is considerable (Dyson 1998; Helin 1998). In Drosphila, two E2F genes and one DP gene have been identified (Dynlacht *et al.* 1994; Ohtani and Nevins 1994; Hao *et al.* 1995; Sawado *et al.* 1998). E2F/DP heterodimers bind to specific DNA sequences in promoters of target genes encoding proteins required for DNA synthesis and cell cycle progression like cyclin E, cyclin A, cdk1, ribonucleotide reductase, DNA polimerase α, and proliferating cell nuclear antigen. In many promoters, E2F-binding sites confer repression during G0 or G1. In these promoters, E2F/DP presumably recruits pRB protein, which is responsible for inhi-

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bition of target gene transcription. pRB appears to repress transcription by altering chromatin structure as it can bind to histone deacetylases and E2F/DP simultaneously (Brehm *et al.* 1998; Luo *et al.* 1998; Magnaghi-Jaulin *et al.* 1998; Zhang *et al.* 1999). In some target genes, however, E2F/DP-mediated transcriptional activation has been observed, and pRB can interfere with activation at these promoters independent of histone deacetylase recruitment (Harbour *et al.* 1999).

As a result of sequential pRB phosphorylation in G1, hyperphosphorylated pRB dissociates from histone deacetylase and E2F/DP and inhibition of target gene expression is relieved. While complexes of D-type cyclins with cdks 4 or 6 are thought to provide the major pRB kinase activity in mid G1, cyclin E/cdk2 contributes to pRB phosphorylation in late G1 as well (Hinds *et al.* 1992; Kitagawa *et al.* 1996; Zarkowska and Mittnacht 1997; Kelly *et al.* 1998; Lundberg and Weinberg 1998; Mittnacht 1998; Brown *et al.* 1999; Harbour *et al.* 1999). In Drosophila, RBF is phosphorylated by Cyclin E/Cdk2 *in vitro* and genetic interactions have been demonstrated *in vivo* (Du *et al.* 1996; Du and Dyson 1999). Moreover, ectopic *Cyclin E* expression can generate E2F activity and induce expression of E2F/DP target genes in a number of cell types (Duronio and O'Farrell 1995; Sauer *et al.* 1995; Weinberg 1995).

Evidence from a number of organisms has indicated that pRB is not the only physiological target of cyclin E/cdk2 activity (Resnitzky and Reed 1995; Duronio *et al.* 1996; Leng *et al.* 1997; Lukas *et al.* 1997; Geng *et al.* 1999). Moreover, experiments with Xenopus egg extracts have demonstrated that cyclin E/cdk2 controls entry into S phase at a post-transcriptional level (Jackson *et al.* 1995; Strausfeld *et al.* 1996; Hua *et al.* 1997; Hua and Newport 1998). Recently, centrosome duplication was found to be dependent on cyclin E/cdk2 in mammalian cells and Xenopus egg extracts (Hinchcliffe *et al.* 1999; Lacey *et al.* 1999; Matsumoto *et al.* 1999). In these extracts, activation of cyclin B/cdk1 complexes is also dependent on cyclin E/cdk2 (Guadagno and Newport 1996). The relevant substrates regulated by cyclin E/cdk2 in these cases are completely unknown.

Interestingly, cyclin E and p27kip1, a cdk inhibitor (cdi) that inhibits the activity of cdk2 complexes, are phosphorylated by cyclin E/cdk2 (Clurman *et al.* 1996; Won and Reed 1996; Xu and Burke 1996; Sheaff *et al.* 1997; Vlach *et al.* 1997; Montagnoli *et al.* 1999). In both proteins, this phosphorylation triggers their subsequent proteolytic destruction in cultured mammalian cells. It is not known whether this control of cyclin E and cdi degradation also occurs in Drosophila, where *dacapo* (*dap*) has been shown to encode a p27kip1-related cdi specific for Cyclin E/cdk2 complexes (DeNooij *et al.* 1996; Lane *et al.* 1996).

Additional regulation of cyclin E/cdk2 activity involves phosphorylation and dephosphorylation of the cdk2 subunit. Apart from the activating phosphorylation of a threonine residue in the activation loop by cdk activating kinase activity, inhibitory phosphorylation of vertebrate cdk2 on a tyrosine residue and on a neighboring threonine residue has also been observed (Gu *et al.* 1992; Hoffmann *et al.* 1994; Jinno *et al.* 1994; Blomberg and Hoffmann 1999; Kim *et al.* 1999). Phosphorylation at the corresponding site in cdk1 is brought about by myt1 and wee1 kinases and is reversed by the activity of cdc25 phosphatases. While this inhibitory phosphorylation is known to be of crucial importance for the regulation of cdk1 *in vivo*, the physiological significance in the case of cdk2 is not understood. Vertebrate cdc25A phosphatase can dephosphorylate and activate cyclin E/cdk2 complexes *in vitro* and is expressed late in G1 and required for progression into S phase (Hoffmann *et al.* 1994; Blomberg and Hoffmann 1999; Kim *et al.* 1999). Experiments involving premature cdc25A expression during G1 to test the idea that cdk2 is a relevant physiological substrate of cdc25A appear to have given ambivalent results (Blomberg and Hoffmann 1999; Sexl *et al.* 1999).

In summary, the functional characterization of cyclin E has clearly established its central role in cell cycle regulation. Moreover, multiple positive and negative autoregulatory loops involving RB family proteins, E2F/DP heterodimers, cdc25A phosphatase, and cdk inhibitors have been implicated in the control of cyclin E/cdk2 activity. However, the significance of the different regulatory loops *in vivo* is poorly understood. Finally, except for pRB, p27kip1, and cyclin E, little is known about physiological substrates of cyclin E/cdk2 activity.

The design of our genetic approach in Drosophila was based on the finding that ectopic *Cyclin E* expression in embryos prevents the arrest of cell cycle progression at the appropriate developmental stage (Knoblich *et al.* 1994). Therefore, we expected that ectopic *Cyclin E* expression in postmitotic cells of developing ommatidia would also result in extra cell cycle progression and thereby cause defects in the extremely regular eye pattern that is observed in wild-type flies. Ectopic *Cyclin E* expression in postmitotic cells during eye development was achieved with a transgene (*Sev-CycE*) regulated by an enhancer from the *sevenless* gene. By screening for mutations that dominantly modified the resulting rough eye phenotype, we identified genes encoding proteins known to interact with Cyclin E, like *dap*, *dE2F1*, and *Cdk2*, as well as additional genes like *split ends (spen)*. Moreover, the *Cdk2* mutations allowed a demonstration that Cdk2 phosphorylation on threonine 18 and tyrosine 19 (corresponding to the phosphorylation sites controlling Cdk1 activity) does not play an essential role during Drosophila development.

