The transcription factor E2F is required for S phase during *Drosophila* embryogenesis

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Overexpression of the E2F-1 cDNA in mammalian cells disrupts normal control of the cell cycle and drives cells into S phase. Whereas eliminating E2F activity would test its inferred involvement in the G₁-S transition, elimination is complicated by the existence of gene families encoding mammalian E2F. Here we identify mutations in a single essential *Drosophila* gene, *dE2F*, that encodes a homolog of the mammalian E2F gene family. Embryos homozygous for null mutations of *dE2F* complete early cell cycles, presumably using maternal contributions of gene products, but DNA synthesis falls to virtually undetectable levels in cycle 17. Mutant embryos also lack the pulses of coordinate transcription of genes encoding replication functions that usually accompany each transition from quiescence to S phase. We conclude that in most cells *dE2F* is essential for a G₁-S transcriptional program and for G₁-S progression.

[Key Words: *Drosophila*; E2F; S phase; cell cycle; embryogenesis]

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The E2F transcription factor was originally identified through its role in the regulation of the adenovirus E2 promoter during viral infection. E2F is a heterodimer composed of a polypeptide encoded by *DP-1* (or *DP-1*-related genes) and a polypeptide encoded by *E2F-1* (or *E2F-1*-related genes). Studies in mammalian cells have suggested that E2F provides a nexus between the cyclin-dependent kinases, the retinoblastoma tumor suppressor gene, and the cell cycle control of transcription. A favored model of E2F regulation [for review, see Nevins 1992; Helin and Harlow 1993; Farnham 1995] is summarized below.

In mammalian cells, the transcriptional activity of E2F is constrained by its physical association with the retinoblastoma protein (pRB) and the pRB-related proteins p107 and p130. The pRB-E2F interaction is regulated through phosphorylation by cyclin-dependent kinases (cdks). pRB has been shown to be phosphorylated by cdks that become activated during G₁ phase of the cell cycle, such as cyclin D/cdk4, cyclin D/cdk6, and cyclin E/cdk2 kinases. Phosphorylation of pRB is thought to lead to the release of E2F, the activation of E2F-dependent transcription, and the promotion of entry into S phase. In this model, E2F and pRB are proposed to act antagonistically to regulate entry into S phase. This scheme provides an appealing common rationale for the action of oncogenes and tumor suppressor genes. Functions that interfere with the inhibitory interaction of

pRB would act dominantly to activate E2F, and mutations of the *RB-1* gene would act recessively to the same end.

This model assumes that the elevation of E2F activity would drive the cell cycle. Transfection and microinjection experiments using the recently cloned mammalian *E2F* genes show that artificially raising the level of E2F activity in tissue culture cells can drive cells into S phase [Johnson et al. 1993; Qin et al. 1994; Shan and Lee 1994; Wu and Levine 1994]. Furthermore, the overexpression of E2F-1 will overcome pRB- or p53-mediated growth arrest [Zhu et al. 1993; Wu and Levine 1994; Qin et al. 1995]. The consequences of E2F overexpression appear to vary, depending on cell type. High-level expression of E2F-1 has been shown to lead to apoptosis [Qin et al. 1994; Shan and Lee 1994; Wu and Levine 1994]. Overexpression of E2F-1 or E2F-4 transforms rat embryo fibroblasts [Beijersbergen et al. 1994; Ginsberg et al. 1994; Singh et al. 1994].

Investigations of E2F action suggest how E2F could trigger S phase. E2F-binding sites have been found in promoters of genes whose expression is important to cell proliferation. Such genes include those for *DHFR*, *c-myc*, *cdc2*, *cyclin A*, *DNA polymerase α (POLα)*, *thymidine kinase*, *B-myb*, *E2F-1*, and *RB-1* [for review, see Nevins 1992; Helin and Harlow 1993]. In several cases, promoter mutation studies have suggested that these E2F-binding sites are important for the temporal regulation of gene expression in late G₁ [Pearson et al. 1991; Dalton 1992; Means et al. 1992; Lam and Watson 1993; Hsiao et al.

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1994; Johnson et al. 1994). Implicit in models of the G_1 -S transition is the hypothesis that E2F-dependent gene products limit progress to S phase and that stimulation of their expression triggers entry into S phase.

An important caveat to these hypotheses is that the role of E2F has been inferred from situations in which E2F is abnormally activated or overexpressed. It is a concern that this activation bypasses rather than mimics the normal route leading to S phase. The finding that cells driven into S phase by overexpression of E2F-1 die by apoptosis instead of completing the cell cycle heightens these concerns. To test whether E2F is normally involved in replication control, it is necessary to examine the consequences of loss of E2F function. Removal of the mammalian E2F is problematic because it is encoded by many genes. Current information suggests that the human E2F family contains at least five members, whereas two DP family members 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; Ginsberg et al. 1994; Sardet et al. 1995; Wu et al. 1995). In contrast, only one representative of each class of gene has been isolated from *Drosophila* (Dynlacht et al. 1994; Ohtani and Nevins 1994). The products of the *dE2F* and *dDP* genes are structurally related to their human counterparts and heterodimerize to generate a sequence-specific factor that activates transcription through E2F-binding sites (Dynlacht et al. 1994).

