E2F-induced S phase requires cyclin E

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Both the heterodimeric transcription factor, E2F, and the G1 cyclin, cyclin E, are required for the G1–S transition at the start of the metazoan cell cycle. It has been established that cyclin E can act as an upstream activator of E2F. In addition to this action, we show here that cyclin E has an essential role in DNA replication distinct from activating E2F. We have created transgenic Drosophila capable of inducible, ectopic production of E2F activity. Simultaneous overexpression of both Drosophila E2F subunits, dE2F and dDP, in embryos stimulated the expression of multiple E2F-target genes including cyclin E, and also caused the initiation of S phase. Mutation of cyclin E prevented the initiation of S phase after overexpression of dE2F/dDP without affecting induction of target gene expression. Thus, E2F-directed transcription cannot bypass loss of cyclin E in Drosophila embryos.

[Key Words: Drosophila; E2F; cyclin E; S phase; cell cycle]

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Many eukaryotic cells make a commitment to divide during the G1 phase of the cell cycle (Pardee 1989). The process of commitment can be described as the execution of a series of molecular events that lead ultimately to the initiation of DNA replication. In mammals and Drosophila, two such events are the activation of the cyclin E/cdk2 kinase and the activation of the E2F transcription factor. Much of the regulation that governs progression through G1 ultimately controls these two events [reviewed in Sherr 1993, 1994; Weinberg 1995]. In this report we explore the nature of the regulatory relationship linking cyclin E and E2F.

E2F is a heterodimeric transcription factor (composed of subunits called E2F and DP) that has been implicated in cell-cycle control in many organisms (for review, see Dyson 1994; La Thangue 1994). In mammals, each subunit is encoded by several genes, creating a family of different E2Fs, whereas in Drosophila only single dE2F and dDP genes have been identified [Helin et al. 1992; Kaelin et al. 1992; Shan et al. 1992; Girling et al. 1993; Ivey-Hoyle et al. 1993; Lees et al. 1993; Beijersbergen et al. 1994; Dynlacht et al. 1994; Ginsberg et al. 1994; Ohtani and Nevins 1994; Hao et al. 1995; Sardet et al. 1995; Wu et al. 1995]. Activation of normally quiescent cells by overexpression of mammalian E2F-1 shows that E2F can trigger S phase [Johnson et al. 1993; Qin et al. 1994; Shan and Lee 1994]. The arrest of Drosophila dE2F mutant embryos prior to S phase shows that E2F is required [Duronio et al. 1995]. E2F is thought to control cell-cycle progression via transcriptional activation of genes required for DNA synthesis [Nevins 1992; Helin and Harlow 1993; La Thangue 1994]. Consistent with this transcriptional role, dE2F mutant embryos lack expression of several genes encoding components of the replication machinery (e.g., PCNA and ribonucleotide reductase) [Duronio et al. 1995]. In addition, E2F binding sites are found in the promoters of many mammalian replication genes, and in some instances these sites have been shown to be required for gene expression at the G1–S transition [Blake and Azizkhan 1989; Pearson et al. 1991; Dalton 1992; Means et al. 1992; Lam and Watson 1993; Slansky et al. 1993; Dou et al. 1994; Hsiao et al. 1994; Johnson et al. 1994; Neuman et al. 1994]. However, because many of the replication proteins are stable, it remains unclear whether a new round of E2F-directed transcription of these genes is required in each cell cycle for S phase to begin.

Several studies have suggested that cyclin E transcription during G1 limits execution of the G1–S transition. Cyclin E is an activator of the cdk2 kinase [Dulic et al. 1992; Koff et al. 1992; Sauer et al. 1995], which is required for the G1–S transition [Pagano et al. 1993; Tsai et al. 1993; van den Heuvel and Harlow 1993]. Expression of cyclin E appears to be a major contributor to the peak of cyclin E/cdk2 activity observed as cells enter S phase [Dulic et al. 1992; Koff et al. 1992; Ohtsubo et al. 1995]. In cultured mammalian cells, overexpression of cyclin E shortens the duration of the G1 phase and reduces growth factor dependency [Ohtsubo and Roberts 1993; Resnitzky et al. 1994; Wimmel et al. 1994]. In Drosophila, heat shock induction of cyclin E transcription causes cells normally arrested in G1 to begin DNA replication [Knoblich et al. 1994; Richardson et al. 1995]. What is the relationship between E2F and cyclin E transcription at the G1–S transition? One view that has dominated recent thinking is that G1 cyclins act as up-