MATERIALS AND METHODS

Drosophila stocks: *Hs-Cdk1*(*Cdc2*) (Stern *et al.* 1993), *Df(3R)H81* (Stern *et al.* 1993), dap^{4} (Lane *et al.* 1996), $dE2F1^{91}$ and $dE2F1^{7172}$ (Duronio *et al.* 1995), and dDP^{a2} and dDP^{a4}

(Royzman *et al.* 1997) have been described previously as well as the *spen* alleles *E(Raf)2A*^{16H1} and *E(Raf)2A*^{16T1} (Dickson *et al.* 1996), *poc*^{361} and *poc*^{231} (Gellon *et al.* 1997), and *l(2)03350* and *l(2)k13624* (Spradling *et al.* 1995).

Fly stocks carrying *Sev-CycE* transgenes were obtained after *P*-element-mediated germ-line transformation with pKB267 constructs. pKB267 (kindly provided by Konrad Basler, University of Zurich) contains between a 5′ and a 3′ *P*-element end two copies of the *sevenless* enhancer (Basler *et al.* 1991), the *hsp 70* promoter/leader, a tubulin trailer, and a mini-*white*$^{+}$ gene. *Cyclin E* type I or type II cDNA fragments (Richardson *et al.* 1993) comprising the entire coding regions were inserted into the unique *Kpn*I and *Eco*RI sites between leader and trailer. Several independent lines carrying either the *Sev-CycE I* or the *Sev-CycE II* transgene were established. Both transgenes were found to cause a rough eye phenotype, although its expressivity varied with different transgene insertions. The *Sev-CycE I* insertion *III.1* on the third chromosome, which results in a strong phenotype, was used in the interaction screen and in the experiments described here.

Fly stocks carrying a *Cdk2*$^{+}$ transgene were obtained by *P*-element-mediated germ-line transformation with a pCaSpeR 4 (Pirrotta 1988) construct containing a 10-kb genomic *Sal*I fragment inserted into the *Xho*I site of the vector.

Fly stocks with transgenes allowing the expression of wild-type *Cdk2* under the control of the heat-shock promoter (*Hs-Cdk2(Cdc2c)*) have been described previously (Stern *et al.* 1993). Site-directed mutagenesis following the method of Chen and Przybyla (1994) was used to mutate the codons for potential phosphorylation sites in the parental pCaSpeR-hs construct, which contained an *Eco*RI-*Sna*BI *Cdk2* cDNA fragment inserted into the *Eco*RI and *Stu*I sites of the vector. In a first mutant transgene (*Hs-Cdk2T18A*), the ACC codon for threonine 18 was changed into GCC encoding alanine. In a second mutant transgene (*Hs-Cdk2Y19F*), the TTC codon for tyrosine 19 was changed into TAC encoding phenylalanine. In the third mutant transgene (*Hs-Cdk2AF*), we introduced both these codon changes. In the first step of the mutagenesis, we used the primer P1 (5′-CAAA GAATTC GTT TAT TTT GCC AAC ATC-3′) in combination with either the primer PmT18A (5′-ACC GTA GGC GCC CTC GCC AAT TCT-3′), PmY19F (5′-TAT ACC GAA GGT GCC CTC GCC AAT-3′), or PmAF (5′-TAT ACC GAA GGC GCC CTC GCC AAT TCT-3′) for enzymatic amplification of short fragments from the *Cdk2* cDNA. These fragments were extended during a second polymerase chain reaction (PCR) using a third primer P2 (5′-GCC CAA CAG AAT CTC TGG AGC-3′). The resulting fragments were digested with *Eco*RI and *Kpn*I and used to replace the corresponding fragment in the *Hs-Cdk2* construct. The replaced regions were sequenced to verify their correctness. Several independent transgene insertions were established and none of the mutant transgenes were found to induce lethality after expression during embryogenesis. In subsequent experiments, we only characterized *Hs-Cdk2AF* transgene insertions. Fly stocks (*Hs-Cdk1(Cdc2)AF*(*III.72*) and (*III.74*)) with an analogous transgene allowing the expression of mutant Cdk1 with alanine and phenylalanine instead of threonine and tyrosine at position 14 and 15, respectively, were kindly provided by Patrick O'Farrell, University of California, San Francisco (Sprenger *et al.* 1997).

CyO and *TM3* balancer chromosomes with *Act-GFP* and *Hs-hid* transgenes were obtained from the Bloomington Stock Center (Indiana University).

Screen for dominant *Sev-CycE* modifier mutations: For EMS mutagenesis, *w*; *iso2*; *iso3* males were starved for 12 hr and then fed a 1% sucrose solution containing 25 mm EMS for 20 hr. Mutagenized males were crossed to *Sev-CycE I* (*III.1*) females. The eye phenotype of 38,000 F_1 progeny was scored under a dissection microscope. Flies with either a stronger or weaker phenotype than normally observed with *Sev-CycE I* (*III.1*) were backcrossed individually to *w*; +/*CyO*; *Sev-CycE I* (*III.1*)/*TM2* flies. In cases where eye phenotypes of progeny fell into two distinct classes, linkage tests were performed and balanced lines were established. Further characterization was restricted to chromosomes associated with recessive lethality. Therefore, three chromosomes resulting in enhancement were eliminated from subsequent analyses. By *inter se* crosses, the recessive lethal mutations were assigned to different complementation groups. The enhancer mutations *E(Sev-CycE) A14*, *E(Sev-CycE)E93*, *E(Sev-CycE)G36*, and *E(Sev-CycE)G66* on the second chromosome were found to behave as single hits. The enhancer mutations *E(Sev-CycE)D45*, *E(Sev-CycE)D50*, *E(Sev-CycE)E19*, and *E(Sev-CycE)E44* as well as the suppressor mutations *S(Sev-CycE)D2*, *S(Sev-CycE)D16*, *S(Sev-CycE)D17*, *S(Sev-CycE) D28*, and *S(Sev-CycE)D30* on the third chromosome were also found to behave as single hits. Representative alleles of complementation groups with multiple alleles were meiotically mapped by analyzing linkage between recessive visible markers and the dominant interaction with *Sev-CycE*. An *al b c* sp chromosome and a *ru h th st cu sr e ca* chromosome were used to map mutations on the second and third chromosome, respectively. Deficiency mapping was used to verify meiotic map positions.

BrdU pulse labeling, scanning electron microscopy, and immunolabeling: BrdU pulse labeling of embryos and eye imaginal discs followed by immunolabeling with anti-BrdU antibodies (Becton-Dickinson, San Jose, CA) was done as described (Lehner *et al.* 1991; Staehling-Hampton *et al.* 1999). The monoclonal antibody AXD5 against a sperm tail antigen (Karr 1991) was used for immunolabeling and Hoechst 33258 for DNA staining of early embryos (Lehner and O'Farrell 1989). Scanning electron micrographs and plastic sections of adult eyes were prepared as described (Basler *et al.* 1991).