As the cell cycle can be examined in considerable detail in *Drosophila* embryos, the identification of mutations in the *Drosophila* genes should allow detailed characterization of function, including a test of the proposed role of E2F in S-phase transcription. In *Drosophila*, DNA *POL* α , ribonucleotide reductase large (*DmRNR1*) and small (*DmRNR2*) subunits, and *PCNA* are coordinately transcribed in transient pulses that parallel and slightly precede DNA synthesis in cells entering S phase from quiescence (Duronio and O'Farrell 1994). To examine the normal function of E2F, we have identified and characterized embryos homozygous for null mutations in *dE2F*. This analysis shows that the *dE2F* gene is essential for the activation of the transcriptional program at the G_1 -S transition and is required for DNA replication during embryonic development.

Results

Identification of candidate *dE2F* mutations

Hybridization of the previously isolated (Dynlacht et al. 1994) *dE2F* cDNA to polytene chromosomes of wild-type (Fig. 1A) and deficiency-bearing stocks localized *dE2F* sequences to position 93E on chromosome 3 within the interval between the distal breakpoints of *Df(3R)e^{F1}* and *Df(3R)e^{D7}* (Fig. 1B). Because E2F is proposed to play a role in G_1 -S transcription in mammalian cells, we tested whether loss of *dE2F* sequences might eliminate the normal program of S-phase transcription in *Drosophila*. Antisense *PCNA* and *DmRNR2* probes were hybridized to embryos collected from the deficiency

Figure 1. Localization of the *dE2F* gene. (A) Digoxigenin (DIG)-labeled *dE2F* cDNA was hybridized to polytene chromosomes from salivary glands of third-instar larvae. Polytene chromosome bands were visualized by Giemsa staining. The open arrowhead indicates the location of the hybridization signal. *dE2F* maps to position 93E on the right arm of chromosome three. (B) Schematic diagram of the 93E region. In situ hybridization with *Df(3R)e^{D7}/+* and *Df(3R)e^{F1}/+* heterozygous polytene chromosomes localized *dE2F* sequences to the interval between the distal breakpoints of these deficiencies (indicated with a bracket). The polytene map is represented with the open bar and number designations according to Bridges (1935). The solid bars represent the deleted regions of each deficiency chromosome.

stocks. The signal normally present in stage 13 embryos was absent in embryos homozygous for the *Df(3R)e^{D7}* deficiency but not in those homozygous for the *Df(3R)e^{F1}* deficiency (data not shown, but see Fig. 2).

These results indicate that the deficiency interval that contains a molecular homolog of mammalian E2F genes also includes at least one gene essential for transcription of *DmRNR2* and *PCNA*. The failure to express these genes might serve as a marker for *dE2F* mutations. To test this possibility, we next sought mutations within the *Df(3R)e^{D7}* deficiency that are defective in S-phase transcription. Previously, Azpiazu and Frasch (1993) had used ethylmethane sulfonate (EMS) to generate lethal mutations that failed to complement the *Df(3R)e^{D7}* deficiency. They identified six complementation groups within the interval containing *dE2F* and the gene required for *DmRNR2* and *PCNA* transcription. We examined *DmRNR2* expression in embryos collected from fly stocks representing each of the six complementation groups. In complementation group IV, one-quarter of the embryos (the expected frequency of homozygous mutant embryos) failed to express *DmRNR2* by stage 13 (Fig. 2B). This loss of expression was seen in embryo collections from all four group IV alleles (91, 155, 296, and 337) and was not apparent in embryo collections from any of the other complementation groups. Thus, group IV mutations define a gene required for transcription of *DmRNR2* and identify a candidate for the *dE2F* gene.

Figure 2. *dE2F* mutant embryos are defective in a G₁-S transcriptional program. Each panel shows a stage-13 embryo that was hybridized with a DIG-labeled *DmRNR2* probe. In this and all subsequent figures, embryos are oriented with anterior to the left and dorsal to the top. (A) The wild-type *DmRNR2* expression pattern. Note that *DmRNR2* is highly expressed in the mitotic cells of the CNS (open arrow) and in the cells of the anterior and posterior midgut (left and right solid arrows, respectively) that begin endocycles. At this stage, these midgut cells have progressed into S phase from G₁₋₁₇ (see text). (B) In *dE2F*⁹¹/*dE2F*⁹¹ homozygous mutants, nearly all *DmRNR2* expression in the entire embryo is lost by stage 13. (C) The wild-type *DmRNR2* expression pattern is restored in *dE2F*⁷¹⁷² mutant embryos after heat-induced transcription of *dE2F* using the *hsp70* promoter. The *DmRNR2* phenotypes of *dE2F*⁹¹/*dE2F*⁹¹ and *dE2F*⁷¹⁷²/*dE2F*⁷¹⁷² embryos are identical.

