

Cyclin D Does Not Provide Essential Cdk4-Independent Functions in *Drosophila*

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ABSTRACT

The three mammalian D-type cyclins are thought to promote progression through the G₁ phase of the cell cycle as regulatory subunits of cyclin-dependent kinase 4 and 6. In addition, they have been proposed to control the activity of various transcription factors without a partner kinase. Here we describe phenotypic consequences of null mutations in *Cyclin D*, the single D-type cyclin gene in *Drosophila*. As previously observed with null mutations in the single *Drosophila Cdk4* gene, these mutations do not primarily affect progression through the G₁ phase. Moreover, the apparently indistinguishable phenotypes of double (*CycD* and *Cdk4*) and single mutants (*CycD* or *Cdk4*) argue against major independent functions of Cyclin D and Cdk4. The reduced cellular and organismal growth rates observed in both mutants indicate that Cyclin D-Cdk4 acts as a growth driver.

D-TYPE cyclin genes are present in plant and animal genomes. Their products function as regulatory subunits of specific cyclin-dependent kinases (cdks). Major physiological substrates of the D-type cyclin-cdk complexes are the retinoblastoma tumor suppressor protein family members (Rbfs), which are known to act as negative regulators of cell proliferation in plant and animal cells (ADAMS 2001; ORTEGA *et al.* 2002). Rbfs can bind to E2F transcription factors and recruit complexes with nucleosome remodeling, histone deacetylase, and methylase activities (STEVAUX and DYSON 2002). Many E2F target genes are required for progression through the cell cycle and their expression is inhibited by Rbfs (DIMOVA *et al.* 2003). Phosphorylation by D-type cyclin-cdk complexes suppresses this inhibitory activity of Rbfs. In addition, mammalian D-type cyclin-cdk complexes can also suppress Rbfs by titrating cdk inhibitors of the CIP/KIP family away from cdk2 complexes, which phosphorylate and thereby contribute to inhibition of Rbfs (ADAMS 2001; ORTEGA *et al.* 2002). Intriguing observations have argued for D-type cyclin-cdk targets other than Rbfs (DATAR *et al.* 2000; BOXEM and VAN DEN HEUVEL 2001; XIN *et al.* 2002; FREI and EDGAR 2004), although clear cases have yet to be demonstrated.

As suggested by a growing number of reports, D-type cyclins are also thought to function independently of either a cdk partner or its kinase activity (COQUERET 2002). Such functions have been described primarily in the context of transcriptional regulation of various

differentiation programs. Mammalian cyclin D1 and D2 were shown to inhibit myogenic differentiation through inactivation of MyoD transcriptional activity (SKAPEK *et al.* 1995, 1996). Cyclin D1 seems to relocalize cdk4 to the nucleus, leading to an inhibition of the myogenic transcription factor apparently by direct interaction with cdk4 independent of kinase activity (ZHANG *et al.* 1999). More recently cyclin D1 has been shown to bind to Myb, DMP1, STAT3, and Beta2/NeuroD transcription factors and to inhibit their transcriptional activity independent of cdk4 (GANTER *et al.* 1998; INOUE and SHERR 1998; HORSTMANN *et al.* 2000; BIENVENU *et al.* 2001; RATINEAU *et al.* 2002). In addition, cyclin D1 interacts with nuclear receptors such as the androgen and thyroid hormone receptors and represses their transcriptional activity (KNUDSEN *et al.* 1999; REUTENS *et al.* 2001; LIN *et al.* 2002). In contrast, cyclin D1, but not D2 or D3, have been reported to activate estrogen receptor (ER)-mediated transcription by recruitment of coactivators after direct binding to ER (ZWIJSEN *et al.* 1997, 1998).

While it is clear, therefore, that at least some D-type cyclins can effectively modulate transcriptional activation in a cdk-independent manner, the physiological role of these effects remains to be clarified. For instance, the results obtained with knock-in mice in which the cyclin D1 coding regions were replaced by those of cyclin E have questioned the physiological significance of the interaction between cyclin D1 and ER (GENG *et al.* 1999). In these knock-in mice, cyclin-E expression under cyclin D1 control was found to rescue the mammary gland proliferation defect known to result from loss of cyclin D1 function. As cyclin E lacks the ability to stimulate ER transcriptional activity, proliferation of

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the mammary epithelium is unlikely to depend on the interaction between ER and cyclin D1 observed after overexpression in transfection experiments.

For the identification of physiologically relevant cdk-independent D-type cyclin functions, a careful comparison of organismal phenotypes resulting from loss of either D-type cyclin or partner cdk gene functions or both is clearly of interest. While genetic redundancy complicates and has so far precluded such analyses in the mouse, which has three different D-type cyclins and two partner kinases, *cdk4* and *cdk6*, *Caenorhabditis elegans* and *Drosophila melanogaster* have single genes for cyclin D and its partner kinase *cdk4*. In *C. elegans* inactivation of *cyd-1* and *cdk-4* function by mutations or RNA interference results in similar but not identical phenotypes (PARK and KRAUSE 1999; BOXEM and VAN DEN HEUVEL 2001). It remains to be clarified whether the subtle phenotypic differences reflect partially independent *cyd-1* and *cdk-4* functions or differential stabilities of maternal protein contributions.