Induction of heat-inducible transgenes: To test whether periodic expression of the transgenes *Hs-Cdk2* and *Hs-Cdk2AF* suppresses the lethality resulting from the lack of endogenous *Cdk2* function, we recombined transgene insertions on the third chromosome with *Df(3R)H81*. Flies with the recombinant chromosomes balanced over *TM2* were crossed to either *Cdk2*^{2}/*TM3*, *Sb* or *Cdk2*^{3}/*TM3*, *Sb*. Progeny were exposed to periodic heat shocks as described previously (Stern *et al.* 1993). In initial experiments, heat shocks were applied at 3-hr intervals, and the fraction of progeny flies without balancer chromosomes was determined. Under these conditions, rescue of *Cdk2*/*Df(3R)H81* mutants was found to be inefficient, largely because the *Df(3R)H81* chromosome dominantly reduced survival. Therefore, we crossed *Cdk2*^{2}/*TM3*, *Sb* flies carrying either *Hs-Cdk2 (III.70.1)* or *Hs-Cdk2AF (II.2)* to *Cdk2*^{1}/*TM3*, *Sb* or *Cdk2*^{3}/*TM3*, *Sb* for the experiments described in detail (see Table 3 and Figure 4).

To address the role of *Cdk2* during oogenesis and early embryogenesis, we crossed *Cdk2*^{2}, *Hs-Cdk2*/*TM3*, *Hs-hid* with either *Cdk2*^{1}/*TM3*, *Hs-hid* or *Cdk2*^{3}/*TM3*, *Hs-hid* and exposed the progeny to 30-min heat shocks at 12-hr intervals. The resulting *Cdk2*^{2}, *Hs-Cdk2*/*Cdk2*^{1} or *Cdk2*^{2}, *Hs-Cdk2*/*Cdk2*^{3} females were crossed to male flies that had been raised at 25° to ensure maximal male fertility. For control experiments, such males were also crossed to *w* females that had been exposed to 30-min heat shocks at 12-hr intervals during development. One-half of the crosses were kept at 25°, while the other half of the crosses was continuously exposed to periodic heat shocks at 12-hr intervals. After 4 days, the flies exposed to periodic heat shocks were distributed into two fresh bottles. One of these bottles was subsequently kept at 25°, while the other was further subjected to periodic heat shocks. During each day of the experiment, egg collections were performed for 5 hr on apple agar plates for counts of laid eggs and for the subsequent 7 hr for fixation and analysis by double labeling with anti-sperm tail antibodies and Hoechst 33258.

To determine the lethality of *Hs*-transgene expression during embryogenesis, we crossed males homozygous for one of the transgenes (see Table 3) to *w* virgins. From these crosses, eggs were collected for 2 hr at 25°. After aging for 3 hr, a heat shock was applied by floating the collection plates on a 37° water bath for 30 min. The collection plates were returned to 25° and after a 3-hr incubation, one-half of the collection was exposed to a second heat shock. After an additional incubation for 24 hr at 25°, the fraction of unhatched eggs was determined.

Larval growth measurement: Flies carrying different *Cdk2* alleles balanced with *TM3, Act-GFP* were crossed and eggs were collected during 3 hr at 25°. Larval progeny was isolated at different time points after egg deposition as described (Britton and Edgar 1998). After collection of larvae in 2 m sucrose and freezing in 86% glycerol, green fluorescent protein-positive *Cdk2*$^+$ and GFP-negative *Cdk2* mutant larvae were sorted using a Leica MZFLIII microscope equipped with a video camera. Video frames were captured using IPLab Spectrum software and the pixel area covered by individual larvae was determined.

RESULTS

A screen for modifiers of the rough eye phenotype resulting from ectopic expression of *Cyclin E* under the control of the *sevenless* enhancer: During wild-type development, *Cyclin E* expression is no longer observed in eye imaginal disc cells after their recruitment into differentiating ommatidia (Richardson *et al.* 1995). To achieve ectopic *Cyclin E* expression in these postmitotic cells of differentiating ommatidia, we used an enhancer element from the *sevenless* (*sev*) gene that directs expression in a subset of photoreceptor cells and in cone cells (Dickson and Hafen 1993). Several lines with a *Sev-CycE* transgene were established and pulse labeling with BrdU was used to determine whether the ectopic *Cyclin E* expression from this transgene resulted in extra proliferation in eye imaginal discs. In wild-type eye discs from third instar larvae, we observed the expected, characteristic pattern of BrdU incorporation [Figure 1A; for a description of cell proliferation during Drosophila eye development see Wolff and Ready (1993)]. In eye discs from transgenic lines, we obtained an additional posterior band of BrdU incorporation (Figure 1B, arrow). Double labeling of *Sev-CycE* eye imaginal discs with a DNA stain and antibodies against Elav, a marker for neuronal differentiation that is only expressed in postmitotic photoreceptor cells during wild-type development, revealed mitotic figures in some of the *elav*-expressing cells (data not shown). These observations indicate that *Sev-CycE* transgene expression interferes with the arrest of cell cycle progression at the correct developmental stage. The ectopic *Cyclin E* expression forces many cells into an extra S phase and at least some also through an extra M phase.

In addition, the *Sev-CycE* transgene was found to cause pattern defects in the eyes of adult flies (Figure 2, A–C). Ommatidia containing more than the normal complement of photoreceptor cells were frequently observed in sections through these eyes (Figure 3B). However, ommatidia with fewer photoreceptor cells were also observed, as well as other pattern irregularities. Loss of cells from ommatidia might result as a secondary consequence of mitotic divisions of differentiating cells, perhaps because extra cells cause difficulties for the establishment of the correct cell-cell contacts required for cell survival. Alternative explanations are not excluded and neomorphic activity of ectopic Cyclin E that is unrelated to its physiological role in proliferating cells might contribute to the rough eye phenotype. Pattern defects in the adult eye have also been observed after ectopic Cyclin E expression from a heat-inducible transgene (Richardson *et al.* 1995).

While the expressivity of the rough eye phenotype was very similar in flies carrying a particular *Sev-CycE* insertion, eye appearance clearly varied with different insertions, suggesting that the eye phenotype was sensitive to differences in expression levels resulting from transgene position effects. The enhancement of eye roughening resulting from increases in transgene copy number confirmed this notion (Figure 2, A–C). Conversely, heterozygosity for the deficiency *Df(3R)H81*, which deletes the *Cdk2* gene, suppressed the eye roughening in *Sev-CycE* flies partially. Since a *Cdk2*$^+$ transgene reversed this partial suppression by *Df(3R)H81*, we conclude that the *Sev-CycE* phenotype is also sensitive to the *Cdk2*$^+$ dose (data not shown, but see below). The sensitivity of the *Sev-CycE* phenotype, therefore, appeared suitable for the identification of mutations interacting with Cyclin E/Cdk2 function.