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stream activators of E2F. Overexpression of cyclin E stimulates E2F-dependent transcription in cultured mammalian cells [Johnson et al. 1994] and in Drosophila embryos [Duronio and O’Farrell 1994, 1995]. This stimulation seems to be mediated by phosphorylation of pRB, which is an in vitro substrate for cyclin E/cdk2 [Hinds et al. 1992; Dowdy et al. 1993; Ewen et al. 1993; Kato et al. 1992; Du et al. 1996a]. Hypophosphorylated forms of mammalian pRB (and its relatives p107 and p130) bind to and inhibit E2F [Chellappan et al. 1991; Zhu et al. 1993; Smith and Nevins 1995; Vairo et al. 1995]. This inhibition is relieved after pRB is phosphorylated in response to mitogenic stimuli [reviewed in Weinberg 1995]. Mutation of cyclin E causes loss of E2F-dependent transcription in the CNS of Drosophila embryos, demonstrating that cyclin E does function as an E2F activator, at least in this specific situation [Duronio and O’Farrell 1995].

Recent work by DeGregori et al. [1995b] suggested that E2F-1 overexpression in rat fibroblasts is capable of bypassing the need for activation of cyclin E/cdk2 kinase. This raised the possibility that the only essential role for cyclin E in S phase is to activate E2F. However, in a different biological setting cyclin E might perform additional functions that are required for S phase. Our previous genetic analysis of Drosophila embryos suggested that in certain tissues cyclin E is required for DNA replication downstream of E2F activity [Duronio and O’Farrell 1995].

We wished to explore further this potential role for cyclin E. Drosophila offers the possibility of directly testing whether cyclin E function is required for E2F-driven S phase by using a cyclin E null mutation. We ectopically activated E2F in Drosophila embryos using heat-inducible hsp70-dE2F and hsp70-dDP transgenes, and caused epidermal cells to enter S phase inappropriately. Mutation of cyclin E blocked the ability of E2F to induce S phase, demonstrating that cyclin E is required for DNA replication independently of E2F. We propose that cyclin E and E2F can interact in two ways: the first is at the level of their activation, and the second is at the level of their action in downstream events that lead to the initiation of DNA replication.

**Results**

**Simultaneous overexpression of dE2F and dDP induces DNA replication**

Drosophila embryogenesis proceeds via a stereotypic cell-cycle program (Fig. 1; Foe et al. 1993). During the first sixteen cycles, DNA replication begins immediately after the completion of mitosis. The first G1 phase appears in interphase of cell cycle 17 (~7 hr after egg deposition at 25°C) [Edgar and O’Farrell 1990]. With some notable exceptions [e.g., the central and peripheral nervous systems] [Hartenstein et al. 1987; Bodmer et al. 1989], cell proliferation ceases at this time. Subsequent growth occurs by a process called endoreduplication in which the genome is amplified through successive rounds of S phase followed by a gap phase without an intervening mitosis [endocycles]. Some cells, including those of the developing midgut, begin endocycles during embryogenesis in a characteristic spatiotemporal pattern [Smith and Orr-Weaver 1991]. In contrast, epidermal cells remain arrested in G1 of cycle 17 (G117) and do not begin endocycles until after hatching (22–24 hr AED at 25°C).

E2F is inactive during G117, and must be activated for cells to enter S phase [Duronio et al. 1995]. We tested whether overexpression of the E2F subunits, individually or together, could induce entry into S phase. Embryos carrying two copies of either a hsp70-dE2F transgene, a hsp70-dDP transgene, or both transgenes were aged until stage 13 or 14, a time at which the epidermal cells are arrested in G117. The staged embryos were heat-shocked, and then pulse labeled with bromodeoxyuridine (BrdU) at various times after the heat treatment (see Materials and Methods). Expression of either dE2F or dDP alone had no effect on the G117-arrested epidermal cells [Fig. 2A,B, respectively]. However, simultaneous expression of dE2F and dDP induced DNA replication in cycle 17 epidermal cells [Fig. 2C]. S phase began by 30 min after the end of the heat shock, and was completed.
by 3 hr, dE2F/dDP overexpression also affected the normal program of embryonic endocycles in tissues like the midgut and salivary gland. This program is disturbed by induction of dE2F/dDP, such that normally quiescent cells (in between endo S phases) begin DNA replication (e.g., see Fig 5C, below). We conclude that coexpression of dE2F and dDP is capable of inducing S phase in G1, and in the quiescent phase of endocycles. Duet al. (1996b) also report that simultaneous overexpression of dE2F and dDP induces ectopic S phase in eye imaginal cells (in between endo S phases) begin DNA replication (e.g., see Fig 5C, below). We conclude that coexpression of dE2F and dDP is capable of inducing S phase in G1.