In *Drosophila*, we have isolated *Cdk4* null alleles and have demonstrated that some mutant progeny can develop to the adult stage in complete absence of maternal and zygotic *Cdk4* function (MEYER *et al.* 2000). Moreover, in addition to the expected involvement in the E2F-Rbf pathway, our findings demonstrated that Cdk4 is required for normal growth (accumulation of mass) of cells and the organism (DATAR *et al.* 2000; MEYER *et al.* 2000). Consistent with this, overexpressed Cyclin D (CycD)-Cdk4 complexes stimulate extra growth in many cell types in the fly (DATAR *et al.* 2000). Here we describe the isolation and phenotypic characterization of *CycD* null mutations. The consequences of these mutations are extremely similar if not identical to those reported for *Cdk4* mutations. Moreover, double mutants do not display a more severe phenotype than single mutants. Our results therefore indicate that *Drosophila* Cyclin D and Cdk4 do not provide independent functions that are vitally important for cell proliferation and differentiation. In addition, they further confirm the crucial role of Cyclin D-Cdk4 in the regulation of cellular growth rates.

MATERIALS AND METHODS

Fly stocks: The $y^{-}, w^{+}, P\{y^{+}mDm12\}, w^{+BR.E.BR} = SUPor-P\} KG04817$ stock was isolated by the gene disruption project of the Berkeley *Drosophila* Genome Project (BDGP) and kindly provided by Hugo Bellen (Baylor College of Medicine, Houston). Polymerase chain reaction (PCR) experiments confirmed the presence of a *P*-element insertion in this stock at the chromosomal position determined by the BDGP. For the mobilization of the transposon, we used *CyO*, *HoPI(w⁺)*, a balancer chromosome with a *hobo* element containing a $\Delta 2-3$ *P*-transposase gene (O'KANE 1998). $y^{-}, w^{+}, P\{SUPor-P\} KG04817$ males were therefore crossed with $w^{-} y^{-}/FM7i; +/CyO, HoPI(w^{+})$ females and the resulting $y^{-}, w^{+}, P\{SUPor-P\} KG04817/FM7i; +/CyO, HoPI(w^{+})$ progeny were mated with *FM7i/Y* males. From the

next generation, 258 virgin females carrying the *FM7i* balancer and lacking the *yellow⁺* marker were selected and mated individually with *FM7i/Y* males. The y^{-} revertant X chromosome was found to be associated with recessive lethality in only four of the resulting stocks. While we have no hints about the cause of the lethality in two of these cases, our molecular analyses of the two other chromosomes revealed deletions removing sequences of not only *CycD* but also *CG8909*, which therefore is likely an essential gene. A total of 161 of the residual stocks with y^{-} revertant X chromosomes that were not associated with lethality were analyzed by PCR for the presence of *CycD* gene regions close to the *P\{SUPor-P\} KG04817* insertion site. Males carrying revertant chromosomes from six independent stocks failed to yield the corresponding *CycD* fragment. With analogous PCR assays these six revertant chromosomes were further analyzed for the absence of other *CycD* gene regions. Finally, by sequence analysis of PCR fragments amplified from the two revertant chromosomes with the largest intragenic *CycD* deletions, *CycD¹* and *CycD²*, we determined the exact breakpoints. In the case of *CycD¹*, the proximal and distal breakpoints were found to be 1011 and 892 bp upstream and downstream of the start of the *CycD* coding region, respectively, with 29-bp residual *P*-element sequences in between. The *CycD²* breakpoints were 1243 and 337 bp upstream and downstream of the translational start, respectively, with two extra bases in between.

Cdk4³, UAS-CycD II.1, da-GAL4 G32, Cdk2², Cdk2³, CycE^{AR95}, CycE^{PZ5}, Cdk1^{E1-25}, Cdk1^{B47}, CycA^{CSLR1}, CycB², CycB3³, DP^{a2}, DP^{a4}, E2F1⁹¹, dap⁴, and dap^{g36} have been described previously (STERN *et al.* 1993; WODARZ *et al.* 1993; KNOBLICH *et al.* 1994; DURONIO and O'FARRELL 1995; ROYZMAN *et al.* 1997; JACOBS *et al.* 1998; LANE *et al.* 2000; MEYER *et al.* 2000).

Southern blot and genotyping by PCR: Genomic DNA from *w¹, CycD¹, or CycD²* flies was isolated and analyzed with a Southern blot according to standard procedures. Probe e and probe i (Figure 1A) were enzymatically amplified from *FM7i* genomic DNA using either the primer pair SA1 (5'-ATC AAG AGT AAG TTC GTA AGA TCG-3') and SA2 (5'-CAT TAT ATC GGC CAT ACG TTCC-3') or HJ30 (5'-GGC CTA AAG TGG CAT CTG-3') and SA4 (5'-TGA TAT TGG CCA ATC CTA TAG TG-3') followed by random primer labeling.

For genotype determination of progeny resulting from the cross illustrated in Figure 3C, genomic DNA from single flies was prepared and used as a template in a multiplex PCR containing the primers df (5'-GTA CAG GAT CTT TAA GTG CAG C-3'), dr (5'-ATC TCT TGC TCA CTG CGA TCA G-3'), d1r (5'-CGA CTT AGC ACA TAC AGC TCC-3'), k4f (5'-GAG AAC GGT GTG CCA ATG-3'), and k4r (5'-GAG CGG ATC GAC TTG CTT CAG-3').

Clonal analyses: Meiotic recombination was used for the construction of X chromosomes carrying *P\{neoFRT\}18A* (XU and RUBIN 1993) and either the *CycD¹* or the *CycD²* mutation. These FLP recombinase target site (FRT) chromosomes were crossed over an FRT chromosome with *P\{Ubi-GFP(S65T)nls\}X* and *P\{neoFRT\}18A*. In addition, *P\{hsFLP\}86E* was crossed in and mitotic recombination between the X chromosomes was induced by a heat shock (25 min in a 37° water bath). Resulting clones in wing-imaginal discs were analyzed as described previously (NEUFELD *et al.* 1998; DATAR *et al.* 2000). Parallel control experiments were done with larvae carrying an X chromosome with *P\{neoFRT\}18A* and the wild-type *CycD* allele over *P\{Ubi-GFP(S65T)nls\}X P\{neoFRT\}18A*. Anticlasted Caspase-3 antibodies (ASP 175) were obtained from Cell Signaling Technology and used at 1:1000.