In addition to the EMS alleles, several lethal P-element insertion mutations have been mapped to the 93E region. Complementation tests revealed that two of these, 7172 and rM729, belong to group IV. Like the four EMS alleles, these P alleles are defective in *DmRNR2* and *PCNA* expression. Both P alleles and two of the EMS alleles were examined for alterations in *dE2F* sequences.

Lesions in the *dE2F* gene

Genomic Southern blots probed with 5' *dE2F* sequences detected novel bands in DNA from each P-element line (Fig. 3A). PCR and DNA sequence analyses (see Materials and methods) demonstrated that the rM729 P element is inserted 48 nucleotides upstream of the initiator methionine, with the inserted *lacZ* gene in the same orientation as the *dE2F* gene. The 7172 P element is inserted 33 nucleotides upstream of the initiator methi-

onine, with *lacZ* in the opposite orientation (Fig. 3B). Transposase-mediated excision of either of these P elements gave *ry*⁻ flies that lacked the P element and frequently reverted the lethal phenotype (data not shown). Thus, these insertions in the *dE2F* sequences are responsible for the lethality, perhaps because they disturb *dE2F* expression. The zygotic *dE2F* RNA is greatly reduced in 7172 mutant embryos (data not shown). Although *dE2F* RNA levels appear normal in rM729, the position of insertion suggests that translation may be compromised.

We examined two group IV EMS alleles to determine whether they are associated with altered *dE2F*-coding sequences. First, the genomic sequence that encodes the open reading frame of wild-type *dE2F* cDNA was isolated and characterized. The *dE2F* open reading frame is divided into five exons that span ~5 kb of genomic DNA (Fig. 3B). Next, fragments of the *dE2F* gene amplified from the DNA of flies heterozygous for the EMS-generated alleles were cloned and sequenced. The 91 allele contained a C-to-T transition at nucleotide +91 that converted amino acid Gln-31 of *dE2F* to a stop codon. The 296 allele contained a similar C-to-T mutation at nucleotide +532 that converted amino acid Gln-178 of the wild-type sequence to a stop codon. In both cases, premature translation termination would result in the synthesis of a short peptide that lacks the recognized functional domains of the *dE2F* gene product (Fig. 3B).

A cDNA encoding *dE2F* rescues the mutant phenotype

To test whether the mutant phenotype is attributable to the alterations in *dE2F* sequences, we introduced a transgene (*hs-dE2F*) allowing heat-inducible expression of the *dE2F* cDNA into the background of the 7172 mutation. Stage 13 embryos were induced with a 30-min 37°C heat treatment. One hour after heat shock, the embryos were fixed and subjected to in situ hybridization with a *DmRNR2* probe. Heat-induced *dE2F* had very little effect on the pattern of *DmRNR2* expression in wild-type embryos (data not shown). However, *hs-dE2F* expression restored *DmRNR2* expression in homozygous mutant (*dE2F*⁷¹⁷²/*dE2F*⁷¹⁷²) embryos. Interestingly, the restored gene expression occurred in a pattern that closely resembled the wild-type pattern (Fig. 2C). No *DmRNR2* expression was observed in embryos from the same line that were not heat treated. Thus, ectopically provided *dE2F* rescues the *DmRNR2* expression defect of the 7172 mutation.

Considered together, the location of the P-element insertions, the loss of *dE2F* expression in the 7172 allele, the detection of inactivating point mutations in the *dE2F* gene in two EMS alleles, and the phenotypic rescue by the *dE2F* cDNA indicate that we have identified mutations of the *dE2F* gene.

Cessation of DNA synthesis in *dE2F* mutant embryos

We examined *dE2F* mutants to define when zygotic *dE2F* is first required for cell cycle progression. Maternally supplied products are sufficient to support cell cycle pro-

Figure 3. Mutation of the *dE2F* gene. (A) Southern blots of DNA from the P-element lines 7172 and rM729 and a control stock (TM3/TM6B) were hybridized with a 845-bp probe prepared from the 5' end of the *dE2F* open reading frame. (B) Structure of the *dE2F* gene, including an illustration of the position and orientation of the P elements and EMS mutations relative to the *dE2F* gene. The *dE2F* open reading frame is encoded by sequences spanning ~5 kb of genomic DNA, as indicated by the shaded regions. Intron sequences are shown as single lines. Glutamine (CAG) codons are mutated to stop (TAG) codons at amino acid 31 of allele *dE2F*⁹¹ and at amino acid 178 of allele *dE2F*²⁹⁶.

gression in the *Drosophila* embryo until G₂ of cycle 14, when *string* gene expression is required. Whereas expression of additional cell cycle genes becomes essential as embryogenesis progresses, to date only five loci have been shown to be essential prior to cycle 17 (*string*, *three rows*, *pimples*, *pebble*, and the histone locus) (Edgar and O'Farrell 1989; Lehner 1992; D'Andrea et al. 1993; Smith et al. 1993).

Immunofluorescent staining of *dE2F* mutant embryos for β -tubulin, which reveals mitoses, failed to show early defects and demonstrated that all epidermal cells complete mitosis 16 as in the wild type (Fig. 4C,D). Similarly, immunofluorescent detection of bromodeoxyuridine (BrdU) incorporated during a pulse failed to detect defects in S-phase 15 or 16 (data not shown). Thus, there is no apparent zygotic requirement for *dE2F* during cycles 15 and 16. After the epidermal cells have completed mitosis 16, virtually all incorporation of BrdU is eliminated in the *dE2F* mutant embryos (Fig. 5).

