Developmental control of the G₁ to S transition in *Drosophila*: cyclin E is a limiting downstream target of E2F

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The E2F transcription factor is required for S phase in *Drosophila*. While it also triggers expression of replication genes at the G₁–S transition, the relevance of this transcription is not clear because many of the induced gene products are sufficiently stable that new expression is not required for S phase. However, one unstable product could couple S phase to E2F activation. Here we show that cyclin E expression at G₁–S requires E2F, that activation of E2F without cyclin E is not sufficient for S phase, and that early in G₁ ectopic expression of cyclin E alone can bypass E2F and induce S phase. We conclude that cyclin E is the downstream gene that couples E2F activity to G₁ control. Not all embryonic cycles are similarly coupled to E2F activation, however. The rapidly proliferating CNS cells, which exhibit no obvious G₁, express cyclin E constitutively and independently of E2F. Instead, cyclin E expression activates E2F in the CNS. Thus, this tissue-specific E2F-independent transcription of cyclin E reverses the hierarchical relationship between cyclin E and E2F. Both hierarchies activate expression of the full complement of replication functions controlled by E2F; however, whereas inactivation of E2F can produce a G₁ when cyclin E is downstream of E2F, we propose that an E2F-independent source of cyclin E eliminates G₁.

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The coordinated expression of genes during G₁–S progression is a feature of the cell cycle in many different eukaryotic species (Andrews and Herskowitz 1990; McKinney and Heintz 1991; Lowndes et al. 1992; Duronio and O'Farrell 1994). Many of the enzymes needed for DNA replication are expressed at this time. In several species the factors involved in transcription at the G₁–S boundary have been identified. Mutation of the genes encoding these factors in budding and fission yeast causes cell cycle arrest in G₁ (Nurse and Bissett 1981; Lowndes et al. 1992; Koch et al. 1993). In mammals and in *Drosophila*, the E2F transcription factor appears to control gene expression at G₁–S (Duronio et al. 1995; for review, see Nevins 1992; La Thangue 1994a,b). Mutation of *Drosophila* E2F blocks G₁–S transcription and DNA synthesis (Duronio et al. 1995). Such genetic results imply a causal connection between the activation of new transcription during late G₁ and cell cycle progress to S phase.

The idea that transcription triggers DNA replication is particularly appealing because of the universality of G₁–S transcription. However, one of the most important correlates of this model has not been verified. If transcription triggers entry into S-phase, then the availabil-

ity of one of the expressed gene products must limit DNA synthesis. Even though many genes expressed at the G₁–S transition encode essential replication functions, their products might be sufficiently abundant and stable that new transcription is not essential for DNA replication (Engstrom et al. 1985; Mann et al. 1988; Bjorklund et al. 1990; Feder et al. 1990). This appears to be the case in *Saccharomyces cerevisiae*, where thorough analyses have demonstrated that sufficient amounts of the products of most cell division cycle (CDC) genes expressed at the G₁–S transition can be provided from expression in previous cell cycles (B. Byers, pers. comm.). But if the product of just one of the genes transcribed only at the G₁–S transition became limiting after completion of every cell cycle, then entry into S phase would be coupled with a requirement for new transcription. In *S. cerevisiae*, this appears to be true for the *CLN* genes, which encode G₁ cyclins (Cross 1988; Hadwiger et al. 1989). New *CLN* expression at the G₁–S transition is required in each cell cycle (Richardson et al. 1989; Nasmyth and Dirick 1991; Ogas et al. 1991; Koch et al. 1993).

Results in *Drosophila* suggest a similar situation. Most of the gene products required for early cell cycle events are provided maternally (Edgar and Schubiger 1986; Merrill et al. 1988; Wieschaus and Sweeton 1988; Edgar et al. 1994). For example, when embryos are ho-

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mozygous mutant for the gene encoding the essential replication function proliferating cell nuclear antigen (PCNA), they nonetheless complete embryogenesis and die a few days later as larvae (Henderson et al. 1994). The generality of this finding was examined by the analysis of numerous deletion mutations, which together covered 34% of the genome (Smith et al. 1993). This analysis uncovered only three loci necessary for embryonic S phases, only one of which has currently been identified (the histone gene repeats). In addition, directed studies of cloned loci have identified two other genes required for S phase by the time the embryo has reached cell cycle 17. One of these genes is *cyclin E*, which encodes a G₁ cyclin (Richardson et al. 1993). Entry into S phase of embryonic cycle 17 requires zygotic transcription of this G₁ cyclin (Knoblich et al. 1994).

The other gene identified in the directed studies was *dE2F* (Duronio et al. 1995), which encodes a subunit of the heterodimeric dE2F transcription factor (Dynlacht et al. 1994a; Ohtani and Nevins 1994). The activation of E2F-dependent transcription is thought to be an important event in the transition from G₁ to S phase (for review, see Nevins 1992; Dyson 1994; La Thangue 1994a,b). Studies in mammalian systems have identified cyclin E as a potential activator of E2F. During G₁, E2F is in a complex with the retinoblastoma tumor suppressor gene product (pRb) (Bagchi et al. 1991; Chellappan et al. 1991; Chittenden et al. 1991; Shirodkar et al. 1992). This complex does not activate transcription but appears to bind E2F sites in DNA and may act as a repressor (Hiebert et al. 1992; Weintraub et al. 1992; Flemington et al. 1993; Helin et al. 1993a,b; Zamanian and La Thangue 1993). Phosphorylation of pRb causes dissociation from E2F and accompanies activation of E2F-dependent transcription and entry into S phase. Coexpression studies suggest that cyclin-dependent kinase (cdk) activities drive the phosphorylation of pRb and, hence, activation of E2F. For example, transfection of the *Rb*⁻ cell line SAOS-2 with *Rb* leads to growth inhibition, whereas cotransfection of *Rb* with either cyclin A, cyclin D2, or cyclin E results in pRb hyperphosphorylation and failure to inhibit growth (Hinds et al. 1992; Dowdy et al. 1993; Ewen et al. 1993; Kato et al. 1993). Thus, one of these cyclins, together with a cdk partner, seems responsible for activating E2F. Because D-type cyclins are present throughout G₁ and their activation appears to contribute to the stimulation of quiescent cells (for review, see Sherr 1993; Sherr 1994), they are excellent candidates for primary activators of E2F. However, similar results suggest that cyclin E-cdk2, whose levels peak at the G₁-S transition (Dulic et al. 1992; Koff et al. 1992), may play an activator role. Without mutations that remove each of these functions, it is difficult to determine which cyclin might be required for E2F activation.

We have examined genetic interactions between *Drosophila cyclin E* and *dE2F* at the embryonic stage when mutations in these genes first show phenotypes. During development there is a progressive addition of cell cycle controls (O'Farrell et al. 1989; Foe et al. 1993): Following early cycles that lack gap phases, a G₂ phase is

added in cycle 14, and in most cells a G₁ phase appears in cycle 17 (Edgar and O'Farrell 1989, 1990; O'Farrell 1989). While maternally provided gene products suffice for the early cycles (1–13), progressively more genes are required for later cycles. These requirements appear when new modes of control are added to the cell cycle. Zygotic *cyclin E* and *dE2F* are not required until after mitosis 16 (Knoblich et al. 1994; Duronio et al. 1995), at which point there has been an important divergence of the cell cycle program in different tissues. The neuroblast precursors of the central nervous system (CNS) begin to segregate during G₂ of cycle 14 (Foe 1989) and initiate a stem-cell type of lineage dividing asymmetrically as many as eight times in a rapid cycle with a total cycle time roughly equal to the length of S phase at this stage (~40 min; Hartenstein et al. 1987; Edgar and O'Farrell 1990). Most non-CNS cells complete two additional G₂-regulated cycles and enter a period of quiescence after mitosis 16. A few mitotic cell types, such as the peripheral nervous system (PNS) precursors, pause only briefly or not at all in G₁₋₁₇ (Bodmer et al. 1989). On the other hand, the mitotic cells of the imaginal disks have a prolonged period of quiescence: They are excluded from the present analysis because they reinitiate proliferation only after hatching (reviewed in Cohen 1993), well after the embryonic arrest point of the *cyclin E* and *dE2F* mutants. However, many of the quiescent cells never proliferate. Instead, they enter endoreduplication cycles (endocycles) during embryogenesis (Smith and Orr-Weaver 1991). In these cycles, DNA is amplified in regulated cycles having distinct S-phase periods and gap phases without intervening mitosis. Thus, we examine the interactions of *cyclin E* and *dE2F* in the control of the two major classes of cell cycle at the time of arrest: the rapid CNS cycles and the endocycling cells when they progress from G₁₋₁₇ into S phase.

