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roughex down-regulates G₂ cyclins in G₁

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Cell cycle arrest in G₁ at the onset of patterning in the Drosophila eye is mediated by roughex. In roughex mutants, cells accumulate Cyclin A protein in early G₁ and progress into S phase precociously. When Roughex is overexpressed in S/G₂ cells, Cyclin A is mislocalized to the nucleus and degraded, preventing mitosis. Whereas Roughex inhibits Cyclin A accumulation, Cyclin E down-regulates Roughex protein in vivo. Roughex binds to Cyclin E and is a substrate for a Cyclin E-Cdk complex in vitro. These data argue that Roughex inactivation in early G₁ by targeting Cyclin E for destruction. In late G₁, Roughex is destabilized in a Cyclin E-dependent process, releasing Cyclin A for its role in S/G₂.

[Key Words: Cell cycle; cyclin A; eye development; G₁; Drosophila; protein stability]

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Most somatic cell cycles consist of four stages—S phase, where DNA synthesis occurs; M phase (mitosis), where chromosomes segregate and cell division takes place; and two intervening gap phases, G₁ and G₂. Progression through the various cell cycle stages occurs largely through the activity of different isoforms of cyclin-dependent kinases (Cdks) and their associated positive regulatory subunits, the cyclins (Cycls) [Norbury and Nurse 1992; Morgan 1995]. In addition to positive controls that ensure the specific activation of Cyc-Cdk complexes during each stage of the cell cycle, regulatory mechanisms prevent their activity in inappropriate stages. Cyclin expression is regulated in part at the level of transcription, with mRNA levels of most cyclins peaking at specific times during the cell cycle [Sherr 1994]. In addition to transcriptional controls, Cyclics are regulated at the post-translational level. This is illustrated most dramatically by the massive destruction of G₂ cyclins by ubiquitin-dependent proteolysis as cells progress through mitosis [King et al. 1994]. In budding yeast, proteolysis of G₂ cyclins persists in G₁, preventing the accumulation of G₂ cyclins [Amon et al. 1994], whereas in both fission and budding yeasts, G₂ cyclins are synthesized in G₁ [Schwob et al. 1994; Correa-Bordes and Nurse 1995]. In this paper we consider the regulation of the Drosophila G₂ cyclin, CycA, in G₁.

Several lines of evidence indicate that CycE-Cdk complexes have a key role in regulating the G₁/S transition in higher eukaryotic cells. CycE-Cdk2 activity increases in late G₁. In Drosophila and mammalian cells, overexpression of CycE shortens G₁ and advances entry into S phase [Ohtsubo and Roberts 1993; Knoblich et al. 1994; Resnitzky et al. 1994; Richardson et al. 1995]. In Drosophila, cycE mutants arrest cell cycle progression in G₁, and down-regulation of CycE is required for G₁ arrest [Knoblich et al. 1994; Richardson et al. 1995]. CycA-Cdk2 also may have a role in S-phase entry in mammalian cells. CycA-Cdk2 activity appears toward the end of G₁, peaks during S phase, and can activate DNA replication in extracts from G₁ cells [D’Urso et al. 1990]. In cultured human cells, inactivation of CycA blocks entry into and progression through S phase [Girard et al. 1991; Pagano et al. 1992], although its ectopic expression can drive precocious entry into S phase [Resnitzky et al. 1995]. However, in Drosophila, a CycA requirement for S-phase entry has not been demonstrated.

CycA also is required for mitosis. Like CycB, the canonical G₂ cyclin, CycA mRNA or protein added to Xenopus oocytes or egg extracts can activate the G₂ Cdk, Cdc2, inducing events such as nuclear envelope breakdown and chromatin condensation [Swenson et al. 1986]. In Drosophila, mutations in cycA arrest embryonic cell cycle progression in G₂ [Lehner and O’Farrell 1989]. In late G₂, mitosis is triggered by the activation of a CycB-Cdc2 complex by Cdc25 phosphatase, encoded by the string [stg] gene in Drosophila [Russell and Nurse 1986; Edgar and O’Farrell 1989; Kumagai and Dunphy 1991; Gabrielli et al. 1992].

The Drosophila compound eye is well suited to the study of cell cycle regulation in higher eukaryotes. The eye develops from a columnar epithelium called the eye

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imaginal disc. During the third and final stage of larval development, a wave of differentiation sweeps across the eye disc anteriorly. The front of this wave is marked by a depression in the disc epithelium, the morphogenetic furrow (MF). Ahead of the MF, cells are unpatterned and undifferentiated, and progress through the cell cycle asynchronously. All cells become synchronized in G$_1$ just anterior to the MF [Thomas et al. 1994], such that cells at the anterior edge of the MF are in early G$_1$. As cells proceed through G$_1$, their positions shift posteriorly relative to the MF. Cells emerging from the posterior edge of the MF either become postmitotic and differentiate into neurons, or up-regulate CycE before entering a final synchronous S phase [Richardson et al. 1995]. As cells transit S and G$_2$, CycA and CycB proteins accumulate. Hence, progression through the cell cycle can be visualized as a continuum from the anterior edge of the MF extending posteriorly (for summary diagram, see Fig. 2A, below). Specific mutations disrupting G$_1$ progression in the MF can be used as a genetic background in which to search for additional genes regulating cell cycle progression [Thomas et al. 1994; Dong et al. 1997].