By screening 38,000 flies carrying *Sev-CycE* and a set of EMS-mutagenized chromosomes, we recovered 32 chromosomes that dominantly modified the rough eye phenotype and were also associated with recessive lethal-

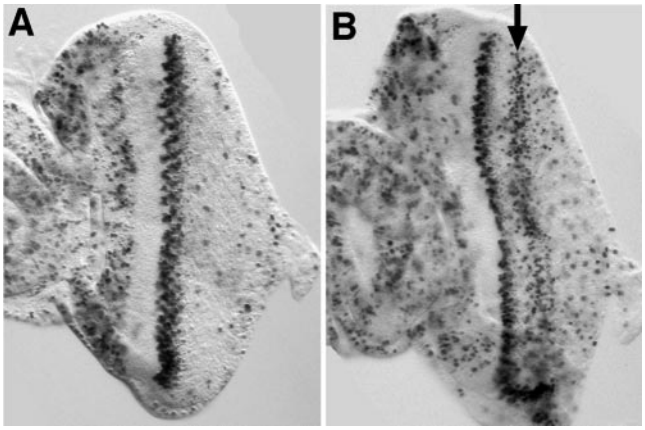

Figure 1.—*Sev-CycE* induces ectopic cell cycle progression. Eye-antennal imaginal discs from wild-type (A) and *Sev-CycE* (B) larvae at the wandering stage were labeled with BrdU. In *Sev-CycE* discs, an additional band of BrdU incorporation is observed in the posterior region and is indicated by the solid arrow. The anterior of the discs is oriented to the left, the posterior to the right.

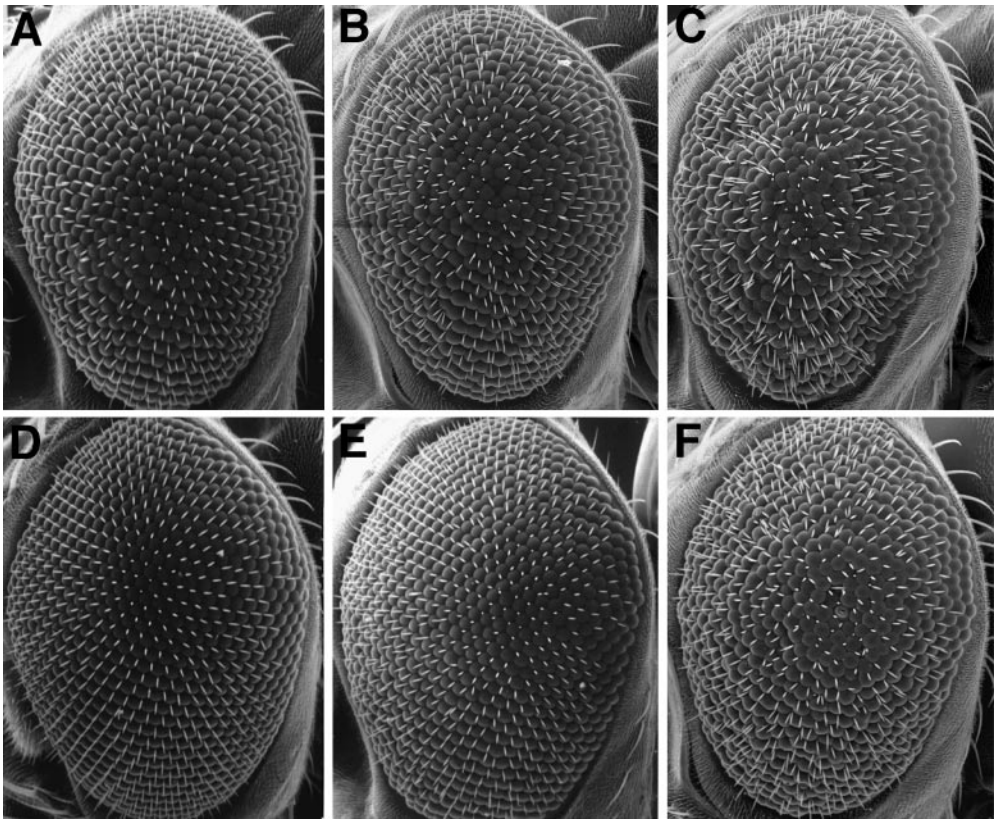

Figure 2.—*Sev-CycE* induces a rough eye phenotype sensitive to transgene dose and genetic interactions. Scanning electron micrographs of eyes from adult flies with the genotypes *Sev-CycE I* (*III.1*)/+ (A), *Sev-CycE I* (*III.1*)/*Sev-CycE I* (*III.1*) (B), *Sev-CycE I* (*III.1*), *Sev-CycE I* (*III.2*)/*Sev-CycE I* (*III.1*), *Sev-CycE I* (*III.2*) (C), wild type (D), *Sev-CycE I* (*III.1*)/*Cdk2*^{2} (E), and *spen*$^{E(Sev\text{-}CycE)E9}$/+; *Sev-CycE I* (*III.1*)/+ (F).

ity. A total of 24 mutations acted as enhancers [*E(Sev-CycE)*; for an example see Figure 2F]. A total of 8 mutations behaved as dominant suppressors [*S(Sev-CycE)*; for an example see Figure 2E]. Based on linkage and complementation tests, 13 mutations qualified as single hits, while the rest could be assigned to one of three different complementation groups with multiple alleles (Table 1). One of the single hits and the three complementa-

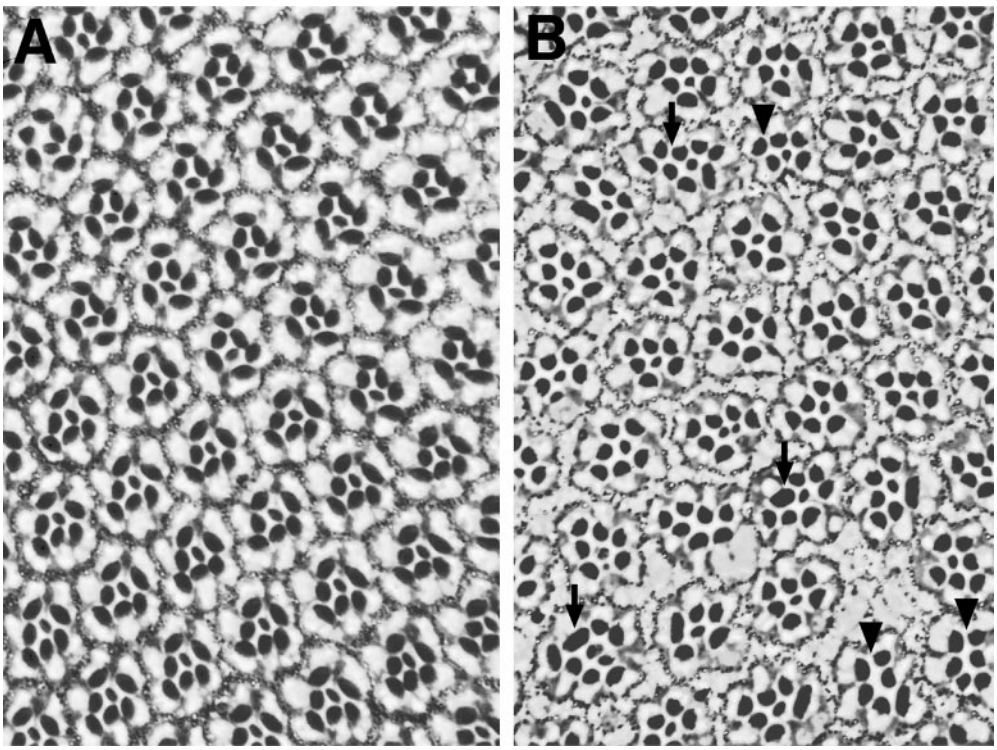

Figure 3.—*Sev-CycE* induces ommatidial irregularities including alterations in photoreceptor cell numbers. Tangential sections of either wild-type (A) or *Sev-CycE I* (*III.1*)/*Sev-CycE I* (*III.1*) (B) flies are shown. Some of the ommatidial clusters with supernumerary photoreceptor cells are indicated by solid arrows and some with decreased photoreceptor cell numbers are indicated by solid arrowheads.