Our analysis shows that during G₁₋₁₇ in the endocycling cells, dE2F activation is independent of *cyclin E*, whereas *cyclin E* expression requires *dE2F*. Though *cyclin E* is downstream of transcriptional activation in endocycling tissue, it is still required for S phase. We show that it is the only dE2F-dependent gene product that limits entry into S phase early during G₁₋₁₇. In contrast to these results, we find that *cyclin E* is expressed in the CNS by a route that is independent of *dE2F*, and in this tissue the activation of dE2F depends on *cyclin E*. Thus, the hierarchical relationship of cyclin E and dE2F is reversed by tissue-specific distinctions in the mode of expression of *cyclin E*.

Results

Cyclin E expression is dE2F-dependent in endocycling cells but not in the CNS

Consistent with studies in mammalian cells, which suggested that cyclin E and E2F function in a pathway (Fig. 1A), mutations of the *Drosophila dE2F* and *cyclin E* genes cause a very similar phenotype: arrest in G₁ of

Figure 1. Different regulatory hierarchies for E2F and cyclin E. Current data suggest that cyclin E and E2F are in a pathway leading to S phase. Cyclin E may contribute to the activation of E2F, thereby functioning upstream (A). This type of control appears to operate in the CNS. Alternatively, cyclin E may act downstream of E2F (B), having an essential role in DNA replication other than via E2F-dependent transcription. (C) In situations when E2F-controlled gene products in addition to cyclin E become limiting for S phase (e.g., in late G_{1-17} ; see text), new E2F-mediated transcription of RNA encoding replication factors is required for S phase.

embryonic cycle 17 (Knoblich et al. 1994; Duronio et al. 1995). Furthermore, *cyclin E* expression parallels the dE2F-dependent transcription of other genes such as *PCNA* and *DmRNR2* (encoding the small subunit of ribonucleotide reductase) that is coordinated with entry into S phase of cycle 17 (Duronio and O'Farrell 1994; Knoblich et al. 1994; Duronio et al. 1995). The coordination of transcription and S phase results in an embryonic pattern of *cyclin E* and *DmRNR2* expression that is identical to the pattern obtained by pulse-labeling embryos with BrdU (cf. Figs. 2A and 3A with 3C). These results can be explained if cyclin E is either an upstream

activator of dE2F (Fig. 1A), or an essential downstream product of dE2F-dependent transcription (Fig. 1B). We therefore tested whether *dE2F* is required for *cyclin E* expression. In situ hybridization of *dE2F* mutant embryos (Fig. 2; Table 1) revealed two means of *cyclin E* expression: one that requires *dE2F* and one that does not.

Early, constitutive zygotic *cyclin E* expression (i.e., during cycles 14–16) was similar between wild-type and mutant embryos (data not shown) and was extinguished normally in those cells entering G_{1-17} . However, whereas wild-type embryos exhibited a dynamic pattern of *cyclin E* transcription in endocycling tissues as quiescent cells entered S phase 17 (Fig. 2A, arrows), none of these tissues showed detectable accumulation of *cyclin E* RNA in the *dE2F* mutant embryos (Fig. 2B). This result indicates that *cyclin E* expression at the G_1 –S transition of cycle 17 requires dE2F, either directly or indirectly. In contrast, the level of *cyclin E* expression in the CNS (Fig. 2, open arrows) of *dE2F* mutant embryos was identical to the wild-type level at all stages examined (up to late stage 16). The persistent *cyclin E* expression in the CNS does not appear to be attributable to prolonged perdurance of maternal dE2F in this tissue, because *dE2F* mutants lose CNS expression of other dE2F-dependent genes (e.g., *DmRNR2* and *PCNA*; Duronio et al. 1995). Thus, a dE2F-independent route of cyclin E expression is used in the CNS.

Expression of several cell cycle genes in the rapidly proliferating CNS cells differs from expression in the endocycling cells. For instance, the mitotic cyclins A and B are expressed continuously in the CNS but are not expressed in endocycling tissues (Lehner and O'Farrell 1989, 1990). Additionally, whereas expression of dE2F-dependent genes like *DmRNR2* and *PCNA* is periodic in endocycling cells, their expression appears to be uninterrupted in the CNS (Duronio et al. 1994). The particularly rapid proliferative cycles of the neuroblasts in the CNS may be related to these differences in cell cycle gene expression. We first consider the relationship of dE2F and cyclin E in the endocycling tissue (i.e., G_{1-17}), and then the CNS. The relationships that we describe lead us to the suggestion that CNS expression of cyclin E by a

Figure 2. *cyclin E* expression in *dE2F* mutant embryos. (A) In situ hybridization of a stage 13 wild-type embryo using a digoxigenin-labeled *cyclin E* probe. In this and all subsequent figures, embryos are oriented with anterior to the left and dorsal to the top. The solid arrows indicate *cyclin E* expression during S_{17} of the endocycling anterior and posterior midgut cells. The open arrow indicates *cyclin E* expression in the proliferating cells of the CNS (brain and ventral nerve cord). (B) In *dE2F*⁹¹ homozygous mutant embryos, *cyclin E* expression is normal in the CNS (open arrow) but is undetectable in endocycling tissues like the midgut (solid arrows).

Figure 3. dE2F in G_{1-17} activation does not require cyclin E. (A) In situ hybridization of a stage 13 wild-type embryo using a digoxigenin-labeled *DmRNR2* probe. At this stage, *DmRNR2* is expressed in the proliferating CNS (open arrow) and in endocycling tissues during S_{17} (e.g., the anterior midgut indicated by the solid arrow). Note that the pattern of expression is identical to that of *cyclin E* at the same stage (Fig. 2A). (B) *DmRNR2* in situ hybridization of a stage 13 *cycE^{AR95}* mutant embryo. *DmRNR2* expression in the CNS is nearly extinguished (open arrow), whereas that in the endocycling cells is initiated in the normal S_{17} pattern (solid

arrow). (C) A wild-type stage 13 embryo pulse-labeled for 15 min with BrdU. Incorporated BrdU was detected by immunofluorescence. Note that replicating nuclei occur in the same pattern as *DmRNR2* expression. The arrows are the same as in A. (D) A stage 13 *cyclin E*-deficient embryo pulse-labeled for 15 min with BrdU. There are no replicating nuclei in the entire embryo, indicating an absence of cyclin E function.

route that is independent of E2F results in specialization of the cell cycle: Specifically, we suggest that this constitutive expression of cyclin E prevents oscillations in E2F activity and thereby bypasses G_1 quiescence in the CNS.

Activation of dE2F in endocycling cells is independent of cyclin E

The finding that *cyclin E* requires dE2F for expression in endocycling cells suggests that cyclin E acts downstream of dE2F and therefore does not play an upstream regulatory role in these cells (Fig. 1B). To assess more directly the role of cyclin E in activation of dE2F, we examined

the influence of *cyclin E* mutations on the expression of other dE2F-dependent transcripts (Fig. 3; Table 1). In situ hybridization of wild-type embryos with *DmRNR2* and *PCNA* probes detected expression just prior to and during S_{17} (e.g., in the anterior midgut; Fig. 3A,C, solid arrow). In *cyclin E*-deficient embryos, these cells remain arrested in G_{1-17} and fail to incorporate BrdU, indicating a lack of cyclin E function (Fig. 3D). However, in spite of the failure to enter S phase, the G_{1-17} cells activated transcription of *DmRNR2* (Fig. 3B, solid arrow), *PCNA* (data not shown), and the *cyclin E* gene itself (not shown for this stage, but see Fig. 7B) with the correct spatiotemporal pattern. The expression of these three genes in these cells requires dE2F (Fig. 2 and Duronio et al. 1995). We

Table 1. Summary of phenotypes

Strain	Endocycle cells (G_{1-17})			CNS		
	<i>cycE</i> expression	<i>DmRNR2</i> expression	BrdU incorporation	<i>cycE</i> expression	<i>DmRNR2</i> expression	BrdU incorporation
Wild type	yes ^a	yes ^a	yes ^a	yes ^b	yes ^b	yes
<i>cycE</i> ⁻	yes ^c	yes ^c	no	no	no	no
<i>dE2F</i> ^{-d}	no	no	no	yes ^b	no	no
Treatment	Early G_{1-17}		Late G_{1-17}			
	ectopic <i>DmRNR2</i> expression	ectopic BrdU incorporation	ectopic <i>DmRNR2</i> expression	ectopic BrdU incorporation		
HS <i>cycE</i> in wild type	yes	yes ^e	yes	yes ^f		
HS <i>cycE</i> in <i>dE2F</i> ⁻	no	yes ^e	no	no		

Data from analysis of mutant embryos and heat shock induction of *cyclin E* are presented qualitatively as yes or no (see text for a more accurate description of the phenotypes).