In situ hybridization and BrdU pulse labeling: Embryos collected from stocks with *CycD¹* or *CycD²* over the *FM7c, P\{ry⁺U2 = ftz/lacC\}YH1* blue balancer chromosome were fixed

as previously described (WHITE 1998). For pulse labeling with 5-bromo-deoxyuridine (BrdU), embryos were permeabilized with octane and incubated for 20 min in Schneider's medium containing 1 mg/ml BrdU before fixation (KNOBLICH *et al.* 1994). Immunolabeling with mouse monoclonal antibodies against BrdU (Becton-Dickinson) in combination with secondary goat anti-mouse antibodies conjugated with Cy3 (Jackson), as well as *in situ* hybridizations using red fluorescent probe detection after tyramide signal amplification (TSA kit, NEN) were performed as previously described (KNIRR *et al.* 1999; MEYER *et al.* 2002). For the identification of *CycD* mutant embryos, we applied double labeling with rabbit antibodies against β -galactosidase (ICN, Cappel) in combination with secondary goat anti-rabbit antibodies conjugated with Alexa488 (Molecular Probes, Eugene, OR).

Analysis of fly weight and wings: Newly eclosed progeny were collected every 24 hr, transferred into fresh yeast vials, and cultured for an additional 3 days before freezing at -70° in tightly capped vials for a maximum of 3 days. After warming to room temperature, individual flies were weighed on a AG135 Mettler Toledo balance. Wings were dissected, deposited on a slide, and covered with a coverslip, which was fixed with nail polish after gentle flattening of the preparation with a weight. Microscopic images were captured with a CCD camera. The total wing area and the wing-hair count in a defined area of constant size was determined using IPLab software. An average cell size was estimated by dividing the analyzed area by the obtained wing-hair count. The total number of cells in the wing was estimated by multiplying the wing-hair count

obtained in the defined area with the ratio between this defined area and the total wing area.

RESULTS

For the isolation of mutant *CycD* alleles we mobilized a *P*-element *P*{*SUPor-P*}*KG04817* located on the X chromosome within an intergenic region ~ 470 bp upstream of the major transcriptional start site of *CycD* (Figure 1A). Molecular characterizations by PCR, DNA sequencing, and Southern blotting (Figure 1B; see also MATERIALS AND METHODS) revealed the presence of small deletions in two of the resulting revertant chromosomes. These deletions, *CycD*¹ and *CycD*², remove the promoter and parts of the coding region and are therefore considered to represent *CycD* null alleles (Figure 1). We emphasize that even the region encoding the conserved cyclin box, which mediates the association with Cdk4, is partially deleted in *CycD*¹.

The *CycD* null alleles did not interfere with development of hemizygous males and homozygous females to the adult stage. The fertility of these mutant adults was severely reduced, however, precluding their propagation as a stock. Nevertheless, a few adult progeny could be obtained from *CycD*¹ or *CycD*² mutant parents, indi-