Onset of the *dE2F* phenotype occurs at a stage when we distinguish three tissue-specific programs of cell cycle control in wild-type embryos. One notable group of cells, the neuroblasts of the central nervous system (CNS), delaminates from the epidermis in three successive waves beginning in cycle 14 (Foe 1989). They then proliferate with a rapid cycle (~40 min) until mid-embryogenesis (Hartenstein et al. 1987). These proliferating

cells lack an obvious G₁ or G₂ and are easily visualized in the brain and ventral nerve cord after pulse-labeling wild-type embryos with BrdU (open arrow, Fig 5A). Outside the CNS, most cells progress through three slower cycles having a G₂ and, following mitosis 16, enter a period of quiescence referred to as G₁₋₁₇. This G₁ can be lengthy [e.g., abdominal histoblasts remain quiescent for 5 days prior to rapid proliferation during pupal development (Cohen 1993)] or short [e.g., cells of the developing anal pads enter S phase ~1 hr after mitosis 16 (Smith and Orr-Weaver 1991; Duronio and O'Farrell 1994)]. Following entry into S-phase 17, some cells proliferate mitotically [e.g., cells of the peripheral nervous system (PNS) and of the imaginal disks], whereas others bypass mitosis and amplify their DNA via a sequence of distinct endoreduplication S phases (Smith and Orr-Weaver 1991). Thus, the three types of cycles are the rapid CNS cycles without obvious gap phases, proliferative cycles that begin from G₁, and the endocycles that have a G₁-like phase but bypass mitosis. Of these three types of cycles, we focus on two: the CNS cycles and the endocycles. Embryonic lethality of *dE2F* mutations precludes an analysis of the postembryonic reactivation of imaginal disk cell proliferation.

In *dE2F* mutant embryos, BrdU incorporation in the CNS decreases gradually beginning during stage 12 (data not shown). By stage 13, BrdU incorporation in the CNS

Figure 4. The effect of *dE2F* mutations on the development of PNS structures and epidermal proliferation. (A,B) Stage-16 wild-type (A) and *dE2F*⁹¹ mutant (B) embryos were stained with monoclonal antibody 22C10, which stains neuronal cell bodies, dendrites, and axons (Zipursky et al. 1984; Canal and Ferrus 1986). A close-up view of the lateral pentascolopodial chordotonal organ (stretch receptor) present in each of the first seven abdominal segments is shown. A characteristic feature of this structure is the array of five cell bodies (arrow). Note that this array is disrupted in the *dE2F*⁹¹ mutant (B). (C,D) β -Tubulin staining of late stage-12 wild-type (C) and *dE2F*⁷¹² mutant (D) embryos. A portion of the lateral epidermis in the three thoracic segments (T1, T2, and T3) is shown. At this stage, the cells in segment T3 have completed mitosis 16 (asterisks). Note the similar number of G₁₋₁₇-arrested cells in T3 in wild-type vs. mutant. Rather than arresting after cycle 16, a group of cells in segment T1 continues through a seventeenth division cycle (Knoblich et al. 1994). Mitosis 17 in these cells is indicated by an arrow in each panel. *dE2F* mutant embryos complete this cell cycle as well.

was barely detectable (open arrow, Fig. 5B). Cells that normally enter endocycles fail at S₁₇. Replication either does not occur or occurs with such a dramatically reduced rate of nucleotide incorporation that it is near the limit of our sensitivity (solid arrows, Fig. 5B). We conclude that E2F is required for normal DNA replication in both mitotic CNS cycles and in endocycles.

Expression of the *hs-dE2F* transgene restored DNA synthesis in *dE2F* mutant embryos (7172 allele) (Fig. 5C). Rescued replication, like rescued *DmRNR2* transcription, closely followed the pattern seen in wild-type embryos. Thus, induced expression of *dE2F* did not provoke unscheduled DNA synthesis in quiescent cells. The rescue of DNA synthesis confirms that the replication defect is attributable to mutation of *dE2F*, whereas the failure of ubiquitous ectopic expression of *dE2F* to override the controls that determine the time of replication in different tissues suggests that *dE2F* transcription does not regulate this timing.

dE2F expression in the *Drosophila* embryo

The lack of an early requirement for the *dE2F* gene might mean that there is no requirement for this transcription

factor during early divisions or that such function is essential but supplied maternally. In situ hybridization reveals intense *dE2F* hybridization throughout syntitial stages (nuclear cycles 1–13) (data not shown). As there is little or no transcription prior to cycle 10 (Edgar and Schubiger 1986), this signal is attributable to maternal *dE2F* RNA.