^aRegulated rather than continuous.

^bContinuous.

^cContinuous after induction during G_{1-17} .

^dExcept for *cyclin E* expression, see Duronio et al. (1995).

^eRapid (15–30 min) response.

^fSlower (30–45 min) response.

conclude that activation of dE2F in G_{1-17} is independent of cyclin E and that cyclin E acts downstream of dE2F in these cells. Furthermore, because the *cyclin E* mutant blocked S phase without blocking dE2F-dependent transcription, this transcription is not sufficient to drive DNA replication in the absence of cyclin E (Fig. 1B).

cyclin E expression can bypass the S-phase requirement for dE2F in early G_{1-17}

Because dE2F is required for *cyclin E* expression in G_{1-17} and *cyclin E* is required for S phase, *cyclin E* expression might couple S phase to dE2F activation. Previous experiments have raised the possibility that cyclin E might be the only factor limiting S phase (Knoblich et al. 1994). Epidermal cells normally arrest in G_{1-17} (~7 hr of development) for the remainder of embryogenesis (~22 hr total) and enter the first endoreduplication S phase only after hatching (Edgar and O'Farrell 1990; Smith and Orr-Weaver 1991). Induced, ubiquitous expression of an *hsp70-cyclin E* transgene will cause epidermal cells arrested in G_1 to initiate DNA synthesis (Knoblich et al. 1994). Shortly (<1 hr) after the cells have completed mitosis 16 (germ band extended; early stage 12), they respond rapidly to ectopic cyclin E, inducing a new round of DNA synthesis within 15–30 min after heat shock (data not shown, but see Fig. 4A).

If cyclin E is the only gene product limiting entry into S phase, then expression of *cyclin E* independently of dE2F should bypass the requirement for dE2F to enter S_{17} (Fig. 1B). To test this idea, we asked whether induction of cyclin E from an *hsp70-cyclin E* transgene can induce S phase in a dE2F mutant (dE2F⁷¹⁷²). Appropriately aged embryo collections from a wild-type stock and a dE2F mu-

tant stock each carrying the *cyclin E* transgene were pulse labeled for 15 min with BrdU at 1 hr after a 30-min, 37°C heat shock. At stage 12, this treatment caused extensive, ectopic labeling throughout the epidermis of both control and dE2F mutant embryos (Fig. 4A,B). Activation of persistent maternal dE2F might potentially explain the successful induction of S phase in early G_{1-17} . However, dE2F activity seems to be greatly reduced in mutant embryos at this stage as judged by the failure of induced cyclin E to stimulate expression of *DmRNR2* (shown below). Nonetheless, the induction of S phase in early G_{1-17} by cyclin E is similar whether the embryos are dE2F⁺ or dE2F⁻. Consequently, we suggest that induction of S phase by cyclin E bypasses dE2F, implying that cyclin E is the only limiting dE2F-dependent gene product at this stage (Fig. 1B).

Mutation of dE2F blocks cyclin E-induced S phase in late G_{1-17}

Whereas ectopically expressed cyclin E induces DNA replication in wild-type embryos within 15–30 min when the embryos are aged to stage 12 (early G_{1-17}), induction of replication requires 30–45 min in older embryos (germ band retracted, stage 13 and later; data not shown, but see Fig. 4C). These results imply changes in the sensitivity to cyclin E as the G_1 period lengthens. We tested whether dE2F might be involved in the response to cyclin E at these later stages (Fig. 4; Table 1). Heat shock expression of cyclin E (identical to that described above) in stage 13 and older embryos induced extensive DNA replication in the epidermis of control but not dE2F mutant embryos (Fig. 4C,D). Although the dE2F⁷¹⁷² mutation blocked nearly all BrdU incorpora-

Figure 4. Mutation of dE2F blocks cyclin E-induced S phase in late, but not early, G_{1-17} . The embryos in each panel were pulse-labeled for 15 min with BrdU 1 hr after a 30-min, 37°C induction of the *hsp70-cyclin E* transgene. Incorporated BrdU was detected by immunofluorescence. Only the region of each embryo containing the lateral epidermis is shown. (A) DNA replication is induced in the epidermis by expression of *hsp70-cyclin E* during early G_1 (the beginning of embryonic stage 12) in a wild-type dE2F embryo. Most of the labeled cells in the field of view were arrested in G_{1-17} prior to heat shock and incorporated BrdU in response to ectopic cyclin E. (B) DNA replication induced by expression of *hsp70-cyclin E* during early G_1 (stage 12) in a dE2F⁷¹⁷² mutant background. The ectopic BrdU incorporation is as extensive as in wild-type. The exposure is the same as in A. (C) DNA replication induced by expression of *hsp70-cyclin E* during late G_1 (embryonic stage 13) in a wild-type dE2F embryo. By this stage, the germ band is fully retracted and the segmental furrows in the epidermis are obvious. [See Fig. 3C for an untreated wild-type in which only the CNS and endocycling cells incorporated BrdU.] (D) Expression of *hsp70-cyclin E* during late G_1 (stage 13) on a dE2F⁷¹⁷² mutant background fails to induce ectopic DNA synthesis. The exposure is the same as in C. With longer exposures, a very low level of BrdU incorporation can be resolved from background staining.

tion, a small amount could still be detected. Nevertheless, this result indicates that loss of dE2F function can limit the progress of cyclin E-induced DNA replication during late G_{1-17} .

Late in G_1 , DNA synthesis requires new dE2F-dependent expression of genes other than cyclin E

The epistasis data indicate that after prolonged G_1 arrest, dE2F can limit progress to S phase in the presence of ubiquitous cyclin E. This finding argues that cyclin E is not always the only component limiting DNA synthesis during G_1 (Fig. 1C). We therefore reexamined the consequences of uniformly expressing *cyclin E* using the *hsp70* promoter. To avoid a contribution of *cyclin E* expression from the endogenous gene, these experiments were performed in a *cyclin E* mutant (see Materials and methods). DNA replication was monitored by pulse labeling with BrdU from 15–30 min after a 30-min, 37°C heat treatment. Stage 14 (late G_{1-17}) *cyclin E* mutant embryos either not heat-shocked or heat-shocked without the *hsp70-cyclin E* transgene failed to incorporate BrdU (data not shown). In experimental embryos, heat shock induced uniform expression of *cyclin E* (data not shown), but BrdU incorporation (Fig. 5B) was confined to positions that precisely matched the pattern of *DmRNR2* expression in a *cyclin E*-deficient embryo (Fig. 5A). Thus,

Figure 5. Late in G_1 , DNA synthesis requires new expression of genes in addition to *cyclin E*. (A) In situ hybridization of a stage 14 *cycE^{AR95}* mutant embryo with a *DmRNR2* probe. By this stage, *DmRNR2* is no longer expressed in the CNS (open arrow). Expression that was initiated normally during G_{1-17} (e.g., see Fig. 3B) persists in tissues such as the anterior midgut (solid arrow). In wild-type embryos, *DmRNR2* expression in the endocycling anterior midgut cells is down-regulated by this stage (Duronio and O'Farrell 1994). (B) A stage 14 *cycE^{AR95}* mutant embryo containing a homozygous copy of the *hsp70-cyclin E* transgene was pulse labeled with BrdU from 15 to 30 min after receiving a 30-min, 37°C heat shock, and then immediately fixed. The resulting pattern of replicating nuclei is identical to the pattern of *DmRNR2* expression shown in A. Thus, DNA synthesis is rapidly induced only in those cells that already express dE2F-dependent genes such as *DmRNR2*.

cells expressing dE2F-dependent genes are particularly responsive to cyclin E, suggesting that transcription by dE2F contributes other functions stimulating DNA synthesis (Fig. 1C). This notion is supported by the failure of induced *cyclin E* expression to bypass the replication block of a *dE2F* mutant late in G_{1-17} (i.e., stage 14; see above) and by the finding that DNA synthesis in the CNS depends on *dE2F* even though expression of *cyclin E* does not (Fig. 2; Duronio et al. 1995). These data indicate that by late G_{1-17} , new expression of at least one dE2F-regulated gene in addition to *cyclin E* limits DNA synthesis (Figs. 1C and 8).