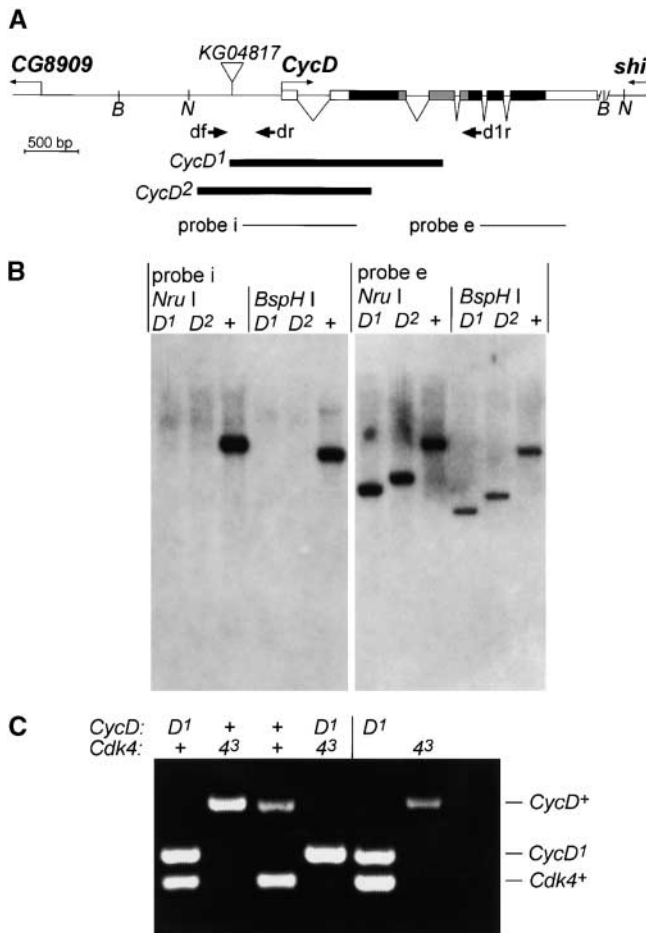


FIGURE 1.—Mutations in *CycD*. (A) The genomic region with *CycD* and the flanking genes *CG8909* and *shibire* (*shi*) is shown schematically. Exons of the major *CycD* transcript are indicated by boxes. Solid boxes represent coding regions. The most conserved cyclin box region is shaded. The deleted regions in the alleles *CycD*¹ and *CycD*², which were isolated after mobilization of the *P*-element insertion *KG04817*, are indicated by solid bars. *Bsp*HI (B) and *Nru*I (N) sites relevant for the Southern blot experiment (B) are illustrated. In addition, the hybridization probes derived from sequences either internal (probe i) or external (probe e) of the regions deleted in *CycD*¹ and *CycD*² are indicated by black lines. Finally, position and orientation of primers (df, dr, d1r) used for genotype determinations by PCR (see C) are indicated by arrows. (B) Southern blots with genomic DNA from *CycD*¹ (*D*¹), *CycD*² (*D*²), or control *w*¹ flies (+) digested with either *Nru*I or *Bsp*HI were hybridized with either probe i or probe e (see A). (C) Genotype determination with a multiplex PCR assay. Genomic DNA from single flies was used as a template for enzymatic amplification with a primer mix (df, dr, d1r, k4f, k4r). The pair df-dr results in amplification of a 630-bp fragment (*CycD*⁺) but from only the *CycD*⁺ and not from the *CycD*¹ allele (see also A). The pair df-d1r efficiently amplifies a 470-bp fragment from the *CycD*¹ allele (*CycD*¹) and inefficiently amplifies a much larger fragment from *CycD*⁺ (not shown). The pair k4f-k4r yields a 400-bp fragment (*Cdk4*⁺) from the *Cdk4*⁺ but not from the *Cdk4*³ allele where the k4r annealing site is eliminated by an intragenic deletion (MEYER *et al.* 2000). The first four lanes show the PCR products obtained from representative male progeny of a cross of *Cdk4*³ males with *CycD*¹/+; *Cdk4*³/+ females (Figure 3C). The last three lanes show control reactions with genomic DNA from *CycD*¹ or *Cdk4*³ homozygous flies or without genomic DNA template.

TABLE 1
Reduced weight of *CycD* mutants

Cross ^a	Genotype of male progeny	Weight ^b	
		(mg)	(%)
1	<i>CycD</i> ⁺ ; +; <i>da-GAL4</i>	0.75 ± 0.07	100
1	<i>CycD</i> ⁺ ; <i>UAS-CycD</i> ; <i>da-GAL4</i>	0.83 ± 0.07	111
2	<i>CycD</i> ¹ ; +; <i>da-GAL4</i>	0.64 ± 0.06	85
2	<i>CycD</i> ¹ ; <i>UAS-CycD</i> ; <i>da-GAL4</i>	0.83 ± 0.07	111
3	<i>CycD</i> ² ; +; <i>da-GAL4</i>	0.70 ± 0.08	93
3	<i>CycD</i> ² ; <i>UAS-CycD</i> ; <i>da-GAL4</i>	0.89 ± 0.07	118

^a Three crosses were set up as illustrated in Figure 3A. Females with the following genotypes were used: +/*FM7i*; *UAS-CycD*/+ (cross 1), *CycD*¹/*FM7i*; *UAS-CycD*/+ (cross 2), and *CycD*²/*FM7i*; *UAS-CycD*/+ (cross 3).

^b At least 70 flies for each genotype were weighed individually before calculation of an average weight ± standard deviation. The average weight obtained for *CycD*⁺; +; *da-GAL4* was set as 100%. The average weight difference of siblings with or without *UAS-CycD* expression was significant in all three crosses according to *t*-tests ($P < 10^{-10}$).

cating that development of adults in complete absence of maternal and zygotic *CycD*⁺ function is not absolutely impossible.

CycD mutant flies were significantly smaller than *CycD*⁺ siblings. For quantification we measured the weight of hemizygous male flies. On average these males weighed 10–20% less than *CycD*⁺ control males (Tables 1 and 4). To confirm that this weight deficit resulted from a lack of *CycD*⁺ function, we ubiquitously expressed a *UAS-CycD* transgene in the *CycD* mutants as well as in *CycD*⁺ control males using *da-GAL4*. The resulting *UAS-CycD* expression was found to eliminate the weight differences between *CycD*⁺ and *CycD* mutant males (Table 1).

The reduced size of *CycD* mutants was also confirmed by an analysis of wing areas (Table 2). Moreover, since every wing cell forms a microscopically detectable hair, cell numbers present in these wings can readily be estimated. These were found to be reduced in *CycD* mutants

TABLE 2
Wing area, cell number, and cell size in *CycD* mutants

Cross ^a	Genotype of male progeny	Wing area ^b		Cell number ^b (%)	Cell size ^b (%)
		(mm ²)	(%)		
1	<i>CycD</i> ⁺ ; +; <i>da-GAL4</i>	1.02 ± 0.05	100	100	100
1	<i>CycD</i> ⁺ ; <i>UAS-CycD</i> ; <i>da-GAL4</i>	1.07 ± 0.04	105	113	93
2	<i>CycD</i> ¹ ; +; <i>da-GAL4</i>	0.90 ± 0.06	88	85	105
2	<i>CycD</i> ¹ ; <i>UAS-CycD</i> ; <i>da-GAL4</i>	1.05 ± 0.05	103	114	91
3	<i>CycD</i> ² ; +; <i>da-GAL4</i>	0.95 ± 0.05	93	92	109
3	<i>CycD</i> ² ; <i>UAS-CycD</i> ; <i>da-GAL4</i>	1.11 ± 0.04	109	118	93

^a Three crosses were set up as illustrated in Figure 3A. Females with the following genotypes were used: +/*FM7i*; *UAS-CycD*/+ (cross 1), *CycD*¹/*FM7i*; *UAS-CycD*/+ (cross 2), and *CycD*²/*FM7i*; *UAS-CycD*/+ (cross 3).

^b At least 12 wings were analyzed for each genotype (see MATERIALS AND METHODS). The values obtained for *CycD*⁺; +; *da-GAL4* were set as 100%.