Cells become synchronized in G$_1$ through the activation of the Cdc25 phosphatase, encoded by stg, and the product of the roughex (rux) locus, which encodes a novel protein of unknown biochemical function [Thomas et al. 1994]. A burst of Stg expression occurs in a band of cells just anterior to the MF (the "synchronization domain") under the control of the patterning gene hedgehog [Alphey et al. 1992; Heberlein et al. 1995]. Stg drives G$_2$ cells through mitosis and into G$_1$. G$_1$ cells are prevented from entering S phase by the product of the rux locus. In rux mutants, cells enter S phase precociously in early G$_1$. In this paper, we show that Rux inhibits entry into S phase by preventing accumulation of CycA protein during G$_1$. We show further that CycE promotes down-regulation of Rux, allowing accumulation of CycA for its function in S and G$_2$.

Results

Rux binds to CycE

Because rux inhibits and CycE promotes S phase in the MF, we reasoned that rux may inhibit CycE-Cdk activity. This hypothesis was supported initially by the observation that Rux interacts physically with CycE (Fig. 1).
Binding of Rux to CycE was detected in a variation of a yeast two-hybrid screen in which a strain carrying CycE fused to an activation domain was mated to a panel of strains harboring various cell cycle and signaling proteins, including Rux, fused to LexA. Subsequently, Rux-LexA was tested for interactions with other Cyscs and Cdkks and was shown to interact specifically with Drosophila and human CycE and a fragment of CycE containing the "cyclin box" (Draetta 1990). It did not interact with Drosophila Cycs C, D, and J, or Drosophila Cdkks Cdk2 or Cdk2c (Fig. 1A). We were unable to test whether Rux interacted with CycA in the two-hybrid system because CycA expression is toxic to yeast (R.L. Finley and R. Brent, unpubl.). The fact that Rux binds directly to CycE but not to CycA was demonstrated in vitro (Fig. 1B). 35S-Labeled Drosophila CycE or CycA were incubated with agarose beads linked to GST–Rux or GST alone, and bound proteins were analyzed by SDS-PAGE. GST–Rux also bound human CycE in extracts prepared from Rat-1 cells expressing human CycE [data not shown].

We next asked whether the addition of Rux protein inhibited the kinase activity of Drosophila or human CycE–Cdk complexes. CycE–Cdk2c or CycA–Cdk2 immunoprecipitated from stage-11 embryo extracts were not significantly inhibited by GST–Rux protein [Fig. 1C], however GST–Rux served as an efficient substrate for phosphorylation by both Cyc–Cdk complexes. Similarly, GST–Rux or histidine-tagged Rux had little effect on histone H1 phosphorylation by purified recombinant human CycE–Cdk2 or CycA–Cdk2 complexes [data not shown]. Therefore, Rux binds CycE but does not inhibit CycE–Cdk activity directly.

**Rux inhibits CycA- but not CycE-dependent S phases**

To test whether rux acts as a negative regulator of cycE in vivo, we first assessed whether the rux phenotype [i.e., ectopic S-phase entry in the MF; Fig. 2B,C] could be dominantly suppressed by mutations in cycE. Neither a cycE deficiency nor a strong point mutation dominantly suppressed the rux S-phase phenotype. In addition, the rux phenotype was unaffected in larval homozygous for a weak loss-of-function cycE allele [H. Richardson, pers. comm.; data not shown]. Finally, ectopic S phases driven by Hs–cycE expression in the eye disc [Knoblich et al. 1994, Richardson et al. 1995] were not suppressed by co-expression of Hs–rux [data not shown] under conditions in which Hs–rux expression inhibited ectopic S phases induced by Hs–cycE [see below].

We next assessed the effects of Rux on CycE-induced S phases in the developing embryo. CycE has been shown to be necessary and sufficient to drive cells into S phase in embryonic somatic cell cycles [Knoblich et al. 1994, Duronio and O’Farrell 1995]. Rux was overexpressed in alternating stripes in Hs–cycE embryos using prd–GAL4 and UAS–rux transgenes (Brand and Perrimon 1993). CycE expression was induced and entry into S phase was monitored by 5-bromo-2-deoxyuridine (BrdU) labeling. The fraction of BrdU-positive cells in the Rux-expressing and nonexpressing stripes was identical, indicating that, as in the eye disc, Rux does not inhibit entry into S phase triggered by ectopic CycE [Fig. 2F]. Finally, high levels of Rux did not inhibit the normal endoreduplication in salivary gland cells [data not shown]. Endoreduplication is known to be CycE-dependent [Knoblich et al. 1994] and is inhibited readily by ectopic expression of Dacapo, a Drosophila CIP/KIP family Cdk inhibitor [de Nooij et al. 1996; Lane et al. 1996; C.F. Lehner, unpubl.). Therefore, rux does not appear to inhibit CycE-dependent processes in vivo. The physiological significance of the interaction between CycE and Rux is considered in the last section of the results [see Fig. 6, below].