Cyclin E can induce dE2F-dependent transcription

As stated above, when more time is allowed between the induction of *cyclin E* and analysis of BrdU incorporation, even late G_1 cells are uniformly driven into S phase. We also observe widespread activation of DNA synthesis in stage 14 *cyclin E* mutant embryos if we label 45–60 min (rather than 15–30 min) after heat induction of *cyclin E* (data not shown). How can cyclin E induce this uniform S phase if new expression of other dE2F-controlled gene products is required for DNA replication in late G_1 ? Previously, we showed that production of ectopic cyclin E in wild-type embryos induces expression of genes encoding replication functions, such as *DmRNR2* and *PCNA* (Duronio and O'Farrell 1994). Similar results were obtained in *cyclin E* mutant embryos (data not shown). We propose that this induced synthesis corresponds to induction of dE2F and is responsible for the slow, ubiquitous induction of S phase by cyclin E.

To test whether the cyclin E-induced transcription is dE2F-dependent, we examined the response of *DmRNR2* and *PCNA* to cyclin E in a *dE2F* mutant using the strain described above (Fig. 6; Table 1). In situ hybridization of control embryos showed widespread induction of *DmRNR2* in G_{1-17} at 1 hr after a 30-min, 37°C heat shock production of cyclin E (Fig. 6A,B). Identically treated *dE2F* mutant embryos failed to induce either *DmRNR2* (Fig. 6C,D) or *PCNA* (data not shown) at stages 12 and older. Thus, the *dE2F⁷¹⁷²* mutation prevents cyclin E-induced gene expression. These results indicate that ectopic cyclin E can activate dE2F-dependent transcription. We suggest that after prolonged G_1 arrest, the onset of DNA synthesis in response to ectopic cyclin E is delayed because it must await the accumulation of other dE2F-dependent gene products. Furthermore, while cyclin E normally acts downstream of dE2F in G_1 (Fig. 1B), it can play an activating role when expressed independently of dE2F (e.g., from the *hsp70* promoter; Fig. 1A).

Different cell cycle regulation occurs in the CNS

We have demonstrated that cyclin E acts downstream of dE2F during G_{1-17} of specific tissues. In these cells, the rapid disappearance of cyclin E causes the cells to pause in G_1 upon completion of mitosis 16 (Knoblich et al. 1994). However, pausing in G_1 may not always be a desirable feature of the cell cycle. For example, the rapid

Figure 6. Cyclin E can induce dE2F-dependent transcription. The embryos in each panel were fixed 1 hr after a 30-min, 37°C induction of the *hsp70-cyclin E* transgene and subsequently subjected to in situ hybridization with a digoxigenin-labeled *DmRNR2* probe. (A,B) Wild-type *dE2F* embryos. Widespread induction of *DmRNR2* in the epidermis occurs during both early (A; stage 12) and late (B; stage 13) G_{1-17} . (C,D) Similarly staged *dE2F*⁷¹⁷² mutant embryos. Cyclin E-induced *DmRNR2* expression is blocked by the *dE2F* mutation in both early (C) and late (D) G_{1-17} . These embryos are virtually indistinguishable from untreated *dE2F* mutants stained for *DmRNR2* expression [Duronio et al. 1995].

postblastoderm mitotic cycles (14–16) in the epidermis lack a G_1 phase [Edgar et al. 1989]. Such a cycle could be created if entry into S phase were uncoupled from activation of new transcription, which could be achieved by the production of cyclin E via a dE2F-independent route or by constitutively activating dE2F. Both alternatives seem to occur in the rapidly proliferating cells of the CNS (Table 1). As shown above (Fig. 2B), *cyclin E* expression in the CNS does not require *dE2F*, indicating that there is an alternate means of expressing *cyclin E* here. In contrast, *DmRNR2* and *PCNA* expression remains dE2F-dependent in the CNS [Duronio et al. 1995]. However, unlike the pulses of expression seen at the G_1 –S transition in other cells, *DmRNR2* and *PCNA* RNAs are present continuously in the CNS (Table 1; Duronio and O'Farrell 1994). This circumstance suggests that dE2F is persistently active in these cells.

In marked contrast to what was observed in the endocycling tissues, *cyclin E* mutation blocked expression of *DmRNR2* and *PCNA* in the CNS. In mutant embryos, CNS levels of *DmRNR2* mRNA fall during stages 12 and 13 (Fig. 3B, open arrow) and are undetectable by stage 14 (Fig. 5A, open arrow). Similar results were obtained with *PCNA* (data not shown). Cyclin E is also required to maintain high levels of its own expression in the CNS (Fig. 7, cf. C with D), although the kinetics of decay are slower than with *DmRNR2* and *PCNA*. These results suggest that cyclin E is required upstream of dE2F to maintain dE2F activity.

All of these changes in the cycle could be the consequence of expression of *cyclin E* by a dE2F-independent route (Fig. 2B), as ectopically produced cyclin E during G_{1-17} can activate dE2F-dependent transcription (Fig. 6). If cyclin E has the same capacity to activate dE2F in the CNS, its constitutive expression should also lead to continuous dE2F activity. These results indicate a plasticity in the relationship between dE2F and cyclin E and suggest that the hierarchy of their relationship is reversed in different developmental situations (Fig. 1, A vs. B). Furthermore, if all of the other replication functions that are expressed under the control of dE2F are more stable than cyclin E, then expressing *cyclin E* independently of dE2F

might prolong the time that dE2F-deficient cells can continue to replicate. BrdU incorporation in the CNS persists longer in a *dE2F* mutant than in a *cyclin E* mutant, despite the similar rates of decay of *DmRNR2* and *PCNA* expression in the two mutants (data not shown). The earlier decline in the *cyclin E* mutant presumably reflects a requirement for cyclin E in DNA synthesis other than for induction of dE2F-dependent transcription; otherwise, the two mutants would behave similarly when examined for both dE2F-dependent transcription and DNA replication. The existence of a CNS requirement for cyclin E that is independent of its role in inducing transcription is consistent with the finding that cyclin E is required for replication, but not G_1 –S transcription, in endocycling tissues.

Negative feedback regulation of dE2F and the endocycle

Transcription of replication genes in endocycling cells is transient, terminating prior to completion of DNA synthesis [Duronio and O'Farrell 1994; Knoblich et al. 1994]. *cyclin E*-deficient embryos revealed a defect in the down-regulation of this dE2F-dependent transcription. Expression of *DmRNR2* (Fig. 5A, solid arrow), *PCNA* (data not shown), and *cyclin E* (Fig. 7B, solid arrow) persisted well beyond the time that these RNAs disappear in wild-type embryos (shown for *cyclin E*; Fig. 7A, solid arrow). Sustained transcription of dE2F-dependent genes is not attributable to the lack of DNA synthesis in the *cyclin E* mutant, because blocking replication in wild-type embryos with aphidicolin failed to mimic the effect (data not shown). Thus, cyclin E appears to have a role in down-regulating dE2F-dependent transcription in endocycling cells.