New transcription of dE2F and dDP does not appear to be the mechanism by which E2F is normally activated in the embryo, because dE2F and dDP transcripts are ubiquitously and continuously present during G1 (Duronio et al. 1995; R.I. Duronio and P.H. O’Farrell, unpubl.). We suggest that dE2F/dDP complexes formed from newly synthesized protein bypass normal constraints on E2F activity, perhaps by evading inhibition from limiting amounts of RBF, a recently identified Drosophila homolog of the mammalian pRB family of proteins (Du et al. 1996a). The failure of singly overexpressed dE2F or dDP to induce S phase in the epidermal cells was not attributable to a failure to accumulate the induced protein because accumulation of each subunit was similar whether induced alone or together with the complementary subunit (data not shown). Thus, if either subunit has a pool of free monomer in the epidermal cells at this stage of development, this pool is small, and the amount of heterodimer formed upon induction of the complementary subunit is not sufficient to titrate inhibitors. Whereas the vast majority of the G1-quiescent cells of the embryo are similarly unaffected by singly overexpressed dE2F and dDP, the central midgut is induced to enter S phase by induction of dE2F alone (arrowhead, Fig. 5B, below; see also Asano et al. 1996). This might be rationalized either in terms of a tissue-specific difference in the levels of free dDP, or in terms of a lower threshold required for triggering S phase.

Mammalian cells stimulated by E2F-1 overexpression can progress from G1 through S phase and into mitosis (DeGregori et al. 1995b). Because the cells we were examining usually undergo endocycles, it was questionable whether they could undergo mitosis. β-tubulin staining revealed no evidence of mitotic figures in the embryonic epidermis up to 4 hr after heat shock production of dE2F/dDP. The lack of evidence for an ectopic mitotic phase is consistent with the fact that cells destined to begin endoreduplication cycles terminate the expression of genes required for mitosis (e.g., cyclins A and B; Lehner and O’Farrell 1990). Similarly, an induction of cyclin E at these embryonic stages drove cells into S phase without subsequent mitosis (Knoblich et al. 1994).

Simultaneous overexpression of dE2F and dDP induces target gene expression, including cyclin E

One way in which E2F might advance cells into S phase is by trans-activating genes encoding essential replication factors. Therefore, we tested whether E2F overexpression activated the expression of known target genes. The small subunit of ribonucleotide reductase (DmRNR2), PCNA, and cyclin E were defined as E2F target genes based on the finding that their expression, which occurs at the G1–S transition, is eliminated in a dE2F mutant (Duronio et al. 1995). In addition, E2F binding sites contribute to PCNA promoter activity in vivo (Yamaguchi et al. 1995). Embryos from hsp70–dE2F, hsp70–dDP, or hsp70–dE2F/hsp70–dDP flies were collected, aged, and heat-shocked as before, and then fixed and analyzed by in situ hybridization. Heat shock coexpression of dE2F- and dDP-induced DmRNR2 (Fig. 3C), PCNA (not shown), and cyclin E (Fig. 4C) transcription uniformly throughout the G1–S transition, whereas expression of either dE2F (Figs. 3A,4A) or dDP (Figs. 3B,4B) alone did not. Detectable RNA accumulation began by 30 min after heat shock, and was maximal by 60 min. Cyclin E RNA disappeared by 120 min after heat shock, whereas DmRNR2 and PCNA disappeared between 3 and 4 hr after heat shock. Thus, as with the
hs E2F

hs DP

hs E2F/DP

Figure 3. Simultaneous ectopic production of dE2F and dDP induces DmRNR2 expression. Staged embryo collections from flies carrying hsp70–dE2F (A), hsp70–dDP (B), or hsp70–dE2F and hsp70–dDP (C) transgenes were heat-shocked and subsequently fixed at 1 hr after the end of the heat pulse. DmRNR2 expression was determined by in situ hybridization with a digoxigenin-labeled antisense RNA probe. Each panel shows a whole embryo from the lateral perspective (orientation as in Fig. 2), with the epidermal cells in the focal plane. Simultaneous production of dE2F and dDP induces massive expression of DmRNR2 in the otherwise quiescent epidermis (C), whereas production of just dE2F (A) or just dDP (B) does not. The embryos in A and B are essentially identical to control embryos that have not been heat-shocked or that have been heat-shocked without the transgenes [see Duronio and O’Farrell 1994, 1995; Sauer et al. 1995]. Thus, the out-of-focus staining seen in A and B is attributable to normal DmRNR2 expression in internal tissues.