TABLE 1

Complementation groups of mutations modifying the *Sev-CycE* phenotype

Complementation group	Alleles	Gene
E(Sev-CycE)2A	11	*split ends* (*spen*)
E(Sev-CycE)2B	1	*dacapo* (*dap*)
E(Sev-CycE)3A	5	*dE2F1*
S(*Sev-CycE*)*3A*	3	*Cdk2*

tion groups could be assigned to defined genes as described in the following.

***split ends (spen)*:** The largest complementation group *E(Sev-CycE)2A* comprised 11 mutations. Meiotic recombination mapping placed this complementation group close to the left end of chromosome 2. Subsequent complementation tests with a series of deficiencies refined the map location to 21B. Additional complementation tests revealed that several alleles of this complementation group failed to complement two *P*-element insertions, *l(2)03350* and *l(2)k13624*, which had been mapped to this region. These two *P* elements were found to be inserted 1.5 kb apart from each other and 5′ of a large open reading frame. Complementation tests with additional mutations that had been previously assigned to this region revealed that *E(Sev-CycE)2A* is allelic to *E(Raf)2A* (Dickson *et al.* 1996), *E(E2F)2A* (Staehling-Hampton *et al.* 1999), and *polycephalon* (Gellon *et al.* 1997), which has recently been characterized molecularly and found to correspond to *spen* (Wiellette *et al.* 1999; see discussion).

***dE2F1*:** The complementation group *E(Sev-CycE)3A* with five alleles was mapped meiotically to the region that contains the Drosophila *dE2F1* gene. Complementation tests indicated that the two analyzed *E(Sev-CycE)3A* mutations failed to complement $dE2F1^{91}$ and $dE2F1^{7172}$.

***dap*:** One *E(Sev-CycE)* mutation (G36) failed to complement the *P*-element insertion B13-2nd-10 (Bier *et al.* 1989). We have already described a detailed analysis of the corresponding gene, which we have named *dacapo* (*dap*) since epidermal cells in *dap* mutant embryos resume progression through an additional division cycle instead of becoming postmitotic (DeNooij *et al.* 1996; Lane *et al.* 1996). *dap* encodes a cdk inhibitor related to vertebrate CIP/KIP-type inhibitors and the DAP protein has been shown to bind and inhibit specifically Cyclin E/Cdk2 complexes (DeNooij *et al.* 1996; Lane *et al.* 1996).

***Cdk2*:** The complementation group *S(Sev-CycE)3A* with three alleles was mapped meiotically to the right arm of chromosome III in the genomic region containing the *Cdk2* gene. Therefore, we tested whether the lethality that was caused by hemizygosity of these alleles over *Df(3R)H81* could be prevented by a $Cdk2^{+}$ transgene. The $Cdk2^{+}$ transgene was found to confer full viability to the three different hemizygotes. Moreover, periodic expression of a heat-inducible transgene (*Hs-Cdk2*) containing a *Cdk2* cDNA under control of a heat-shock promoter was found to prevent the lethality associated with transheterozygous combinations of the three *S(Sev-CycE)3A* alleles (Table 3). Finally, sequence analysis of the *Cdk2* sequence isolated from one of the *S(Sev-CycE)3A* chromosomes revealed the presence of a premature stop codon (TAG) instead of a glutamine codon (CAG) at position 134 of the predicted amino acid sequence. Based on these results, we conclude that *S(Sev-CycE)3A* corresponds to the *Cdk2* gene. In the following, the alleles are designated as $Cdk2^{1}$, $Cdk2^{2}$, and the sequenced allele $Cdk2^{3}$. The $Cdk2^{3}$ mutation is likely to eliminate *Cdk2* function completely, since translational termination at the premature stop codon at position 134 results in deletion of the C-terminal lobe of the protein kinase domain.

***Cdk2* is required zygotically for larval growth and maternally for early embryogenesis:** To characterize the effects of *Cdk2* mutations on development, we first analyzed embryogenesis. However, we were unable to identify a requirement for zygotic *Cdk2* expression during this developmental phase. Embryos hemizygous or transheterozygous for *Cdk2* allele combinations appeared to have wild-type morphology. They incorporated BrdU with normal efficiency and in normal patterns throughout embryogenesis. Hatching of larvae was observed to occur at the same rate as in control embryos.

For the characterization of larval development, we analyzed progeny from parents with *Cdk2* alleles over a balancer chromosome carrying *Tb*. Scoring for the *Tb* phenotype, which can readily be distinguished from wild type after development beyond the second larval instar, suggested that *Cdk2* mutants do not reach this stage. However, experiments involving the expression of *Hs-Cdk2* in *Cdk2* mutant larvae indicated that these mutants survive for longer time periods than what is normally required to reach second instar (Figure 4A). As indicated above, periodic *Hs-Cdk2* expression by heat shocks allowed *Cdk2* mutants to develop into morphologically normal and fertile adults. Some *Cdk2* mutants were still observed to develop into adults even if the onset of the periodic *Hs-Cdk2* expression was delayed until 116 hr after egg deposition (Figure 4A). *Cdk2* mutant larvae, therefore, fail to grow but some survive for several days and can be rescued by providing Cdk2 again. The dependence of larval growth on zygotic *Cdk2* expression was confirmed by comparing the size of $Cdk2^{+}$ and *Cdk2* mutant larvae derived from parents with *Cdk2* alleles over a balancer chromosome marked by an *Act-GFP* transgene. GFP-negative *Cdk2* mutant larvae were observed in decreasing numbers for at least 4 days after egg deposition, but their size failed to increase significantly after 1.5 days (Figure 4B).