How can cyclin E both activate dE2F-dependent transcription, as revealed in the heat shock induction experiments, and down-regulate this expression? There is a precedent for such opposing effects in mammalian systems, where cyclin A–cdk2 kinase can activate E2F, apparently by phosphorylation of the pRb inhibitor, and can also inhibit E2F by phosphorylation of the DP-1 sub-

Figure 7. *cyclin E* is required for the down-regulation of dE2F-dependent transcription in endocycling cells but is also needed to maintain high levels of its own expression in the CNS. Each panel shows an embryo hybridized with a digoxigenin-labeled *cyclin E* probe. (A) A stage 14 wild-type embryo. High-level *cyclin E* expression continues in the CNS (open arrow). *cyclin E* transcription is terminated in the anterior midgut (solid arrow), which has exited S₁₇ by this time. (cf. the expression pattern to that shown in Fig. 2A.) (B) A stage 14 *cycE^{AR95}* mutant. *cyclin E* expression inappropriately persists in the anterior midgut (solid arrow). In contrast, CNS levels of *cyclin E* RNA have declined. This is shown in more detail in C and D. (C,D) A higher-magnification view of the CNS expression of *cyclin E* in the last three abdominal segments of wild-type (C) and *cycE^{AR95}* mutant (D) embryos (shown from a ventral perspective at the position of the open arrows in A and B).

unit of E2F itself (Hinds et al. 1992; Dynlacht et al. 1994b; Krek et al. 1994; Xu et al. 1994). Although human cyclin E-cdk2 did not cause inhibitory phosphorylation, perhaps in *Drosophila* embryos a cyclin E-dependent kinase normally plays this role.

Whatever the mechanism of inhibition, it limits dE2F activity to pulses. Activation of dE2F in G₁₋₁₇ is followed by cyclin E expression, which then extinguishes dE2F activity and its own transcription. Such interactions could provide the basis for regulation of the endocycles: Following a pulse, the decay of unstable cyclin E protein (Richardson et al. 1993) might simultaneously end S phase and allow dE2F to become re-activatable. Other regulation (e.g., a pRb homolog) might keep dE2F inactive until the next developmental signal begins the process again. Although this proposal does not suggest how these cycles bypass the normal requirement for mitosis, other work suggests that this might occur simply as a result of the absence of G₂ regulators in the endocycling cells (Lehner and O'Farrell 1989, 1990; Hayles et al. 1994; Nurse 1994; N. Yakubovich, F. Sprenger, and P. O'Farrell, unpubl.). In addition, the results described in the preceding section suggest that inhibition of dE2F activity by cyclin E presumably does not occur in the rapidly cycling cells of the CNS.

Discussion

Genes encoding replication functions are selectively transcribed at the transition from G₁ to S in the budding

yeast *S. cerevisiae* (for review, see Andrews and Herskowitz 1990), in the fission yeast *Schizosaccharomyces pombe* (Lowndes et al. 1992), in *Drosophila* (Duronio and O'Farrell 1994), and in mammals (for review, see McKinney and Heintz 1991; Johnson 1992). In all four systems, there are strong suggestions that this transcription might cause the transition to S phase. However, a model that contends that S phase is controlled by transcriptional regulation of replication proteins must consider the lifetimes of the RNA and protein products of the regulated genes. If cells fail to synthesize DNA and arrest in G₁ because of limiting levels of a replication protein, then that gene product must decay from sufficient levels to limiting levels in the time between the preceding S phase and passage through mitosis (Fig. 8). However, in both *Drosophila* and yeast, most of the replication functions expressed at the G₁-S interface are relatively stable, and new expression is not required for entry into S phase. Nonetheless, S phase could be coupled to transcription if just one member of the group of genes expressed specifically at the G₁-S interval is unstable (Fig. 8). Our data show that cells of the *Drosophila* embryo arrest in G₁ because of depletion of the dE2F-regulated gene product cyclin E and lead to a model in which an S-phase requirement for the unstable cyclin E gene product provides the means of coupling S phase to transcription.

Cyclin E is downstream of dE2F

Contrary to expectations built on demonstrations that cyclin E can contribute to the activation of E2F, we found that new *cyclin E* expression in cells arrested in G₁₋₁₇ required dE2F. Therefore, cyclin E acts downstream of dE2F in these cells. Furthermore, the timing of expression of other dE2F-dependent genes during G₁₋₁₇, such as *DmRNR2* and *PCNA*, was unaffected by *cyclin E* mutations. Thus, we suggest that during G₁ the initial activation of dE2F does not require cyclin E (Fig. 1B,C).

Because activation of dE2F drives cyclin E expression and ectopic expression of cyclin E can activate dE2F, we might expect that initial activation of dE2F would be amplified via a positive feedback loop involving cyclin E. However, cyclin E is not required for high-level expression of dE2F-dependent transcripts in endocycling tissues. In these cells, the level of these transcripts rises and persists longer in a *cyclin E* mutant than in the wild type, suggesting that cyclin E is involved in a negative rather than a positive feedback loop (see Results). Similarly, although sustained dE2F activity in the CNS appears to require cyclin E, it does not require a positive feedback loop involving cyclin E transcription, because cyclin E expression in the CNS does not rely on dE2F. In contrast, a positive feedback loop involving transcription of G₁ cyclins and activation of the SWI4/SWI6 transcription factor appears to be important for progression to S phase in *S. cerevisiae* (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991; Nasmyth and Dirick 1991; Ogas et al. 1991; Koch et al. 1993).

Figure 8. Prolonged quiescence requires dE2F activity to re-enter S phase. The schematic represents how different replication components (*a, b, c*) might become limiting at different times after entry into quiescence (G_{1-17}) as a result of differing rates of decay. dE2F-controlled transcripts, which encode proteins required for DNA synthesis, including cyclin E, are synthesized continuously during the early, zygotically controlled cell cycles that lack a G_1 phase (i.e., cycles 14–16). Thus, dE2F activity (broken line) appears to be constitutive in these cycles. During interphase 16, dE2F is inactivated and transcription of RNA encoding replication proteins is terminated. Because these messages are unstable, the protein stability dictates how fast a particular factor will fall below a level required for entry into S phase. Any gene product that falls below this threshold before the end of cycle 16 will prevent entry into the subsequent S phase, causing G_1 to ensue in the next cycle. Our data indicate that cyclin E is the first, and initially the only, dE2F-dependent gene product that exhibits this behavior (represented by the *a* curve). As the length of G_{1-17} increases, successively more and more gene products decay below the threshold, such that entry into S phase requires new gene expression. For instance, before S_{17} can begin in our hypothetical cell type, protein *b*, in addition to cyclin E [*a*], must reaccumulate via new dE2F-directed expression. In contrast, relatively stable proteins (e.g., *c*) do not have this requirement for new transcription at the time of S_{17} . PCNA apparently falls into this latter category (see introductory section). *dE2F* mutants cannot activate new transcription in cycle 17 and, hence, fail to initiate S_{17} .

The S-phase requirement for cyclin E

Because cyclin E is downstream of dE2F and yet is required for DNA replication, we suggest that S phase has a requirement for this G_1 cyclin that is independent of transcription (Fig. 1C). This suggestion is consistent with other aspects of our data, including cyclin E induction of S phase in the absence of dE2F-dependent transcription (early G_{1-17}). Experiments in *Xenopus* extract systems are also consistent with a requirement for cyclin E that is independent of transcription (reviewed in Heichman and Roberts 1994). Because these extracts replicate DNA without transcription (Newport and Kirschner 1982), replication ought to be independent of functions required for transcriptional events. In these extracts, the p21 cyclin-dependent kinase inhibitor (Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993) prevents DNA replication, which is restored by addition of cyclin E to p21-arrested extracts (Strausfeld et al. 1994). One possible interpretation of this result is that cyclin E is required for replication in this system and that this requirement is not for induction of transcription.

Cyclin E couples S phase to G_1 –S transcription

In mammalian cells, cyclin E is an unstable protein that is present transiently near the time of the G_1 –S transition (Dulic et al. 1992; Koff et al. 1992), and recent data suggest that in *Drosophila* embryos cyclin E is unstable as well (H. Richardson, pers. comm.). This instability, coupled with a possible replication requirement for cyclin E, suggests that new transcription of this gene might be required in each cell cycle. This idea is supported by

findings that new transcription of cyclin E is required for S_{17} . Here we show that expression of cyclin E from an inducible transgene during early G_{1-17} can bypass the dE2F requirement for S phase. This finding suggests that periodic inactivation of dE2F arrests progress to S phase because of the instability of cyclin E. We suggest that cyclin E may couple S phase to transcription in many G_1 -regulated cycles.

The coordinate provision of S-phase functions

Why are so many replication functions expressed in an S-phase program if they are so stable that this regulation cannot couple the cell cycle to the regulated transcription? We suggest that this scheme is an effective way of ensuring that all of the functions required for DNA replication are provided in concert. Once cells arrest in G_1 , they may remain quiescent for extended periods. For example, endothelial cells lining an undisturbed mammalian blood vessel divide only once every several years (Hobson and Denekamp 1984) but will proliferate rapidly following injury. During protracted G_1 periods, even very stable gene products are likely to decay. Under these circumstances, the coordinate expression of all the components required for DNA replication might be required to ensure successful proliferation (Fig. 8).