Figure 4. Simultaneous ectopic production of dE2F and dDP induces cyclin E expression. Staged embryo collections from flies carrying hsp70–dE2F (A), hsp70–dDP (B), or hsp70–dE2F and hsp70–dDP (C) transgenes were heat-shocked and subsequently fixed at 1 hr after the end of the heat pulse. Cyclin E expression was determined by in situ hybridization with a digoxigenin-labeled antisense RNA probe. Each panel shows a whole embryo from the lateral perspective (orientation as in Fig. 2). As seen with DmRNR2 (Fig. 3), coproduction of dE2F and dDP (C) causes massive accumulation of cyclin E RNA in the otherwise quiescent epidermis. hsp70–dDP embryos (B) appear identical to control embryos, where the staining of out-of-focus internal tissues is less intense than with DmRNR2 (Fig. 3), and therefore less obvious (see Duronio and O’Farrell 1995, Sauer et al. 1995). In hsp70–dE2F embryos (A) cyclin E RNA staining is more intense in the midgut relative to control embryos, accounting for the obvious out-of-focus staining. Whereas the basis for this is unknown, it may be related to the sensitivity of this tissue to overexpression of just dE2F [see text and Fig. 5B].

BrdU labeling experiments, induction of E2F-target gene transcription in G₁-arrested cells required simultaneous expression of dE2F and dDP.

E2F overexpression cannot induce S phase in the absence of cyclin E

How can E2F-directed transcription trigger S phase? If an essential replication factor encoded by an E2F target gene was absent during G₁ and therefore limited a cell’s capacity to synthesize DNA, then new, E2F-mediated synthesis not only would be required for S phase, but might actually trigger it. Maternal stores are likely to fulfill the need for most replication factors early in development (Smith et al. 1993, Duronio and O’Farrell 1994). Consequently, no embryonic expression of the genes encoding these factors would be required. For instance, zygotic PCNA function is not required until postembryonic larval stages [Henderson et al. 1994]. In contrast, genetic analyses have indicated that new cyclin E transcription is required for the G₁–S transition during cycle 17 [Knoblich et al. 1994]. We tested, therefore, whether E2F-induced S phase requires cyclin E production by coexpressing dE2F and dDP in null cyclin E<sup>AR95</sup> mutant embryos. cyclin E<sup>AR95</sup> is a nonsense allele [Knoblich et al. 1994] that allowed us to monitor expression of the mutant cyclin E transcript in the absence of cyclin E function. Both cyclin E and DmRNR2 transcription were induced by heat shock-produced dE2F/dDP in cyclin E<sup>AR95</sup> mutant embryos to the same extent as in wild-type embryos (not shown, but see Figs. 3C, 4C). In spite of this, BrdU labeling indicated that DNA replication was not induced in the mutant embryos (Fig. 5D). Thus, mutation of cyclin E blocked completely the ability of E2F to induce S phase. We conclude from this result that activation of...
E2F-mediated transcription is incapable of bypassing the loss of cyclin E function at these embryonic stages.

**Discussion**

We have demonstrated here that ectopic expression of *Drosophila* dE2F/dDP during embryonic G1-17 induces both E2F-dependent transcripts and DNA replication. Additionally, induction of E2F and E2F-dependent transcripts does not bypass the replication block caused by mutation of cyclin E. We conclude that cyclin E has an essential in vivo role in DNA replication other than induction of E2F activity.

Our conclusion is consistent with several other observations. First, in vitro replication of sperm chromatin in *Xenopus* egg extracts does not require transcription, but is still blocked by immunodepletion of cyclin E [Jackson et al. 1995]. Second, microinjection of anticyclin E antibodies into cells lacking functional pRb prevents entry into S phase [Ohtsubo et al. 1995], suggesting that pRb phosphorylation, and consequently E2F activation, is not the only essential role of cyclin E [see also Beijersbergen et al. 1995; Resnitzky and Reed 1995]. Third, mutation of *Drosophila* cyclin E does not prevent the activation of E2F in all cells [Duronio and O’Farrell 1995] in spite of causing cells to arrest in G1 [Knoblich et al. 1994]. Similarly, Hofmann and Livingston [1996] reported that expression of a dominant negative cdk2 causes G1 arrest in cultured cells, even in the presence of active E2F.