Genetic studies revealed that rux inhibits CycA-dependent processes. In screens for dominant suppressors of the rux rough-eye phenotype, we identified three regulators of the CycA activity—cycA, stg, and regulator of cyclin A-1 (rca1) [Thomas et al. 1994; Dong et al. 1997]. Because Drosophila Stg activates mammalian CycA–Cdk complexes in vitro [Gu et al. 1992] and rca1 encodes an upstream positive regulator of CycA [Dong et al. 1997], we considered whether rux suppresses entry into S phase by preventing ectopic activation (either directly or indirectly) of a CycA–Cdk complex in the G1 domain within the MF. Consistent with this interpretation, overexpression of CycA from a heat-inducible transgene [Hs–cycA] mimicked the rux mutant phenotype [Fig. 2B], showing extensive induction of S-phase cells just anterior to and within the MF (Fig. 2D). In addition, as in rux, a broad domain of BrdU-labeled cells was observed extending throughout the normally quiescent region of the disc posterior to the MF [data not shown]. Coexpression of Hs–rux resulted in suppression of the ectopic S phases induced by Hs–cycA in all discs assayed (n = 10) [Fig. 2E]. Interestingly, the wild-type pattern of asynchronous S phases anterior and synchronous S phases posterior to the MF were not inhibited by overexpression of Rux. Therefore, ectopic CycA expression can drive G1 cells into S phase, and coexpression of Rux inhibits this phenotype. We conclude that rux acts as a negative regulator of CycA- but not CycE-dependent S phases.

**Ectopic Rux inhibits mitosis**

Because purified GST–Rux neither binds to CycA nor inhibits CycA–Cdk activity [Fig. 1], a series of experiments were pursued to evaluate the relationship between CycA and Rux in vivo. We tested the effect of misexpressing Rux under the control of the eye-specific GMR enhancer (GMR–rux), which drives expression in all cells beginning in late G1 in the MF and continuing to the posterior edge of the disc [Ellis et al. 1993; Hay et al. 1994; Fig. 6D, below]. S phases posterior to the MF were not inhibited by GMR–rux [Fig. 3A]. However, a marked decrease in the number of dividing cells was observed; some 21 (±9) mitotic figures were detected in wild-type discs in the region behind the MF compared with 1 (±1) in GMR–rux (n = 8 for both wild-type and GMR–rux). Similarly, in embryos expressing UAS–rux under the control of prd–GAL4, cell division in the Rux-expressing
Figure 2. CycA-induced S phases are inhibited by Rux. (A) A schematic representation of cell division in the eye imaginal disc. During eye development, the morphogenetic furrow (MF, arrow) moves anteriorly across the eye disc. Anterior to the MF, cell division proceeds asynchronously, depicted by scattered S-phase cells (brown circles), mitotic figures and broad expression of CycA, CycB, and CycE (off-white). Just anterior to the MF, stg mRNA (blue) is expressed in a band defining the synchronization domain. Approximately 1–2 cell diameters posterior to the onset of stg expression, cycD mRNA also is expressed in a stripe (stippled region), defining early G1 (Finley et al. 1996).

Within the MF, groups of cells segregate into preclusters, the precursors of photoreceptor cells. In late G1, cycE mRNA is expressed in cells that subsequently enter a synchronous S phase. CycA and CycB expression occurs in S/G2 cells posterior to the MF. (B–E) BrdU labeling and subsequent immunohistochemical detection of S-phase nuclei in the developing eye disc. Anterior is to the right. In wild type [C], all cells in the MF (arrow, B–E) are in G1. In rux+ [B] and in heat-shock-induced Hs-cycA eye discs [D], most if not all cells in the MF are in S phase. (E) Hs-rux inhibits Hs-cycA-induced S phases. Hs-rux does not inhibit the normal pattern of S phases anterior or posterior to the MF. (F,G) Rux does not inhibit CycE-induced S phases in the embryo. Embryos in which CycE is expressed ubiquitously under heat shock control express Rux in alternating segments. These were labeled with BrdU [F] or stained with anti-tubulin antibodies [G]. Rux-expressing segments [left of the broken line] flanking Rux-nonexpressing segments [right of the broken line] are shown. Whereas Rux expression does not reduce the proportion of cells induced into S phase by CycE in each segment, it inhibits M phase [data not shown] resulting in a lower cell density and larger nuclei in the Rux-expressing stripes. Scale bar, 25 μm (B–E) and 7.5 μm (F,G).

strips arrested during G2 of cycle 15 in some epidermal cells and during G1 of cycle 16 in the remaining cells, resulting in a lower cell density [Fig. 2G; data not shown]. Using an Hs-rux transgene, an average of 149 (±53) mitotic figures was observed in heat-pulsed wild-type embryos, whereas no mitotic figures were seen in the Hs-rux embryos after a brief heat pulse in G2 of cycle 16. Therefore, ectopic Rux blocks mitosis.