This view, in which cells come to rely on expression of more and more dE2F-dependent transcripts during the course of G_1 , is supported by our finding that in addition to cyclin E, one or more dE2F-dependent gene products become limiting during G_{1-17} (Figs. 1C and 8). When ectopic cyclin E is provided to *dE2F* mutant embryos late

during G_{1-17} , very little BrdU incorporation is seen. That this situation represents a requirement for dE2F-dependent transcription is supported by the analysis of the induction of S phase in *cyclin E* mutants in response to ubiquitous, ectopic expression of a *cyclin E* transgene. In this experiment, only cells that had already activated dE2F showed a rapid induction of S phase.

An alternative route to cyclin E expression

dE2F regulation in the CNS differs from that in cells that pause in G_1 during cycle 17. In wild-type embryos, dE2F-dependent genes appear to be expressed continuously in CNS cells but are highly regulated in G_{1-17} cells (Duronio and O'Farrell 1994). In a *dE2F* mutant, CNS expression of *cyclin E* continues, revealing a dE2F-independent mode of expression in these cells. This *cyclin E* expression seems to influence CNS activity of dE2F, because dE2F-dependent transcripts decay in the CNS of a *cyclin E* mutant. Thus, the data suggest that the hierarchy of interactions is reversed in the different tissues: Cyclin E activates dE2F in the CNS, whereas dE2F activation triggers *cyclin E* expression in G_{1-17} (see Table 1; Fig. 1). Consequently, in the CNS, cyclin E transcription does not couple dE2F activity to S phase, as it does during G_{1-17} . Because the neuroblasts of the CNS have cell cycles of about the same length as S phase (Hartenstein et al. 1987; Edgar and O'Farrell 1990), they either lack a G_1 phase entirely or pause for a period of only minutes between mitosis and S phase. We propose that continuous dE2F-independent cyclin E expression eliminates a G_1 in CNS cell cycles.

Whereas the inversion of the regulatory hierarchy represents dramatic tissue-specific remodeling of cell cycle controls, it may have a relatively simple foundation. Though cyclin E ordinarily acts downstream of dE2F in G_{1-17} , induction of ectopic *cyclin E* expression nonetheless activates dE2F in these cells. Thus, the ability of cyclin E to activate dE2F may be an intrinsic feature of the regulatory circuitry, but cyclin E does not perform this activating role if it is not expressed. Accordingly, a CNS-specific mode of cyclin E transcription would be sufficient to give cyclin E an upstream activating role.

In addition to activating dE2F in the CNS, cyclin E may also function downstream of transcriptional controls during replication, much as it does in the endocycling cells. This possibility is suggested by the kinetics of loss of BrdU incorporation in mutant embryos. In both *dE2F* and *cyclin E* mutants, DNA replication and expression of dE2F-dependent genes decline in the CNS as the influence of maternally contributed gene products diminishes. Whereas the decline of dE2F-dependent transcripts is similar in both mutants, the decline of DNA replication occurs earlier in the *cyclin E* mutant than in the *dE2F* mutant. This finding is explained most easily if cyclin E has a relatively direct role in DNA replication, whereas loss of dE2F-dependent transcription has no immediate consequences as long as the unstable cyclin E is provided independently.

Regulation of the timing of the G_1 -S transition

One of the special features of the cell cycle in an embryonic system is the detailed and precise temporal control. The tissue-specific temporal control of the transition from G_{1-17} to S phase is visualized by BrdU incorporation into certain tissues at precise times during embryogenesis. The precise correlation of dE2F-dependent transcription and S-phase suggests that dE2F activation is an integral part of S-phase timing. What signals control spatially and temporally patterned dE2F activation? Initially, it seemed that cyclin E might perform this function (Duronio and O'Farrell 1994; Knoblich et al. 1994); however, our present work suggests that activation of dE2F-dependent transcription by ectopic cyclin E reverses the normal hierarchy of regulation for cyclin E and dE2F during G_1 . Similarities with mammalian systems suggest that activation of cyclin D-dependent kinases might phosphorylate a pRb homolog, hence activating dE2F. Testing of this proposal awaits the isolation of the upstream regulators in *Drosophila*.

Although its mechanism is unknown, dE2F activation is clearly triggered at specific developmental times irrespective of the cell cycle. This conclusion follows from the failure of cell cycle arrest to disturb the program of dE2F-dependent transcription (Duronio and O'Farrell 1994; Knoblich et al. 1994). We suggest that the cascade of developmental regulators provides a timing mechanism. Among the things controlled by this developmental clock is activity of dE2F. Inactivation of dE2F in cycle 16 is followed by a rapid decline in cyclin E, which prevents cells from entering S_{17} immediately upon completion of mitosis 16. Having created a G_1 phase in this way, the developmental program can overcome the newly introduced restriction point by triggering activation of dE2F at specific times in specific tissues. Accordingly, the signals that activate dE2F would be extrinsic to the cell cycle and would place the cell cycle under the control of the developmental program.

Materials and methods

Drosophila stocks and genetics

Embryos designated wild type were either Oregon-R or heterozygous siblings from balanced mutant stocks. Embryos completely lacking zygotic cyclin E were collected from a cross of two deficiency stocks: *w; Df(2R)GW1/CyO P[wg-lacZ]* and *w; Df(2R)GW3/CyO P[wg-lacZ]* (Ashburner et al. 1990). The only known locus missing in *GW1/GW3* trans-heterozygous embryos is *cyclin E* (Knoblich et al. 1994). In other experiments we used the *AR95* nonsense allele of *cyclin E*, which has a phenotype virtually indistinguishable from the deficiency mutations (Knoblich et al. 1994). A homozygous-viable, third chromosome P element (designated *E3*) containing the *hsp70-cyclin E* construct was used for heat-inducible expression of *cyclin E* cDNA (Knoblich et al. 1994). *lacZ*-Containing balancer chromosomes in these and all other mutant stocks allowed unambiguous identification of homozygous mutant embryos. Embryos were staged according to Campos-Ortega and Hartenstein (1985).

For the epistasis experiments, a recombinant third chromosome was constructed using the lethal *P[rosy⁺]* element inser-

tion allele *dE2F⁷¹⁷²* (Duronio et al. 1995) and the *E3* heat shock construct. Individual *E3/Sb* males were collected from the following cross: *w/w; +/+; dE2F⁷¹⁷²/E3* virgin females $\times$ *w; +/+; D/TM3 Sb P[w⁺; Ubx-lacZ]* males (the *E3* P element causes darker eye color than the *Ubx-lacZ* P element, allowing them to be distinguished from each other). These flies were backcrossed to *w/w; +/+; dE2F⁷¹⁷²/TM3 Sb P[w⁺; Ubx-lacZ]* virgin females. Crosses that gave only *Sb* progeny presumably contained a recombinant third chromosome with the *dE2F* mutation and were used to make balanced stocks containing *E3*. The presence of the *dE2F⁷¹⁷²* mutation was confirmed phenotypically by the lack of *DmRNR2* expression.

For the induction of *cyclin E* in a *cyclin E⁻* background, a *y w⁶⁷; cycE^{AR95}/CyO P[w⁺; ftz-lacZ]; P[w⁺; hsp70:cyclin E]/P[w⁺; hsp70:cyclin E]* stock was constructed in four steps from pre-existing stocks as follows: (1) virgin females *y w⁶⁷; Pin^{88K}/CyO; E3/E3* $\times$ males *w; cycE^{AR95}/CyO P[w⁺; ftz-lacZ]; +/+*; (2) males *y w⁶⁷; Pin^{88K}/cycE^{AR95}; E3/+* from step 1 $\times$ virgin females *w/w; cycE^{AR95}/CyO P[w⁺; ftz-lacZ]; +/+*, and (3) males $\times$ virgin females *y w⁶⁷/w; cycE^{AR95}/CyO P[w⁺; ftz-lacZ]; E3/+* flies from step 2; and (4) *E3/E3* progeny from step 3 were self-crossed to generate the desired stock. Eggs were collected for 1 hr at 25°C and then aged at 17°C until stage 14 (20 hr), at which time they were warmed to room temperature and heat-shocked.