In contrast, our conclusion appears to conflict with the recent observations of DeGregori et al. [1995b], who report that expression of human E2F-1 in rat embryonic fibroblasts using a recombinant adenoviral vector stimulates S phase and cell division without activation of cyclin E/cdk2. The simplest explanation is that a low level of cyclin E/cdk2 activity that seems to persist in their system is sufficient to supply the essential S-phase function that is independent of E2F activation. However, we would like to highlight a second possible explanation that comes from the realization that cyclin E and E2F might interact twice, once at the level of their activation and once at the level of their action to trigger S phase.

There does not appear to be a defined order of cyclin E and E2F activation, because each activates the other. In mammals, extensive biochemical characterization suggests that cyclin E activation of E2F occurs by phospho-
cyclin E. Expression of human E2F-1 in rat fibroblasts induces transcription of the endogenous cyclin E gene (DeGregori et al. 1995a), and mutation of dE2F prevents cyclin E expression at the G1-S transition in Drosophila embryos (Duronio and O'Farrell 1995). Thus, each regulator has the capability of activating the other (Fig. 6).

Because cyclin E and E2F activate each other, one cannot define an a priori order in the pathway of their activation. Indeed, as described previously (Duronio and O'Farrell 1995), the order of activation is different in different cells of the Drosophila embryo. In the transition from G1 to S phase, E2F is activated first, and this E2F activity is required for expression of cyclin E; in the rapidly cycling cells of the central nervous system, constitutive expression of cyclin E is responsible for the activation of E2F. Additionally, whereas ectopic expression can often be used to define the order of action of genes in a pathway, mutual activation leads to ambiguity. Thus, cells arrested in G17 can be advanced into S phase by ectopic expression of either cyclin E or dE2F/dDP (Knoblich et al. 1994; data presented here).

In analyzing the order of action of these two gene products, we should consider separately the pathway of their activation and their involvement in the pathway that promotes S phase (Fig. 6). Once activated, cyclin E and E2F are presumed to carry out some function to promote S phase. The order of their action to promote S phase can influence the results of an attempt to bypass the function of cyclin E. Because we do not know the function that cyclin E performs to promote S phase, it may be able to act prior to E2F-driven accumulation of essential replication gene products. Thus, if cyclin E/cdk2 is inactivated and then E2F is activated independently, the cyclin E-dependent step might have been completed already. Consequently, an experimental demonstration that cyclin E function can be bypassed for a single cycle does not exclude the possibility that it has an essential S phase function. It is possible that the bypass experiments of DeGregori et al. (1995b) might have been influenced by earlier action of cyclin E. Their experiments involved use of cyclin/cdk inhibitors or γ-irradiation to block cell cycle progression, and expression of E2F-1 to activate E2F independently. In G1 of Drosophila embryonic cycle 17, there is no cyclin E expression, and entry into S phase is limited by this absence. Thus, in our experimental setting, cyclin E has not had an opportunity to perform its essential S-phase function and the mutant cannot be bypassed by induction of E2F. Accordingly, cyclin E does have an S-phase role other than activation of E2F in G17 of Drosophila embryos, and we do not consider the evidence in mammalian cells as a challenge to the generalization of this conclusion. Indeed, we note that parallels between results in mammalian cells, Xenopus, Drosophila, and yeasts suggest that there is a universal requirement for cyclin-dependent kinase in the S phase of eukaryotes (see Heichman and Roberts 1994). In Saccharomyces cerevisiae it has been proposed that one requirement for G1 cyclins is to effect a change in protein turnover by ubiquitin-dependent proteolysis of an S-phase inhibitor (Tyers et al. 1992; Schwob et al. 1994; Dirick et al. 1995). Perhaps cyclin E will be similarly involved in triggering changes in the stability of key cell cycle regulators in the metazoa.

**Figure 6.** Schematic of the relationship between cyclin E, E2F, and S phase. Cyclin E and E2F are capable of inducing each other (solid horizontal arrows). Each also has a role in an essential step or steps) leading to DNA synthesis [broken arrows] that is separate from their capacity for mutual induction. We propose that the order of activation of cyclin E and E2F can change in different developmental situations or different cell types. According to either E2F [A] or cyclin E [B] by natural or engineered (e.g., overexpression) signals results in activation of both, and triggers the G1-S transition. Note that mutual induction does not necessarily establish that positive feedback amplification is what leads to full activation of cyclin E and E2F (Dirick et al. 1995; Duronio and O'Farrell 1995; Stuart and Wittenberg 1995). After their activation, E2F and cyclin E must perform other functions for DNA synthesis to ensue [broken arrows]. The order of execution of these functions can influence the results of bypass experiments: They may need to occur in an obligate sequence to trigger DNA synthesis, or they may occur independently of one another.