Several lines of evidence are consistent with Rux not only inhibiting mitosis but also leading to alterations in S-phase regulation subsequent to the mitotic block. In embryos ubiquitously expressing UAS-rux under the control of an armadillo-GAL4 transgene [a gift of J.P. Vincent, Medical Research Council, Cambridge, UK], a fraction of cells entered into another S phase without a preceding mitosis [i.e., endoreduplication; data not shown]. Rux overexpression also induces endoreduplication cycles in the wing disc. UAS-rux was expressed under the control of the enhancer trap line 30A [Brand and Perrimon 1993], which results in expression of Rux in a proximal ring in wing imaginal discs during a period of continuous proliferative growth. DNA labeling revealed a severe reduction in the number of cells in the expression domain, reflecting inhibition of mitosis, and a marked increase in nuclear size, consistent with endoreduplication [Fig. 3D,E]. In the eye, an increase in BrdU incorporation and larger nuclei were consistently observed after heat-induced expression of Hs-rux [Fig. 3B,C]. Whether this reflects increased rates of DNA synthesis, multiple initiations, or activation of additional origins is not known. Conversion of mitotic cycles into endoreduplication cycles has been described previously in cycA, cdc2, and rca1 mutants [Sauer et al. 1995; Haya-shi 1996; Dong et al. 1997; K. Weigmann and C.F. Leh-ner, in prep.). We propose that ectopic Rux converts the mitotic cycle into an endoreduplication cycle by down-regulating CycA-associated kinase activity.

Rux controls CycA accumulation

Striking defects in the level and subcellular distribution of CycA were observed in cells ectopically expressing high levels of Rux [Fig. 4A,B,E–G; and Fig. 6, below]. In the eye, dividing cells lose their connection to the basal lamina and mitotic nuclei are found at the extreme apical surface of the disc epithelium, immediately below the peripodial membrane [Tomlinson 1983]. In wild type, CycA accumulates in the cytoplasm of cells with basally located nuclei. As nuclei rise apically on entry into mitosis, CycA localizes transiently to the nucleus and then rapidly disappears [Lehner and O’Farrell 1989; Whitfield et al. 1990]. In apical focal planes, this expression pattern is revealed as a reticular network of thin cytoplasmic processes surrounding the unstained differentiating nuclei [Fig. 4A]. In GMR-rux, there was a marked decrease in the overall number of CycA-expressing cells, and no cytoplasmic CycA staining was detected in apical focal planes [data not shown]. Instead, CycA accumulated transiently in basally located nuclei of cells behind the MF [Figs. 4B and 6E, below]. More-
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tent with its role in negatively regulating CycA levels in early G1. Because Rux is expressed at very low levels, it has not been possible to detect endogenous expression of the gene during development [B.J. Thomas and S.L. Zipsevsky, unpubl.]. As a step toward assessing the normal pattern of Rux expression, localization of a Rux–lacZ protein fusion was determined. Genomic DNA containing the rux open reading frame and 910 bp of upstream sequences were fused in-frame to the Escherichia coli lacZ gene and introduced into flies by P-element-mediated transformation. This transgene rescued the rux mutant phenotype [data not shown], indicating that it is expressed in at least those cells that require Rux normally. Expression of Rux–lacZ was monitored using a monoclonal antibody to LacZ (Fig. 5A).

High levels of expression were observed in the nuclei of cells in a band just anterior to the MF. Lower and variable levels were seen within the MF, whereas behind the MF expression increased. Therefore, Rux is ubiquitously expressed in early G1 cells just anterior to the MF. In rux mutants, it is these cells that accumulate CycA and enter S phase precociously. Rux–lacZ protein is down-regulated in late G1 cells within the MF, whereas behind the MF Rux–lacZ expression increases in many

Figure 3. Ectopic Rux expression does not inhibit normal S phases and promotes endoreduplication. BrdU labeling of eye (A–C) and wing (D,E) imaginal discs. (A–C) Anterior is to the right. (A) A GMR–rux eye disc. Rux expression commences at the posterior edge of the MF (arrow) and extends posteriorly [see Fig. 5]. S phase is not inhibited by ectopic Rux. (C) After heat shock treatment, S-phase nuclei (arrowhead) posterior to the MF in Hs–rux are more strongly labeled and appear larger than non-heat-shocked controls [B]. (D) The 30A–GAL4 enhancer trap line drives UAS–rux expression in a portion of the proximal ring of the wing imaginal disc. Fewer cells with much larger nuclei are seen in this region [left of broken line]. (E) A corresponding region from a wild-type wing disc shows nuclei of uniform size. The marked increase in nuclear size in D is consistent with conversion of mitotic to endoreduplication cell cycles. Scale bar, 10 μm (A,D,E) and 5 μm (B,C).