Heat shock procedure

All heat treatments were performed by floating the grape juice-agar egg collection plates directly on the surface of a 37°C water bath for 30 min and then returning the plates to room temperature (23°C). After various recovery times (see text) at room temperature, the embryos were either fixed for in situ hybridization or prepared for BrdU labeling (see below).

BrdU labeling

Dechorionated and octane-permeabilized embryos were pulse labeled for 15 min with 1 mg/ml of BrdU (Sigma) in Schneider's medium as described previously (Edgar and O'Farrell 1990). Immediately after the BrdU pulse, the embryos were fixed for 5 min in a 1:1 mixture of 37% formaldehyde/heptane and subsequently devitellinized with methanol. To detect incorporated BrdU, the fixed and labeled embryos were hydrolyzed in 2 N HCl for 40 min and subsequently neutralized in 0.1 M Na₂B₄O₇ for 5 min (Schubiger and Palka 1987). Incorporated BrdU was observed by immunofluorescence using mouse monoclonal anti-BrdU (Beckton Dickinson) primary and rhodamine-conjugated goat anti-mouse (Jackson Labs) secondary antibodies.

In situ hybridization

In situ hybridizations (Tautz and Pfeifle 1989) were performed with digoxigenin-labeled RNA probes exactly as described in Duronio and O'Farrell (1994). The *cyclin E^{AR95}* allele was used to assess *cyclin E* expression in the absence of *cyclin E* function. This allele contains an ochre mutation at codon 365 of the 601-amino-acid type I protein and presumably produces a non-functional translation product from an otherwise normal transcript (Richardson et al. 1993; Knoblich et al. 1994).

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References

- Andrews, B.J. and I. Herskowitz. 1990. Regulation of cell cycle-dependent gene expression in yeast. *J. Biol. Chem.* **265**: 14057–14060.
- Ashburner, M., P. Thompson, J. Roote, P.F. Lasko, Y. Grau, M. El Messal, S. Roth, and P. Simpson. 1990. The genetics of a small autosomal region of *Drosophila melanogaster* containing the structural gene for alcohol dehydrogenase. VII. Characterization of the region around the *snail* and *cactus* loci. *Genetics* **126**: 679–694.
- Bagchi, S., R. Weinmann, and P. Raychaudhuri. 1991. The retinoblastoma protein copurifies with E2F-1, an E1A-regulated inhibitor of the transcription factor E2F. *Cell* **65**: 1063–1072.
- Bjorklund, S., S. Skog, B. Tribukait, and L. Thelander. 1990. S-phase-specific expression of mammalian ribonucleotide reductase R1 and R2 subunit mRNAs. *Biochemistry* **29**: 5452–5458.
- Bodmer, R., R. Carretto, and Y.N. Jan. 1989. Neurogenesis of the peripheral nervous system in *Drosophila* embryos: DNA replication patterns and cell lineages. *Neuron* **3**: 21–32.
- Campos-Ortega, J.A. and V. Hartenstein. 1985. *The embryonic development of Drosophila melanogaster*. Springer-Verlag, Berlin, Germany.
- Chellappan, S.P., S. Hiebert, M. Mudryj, J.M. Horowitz, and J.R. Nevins. 1991. The E2F transcription factor is a cellular target for the RB protein. *Cell* **65**: 1053–1061.
- Chittenden, T., D.M. Livingston, and W.J. Kaelin. 1991. The T/E1A-binding domain of the retinoblastoma product can interact selectively with a sequence-specific DNA-binding protein. *Cell* **65**: 1073–1082.
- Cohen, S.M. 1993. Imaginal disc development. In *The development of Drosophila melanogaster* (ed. M. Bate and A. Martinez Arias), pp. 747–842. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Cross, F.R. 1988. DAF1, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **8**: 4675–4684.
- Cross, F.R. and A.H. Tinkelenberg. 1991. A potential positive feedback loop controlling *CLN1* and *CLN2* gene expression at the start of the yeast cell cycle. *Cell* **65**: 875–883.
- Dirick, L. and K. Nasmyth. 1991. Positive feedback in the activation of G1 cyclins in yeast. *Nature* **351**: 754–757.
- Dowdy, S.F., P.W. Hinds, K. Louie, S.I. Reed, A. Arnold, and R.A. Weinberg. 1993. Physical interaction of the retinoblastoma protein with human D cyclins. *Cell* **73**: 499–511.
- Dulic, V., E. Lees, and S.I. Reed. 1992. Association of human cyclin E with a periodic G1-S phase protein kinase. *Science* **257**: 1958–1961.
- Duronio, R.J. and P.H. O'Farrell. 1994. Developmental control of a G1-S transcriptional program in *Drosophila*. *Development* **120**: 1503–1515.
- Duronio, R.J., P.H. O'Farrell, J.-E. Xie, A. Brook, and N. Dyson. 1995. The transcription factor E2F is required for S phase