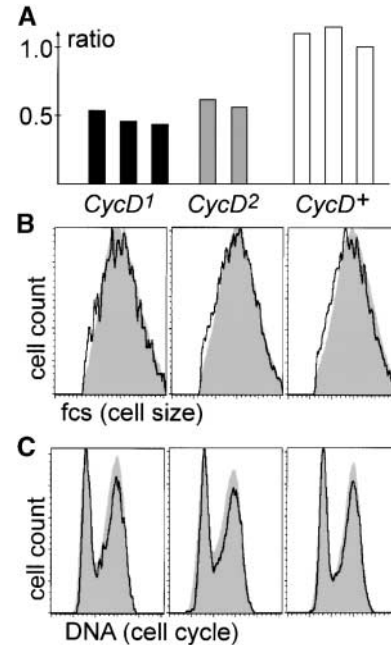


FIGURE 2.—Reduced growth of *CycD* mutant clones. (A) Mitotic recombination was induced 60 hr after egg deposition by *Hs-Flp* expression in *CycD*¹ *FRT*/*Ub-GFP FRT* (*CycD*¹, solid bars), *CycD*² *FRT*/*Ub-GFP FRT* (*CycD*², shaded bars), or + *FRT*/*Ub-GFP FRT* (*CycD*⁺, open bars) larvae. Wing-imaginal discs were dissected 60 hr after clone induction and the ratio between the area covered by *GFP*^{-/-} and *GFP*^{+/+} sister clones was determined. Each bar gives the ratio from an independent experiment after analysis of at least three imaginal discs with multiple twin clones. (B and C) Mitotic clones were induced as in A, followed by dissociation of imaginal discs and analysis of sorted *GFP*^{-/-} (black tracings) and *GFP*^{+/+} (shaded areas) cells by flow cytometry measuring either forward scatter as an estimate for cell size (B) or DNA content (C).

(Table 2). Moreover, the ratios between total wing area and cell number indicated that the size of the differentiated cells in *CycD* mutant wings is slightly increased (Table 2).

To analyze the effects of loss of *CycD*⁺ function on

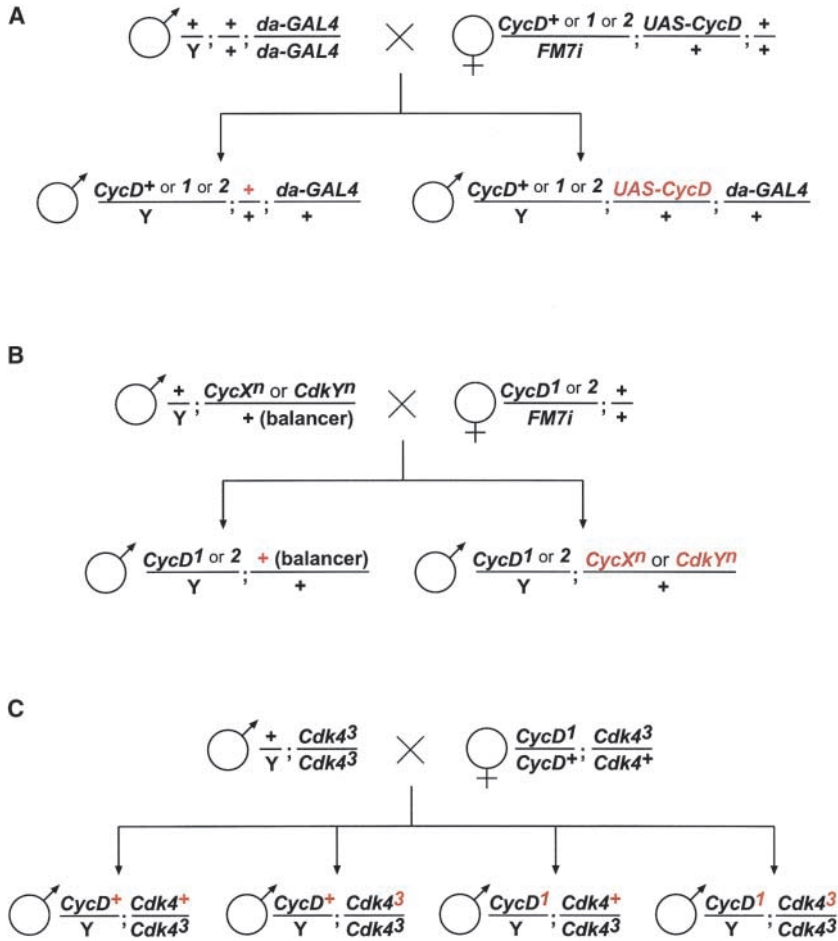


FIGURE 3.—Genotype constructions: the crossing schemes realized for the phenotypic rescue experiments (A; see also Tables 1 and 2) for the analysis of genetic interactions between mutations in *CycD* and other cyclin/cdk genes (B; see also Table 3), as well as for the comparison of single- and double-mutant phenotypes (C; see also Table 4). Only the genotype classes of progeny analyzed phenotypically are indicated, and crucial genetic differences between them are emphasized by red print.