over, there did not show features of cells that are entering mitosis [e.g., condensed mitotic chromosomes; B.J. Thomas, unpubl.]. In strains expressing high levels of Rux [attributable to position effects related to the transposon insertion site], CycB-positive cells were never seen [Fig. 6F, below]. However, when Rux was expressed at lower levels, CycB also was localized to the nucleus [data not shown]. Conversely, in rux mutants CycA protein levels were increased beginning in the region just anterior to the MF and extending posteriorly, corresponding to cells in early G1 [Fig. 4C,D].

Similar results were obtained in embryos expressing UAS–rux with prd–GAL4. Immunolabeling revealed that CycA [Fig. 4E–G], CycB and CycB3 [data not shown] proteins were shifted to a predominantly nuclear localization and eventually became undetectable in Rux-expressing cells. F. Sprenger and P. O’Farrell [pers. comm.] also have shown that, in embryos, a CycA mutant protein lacking the destruction box motif accumulated to high levels in the nucleus in response to ectopic Rux expression. In conclusion, we propose that Rux controls CycA levels by promoting its nuclear localization and thereby its rapid degradation.

Rux is expressed anterior to the MF and is down-regulated in cells that re-enter S phase

The spatiotemporal pattern of Rux expression is consis-

Figure 4. Rux regulates the subcellular distribution and level of CycA protein. (A–D) Eye disc and (E–G) embryo. (A) Apical focal plane. A wild-type eye disc stained with anti-CycA antibody. The stained cytoplasm of cells between developing photoreceptor cell clusters forms a reticular pattern in apical focal planes. (B) Basal focal plane. The GMR enhancer drives Rux expression posterior to the MF. This leads to a marked decrease in CycA levels and localization of CycA to the nuclei of basally localized cells. No CycA staining is seen in apical focal planes. (D) In rux+, increased CycA protein is seen in the MF, and overall CycA levels are higher than in wild type (C). (E) In the embryo, Rux overexpression [using prd–GAL4 driving a UAS–rux transgene] led to mislocalization of CycA to the nucleus. (F) CycA is eventually lost from the Rux-expressing cells [left of the broken line]. CycA appears in the cytoplasm of cells that do not ectopically express Rux [right of the broken line]. (G) Hoechst 33258 staining of DNA of the same region as D. (A–D) Anterior is to the right. Arrow indicates MF. Scale bar, 25 μm (A–D), 2.5 μm (E), and 5 μm (F,G).
in all cells anterior and posterior to the MF. By 6 hr after heat induction, Rux protein becomes localized to a stripe of cells anterior to the MF and in differentiating neurons behind the MF. During this time, Rux disappears from cycling cells both anterior and posterior to and late G1 cells within the MF (data not shown). In summary, studies with the Rux-lacZ fusion indicate that the onset of Rux expression occurs in early G1. Using GMR-rux, Rux protein was shown to be down-regulated in S/G2 cells. Therefore, high levels of Rux correlated with low levels of CycA and conversely, low levels of Rux correlated with high levels of CycA.

**CycE inhibits Rux accumulation**

Rux rapidly disappears from cycling cells at the posterior edge of the MF where the level of CycE increases. That CycE directly binds to Rux and that Rux is phosphorylated by CycE-Cdk2 in vitro, raised the intriguing possibility that CycE-Cdk activity inhibits accumulation of Rux in these cells. To assess this possibility, the Hs-cycE construct was introduced into a GMR-rux background and the levels of Rux, CycA, and CycB proteins were monitored after a heat pulse (Fig. 6). Overexpression of CycE, [Fig. 6G] but not of CycA [i.e., via Hs-cycA; data not shown], inhibits Rux protein accumulation. A marked increase in both CycA and CycB staining also was observed [Fig. 6H,I]. Reversion of the Rux inhibition was not complete; much of the CycA remained in the nucleus and mitosis was only partially restored [data not shown]. These results suggest that CycE targets Rux for destruction in cells that re-enter S phase behind the MF.