The *dE2F* hybridization signal disappeared by the beginning of the cellular blastoderm stage (cycle 14) and subsequently reaccumulated in all cells. Because reaccumulation did not occur in *Df(3R)e*^{D7} deficiency-homozygous embryos, the reappearance of *dE2F* transcripts is attributable to zygotic gene expression (data not shown). Although RNA accumulates more rapidly in some cells, we attach no special significance to the initial pattern (Fig. 6A). Later in development, *dE2F* transcripts remain widespread: Whereas hybridization is clearly evident in cells that are not actively synthesizing DNA, such as the G₁₋₁₇-arrested epidermis (solid arrow, Fig. 6C), the signal is particularly strong in cells of the CNS that are proliferating especially rapidly (open arrow, Fig. 6B–D). Embryos heterozygous for the P-element allele rM729 express β -galactosidase in a similar pattern, presumably because of an enhancer trap effect (Fig. 6D). We conclude from these observations that *dE2F* transcription is not

Figure 5. *dE2F* mutant embryos are defective in DNA replication. (A) Immunofluorescent detection of BrdU incorporated during pulse labeling of a stage-13 wild-type embryo. Both mitotically active CNS cells (open arrow) and endocycling midgut cells (solid arrows, denoting S₁₇) are labeled. (B) Very little BrdU incorporation is observed in a stage-13 *dE2F*^{M729} mutant embryo. S₁₇ in the midgut is absent (solid arrows), whereas DNA replication in the CNS is failing (open arrow). Note that the embryos shown in A and B were photographed in the same field of view, allowing a direct comparison of the level of DNA synthesis. (C) DNA replication in stage-13 mutant embryos is restored in a normal pattern after heat shock provision of *dE2F*.

Figure 6. Expression of the *dE2F* gene during embryogenesis. Digoxigenin-labeled *dE2F* cDNA was hybridized to fixed, wild-type embryos obtained from staged egg collections. (A) By the cellular blastoderm stage (cycle 14; ~2.5 hr of development at 25°C), early zygotic transcription drives the reaccumulation of *dE2F* RNA in all cells after disappearance of maternal *dE2F* RNA. The rate of accumulation is not uniform, however, which accounts for the apparent pattern. (B) After germ-band extension (stage 10; ~5 hr of development), *dE2F* expression is ubiquitous and nearly uniform. High expression in neuroblasts makes these cells readily visible (open arrow). (C) Ubiquitous expression of *dE2F* continues after germ-band retraction (early stage 13; ~8 hr of development), including $G_{1,17}$ -arrested cells (solid arrow). *dE2F* expression in the CNS continues as well (open arrow). (D) Staining of enhancer trap line rM729 with antibodies to β -galactosidase at stage 13. Note that the plane of focus is internal rather than on the surface as in C. (Arrow is the same as in C.)

confined to particular cell cycle phases during the embryonic stages that we have examined, and that the regulation of *dE2F* mRNA levels is unlikely to be a major method of cell cycle control in the embryo.

Coupling of S phase and the G_1 -S transcription program

As maternal *dE2F* activity is depleted in mutant embryos, *dE2F*-dependent transcription and DNA replication decline. If the decline in DNA replication is secondary to deficits in expression of *dE2F*-dependent gene products, then loss of *DmRNR2* RNA might precede the decline in BrdU incorporation.

In wild-type embryos, a maternal supply of *DmRNR2* message disappears by early cycle 14, and zygotic expression, which restores *DmRNR2* RNA levels in all cells during G_2 of cycle 14, is fairly uniform in the epidermis until cycle 16 (Duronio and O'Farrell 1994). During stage 11, *DmRNR2* transcripts decline rapidly as cells progress through S phase of cycle 16. Transcripts are not detected in epidermal cells that are in G_1 of cycle 17. *PCNA* and several other genes encoding replication functions show a similar pattern of expression (Duronio and O'Farrell 1994; P. Follette, T.T. Su, and P. O'Farrell, unpubl.). In *dE2F* mutant embryos, these early features of *DmRNR2* and *PCNA* expression were undisturbed (data not shown).

In late stage-11 wild-type embryos, *DmRNR2* expression persists in the rapidly proliferating CNS cells and in two mitotic populations of cells that we have examined (Fig. 7A). These mitotic populations, the precursors of the PNS (Bodmer et al. 1989) and a group of dorsolateral epidermal cells in the first thoracic segment (T1; Knoblich et al. 1994), pause briefly or not at all after mitosis 16 and then enter S_{17} (Fig. 7A,C). The first defect seen in *dE2F* mutant embryos is a reduction of *DmRNR2* (Fig. 7B) and *PCNA* (data not shown) RNA in all three of these tissues. The disappearance of these transcripts in the *dE2F* mutant suggests that maternally supplied *dE2F* becomes limiting for transcription during embryonic stage 11. Consistent with this notion, the regulated expression of *dE2F*-dependent transcripts associated with endocycle S phases, which begin in stage 12, is eliminated in the *dE2F* mutant (Fig. 2B).