cell growth in proliferating cells, we induced *CycD*^{-/-} and *CycD*^{+/+} sister clones simultaneously by *Hs-Flp*-mediated mitotic recombination in a heterozygous *FRT CycD*⁻/*FRT CycD*⁺ *Ub-GFP* background at ~60 hr after egg deposition. An additional 60 hr after clone induction, wing-imaginal discs were dissected and the area of *Ub-GFP*^{+/+} (*CycD*^{-/-}) and *Ub-GFP*^{-/-} (*CycD*^{+/+}) sister clones was measured and compared (Figure 2A). In three independent experiments, *CycD*¹ clones were found to cover only ~50% of the area encompassed by the control sister clones. *CycD*² clones covered ~60% of the control area in two experiments. In contrast, in three control experi-

ments with an *FRT CycD*⁺/*FRT CycD*⁺, *Ub-GFP* background we did not detect significant size differences between twin spots. To determine whether the decreased size of *CycD* mutant clones was due to increased cell death, we used a DNA stain to assess the abundance of condensed, fragmented nuclei, which are characteristic of apoptotic cells. No increase in apoptotic figures was detected. We also assayed apoptosis by immunofluorescence, using an antibody to cleaved Caspase-3. This analysis also failed to reveal increased numbers of apoptotic cells associated with the *CycD*¹ mutant clones. To analyze the effects of loss of *CycD*⁺ function on cell size

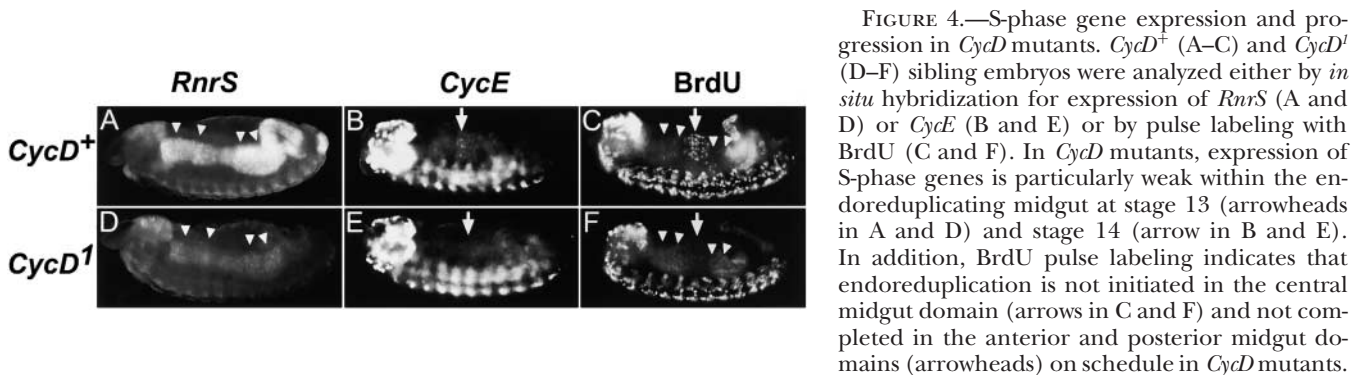


FIGURE 4.—S-phase gene expression and progression in *CycD* mutants. *CycD*⁺ (A–C) and *CycD*¹ (D–F) sibling embryos were analyzed either by *in situ* hybridization for expression of *RnrS* (A and D) or *CycE* (B and E) or by pulse labeling with BrdU (C and F). In *CycD* mutants, expression of S-phase genes is particularly weak within the endoreduplicating midgut at stage 13 (arrowheads in A and D) and stage 14 (arrow in B and E). In addition, BrdU pulse labeling indicates that endoreduplication is not initiated in the central midgut domain (arrows in C and F) and not completed in the anterior and posterior midgut domains (arrowheads) on schedule in *CycD* mutants.

and the cell-cycle profile, we measured forward scatter and DNA content of wing-imaginal disc cells after *CycD*^{-/-} clone induction and fluorescence activated cell sorting (FACS) of GFP- and GFP+ cells (Figure 2, B and C). These experiments failed to detect significant differences in cell size or cell-cycle phasing in the *CycD* mutant cells. In addition, the FACS analysis did not detect a significant fraction of sub-G₁ cells, which would be expected if loss of *CycD* caused high levels of apoptosis. These findings indicate that *CycD*⁺ function is required for normal cell growth in proliferating wing-imaginal disc cells. Moreover, reduced growth in *CycD*^l mutant cells appears to be accompanied by a proportional delay of cell-cycle progression during G₁, S, and G₂ phases, since neither cell size nor cell-cycle profile was altered.

To monitor cell-cycle progression during embryogenesis, we performed BrdU pulse labeling. The pattern and intensity of BrdU incorporation during embryogenesis revealed that zygotic *CycD*⁺ function is particularly important for endoreduplication. During wild-type embryogenesis, endoreduplication occurs during late stages in a spatially and temporally defined pattern in most internal organs (SMITH and ORR-WEAVER 1991). In *CycD* mutants, endoreduplication is not initiated on time in the central domain of the midgut (Figure 4, compare C and F). Moreover, the preceding endoreduplication within the anterior and posterior midgut domains is inefficient and not completed on time (Figure 4, compare C and F). In contrast, BrdU incorporation in the mitotically proliferating cells within the central nervous system appeared to be normal during these stages. *In situ* hybridization with probes for the E2F target genes (DURONIO *et al.* 1995) encoding the ribonucleotide reductase subunit RnrS (Figure 4, A and D) and Cyclin E (Figure 4, B and E) indicated that transcript levels of these S-phase genes are decreased predominantly within endoreduplicating tissues.