**Figure 5.** Rux is expressed anterior to the MF and down-regulated in cycling cells behind the MF. (A) Rux expression assessed using a genomic rescue transgene tagged with lacZ [Rux-lacZ, see text] and an antibody to LacZ. Rux-lacZ expression is low in the nuclei of many cells in the region of asynchronous divisions in the most anterior region of the disc. Rux-lacZ is up-regulated in a stripe corresponding to the region of G1 synchronization anterior to the MF [early G1]. Rux protein is down-regulated in the MF [late G1] and reaccumulates in most cells posterior to the MF. [Inset] Rux expression in a Hs-rux disc anterior to the MF shows nuclear staining of the Rux antigen. (B) The GMR enhancer drives Rux expression commencing at the posterior edge of the MF and continuing to the posterior edge of the disc, as detected with an anti-Rux monoclonal antibody. Rux is initially seen in the nuclei of most cells, but is down-regulated in cycling cells [basally located nuclei] between clusters of differentiating neurons [apically located nuclei, arrowhead]. An apical focal plane is shown. [C] A GMR-lacZ control shows that LacZ remains expressed in both cycling and differentiating cells. A basal focal plane is shown. Arrow indicates position of MF [A] and posterior edge of the MF [B,C]. Anterior is to the right. Scale bar, 20 μm [A, inset] and 10 μm [B,C].

cells and remains detectable in them to the posterior edge of the disc. However, studies with the native Rux protein indicate that the persistence of Rux-lacZ in cycling cells is a consequence of the increased stability of the LacZ moiety relative to Rux itself [see below].

Although Rux cannot be detected in tissue when expressed under its own promoter, it can be detected using an anti-Rux monoclonal antibody in discs in which it is ectopically expressed using the GMR-rux and Hs-rux transgenes. In GMR-rux, Rux protein is observed initially in the nuclei of most cells at the posterior edge of the MF. Just behind the MF, Rux protein remains at high levels within the nuclei of differentiating photoreceptor cells but is rapidly down-regulated in the nuclei of S/G2 cells. As a control, GMR-lacZ expression persists to variable levels in all cells behind the MF [Fig. 5B,C] [Ellis et al. 1993, Hay et al. 1994]. A similar pattern is observed after heat induction of Hs-rux. Rux is initially expressed...
Therefore, during normal eye development, accumulation of CycE in late G₁ cells may down-regulate Rux protein. This, in turn, facilitates the accumulation of CycA, which is required in subsequent cell cycle stages.

Discussion
In both unicellular and multicellular organisms, the coordination of cell cycle progression and development is critical. Analysis of the relationship between these processes provides insights into mechanisms by which the developmental program controls the cell cycle, as well as the ways in which the cell cycle impinges on development. A critical step in Drosophila eye development is the synchronization of cells in G₁ within the MF; failure to maintain G₁ leads to defects in pattern formation (Thomas et al. 1994). In rux mutants, cells enter S phase precociously just anterior to the MF. As we show here, in addition to Stg, Rux protein is markedly up-regulated within this region. It is likely that it is the coordinate expression of Stg and Rux under the control of patterning genes that regulates G₁ synchronization (Thomas and Zipursky 1994; Heberlein et al. 1995). Whereas the function of Stg/Cdc25 in driving the G₂/M transition is well established from studies in fission yeast (Russell and Nurse 1986), frogs (Gabricelli et al. 1992), and Drosophila (Edgar and O'Farrell 1989), little was known previously about the processes regulated by rux.

In earlier studies we showed that loss-of-function mutations in both cycA and stg are dominant suppressors of rux (Thomas et al. 1994). This raised the possibility that early entry into S phase was a consequence of ectopic activation of a CycA-Cdk complex by the elevated Stg levels in the synchronization domain. Indeed, mammalian CycA-Cdk2 complexes can be activated in vitro by Stg (Gu et al. 1992). That ectopic expression of CycA drives G₁ cells in the MF into S phase, and that overexpression of Rux prevents this, is consistent with this notion. In rux mutants, elevated levels of CycA protein were observed in early G₁. During normal eye development an inverse correlation is observed between Rux and CycA levels. In the asynchronously dividing cells anterior to the MF, CycA levels are high and Rux levels are low. Immediately anterior to the MF, CycA protein disappears where Rux is expressed at high levels. Behind the MF, CycA reaccumulates in cells entering S phase. It is in these cells that Rux protein declines rapidly.

The results of ectopic Rux expression are consistent with a role for Rux in controlling CycA stability. Ectopic expression both in the embryo and eye disc led to a block in mitosis and a marked decrease in CycA protein levels coupled to its nuclear localization. Ectopic Rux not only led to a block in mitosis, but to endoreduplication cycles. This is consistent with inhibition of CycA. CycA is not expressed in the normal endoreduplication cycles in the fly embryo and loss-of-function cycA mutants not only fail to undergo mitosis of cell cycle 16, but enter an endoreduplication cycle [Lehner and O'Farrell 1989; Sauer et al. 1995; K. Weigmann and C.F. Lehner, in prep.]. Although levels of stg mRNA and CycB protein also are misregulated in rux (Thomas et al. 1994), several lines of evidence are consistent with the cell cycle phenotype resulting from altered CycA levels. First, heat shock-induced expression of CycA, but neither Stg nor CycB [B.J. Thomas, S.L. Zipursky, and C.F. Lehner, unpubl.], phenocopies loss-of-function rux mutations. Second, overexpression of Rux not only inhibits mitosis but also leads to endoreduplication, a phenotype associated with loss-of-function cycA mutations but not mutations in cycB or stg. Third, although stg mRNA is up-regulated in rux, stg mRNA is not altered by overexpression of rux. And finally, the rca1 gene, which was isolated as a strong suppressor of rux, positively regulates cycA (Dong et al. 1997).