**Materials and methods**

**Drosophila stocks**

All experiments were performed with P[w+; hsp70-dE2F] and P[w+; hsp70-dDP] transgenes, each of which are third chromosome insertions that are homozygous viable. Construction of P[w+; hsp70-dE2F] was described in Duronio et al. (1995). P[w+; hsp70-dDP] was made by subcloning a 2.1-kb dDP cDNA (Dynlacht et al. 1994) into pCaSpeR-hs. This construct produces a polypeptide that has an 8-kD truncation at the amino terminus relative to endogenous dDP [data not shown; Hao et al. 1995]. To coexpress dE2F and dDP, a homozygous viable recombinant third chromosome containing the P[w+; hsp70-dE2F] and P[w+; hsp70-dDP] transgenes was constructed. This recombinant third chromosome was used to construct the following stock for overexpression of dE2F/dDP in cyclin E mutant embryos: w, cycE(865)/CyO P[w+; wg-lacZ], P[w+; hsp70-dE2F] P[w+; hsp70-dDP]/P[w+; hsp70-dE2F] P[w+; hsp70-
Characterization of the cyclin E<sup>AR95</sup> allele is described in Knoblich et al. [1994]. The CyO [P<sup>+</sup>; wg-lacZ] balancer chromosome allowed unambiguous identification of homozygous AR95 mutant embryos, which failed to express β-galactosidase. In addition, the wg phenotype caused by CyO [P<sup>+</sup>; wg-lacZ] allowed us to distinguish +/+ from +/AR95 progeny.

**Heat shock procedure**

Eggs were collected on grape juice agar plates for 1–2 hr at 25°C, and then aged for the appropriate time at 17°C (usually 18–20 hr). Staged embryos [Campos-Ortega and Hartenstein 1985] were heat-shocked by floating the egg collection plates on the surface of a 37°C water bath for 30 min. Heat-shocked embryos were allowed to recover at room temperature for 30, 60, 90, 120, 180, or 240 min, and then either fixed or pulse labeled in Schneider’s medium containing 1 mg/ml BrdU (Sigma) for 15 min at room temperature, followed by immediate fixation as described [Edgar and O’Farrell 1990]. In all experiments fixation was achieved with a 5-min incubation in a 1:1 mixture of 37% paraformaldehyde/heat.

**In situ hybridization and immunofluorescence**

Digoxigenin-labeled antisense RNA probes [Boehringer-Manheim] from DmRNR2 and PCNA genomic clones [Yamaguchi et al. 1990; Duronio and O’Farrell 1994], and full-length type 1 cyclin E cDNA [Richardson et al. 1993] were prepared as described [Duronio and O’Farrell 1994]. In situ hybridizations were performed with fixed embryos as described [Tautz and Pfeifle 1989; Lehrer and O’Farrell 1990; Duronio and O’Farrell 1994]. Incorporation BrdU was detected [Schubiger and Palka 1987] using mouse anti-BrdU monoclonal antibodies [Beckton-Dickinson]. Microtubules were detected with a mouse anti-β-tubulin monoclonal antibody [Amersham]. Mouse monoclonals were transferred electrophoretically to nitrocellulose, and dE2F and dDP polypeptides were detected using mixtures of monoclonal antibodies Hao4 and Hao5, and Yun1, Yun2, and Yun3, respectively [Du et al. 1996a].

**Immunoblot analysis**

Eggs were collected, aged, and heat-shocked as described above. After 10, 30, and 60 min recovery at room temperature, embryos were fixed in a 1:1 mixture of methanol/heptane. Either 4, 8, or 16 methanol-fixed embryos were hand-selected using a dissecting microscope, solubilized by boiling 5 min in 3× gel loading buffer [62.5 mM Tris [pH 6.8], 2% SDS, 5% glycerol, 5% β-mercaptoethanol], and subjected to SDS-Page. Separated proteins were transferred electrophoretically to nitrocellulose, and dE2F and dDP polypeptides were detected using mixtures of monoclonal antibodies Hao4 and Hao5, and Yun1, Yun2, and Yun3, respectively [Du et al. 1996a].

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