Functional redundancies might explain the relatively minor consequences observed to result from a loss of *CycD*⁺ function. In particular, Cyclin E-Cdk2 is thought to provide functions similar to those of Cyclin D-Cdk4. To evaluate this possibility, we analyzed the effects of heterozygosity for loss-of-function mutations in various cell-cycle genes on the viability of *CycD* mutants (Table 3). We observed a clear effect with mutations in *Cdk2* and *CycE*. In particular, heterozygosity for *Cdk2* mutations eliminated the viability of *CycD* mutant males almost completely. Heterozygosity for *CycE* mutations reduced *CycD* mutant male viability as well, but less severely. Moreover, surviving *CycD* mutant males displayed a rough eye phenotype when heterozygous for a *CycE* mutation (data not shown). In contrast to the strong synthetic effects of *CycE* and *Cdk2* mutations, we did not observe clear effects on *CycD* mutant viability with mutations in genes encoding Cdk1 and its cyclin subunits (*CycA*, *CycB*, *CycB3*; Table 3). Similarly, we did not observe clear genetic interactions between muta-

TABLE 3
Genetic interactions of *CycD* mutations

Gene	Allele	Frequency of heterozygous <i>CycD</i> mutant male progeny (%) ^a	
		<i>CycD</i> ^l	<i>CycD</i> ²
<i>Cdk2</i>	2	6	2
	3	0	ND ^b
<i>CycE</i>	AR95	35	18
	PZ5	61	46
<i>Cdk1</i>	E1-23	103	ND
	B47	103	ND
<i>CycA</i>	C8LR1	89	105
<i>CycB</i>	2	110	90
<i>CycB3</i>	3	114	ND
<i>dap</i>	4	128	98
	g36	100	97
<i>E2F1</i>	91	112	126
<i>DP</i>	a2	64	75
	a4	67	61

^a For an analysis of genetic interactions between mutations in *CycD* and genes encoding other cyclins (*CycA*, *CycB*, *CycB3*, *CycE*) or cdks (*Cdk1*, *Cdk2*), crosses were set up as illustrated in Figure 3B. In addition, alleles of *E2F1*, *DP*, and *dacapo* (*dap*) were tested analogously. After eclosion of progeny, the ratio of *CycD* mutant males, which were heterozygous either for another cell-cycle gene mutation or for a balancer chromosome, was determined and given after multiplication with 100. The resulting values are expected to be 100, if the mutation in *CycD* and the cell-cycle gene mutation analyzed do not interact. Lower values indicate that the viability of the *CycD* mutant males is reduced by heterozygosity for the additional cell-cycle gene mutation. At least 200 *CycD* mutant males were analyzed from each cross.

^b ND, not done.

tions in *CycD* and *dacapo* or *E2F1* (Table 3). Finally, mutations in *DP* resulted in a slight reduction of viability (Table 3).

Our characterization of *CycD* mutant phenotypes, described above, yielded results very similar to those found for *Cdk4* mutants (DATAR *et al.* 2000; MEYER *et al.* 2000, 2002). These extensive phenotypic similarities suggest that Cyclin D and Cdk4 provide their major functions exclusively together in a complex. Association of *Drosophila* Cyclin D and Cdk4 was first suggested by yeast two-hybrid experiments (FINLEY *et al.* 1996; SAUER *et al.* 1996) and later confirmed by co-immunoprecipitation experiments (MEYER *et al.* 2000). If Cyclin D and Cdk4 function exclusively as a complex, double-mutant phenotypes would be predicted to correspond to the single-mutant phenotypes. In contrast, if Cyclin D and Cdk4 also provided some major functions independently, double mutants would be expected to have a more severe phenotype than single mutants. For a careful comparison of single and double mutants, we analyzed readily quantifiable aspects including viability, body weight, wing area, wing cell number, and size. Moreover,

TABLE 4
Comparison of *CycD*, *Cdk4* single and double mutants

Genotype ^a <i>CycD</i> ; <i>Cdk4</i>	Survival ^b (%)	Weight ^c		Wing area ^d		Cell number ^d (%)	Cell size ^d (%)
		(mg)	(%)	(mm ²)	(%)		
+; +	100	0.85 ± 0.08	100	1.13 ± 0.04	100	100	100
+; -	87	0.69 ± 0.07	81	1.06 ± 0.02	94	88	107
-; +	100	0.71 ± 0.07	84	1.01 ± 0.03	89	88	108
-; -	91	0.71 ± 0.06	84	1.03 ± 0.06	91	83	111

^a For comparison of single- and double-mutant phenotypes, a cross was set up as illustrated in Figure 3C, resulting in male progeny hemizygous for either the *CycD*⁺ (indicated by +) or the *CycD*⁻ allele (indicated by -). Moreover, the males were either heterozygous (indicated by +) or homozygous (indicated by -) for the *Cdk4*³ allele. The genotype of individual male progeny was determined with a PCR assay (Figure 1C) following measurement of fly weight, wing size, and wing-hair density.

^b For the comparison of the developmental fitness associated with the four different genotypes expected to segregate with equal frequency according to Mendelian rules, all 170 eclosing males from a cross were genotyped. The number of progeny males that survived to the adult stage is given relative to the number of +; + males, which was set as 100%.

^c All males were weighed individually before calculation of an average weight ± standard deviation. The average weight of +; + males was set as 100%.

^d At least five wings were analyzed for each genotype (see MATERIALS AND METHODS). The values obtained with +; + males were set as 100%.

this experiment was designed such that all the different genotypes developed as siblings with the same maternal contribution under identical growth conditions. In addition, for the determination of the different sibling genotypes, we did not rely on adult visible phenotypes induced by dominant marker mutations on balancer chromosomes with potential side effects on the analyzed parameters. Genotypes were therefore determined with a PCR assay (Figure 1C) after completion of the phenotypic analyses. The comparison of single- and double-mutant phenotypes did not reveal major differences (Table 4). A second experiment gave very similar results (data not shown) although in this case the fraction of eclosing double mutants and their average weight was slightly lower compared to single mutants. In both experiments, however, all the differences between weight, wing size, and cell numbers of single and double mutants were not statistically significant (*P*-values obtained with *t*-test >0.05), while the differences between mutants and *CycD*⁺; *Cdk4*⁺ siblings, which also tended to eclose slightly faster than the mutants, were highly significant. Our findings therefore fail to provide evidence for independent functions of Cyclin D or Cdk4.