In contrast to the severe reduction of *DmRNR2* transcripts observed in late stage-11 *dE2F* mutants, BrdU incorporation continues with only a slight quantitative reduction (Fig. 7D). Thus, the decline of maternal *dE2F* function as assessed by analysis of BrdU incorporation is considerably slower than the decline assessed by analysis of transcripts. Although these two assays might reflect a difference in the level of *dE2F* activity required for each process (transcription vs. replication), this result is consistent with models in which the dependence on *dE2F* for DNA replication is secondary to its requirement for gene expression. In this case, the delay in appearance of the BrdU-incorporation phenotype would be attributed to perdurance of previously synthesized *dE2F*-dependent gene products.

The reduction in intensity of BrdU staining in the PNS cells and T1 epidermal cells of mutant embryos suggests that these cell cycles might be compromised. β -Tubulin staining indicated that the latter group of cells completed mitosis 17 on schedule (arrow, Fig. 4C,D). However, it was difficult to assess by BrdU or tubulin staining if the full program of PNS divisions was completed. A failure to complete this division program prevents proper formation of the larval sensory organs, which can be visualized with monoclonal antibodies that stain neurons (Fig. 4A). Such staining of stage-16 *dE2F⁹¹* mutant embryos revealed aberrations in PNS structures that were associated with a reduction in cell number (Fig. 4B). Because cell cycle arrest does not prevent the final differ-

entiation of neuronal cell types in the PNS (Hartenstein and Posakony 1990), this result suggests that the PNS precursor cells do not complete their normal division program in *dE2F* mutant embryos.

Discussion

The identification of inactivating mutations of the essential *dE2F* gene has enabled us to demonstrate that E2F is required for the transcription of genes that provide replication functions, for DNA replication, and for cell proliferation. This is the first analysis of the consequences of loss of E2F function and the first direct determination of the *in vivo* role of this factor.

To prove that we have identified mutations in *dE2F*, we have physically mapped lesions to *dE2F* sequences and demonstrated that mutants with such lesions are rescued by expression of the cloned *dE2F* cDNA sequences. Molecular criteria show that three of the mutant alleles reduce function severely and probably represent nulls: One P-element allele does not produce detectable levels of *dE2F* RNA, and the two characterized EMS alleles are translational stops that interrupt the *dE2F*-coding sequence prior to important conserved domains. Consequently, any E2F function in a homozygous mutant embryo must be derived from perdurance of protein translated from maternally provided *dE2F* RNA, which is fully destroyed by cycle 14.

A potential role for E2F-dependent transcription in DNA replication

In *Drosophila*, as in many organisms, several genes that encode components of the replication machinery are specifically expressed at the time of the G₁-S transition (Andrews and Herskowitz 1990; Lowndes et al. 1992; Duronio and O'Farrell 1994). Here we show that G₁-S transcription in *Drosophila* is dependent on *dE2F*. Furthermore, the timing of events in *dE2F* mutant embryos provides experimental evidence consistent with the model that E2F activity limits S phase because it drives

expression of other genes essential for DNA replication. In the CNS, the decay of *DmRNR2* transcripts follows decay of maternal *dE2F* transcripts by ~3.5 hr and precedes the defect in DNA synthesis by ~3 hr. In this rapidly dividing tissue, five cell cycles could occur between the time of decay of *DmRNR2* RNA and the arrest of DNA replication. This sequence of events is consistent with a replication block that results from depletion of gene products downstream of E2F.

Beyond the demonstration that *dE2F* is required for DNA replication, we would like to know whether its regulation controls the timing of S phase. The analysis of mutant embryos suggests that *dE2F* may play a regulatory role in some lineages and not in others. In cells entering regulated endocycles, *dE2F*-dependent transcripts are expressed in pulses at the start of each S phase, and in the *dE2F* mutant embryos the first failure to express these genes coincides with a great reduction in DNA replication. This close coupling of the failure of *dE2F*-dependent transcription and failure of DNA replication suggests that the level of some *dE2F*-dependent products may be limiting. The accompanying paper (Duronio and O'Farrell 1995) shows that expression of cyclin E is *dE2F*-dependent and that cyclin E is the first *dE2F*-dependent gene product to limit progress to S phase in the endocycling cells.

In contrast to cells having a G₁ period of quiescence, in the rapidly proliferating cells of the CNS, *dE2F*-dependent transcription of *DmRNR2* and *PCNA* appears to be constitutive, and loss of *dE2F*-dependent gene expression does not have an immediate cell cycle consequence. Although there are many possible explanations for these observations, it appears that *dE2F*-dependent transcription in these cells is neither regulated nor limiting for DNA replication.