The normal initial development that was observed in the absence of zygotic *Cdk2* function could be explained

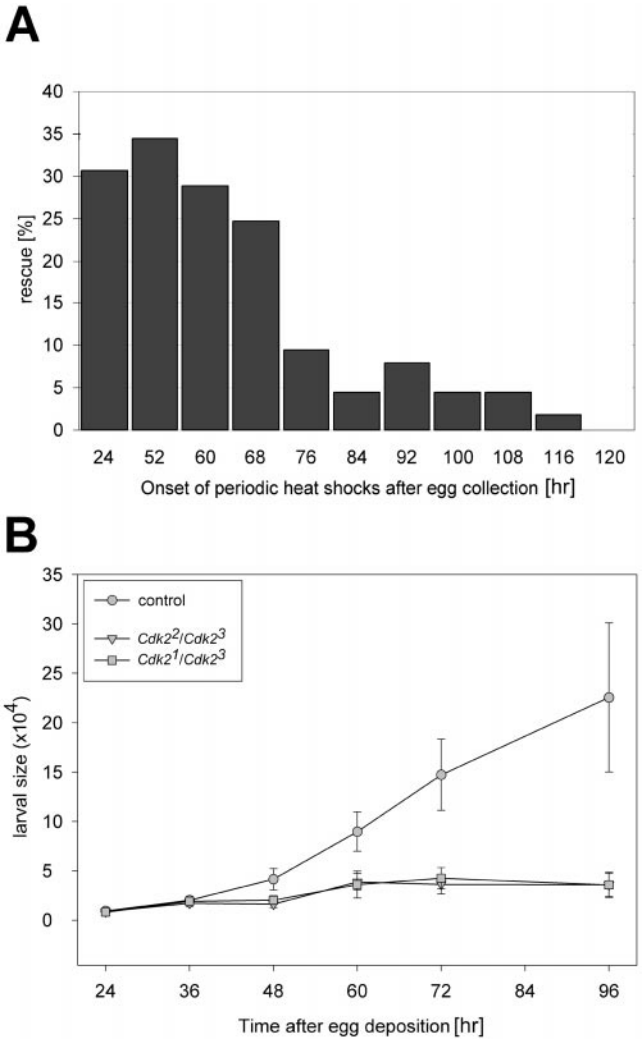

Figure 4.—Zygotic *Cdk2* function is required for larval growth and long-term survival. (A) *Cdk2*^{2}, *Hs-Cdk2*/*TM3*, *Sb* flies were crossed with *Cdk2*^{3}/*TM3*, *Sb* and the progeny collected during a 4-hr egg collection was exposed to periodic heat shocks at 6-hr intervals starting at variable times of development at 25°. The onset of the periodic heat shocks in hours after the end of the egg collection is indicated below the bars. The percentage of *Cdk2*^{2}, *Hs-Cdk2*/*Cdk2*^{3} among the total eclosing flies was determined. While early onset of periodic heat pulses resulted in a full rescue of *Cdk2*^{2}, *Hs-Cdk2*/*Cdk2*^{3} vitality (33% of *Sb*$^+$ flies theoretically), no rescue was observed after late onset of periodic heat pulses. At least 200 flies were scored for each time point. (B) The growth of *Cdk2*^{2}/*Cdk2*^{3} (triangles), *Cdk2*^{1}/*Cdk2*^{3} (squares), and heterozygous sibling larvae marked by an *Act-GFP* balancer chromosome (circles) was analyzed as described in material and methods. At least 18 larvae were analyzed for each genotype and time point.

by maternally derived *Cdk2*. The presence of a maternal *Cdk2* contribution in the Drosophila egg has been demonstrated (Lehner and O'Farrell 1990). To demonstrate the functional role of this maternal contribution, we analyzed the development of eggs derived from *Cdk2* mutant females that had been rescued by periodic *Hs-Cdk2* expression. These mutant females (*Cdk2*^{2}, *Hs-Cdk2*/*Cdk2*^{3} or *Cdk2*^{2}, *Hs-Cdk2*/*Cdk2*^{1}) readily laid eggs as long as they were subjected to periodic heat shocks (Table 2). However, after termination of periodic heat shocks, egg deposition decreased rapidly and stopped completely within 2–3 days (Table 2). This arrest of egg deposition was readily reversed within 7 days after resumption of periodic heat shocks.

The eggs from mutant females collected 1 day after the termination of periodic *Hs-Cdk2* expression were fixed and stained for DNA. For comparison, we also analyzed the eggs from mutant females that had been maintained with periodic *Hs-Cdk2* expression. In addition, we analyzed eggs from *w* control females exposed to periodic heat shocks or 1 day after termination of these heat shocks. The great majority of the eggs from these *w* control females revealed normal DNA staining patterns (Figure 5A). Conversely, the majority of the eggs collected from mutant females (*Cdk2*^{2}, *Hs-Cdk2*/*Cdk2*^{3} or *Cdk2*^{2}, *Hs-Cdk2*/*Cdk2*^{1}) displayed abnormal DNA staining patterns (Figure 5, B–D, data not shown). The spatial distribution of nuclei and the appearance of chromatin was often aberrant, indicating that progression through the syncytial division cycles was severely perturbed in these embryos. This finding suggests that the maternal contribution is required during the syncytial division cycles. In addition, a significant fraction of embryos contained very few nuclei (Figure 5, E and F), suggesting that they had failed to commence progression through the syncytial divisions (although it is not excluded that a minor fraction of these eggs were fixed while progressing normally through the first three cycles). Double labeling with an antibody recognizing a sperm tail epitope (Figure 5, G and H) indicated that about two-thirds of these eggs with less than five nuclei were not fertilized. Compared to continuously heat-pulsed *Cdk2* mutant females, those withdrawn from heat-shock treatment generated a higher fraction of eggs containing less than five nuclei at the expense of the eggs with normal appearance. Termination of periodic *Hs-Cdk2* expression in *Cdk2* mutant females, therefore, is accompanied by a transient production of eggs that cannot be fertilized followed by a rapid arrest of egg laying. A significant fraction (60%) of abnormal eggs were produced even when periodic *Hs-Cdk2* expression was maintained. The production of abnormal eggs despite periodic *Hs-Cdk2* expression is likely to reflect the fact that the germ line is refractory to induction of heat-shock genes during stages 10–12 of oogenesis (Wang and Lindquist 1998). Our observations demonstrate that *Cdk2* expression is crucial for oogenesis and early embryogenesis.

Cdk2 lacking the conserved putative phosphorylation sites Thr18 and Tyr19 can provide all essential functions *in vivo*: Inhibition of Cyclin E/Cdk2 appears to be required for the arrest of cell proliferation at the appropriate developmental stage. In the embryonic epidermis, upregulation of *dap* expression is known to occur immediately before the epidermal cells exit from the

TABLE 2

Dependence of egg laying on *Hs-Cdk2* expression in *Cdk2* mutant females

	Number of eggs laid by:[a]					
	Cdk2², *Hs-Cdk2* / *Cdk2¹*		*Cdk2²*, *Hs-Cdk2* / *Cdk2³*		*Cdk2⁺ (w)*	
Time (days)	+ hs	− hs	+ hs	− hs	+ hs	− hs
1	144	155	56	58	38	43
2	532	12	472	6	277	236
3	410	2	746	0	164	197
4	988	0	892	0	560	600
5 / 1[b]	704	372[b]	344	508[b]	804	1216[b]
6 / 2[b]	860	0[b]	914	1[b]	948	1656[b]
7 / 3[b]	345	0[b]	470	0[b]	442	398[b]

[a] Females with the genotype *Cdk2²*, *Hs-Cdk2* / *Cdk2¹*, or *Cdk2²*, *Hs-Cdk2* / *Cdk2³* or *w* were obtained after applying heat shocks at 12-hr intervals throughout development as described in materials and methods. These females were crossed to males and half of the crosses were incubated at 25° (−hs), while the other half of the crosses was further exposed to periodic heat shocks at 12-hr intervals (+hs). Eggs laid during a 5-hr collection period were counted each day.