DISCUSSION

D-type cyclin-cdk complexes are of crucial importance in human tumorigenesis. Since these complexes have been conserved in evolution, it is readily possible to use model organisms like *D. melanogaster* for functional characterizations. Here we extend our previous characterization of *Drosophila Cdk4* mutants by phenotypic comparisons with *CycD* mutants. As previously observed for *Cdk4* (MEYER *et al.* 2000), we find that Cyclin D is

not required for progression through the G₁ phase of the cell cycle. Some escapers develop to the adult stage even when both maternal and zygotic *Cdk4*⁺ or *CycD*⁺ function is abolished. Moreover, FACS analyses demonstrate that the cell-cycle profile of wing-imaginal disc cells homozygous for null mutations in *Cdk4* (MEYER *et al.* 2000) or *CycD* (Figure 2B) is essentially indistinguishable from that of wild type. Our evidence therefore is not consistent with the prevailing idea that D-type cyclin-cdk complexes primarily regulate progression through the G₁ phase. In cultured mammalian cells, where the most support for this suggestion has accumulated, D-type cyclin-cdk complexes have been shown to act in part by titrating CIP/KIP inhibitors away from CycE/Cdk2 complexes, which are thus freed to stimulate cell-cycle progression (ADAMS 2001; ORTEGA *et al.* 2002). In contrast, binding of Dacapo, the single known *Drosophila* CIP/KIP family member, to *Drosophila* Cyclin D-Cdk4 has not been detectable (MEYER *et al.* 2000). This provides a potential explanation for the apparent discrepancy. It should be noted, however, that the strong genetic interactions we report among *CycD*, *CycE*, and *Cdk2* (Table 3), previous interaction tests performed with Rbf (DATAR *et al.* 2000; XIN *et al.* 2002), and target gene analysis (B. LYNCH, A. F. A. DE LA CRUZ and B. A. EDGAR, unpublished results) indicate that *Drosophila* CyclinD-Cdk4 complexes do play a significant, if redundant, activating role in the E2F/RBF network, just as described in mammals. In the *CycD* and *Cdk4* mutants, CycE/Cdk2 complexes are presumably sufficient to perform this function.

While not revealing a specific role during G₁, the *Drosophila* mutant phenotypes provide compelling evidence that Cyclin D-Cdk4 promotes cellular growth and

thereby accelerates progression through all the cell-cycle phases proportionally. *CycD* and *Cdk4* mutants develop into small but normally proportioned flies with an average weight of $\sim 20\%$ less than that of wild-type siblings (MEYER *et al.* 2000; this work). Conversely, overexpression of Cyclin D and Cdk4 has the opposite effect, causing increased growth in organs such as the eye, wing, and salivary glands (DATAR *et al.* 2000). Moreover, growth regulation by Cyclin D and Cdk4 is also clearly apparent at the cellular level. Clones of wing-imaginal disc cells either lacking one of the Cyclin D-Cdk4 complex partners or overexpressing the complex grow slower or faster, respectively, than wild-type clones (DATAR *et al.* 2000; MEYER *et al.* 2000; this work).

In *Drosophila*, the growth-promoting function of Cyclin D-Cdk4 can be interrogated using genetic approaches. Initial results have so far argued that Cyclin D-Cdk4 is not part of one of the other pathways (insulin/TOR, ras, myc, bantam), which are known to control cellular and organismal growth rates (FREI and EDGAR 2004). However, we have recently identified the Hif-1 prolyl hydroxylase as a key growth stimulator downstream of Cyclin D-Cdk4 (FREI and EDGAR 2004). This raises the possibility that Cyclin D-Cdk4 is interconnected with metabolic pathways sensitive to oxygen levels (LAVISTA-LLANOS *et al.* 2002; FREI and EDGAR 2004). Future analyses might therefore reveal whether an involvement in oxygen-related metabolism represents the evolutionary conserved role of Cyclin D-Cdk4 in multicellular eukaryotes and throw a new light on its significance in human tumors, where oxygen limitation is a known and crucial challenge. Cyclin D-Cdk4 has also recently been implicated in the JAK-STAT pathway by an independent genetic approach in *Drosophila* (CHEN *et al.* 2003).

Our comparison of *CycD* and *Cdk4* mutant phenotypes is also of interest with regard to functions provided by these proteins independently. In particular, D-type cyclins have been proposed to regulate a number of transcription factors without a partner kinase (see Introduction). Moreover, overexpression of *UAS-CycD* alone or *UAS-Cdk4* alone does often have phenotypic consequences that vary in extent with different GAL4 driver lines. For instance, *ey-GAL4*-driven *UAS-CycD* expression suppresses the inhibitory effects of simultaneous *UAS-RBF1* expression dramatically (DATAR *et al.* 2000). Similarly, *da-GAL4*-driven *UAS-CycD* expression during development of otherwise wild-type flies results in an increased adult fly weight (Table 1). However, in these experiments in *Drosophila*, the overexpressed Cyclin D might execute its effect in combination with excess Cdk4 expressed from the endogenous *Cdk4* gene, as suggested by the finding that *da-GAL4*-driven *UAS-CycD* expression in *Cdk4* mutants increases adult fly weight at most marginally (data not shown). Our findings that loss of *CycD*⁺ or *Cdk4*⁺ function, as well as simultaneous loss of both *CycD*⁺ and *Cdk4*⁺ function, results in essentially indistin-

guishable phenotypes and does not necessarily prevent development to the adult stage, demonstrate that neither Cyclin D nor Cdk4 provides essential functions in *Drosophila* independently of each other.

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