rux also regulates CycA post-transcriptionally in meiosis. rux males are sterile—whereas meiosis I and meiosis II occur normally, haploid germ cells attempt a third division with nuclear envelope breakdown and abnormal chromosomal segregation. As in mitosis, CycA and the germ-line isoform of Cdc25, twine, act as dominant suppressors of this phenotype (Gönczy et al. 1994; Thomas et al. 1994), and Rux affects CycA levels during meiosis (Gönczy et al. 1994). In wild-type premeiotic G₂ cells, CycA is found in both the nucleus and cytoplasm. In rux mutants, increased CycA protein accumulates in the cytoplasm but is excluded largely from the nucleus. Conversely, a modest increase in rux gene dosage also leads to nuclear localization of CycA and a block in meiosis II in premeiotic G₂ cells. Therefore, during both meiosis and mitosis, Rux controls CycA levels, presumably by controlling CycA stability. Immunolocalization studies raise the intriguing notion that Rux may control CycA stability by regulating its subcellular distribution.

Whereas genetic studies argue that rux negatively regulates CycA levels, Rux was shown to bind CycE but not CycA, both in vitro and in yeast. Furthermore, Rux does not inhibit the activity of either CycA- or CycE-Cdk complexes. Because CycE expression precedes the up-regulation of CycA protein at the posterior edge of the MF (i.e., in late G₁), we assessed the possibility that CycE negatively regulates Rux in vivo. Overexpression of CycE reversed the effects of Rux overexpression. This led to a marked decrease in Rux, an increase in CycA and CycB protein levels and a restoration of mitosis. We are currently assessing whether phosphorylation by CycE-Cdk regulates Rux stability.

The control of CycA activity in G₁ cells in the MF shows striking similarities to the control of G₂ cyclins during G₁ in yeast. Degradation of the G₂ cyclin, CLB2, in Saccharomyces cerevisiae continues in early G₁ prior to Start, overexpression of CLB2 protein in G₁ drives cells into S phase, and the CLB2 degradation pathway is inhibited by G₁ cyclins in late G₁ [Amon et al. 1994]. In Schizosaccharomyces pombe, the Rum1 protein has a key role in regulating the G₁ Cyc-Cdk complex, Cdc13-Cdc2, in G₁. Genetically, Rum1 shares several features with rux. Rum1 overexpression inhibits mitosis and leads to endoreduplication. Conversely, loss-of-function rum1 mutations shorten G₁ and lead to premature entry into S phase [Correa-Bordes and Nurse 1995]. This is ac-
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...companied by increased levels of Cdc13-Cdc2 in Gt. Whereas Rum1 inhibits the activity of Cdc13-Cdc2 [Correa-Bordes and Nurse 1995], it may also promote proteolytic degradation of the cyclin subunit [P. Nurse, pers. comm.]. Recent studies in mammalian cells also show that Cyclin B is unstable in Gt [Brandes and Hunt 1996]. Therefore, studies from yeast, mammalian cells, and flies argue that negative regulation of Gt cyclins in Gt is a fundamental feature of cell cycle control.

Despite its critical role in regulating CycA in Gt cells within the MF, rux is not an essential gene. This may reflect the existence of redundant mechanisms controlling the activity of Gt Cyc-Cdk complexes in Gt, as described for yeast and mammalian cells. For instance, whereas CLB2 proteolysis is active in early Gt, CLB2/CDC28 activity also is inhibited by the Cki Sic1 [Schwob et al. 1994]. In mammalian cells, not only is CyclicB proteolysis active in early Gt, CyclicB transcription also is down-regulated [Brandes and Hunt 1996]. Redundant mechanisms control CycA activity in the embryo, as supported by the observation that Hs-cycA expression drives cells into S phase more readily in rux-mutant than wild-type embryos [N. Yakubovich and P. H. O’Farrell, pers. comm.]. Given the importance of normal cell cycle progression for cell viability, perhaps it is not surprising that multiple regulatory mechanisms have evolved to ensure the precise control of specific Cyc-Cdk complexes at the appropriate cell cycle stage. The importance of a single mechanism may be revealed by specific constraints imposed on the development or physiology of different tissues. For instance, as a consequence of the cell cycle synchronization mechanism in the eye disc, an abnormally high level of the Cyc-Cdk activator Stg-Cdc25 is induced, thereby revealing the importance of rux-mediated down-regulation of CycA in these cells. As we have shown recently, this unique feature of cell cycle regulation in the developing eye provides a powerful genetic system for identifying genes regulating CycA levels and/or activity [Dong et al. 1997].