[b] After 4 days, the crosses that had been exposed to heat shocks (+hs) were divided and one-half of the crosses was incubated at 25°, while the other half of the crosses was further exposed to periodic heat shocks at 12-hr intervals.

mitotic cell cycle. In *dap* mutants an additional division cycle is observed instead of a proliferation arrest (DeNooij *et al.* 1996; Lane *et al.* 1996). In parallel to *dap* upregulation, downregulation of *Cyclin E* expression is observed in the embryonic epidermis (Knoblich *et al.* 1994). It is not known what triggers this simultaneous up- and downregulation and, given the complexity of the regulatory loops controlling progression into the cell cycle, it was not excluded that inhibition of Cyclin E/Cdk2 by inhibitory phosphorylation of the Cdk2 subunit, as known to occur in cultured mammalian cells, might contribute to Cyclin E/Cdk2 inactivation at the stage when the embryonic epidermal cells exit from the cell cycle. Moreover, upregulation of *dap* expression does not always precede cell proliferation arrest during Drosophila development. A cell proliferation arrest independent of *dap* upregulation, for instance, is observed in the region of the wing margin in third instar imaginal discs and in the region in front of the advancing morphogenetic furrow in the eye imaginal disc (DeNooij *et al.* 1996; Lane *et al.* 1996; Johnston and Edgar 1998). Inhibitory Cdk2 phosphorylation, therefore, might potentially replace inhibition by DAP.

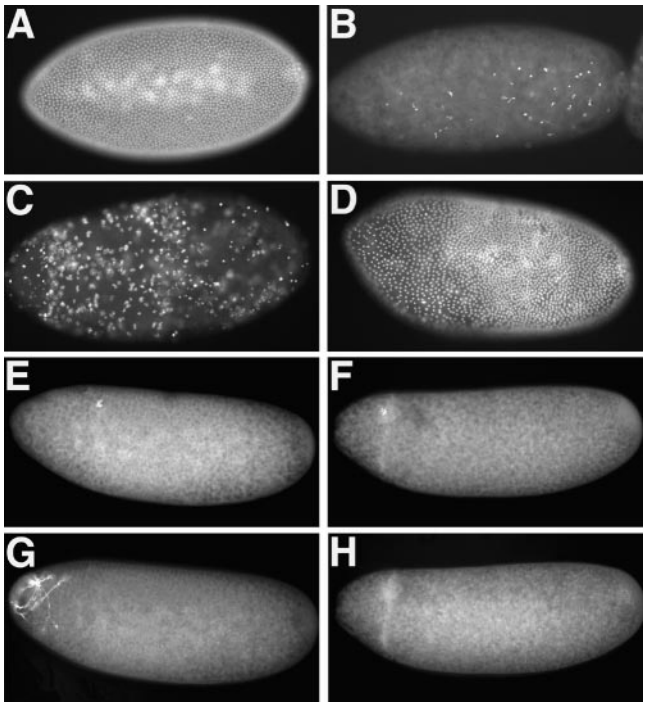

Figure 5.—Maternal *Cdk2* function is required for early embryonic cell cycle progression. Eggs from wild-type females (A) and *Cdk2²*, *Hs-Cdk2* / *Cdk2³* females collected 1 day after the last of periodic heat shocks stained for DNA (A–F) and with anti-sperm tail antibodies (G and H) are shown to illustrate the various phenotypic classes observed. E, G and F, H, respectively, show the same egg. For further details see text.

To address the role of inhibitory Cdk2 phosphorylation, we mutated the conserved amino acid residues at the positions corresponding to the sites of inhibitory phosphorylation in Cdk1 and mammalian cdk2. By mutating the threonine and tyrosine codons at positions 18 and 19 into alanine and phenylalanine codons, respectively, the potential phosphate acceptor sites were eliminated. We established transgenes, allowing conditional expression of the mutant kinase under control of the heat-shock promoter.

In a first experiment, we analyzed whether expression of mutant *Cdk2* at the stage of embryogenesis when epidermal cells exit from the cell cycle prevents a timely arrest of cell proliferation. BrdU pulse labeling failed to reveal extra proliferation (data not shown), sug-

gesting that the cell cycle arrest in the embryonic epidermis is not dependent on inhibitory Cdk2 phosphorylation. In further experiments, we addressed whether expression of the mutant kinase at other developmental stages caused lethality or morphological abnormalities. However, we failed to observe effects of *Hs-Cdk2AF* (Table 3). These negative results raised the question whether the mutant kinase was expressed and active *in vivo*. Therefore, we crossed the *Hs-Cdk2AF* transgene into a *Cdk2* mutant background and tested whether periodic expression could rescue the lethality associated with the *Cdk2* mutant background. As shown in Table 3, *Hs-Cdk2AF* rescued the lethality with the same efficiency as *Hs-Cdk2*. We conclude therefore that Cdk2, which cannot be phosphorylated on the sites corresponding to those that are of crucial importance in the case of Cdk1 regulation, can provide all the essential functions during Drosophila development.

DISCUSSION

Although the crucial role of cyclin E/cdk2 complexes in cell cycle control has been clearly demonstrated, their precise functions have yet to be defined. Thus we have designed a genetic approach in *D. melanogaster* for the identification of genes involved in Cyclin E/cdk2 function. By screening for mutations that act as dominant modifiers of the rough eye phenotype caused by ectopic *Cyclin E* expression under the control of the *sevenless* enhancer, we have identified the genes *dE2F1*, *dap*, and *Cdk2(Cdc2c)*, which are known to encode proteins that are intimately involved in *Cyclin E* function. The future analysis of the additional uncharacterized modifier mutations that we have identified, therefore, can be expected to define novel components involved in Cyclin E/Cdk2 function.

The largest complementation group identified in our screen, *E(Sev-CycE)2A*, has not previously been implicated in Cyclin E function. Our genetic and molecular analysis of *E(Sev-CycE)2A* suggested that it corresponds to the *spen* gene. Independent work has proven this suggestion (Wiellette *et al.* 1999). *spen* encodes a 600-kD ubiquituously expressed nuclear protein containing three RNP-type RNA binding domains and a novel characteristic C-terminal domain defining a family of homologous metazoan genes. Mutations in *spen* result in peripheral nervous system defects (Kolodziej *et al.* 1995; Kuang *et al.* 1999) and interact with raf kinase signaling (Dickson *et al.* 1996) and the function of HOX (Gellon *et al.* 1997; Wiellette *et al.* 1999) and E2F/DP transcription factors (Staehling-Hampton *et al.* 1999) and, as shown here, with *Cyclin E*. It is attractive to speculate that *spen* is particularly important for the transition from cell proliferation to terminal differentiation. *spen* mutant ommatidia in the eye (Dickson *et al.* 1996) display the same defects as those resulting from the *Sev-CycE* transgene. The affected ommatidia are of variable composition, often lacking either R7 or one or more other photoreceptors, but also occasionally containing extra photoreceptors. *Sev-CycE* transgene expression forces differentiating cells through an extra cell cycle, presumably explaining the presence of extra cells. In addition, extra divisions of differentiating cells are likely to disturb the regular arrangement of the ommatidial cluster and consequently might cause apoptosis, potentially explaining the observed loss of cells as well. Similarly,