Regulation of E2F activity

Transcription of *dE2F*-dependent genes is abruptly activated at the time of the G₁-S transition. New transcription of *dE2F* does not underlie these pulses of transcrip-

Figure 7. *dE2F*-dependent transcription declines before the arrest of DNA replication. (A,B) Whole-mount in situ hybridization of early stage 12 embryos with a DIG-labeled *DmRNR2* probe. In wild-type (A) *DmRNR2* is expressed in the CNS (solid arrow) and PNS (open arrow). A *dE2F*⁹¹ mutant (B) fails to express *DmRNR2* in the PNS, whereas only a few cells in the CNS still express *DmRNR2*. (C,D) Immunofluorescent detection of BrdU incorporated during pulse labeling of wild-type (C) and *dE2F*¹⁷² mutant (D) embryos at stage 12. Note that DNA replication in the mutant still occurs at this stage in the CNS (solid arrows), PNS (open arrows), and a region of the epidermis in thoracic segment 1 (open arrowheads) that continues through a seventeenth mitotic cycle before entering G₁ in cycle 18 [see text and Knoblich et al. 1994].

tion, as the embryonic expression of *dE2F* is nearly ubiquitous and is not cell cycle regulated. Furthermore, overexpression of *dE2F* by ~20- to 50-fold as a consequence of induced expression from the ubiquitously expressed heat shock promoter did not cause G_{1-17} cells to induce new transcription of *DmRNR2* and did not drive them into S phase. The failure of overexpression of *dE2F* to advance G_1 cells to S phase differs from reports showing that transfection or microinjection of E2F-1 into mammalian tissue culture cells is sufficient to drive cells into S phase (Johnson et al. 1993; Zhu et al. 1993; Qin et al. 1994; Shan and Lee 1994; Wu and Levine 1994). Although it is unclear whether the discrepancy is attributable to different levels of overexpression, these data raise the possibility that in *Drosophila* additional factors required for *dE2F* activity are present in limiting amounts during G_{1-17} .

These observations indicate that the activity of *dE2F* protein is tightly regulated. In mammalian cells, the activity of E2F/DP heterodimers is inhibited by association with regulatory proteins such as pRB, p107, and p130 and modulated by cyclin-dependent kinases. It remains to be determined whether *Drosophila* E2F is regulated by proteins analogous to pRB, p107, and p130. However, the program of E2F-dependent transcription in *Drosophila* is also activated by a G_1 cyclin (Duronio and O'Farrell 1994 and this issue), suggesting that the regulation of *dE2F* will parallel that of the mammalian transcription factor.

Downstream action of *dE2F*

The *dE2F* mutations block expression of *PCNA* and *DmRNR2*, and induction of *dE2F* with a heat shock promoter restores this expression in the normal pattern of the G_1 -S program. We have shown previously that this program of transcription occurs at the normal developmental time in the absence of cell cycle progression (Duronio and O'Farrell 1994). Therefore, this requirement for *dE2F* is not an indirect consequence of the arrest of cell cycle progression. We speculate that *dE2F* may be required directly to activate transcription from the promoters of this set of genes. This simple model is supported by Ohtani and Nevins (1994), who reported that several consensus E2F-binding sites are positioned upstream of *POL α* and that this promoter is activated by cotransfection with *dE2F*.

Elimination of E2F sites from mammalian promoters has been shown to disturb cell cycle-dependent transcription in two different ways. Mutation of the E2F sites in the *DHFR* promoter prevents the activation of gene expression at the G_1 -S transition (Means et al. 1992). In contrast, mutation of E2F sites in the *cdc2*, *B-myb*, and *E2F-1* promoters causes their derepression during G_0/G_1 phase (Dalton 1992; Lam and Watson 1993; Hsiao et al. 1994; Johnson et al. 1994). This latter result led to the idea that E2F functions as an inhibitor of transcription when complexed with a pRB-like molecule during G_0/G_1 . Null mutants of E2F ought to be deficient in both potential inhibitory and potential stimulatory activities.

Expression of the genes that we have examined to date, *DmRNR2* and *PCNA*, is extinguished in the *dE2F* mutant embryos, suggesting that *dE2F* provides temporal control of gene expression through transcriptional activation rather than by the relief of repression, and further suggesting that these promoters are silenced in vivo by mechanisms that are independent of *dE2F*. Analysis of other *dE2F*-regulated promoters will be necessary to determine whether this is true for all *dE2F* targets or whether *dE2F* mediates repression of other genes as well.

Our results in *Drosophila* show that E2F is essential and is required for G_1 -S transcription and DNA replication. These data, coupled with results from the overexpression of E2F in mammalian cells, suggest that E2F activation drives the G_1 -S transition. Thus, E2F is part of an evolutionarily conserved mechanism that triggers S phase. The genetics of *Drosophila* could provide further insights into the regulatory circuitries that cause E2F activation and that couple E2F activity to DNA replication.

Materials and methods

Fly stocks

Four alleles of *dE2F* (91, 155, 296, and 337) were generated from the EMS mutagenesis described by Azpiazu and Frasch (1993). In addition to the *dE2F* mutation, each chromosome carries eight recessive marker mutations (*ru th st ri roe p^e e^s ca*). The origin of the 7172 and rM729 chromosomes and the structure of the *rosy⁺ lacZ* P element are as described elsewhere (Karpen and Spradling 1992; Mlodzik and Hiromi 1992). The *Df(3R)*e^{F1}** and *Df(3R)*e^{D7}** deficiency chromosomes are described by Lindley and Zimm (1992). All mutants were balanced with a derivative of TM3 containing a P transposon expressing *lacZ* with *Ubx* upstream regulatory sequences (Irvine et al. 1991). In this way, homozygous mutant embryos could be identified unambiguously by the lack of *lacZ* expression in either in situ hybridizations or BrdU labelings (see below). In a typical experiment, eggs were collected on grape juice agar plates for 1 hr and then developed at 18°C until the appropriate age. Overnight collections were used for some experiments. Embryos were staged morphologically according to Campos-Ortega and Hartenstein (1985).