Materials and methods

Fly strains and genetics

A description of genetic markers and balancers used can be found in Lindsley and Zimm (1992). The rux null allele was described previously [Thomas et al. 1994]. The Hs-rux and GMR-rux constructs were made by inserting a 1.3-kb EcoRI fragment containing the entire rux cDNA into the EcoRI site of pCaSpeR-hs [Pirotta 1988] and pGMR [Hay et al. 1994], respectively. UAS-rux was made by cloning a 1.1-kb EcoRI-XbaI fragment containing the 5'-untranslated region and Rux coding sequence up to the translation termination codon into pUAST [Brand and Perrimon 1993]. The transcription termination and polyadenylation sites are provided by the vector. The Rux-lacZ protein fusion was constructed by synthesizing the Rux coding region up to the translation termination codon and 910 bp of upstream regulatory sequences by PCR from rux genomic clone px16. The 5' primer was 5'-CATGGATCCATGCTGCTGAATGCTGGAAATGCTGCCC-3'. The 3' primer was 5'-CATGGGATCCAAACGATCCCGCTTGGCTCCCCATCCACGAGC-3'. The BamHI sites used for cloning are underlined in both primers. An

in-frame protein fusion to lacZ was made by cloning the rux BamHI fragment and a BamHI-EcoRI fragment from pC4-B-gal [Thummel et al. 1988] into pCaSpeR-2 [Pirotta 1988]. The Hs-cycE [Noblich et al. 1994; Richardson et al. 1995], Hs-cycA [Lehner et al. 1991] and pnd-GAL4 [Brand and Perrimon 1993] strains were described previously.

Immunohistochemistry

The Rux monoclonal IgG antibody IC12-H9 was made by immunizing mice with a bacterially-produced full-length Rux-GST fusion protein. Monoclonal supernatants were used at a dilution of 1:5. CycA polyclonal serum and monoclonal antibodies to CycA and CyclicB were a generous gift of P. O’Farrell [University of California, San Francisco]. The CycA serum was diluted 1:100 before use. The CycA and CyclicB monoclonal antibodies were preabsorbed against fixed adult heads and used at a final dilution of 1:5. The anti-β-galactosidase monoclonal antibody [Promega] was used at 1:500. HRP-conjugated antimouse or anti-rabbit secondary antibodies [Bio-Rad] were used at 1:50. Biotinylated anti-rabbit and anti-mouse secondary antibodies and avidin-FITC [Vector labs] were used at 1:200. Heat shock of wandering third-instar larvae was done by placing larva in Drosophila medium into an eppendorf tube plugged with cotton and incubating for 1 hr at 37°C in a Multi-Block heater [Baxter]. Larvae were allowed to recover for 2 hr at room temperature before dissection. For heat shock of embryos, eggs were collected on apple juice agar plates for 1 hr at 25°C and aged appropriately. Plates were transferred to a 37°C humidified chamber for 30 min, and returned to 25°C for the desired recovery period. Confocal microscopy was done using a Bio-Rad MRC 1024.

Two hybrid interaction matings

Interaction mating assays were performed essentially as described [Finley and Brent 1994]. The “prey” strains were EGY48 [MATa his3 ura3 trp1 lys2 leu2:3,loxP-LEU2] containing pG4-5 [Gyuris et al. 1993] expressing activation-tagged Drosophila Cycs or Cdks. LexA fusion “bait” plasmids were derived from pEG202 [Estojak et al. 1995]. Interaction matings were done by streaking haploid yeast containing either plasmid individually onto selective medium and allowing growth for two days at 30°C. Bait and prey grids were replica-mated by pressing both plates to the same replica velvet and lifting the impression with a single YPD plate. Mating was allowed to proceed for one day at 30°C and mated diploids were then replica-plated to two selective X-Gal indicator plates to select for diploids and score for interactions.

In vitro binding and kinase assays

For in vitro binding, 5 μl of reticulocyte translate [Promega] was added to the 0.4-ml binding buffer [20 mM HEPES-KOH pH 7.7] 150 mM NaCl, 0.1% NP-40 10% glycerol, 20 mM NaF, 0.5 mM Na vanadate, 1 mM PMSF, 2 μg/ml leupeptin, and 2 μg/ml of aprotinin] with 10 μl of glutathione–agarose containing bound GST [100 μg] or GST–Rux [20 μg] and rotated for 2 hr at 4°C. The beads were washed four times with 0.5 ml of binding buffer before electrophoresis and autoradiography.

Histone H1 kinase assays were carried out in 25 μl of kinase buffer [50 mM Tris-HCl [pH 7.6], 10 mM MgCl2, 1 mM DTT, 10% glycerol, 100 μg/ml BSA] supplemented with 10 μM ATP, 5 μCi of [γ-32P]ATP, and 2 μg of histone H1 [Sigma]. Reactions were initiated by the addition of ATP, incubated at 30°C for 20 min, and stopped by the addition of 5 μl of 5x gel sample buffer before analysis by SDS-PAGE and autoradiography.
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