- during *Drosophila* embryogenesis. *Genes & Dev.* (this issue).
- Dynlacht, B.D., A. Brook, M. Dembski, L. Yenush, and N. Dyson. 1994a. DNA-binding and trans-activation properties of *Drosophila* E2F and DP proteins. *Proc. Natl. Acad. Sci.* **91**: 6359–6363.
- Dynlacht, B.D., O. Flores, J.A. Lees, and E. Harlow. 1994b. Differential regulation of E2F transactivation by cyclin/cdk2 complexes. *Genes & Dev.* **8**: 1772–1786.
- Dyson, N. 1994. pRB, p107 and the regulation of the E2F transcription factor. *J. Cell Sci.* **18**: 81–87.
- Edgar, B.A., and P.H. O'Farrell. 1989. Genetic control of cell division patterns in the *Drosophila* embryo. *Cell* **57**: 177–187.
- . 1990. The three postblastoderm cell cycles of *Drosophila* embryogenesis are regulated in G2 by string. *Cell* **62**: 469–480.
- Edgar, B.A., and G. Schubiger. 1986. Parameters controlling transcriptional activation during early *Drosophila* development. *Cell* **44**: 871–877.
- Edgar, B.A., F. Sprenger, R.J. Duronio, P. Leopold, and P.H. O'Farrell. 1994. Distinct molecular mechanisms regulate cell cycle timing at successive stages of *Drosophila* embryogenesis. *Genes & Dev.* **8**: 440–452.
- Engstrom, Y., S. Eriksson, I. Jildevik, S. Skog, L. Thelander, and B. Tribukait. 1985. Cell cycle-dependent expression of mammalian ribonucleotide reductase. Differential regulation of the two subunits. *J. Biol. Chem.* **260**: 9114–9116.
- Ewen, M.E., H.K. Sluss, C.J. Sherr, H. Matsushime, J. Kato, and D.M. Livingston. 1993. Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell* **73**: 487–497.
- Feder, J.N., C.J. Guidos, B. Kusler, C. Carswell, D. Lewis, and R.T. Schimke. 1990. A cell cycle analysis of growth-related genes expressed during T lymphocyte maturation. *J. Cell Biol.* **111**: 2693–2701.
- Flemington, E.K., S.H. Speck, and W.J. Kaelin. 1993. E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. *Proc. Natl. Acad. Sci.* **90**: 6914–6918.
- Foe, V.E. 1989. Mitotic domains reveal early commitment of cells in *Drosophila* embryos. *Development* **107**: 1–22.
- Foe, V.E., G.M. Odell, and B.A. Edgar. 1993. Mitosis and morphogenesis in the *Drosophila* embryo: Point and counterpoint. In *The development of Drosophila melanogaster* (ed. M. Bate and A. Martinez Arias), pp. 149–300. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Gu, Y., C.W. Turck, and D.O. Morgan. 1993. Inhibition of CDK2 activity *in vivo* by an associated 20K regulatory subunit. *Nature* **366**: 707–710.
- Hadwiger, J.A., C. Wittenberg, H.E. Richardson, M. de Barros Lopes, and S.I. Reed. 1989. A family of cyclin homologs that control the G1 phase in yeast. *Proc. Natl. Acad. Sci.* **86**: 6255–6259.
- Harper, J.W., G.R. Adami, N. Wei, K. Keyomarsi, and S.J. Elledge. 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75**: 805–816.
- Hartenstein, V., E. Rudloff, and J.A. Campos-Ortega. 1987. The pattern of proliferation of the neuroblasts in the wild-type embryo of *Drosophila melanogaster*. *Wilhelm Roux's Arch. Dev. Biol.* **196**: 473–485.
- Hayles, J., D. Fisher, A. Woollard, and P. Nurse. 1994. Temporal order of S phase and mitosis in fission yeast is determined by the state of the p34cdc2-mitotic B cyclin complex. *Cell* **78**: 813–822.
- Heichman, K.A. and J.M. Roberts. 1994. Rules to replicate by. *Cell* **79**: 557–562.
- Helin, K., E. Harlow, and A. Fattaey. 1993a. Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. *Mol. Cell. Biol.* **13**: 6501–6508.
- Helin, K., C.L. Wu, A.R. Fattaey, J.A. Lees, B.D. Dynlacht, C. Ngwu, and E. Harlow. 1993b. Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative trans-activation. *Genes & Dev.* **7**: 1850–1861.
- Henderson, D.S., S.S. Banga, T.A. Grigliatti, and J.B. Boyd. 1994. Mutagen sensitivity and suppression of position-effect variegation result from mutations in mus209, the *Drosophila* gene encoding PCNA. *EMBO J.* **13**: 1450–1459.
- Hiebert, S.W., S.P. Chellappan, J.M. Horowitz, and J.R. Nevins. 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. *Genes & Dev.* **6**: 177–185.
- Hinds, P.W., S. Mittnacht, V. Dulic, A. Arnold, S.I. Reed, and R.A. Weinberg. 1992. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* **70**: 993–1006.
- Hobson, B. and J. Denekamp. 1984. Endothelial proliferation in tumours and normal tissues: Continuous labelling studies. *Br. J. Cancer* **49**: 405–413.
- Johnson, L.F. 1992. G1 events and the regulation of genes for S-phase enzymes. *Curr. Opin. Cell Biol.* **4**: 149–154.
- Kato, J., H. Matsushime, S.W. Hiebert, M.E. Ewen, and C.J. Sherr. 1993. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes & Dev.* **7**: 331–342.
- Knoblich, J.A., K. Sauer, L. Jones, H. Richardson, R. Saint, and C.F. Lehner. 1994. Cyclin E controls S phase progression and its down-regulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation. *Cell* **77**: 107–120.
- Koch, C., T. Moll, M. Neuberger, H. Ahorn, and K. Nasmyth. 1993. A role for the transcription factors Mbp1 and Swi4 in progression from G1 to S phase. *Science* **261**: 1551–1557.
- Koff, A., A. Giordano, D. Desai, K. Yamashita, J.W. Harper, S. Elledge, T. Nishimoto, D.O. Morgan, B.R. Franza, and J.M. Roberts. 1992. Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. *Science* **257**: 1689–1694.
- Krek, W., M.E. Ewen, S. Shirodkar, Z. Arany, W.G. Kaelin, Jr., and D.M. Livingston. 1994. Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. *Cell* **78**: 161–172.
- La Thangue, N.B. 1994a. DP and E2F proteins: Components of a heterodimeric transcription factor implicated in cell cycle control. *Curr. Opin. Cell Biol.* **6**: 443–450.
- . 1994b. DRTF1/E2F: An expanding family of heterodimeric transcription factors implicated in cell-cycle control. *Trends Biochem. Sci.* **19**: 108–114.
- Lehner, C.F. and P.H. O'Farrell. 1989. Expression and function of *Drosophila* cyclin A during embryonic cell cycle progression. *Cell* **56**: 957–968.
- . 1990. The roles of *Drosophila* cyclins A and B in mitotic control. *Cell* **61**: 535–547.
- Lowndes, N.F., C.J. McInerney, A.L. Johnson, P.A. Fantes, and L.H. Johnston. 1992. Control of DNA synthesis genes in fission yeast by the cell-cycle gene *cdc10⁺*. *Nature* **355**: 449–453.
- Mann, G.J., E.A. Musgrove, R.M. Fox, and L. Thelander. 1988. Ribonucleotide reductase M1 subunit in cellular proliferation, quiescence, and differentiation. *Cancer Res.* **48**: 5151–5156.

- McKinney, J.D. and N. Heintz. 1991. Transcriptional regulation in the eukaryotic cell cycle. *Trends Biochem. Sci.* **16**: 430–435.
- Merrill, P.T., D. Sweeton, and E. Wieschaus. 1988. Requirements for autosomal gene activity during precellular stages of *Drosophila melanogaster*. *Development* **104**: 495–509.
- Nasmyth, K. and L. Dirick. 1991. The role of SW14 and SW16 in the activity of G1 cyclins in yeast. *Cell* **66**: 995–1013.
- Nevins, J.R. 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science* **258**: 424–429.
- Newport, J. and M. Kirschner. 1982. A major developmental transition in early *Xenopus* embryos: I. characterization and timing of cellular changes at the midblastula stage. *Cell* **30**: 675–686.
- Nurse, P. 1994. Ordering S phase and M phase in the cell cycle. *Cell* **79**: 547–550.
- Nurse, P. and Y. Bissett. 1981. Gene required in G1 for commitment to cell cycle and in G2 for control of mitosis in fission yeast. *Nature* **292**: 558–560.
- O'Farrell, P.H., B.A. Edgar, D. Lakich, and C.F. Lehner. 1989. Directing cell division during development. *Science* **246**: 635–640.
- Ogas, J., B.J. Andrews, and I. Herskowitz. 1991. Transcriptional activation of CLN1, CLN2, and a putative new G1 cyclin (HCS26) by SW14, a positive regulator of G1-specific transcription. *Cell* **66**: 1015–1026.
- Ohtani, K. and J.R. Nevins. 1994. Functional properties of a *Drosophila* homolog of the E2F1 gene. *Mol. Cell. Biol.* **14**: 1603–1612.
- Richardson, H.E., C. Wittenberg, F. Cross, and S.I. Reed. 1989. An essential G1 function for cyclin-like proteins in yeast. *Cell* **59**: 1127–1133.
- Richardson, H.E., L.V. O'Keefe, S.I. Reed, and R. Saint. 1993. A *Drosophila* G1-specific cyclin E homolog exhibits different modes of expression during embryogenesis. *Development* **119**: 673–690.
- Schubiger, M. and J. Palka. 1987. Changing spatial patterns of DNA replication in the developing wing of *Drosophila*. *Dev. Biol.* **123**: 145–153.
- Sherr, C.J. 1993. Mammalian G1 cyclins. *Cell* **73**: 1059–1065.
- . 1994. G1 phase progression: Cycling on cue. *Cell* **79**: 551–555.
- Shirodkar, S., M. Ewen, J.A. DeCaprio, J. Morgan, D.M. Livingston, and T. Chittenden. 1992. The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. *Cell* **68**: 157–166.
- Smith, A.V. and T.L. Orr-Weaver. 1991. The regulation of the cell cycle during *Drosophila* embryogenesis: the transition to polyteny. *Development* **112**: 997–1008.
- Smith, A.V., J.A. King, and T.L. Orr-Weaver. 1993. Identification of genomic regions required for DNA replication during *Drosophila* embryogenesis. *Genetics* **135**: 817–829.
- Strausfeld, U.P., M. Howell, R. Rempel, J.L. Maller, T. Hunt, and J.J. Blow. 1994. CIP1 blocks the initiation of DNA replication in *Xenopus* extracts by inhibition of cyclin-dependent kinases. *Curr. Biol.* **4**: 876–883.
- Tautz, D. and C. Pfeifle. 1989. A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene hunchback. *Chromosoma* **98**: 81–85.
- Weintraub, S.J., C.A. Prater, and D.C. Dean. 1992. Retinoblastoma protein switches the E2F site from positive to negative element. *Nature* **358**: 259–261.
- Wieschaus, E. and D. Sweeton. 1988. Requirements for X-linked zygotic gene activity during cellularization of early *Drosophila* embryos. *Development* **104**: 483–493.
- Xiong, Y., G.J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach. 1993. p21 is a universal inhibitor of cyclin kinases. *Nature* **366**: 701–704.
- Xu, M., K.A. Sheppard, C.Y. Peng, A.S. Yee, and H. Piwnicka-Worms. 1994. Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. *Mol. Cell. Biol.* **14**: 8420–8431.
- Zamanian, M. and N.B. La Thangue. 1993. Transcriptional repression by the Rb-related protein p107. *Mol. Biol. Cell* **4**: 389–396.

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