TABLE 3

Effects of *Hs-Cdk2AF* expression during wild-type and *Cdk2* mutant development

	Fraction of unhatched eggs (%)[a]		Extent of rescue (%)[b]			
			$Cdk2^2/Cdk2^1$		$Cdk2^2/Cdk2^3$	
Transgene	One heat shock	Two heat shocks	−Hs	+Hs	−Hs	+Hs
None	4	12	0	ND	0	0
Hs-Cdk2 (*III.70.1*)	28	31	ND	ND	0	31
Hs-Cdk2 (*III.19.3*)	14	25	ND	ND	ND	ND
Hs-Cdk2AF (*II.1*)	9	20	0	30	0	26
Hs-Cdk2AF (*II.2*)	24	19	ND	ND	ND	ND
Hs-Cdk1 (*64.2*)	12	12	ND	ND	ND	ND
Hs-Cdk1 (*68.1*)	29	17	ND	ND	ND	ND
Hs-Cdk1AF (*III.72*)	92	98	ND	ND	ND	ND
Hs-Cdk1AF (*III.74*)	85	100	ND	ND	ND	ND

ND, not done.

[a] Two independent insertions of each transgene were expressed in $Cdk2^+$ embryos and the fraction of unhatched eggs was determined as described in materials and methods. At least 100 eggs were analyzed in each experiment.

[b] Transgenes were crossed into *Cdk2* mutant embryos that fail to develop into adult flies unless rescued by *Cdk2* function expressed from a transgene. Heat shocks to induce transgene expression were applied at 12-hr intervals. At least 500 adult progeny were analyzed in each experiment.

coexpression of *GMR-E2F1* and *GMR-DP* transgenes in all eye imaginal disc cells posterior to the morphogenetic furrow has been shown to result in ectopic BrdU incorporation and apoptosis. *spen* mutations dominantly enhance both the *Sev-CycE* and *GMR-E2F1/DP* rough eye phenotype. Conversely, *spen* mutations suppress the eye phenotypes resulting from *GMR-dap* expression in a *CycE* heterozygous background (Staehling-Hampton *et al.* 1999). While *spen* function opposes the mitogenic activity of *CycE* and *dE2F1*, it remains to be analyzed whether the phenotypic interactions observed between *spen* and *Hox* and *Raf* involve deregulated cell proliferation as well.

The identification of mutations in Drosophila *dE2F1* in our screen was expected on the basis of the large body of evidence demonstrating the tight functional relationship between Cyclin E and E2F/DP transcription factors. However, the fact that *dE2F1* mutations resulted in enhancement rather than suppression of the *Sev-CycE* phenotype would not necessarily have been predicted since the results of genetic analysis in Drosophila so far have suggested that E2F/DP activity has a positive role in stimulating the transcription of S phase genes (*Cyclin E, RNR2, DNA pol* α, *PCNA*, and *Orc1*) and cell proliferation (Duronio *et al.* 1995, 1998; Royzman *et al.* 1997; Asano and Wharton 1999; Secombe *et al.* 1999). In contrast, the enhancement of the *Sev-CycE* phenotype observed with *dE2F1* alleles points to a growth-suppressive role of *dE2F1*. Similarly, the *E2F1* knock-out phenotype observed in mice has clearly demonstrated a tumor-suppressing function (Field *et al.* 1996; Yamasaki *et al.* 1996). Moreover, while vertebrate E2F/DP functions as a transcriptional activator in some promoters, it acts as a corepressor in conjunction with pRB in many other promoters (for a review see Dyson 1998). A decrease in E2F/DP levels, therefore, might also result in derepression of unknown proliferation-stimulating genes and synergy with ectopic *Cyclin E* expression.

An understanding of the observed enhancement will require extensive and careful analysis given the complexity of the interactions between Cyclin E, E2F/DP, and RBF. We point out, however, that enhancement of the *Sev-CycE* phenotype was also observed with the putative null allele $dE2F1^{91}$, suggesting that we have not selected for dominant alleles in our screen (C. Lehner, data not shown). *dDP* alleles were not recovered in our screen, and a modification of the *Sev-CycE* phenotype by putative *dDP* null alleles could not be observed (C. Lehner, data not shown). With the exception of *spen* alleles, we also did not recover mutations in other genes identified in the screen for E2/DP interactors (Staehling-Hampton *et al.* 1999; C. Lehner, data not shown).

Cdk2 alleles were isolated as suppressor mutations, as expected. Our initial characterization of the *Cdk2* mutant phenotypes clearly demonstrates that *Cdk2* is required in females for oogenesis and for provision of a maternal contribution to the egg. This maternal contribution allows development in the absence of zygotic function until the first larval instar. After that stage, zygotic *Cdk2* expression is clearly required for larval growth and survival.

The identification of mutations in *Cdk2* has allowed us to evaluate the physiological significance of potential phosphorylation sites (Thr 18 and Tyr 19) that have been implicated in negative regulation of Cdk2 activity. The corresponding sites in Cdk1 (Thr 14 and Tyr 15) are known to be of paramount importance for physiological regulation. Expression of Cdk1 mutant protein (Cdk1AF) that can no longer be phosphorylated results in premature mitosis and inability to delay entry into mitosis in the presence of unreplicated or damaged DNA. *Cdk1AF* expression from a heat-inducible transgene in Drosophila embryogenesis results in lethality. Conversely, expression of an analogous Cdk2 mutant protein (Cdk2AF) has apparently no effect during Drosophila development. Moreover, *Hs-Cdk2AF* expression can restore normal development in mutants lacking endogenous *Cdk2* completely, demonstrating that Cdk2AF is functional. Our results therefore clearly demonstrate that phosphorylation on Thr 18 and Tyr 19 does not represent an essential level of Cdk2 regulation. The *dap*-dependent cell cycle arrest in G1 after the terminal mitosis 16 occurs at the correct developmental stage in the embryonic epidermis in the presence of Cdk2AF. In addition, the *dap*-independent G1 arrest in front of the approaching morphogenetic furrow in eye imaginal discs does not appear to be abolished by *Hs-Cdk2AF* expression. Failure to arrest in G1 ahead of the advancing morphogenetic furrow is known to result in a rough eye phenotype (Thomas *et al.* 1994), which is not observed after *Cdk2AF* expression.

Our demonstration that phosphorylation of Cdk2 on Thr 18 and Tyr 19 has no essential role during normal development does not exclude its involvement in subtle or stress regulation. Moreover, we also point out that vertebrate cells, in which Cdk2 phosphorylation on Thr 18 and Tyr 19 has been demonstrated to occur, express A-type cdc25 phosphatases that have been implicated in Cdk2 dephosphorylation and that do not appear to exist in Drosophila.

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