In situ hybridization to polytene chromosomes

Salivary gland polytene chromosomes dissected from Oregon-R wild-type, *Df(3R)*e^{F1}** +, or *Df(3R)*e^{D7}** + third-instar larvae were prepared for in situ hybridization as described (Ashburner 1989). Preparation of digoxigenin (DIG)-labeled *dE2F* cDNA probes and hybridization conditions were exactly as described by Duronio and O'Farrell (1994).

BrdU labeling, antibody labeling, and in situ hybridization of embryos

Dechorionated and octane-permeabilized embryos were pulse-labeled with 1 mg/ml of BrdU (Sigma) for 15 min in Schneider's *Drosophila* medium as described (Edgar and O'Farrell 1990) and then fixed immediately in a 1:1 mixture of either PBT + 7% paraformaldehyde/heptane for 25 min or 37% paraformaldehyde/heptane for 5 min. Following fixation, the embryos were devitellinized with methanol. Incorporated BrdU was detected

(Schubiger and Palka 1987) by immunofluorescence using mouse monoclonal anti-BrdU (Beckton Dickinson) primary antibodies followed by rhodamine-conjugated goat anti-mouse (Jackson Labs) secondary antibodies. Prior to detection of BrdU, embryos containing a balancer chromosome were identified using rabbit anti- β -galactosidase (Cappel) and FITC-conjugated goat anti-rabbit antibodies (Jackson Labs). These antibody conjugates were mildly fixed (5% paraformaldehyde + PBT for 15 min) before beginning the BrdU detection protocol. Mouse monoclonal antibody 22C10 binding was detected with the rhodamine-conjugated goat anti-mouse antibodies.

Whole-embryo in situ hybridization was performed as described (Tautz and Pfeifle 1989; Duronio and O'Farrell 1994). DIG-labeled RNA probes were transcribed from *dE2F* cDNAs and *PCNA* and *DmRNR2* genomic clones as described (Duronio and O'Farrell 1994). Mutant embryos were identified by inclusion of DIG-labeled *lacZ* RNA in the hybridization reactions at a concentration equal to that of the target probes.

Molecular analysis of *dE2F* mutants

The approximate position and the orientation of the P elements were established by PCR analysis using primers from the *dE2F* cDNA in combination with primers located either in the terminal repeat sequences or in the *lacZ* gene of the P element. PCR products obtained with primers on both sides of the P elements indicated that the P element was inserted close to the initiator methionine of the *dE2F* gene in both the 7172 and rM729 alleles. These products were cloned and sequenced to determine the precise position of the P elements.

The *dE2F* gene in the EMS-generated alleles was analyzed as follows. Primers from the *dE2F* cDNA were used to amplify fragments of the *dE2F* genes from heterozygous flies. The PCR products were cloned, and initially six clones were selected from each PCR reaction for sequencing. Polymorphisms between the *dE2F* gene on the balancer chromosome and that on the mutagenized chromosome enabled the two copies to be readily distinguished. Sequence alterations in the mutagenized chromosome were confirmed in two additional independent PCR reactions.

Generation of *hs-dE2F* lines

The *hs-dE2F* transgenic lines were isolated following P-element-mediated germ-line transformation with a pCaSpeR-*hs* construct containing the 4.4-kb *dE2F* cDNA. The CaSpeR-*hs-dE2F* construct was coinjected with the pUChs Δ 2-3 helper plasmid into *w*¹¹¹⁸ embryos. Injection and transformation were carried out as described by Spradling and Rubin (1982) and Rubin and Spradling (1982). Heat-inducible *dE2F* expression was verified by Western blotting of embryo extracts prepared from independent lines of transformants.

For the rescue experiments, a recombinant third chromosome was constructed using the lethal P[*rosy*⁺]-element insertion allele *dE2F*⁷¹⁷² and the P[*w*⁺; *hsp70:dE2F*] heat shock construct (*hs-dE2F12*). Individual *hs-dE2F12*/Sb males were collected from the following cross: *w/w*; *+/+*; *dE2F*⁷¹⁷²/*hs-dE2F12* virgin females $\times$ *w/w*; *+/+*; *D/TM3 Sb* P[*w*⁺; *Ubx-lacZ*] males (the *hs-dE2F12* P element causes darker eye color than does the *Ubx-lacZ* P element, allowing them to be distinguished from each other). These flies were backcrossed to *w/w*; *+/+*; *dE2F*⁷¹⁷²/*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 *hs-dE2F12*. The presence of the *dE2F*⁷¹⁷² mutation was confirmed phenotypically by the

lack of *DmRNR2* expression. Heat shocks were performed by floating the grape juice-agar egg collection plates directly on the surface of a 37°C water bath for 30 min. After a 1-hr incubation at 25°C, the embryos were either fixed as above for RNA in situ analysis or pulse-labeled with BrdU prior to fixation.

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