Mutations in Drosophila DP and E2F distinguish G₁-S progression from an associated transcriptional program

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The E2F transcription factor, a heterodimer of E2F and DP subunits, is capable of driving the G₁-S transition of the cell cycle. However, mice in which the E2F-1 gene had been disrupted developed tumors, suggesting a negative role for E2F in controlling cell proliferation in some tissues. The consequences of disrupting the DP genes have not been reported. We screened for mutations that disrupt G₁-S transcription late in Drosophila embryogenesis and identified five mutations in the dDP gene. Although mutations in dDP or dE2F nearly eliminate E2F-dependent G₁-S transcription, S-phase still occurs. Cyclin E has been shown to be essential for S-phase in late embryogenesis, but in dDP and dE2F mutants the peaks of G₁-S transcription of cyclin E are missing. Thus, greatly reduced levels of cyclin E transcript suffice for DNA replication until late in development. Both dDP and dE2F are necessary for viability, and mutations in the genes cause lethality at the late larval/pupal stage. The mutant phenotypes reveal that both genes promote progression of the cell cycle.

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The regulation of the transition between the G₁ gap phase and the S phase is a critical cell cycle control point, because duplication of the DNA commits a cell to division. This transition has been inferred to be controlled both at the post-transcriptional and transcriptional level (Murray and Hunt 1993; Nasymth 1996). In the yeast Saccharomyces cerevisiae the activation of the G₁-cyclin (CLN)/CDC28 kinase complex drives the cells into S phase, whereas in mammalian cells activation of the cyclinE/cyclin-dependent kinase 2 (CDK2) complex is crucial for the onset of DNA replication. Thus, phosphorylation of key substrates by these kinase complexes is one regulatory component of the initiation of S-phase. Regulation also appears to occur at the level of transcription, however, because CLN1 and CLN2 and cyclin E transcripts accumulate in G₁. In addition, many genes encoding proteins necessary for DNA replication are transcribed at the onset of S-phase.

E2F is a transcription factor whose activity has been linked to the G₁-S transition in mammalian cells. This transcription factor is a heterodimer of the E2F protein and the DP protein, and there are several forms of either protein present in mammalian cells (Weinberg 1995). E2F/DP has been shown to activate the transcription of several genes needed for S-phase, and E2F/binding sites are found in the promoters of many other genes (Nevins 1992). In addition, E2F/DP binding may repress the transcription of some of these genes during G₁ when the heterodimer is complexed with one of the family of pocket proteins, one member of which is the retinoblastoma protein (pRB); (Weintraub et al. 1992, 1995; Zwicker et al. 1996). E2F/DP is the focal point of a regulatory loop that links both transcriptional and post-transcriptional control of the cell cycle (for review, see Sherr 1996). Phosphorylation of pRB by either the cyclinD/CDK4,6, cyclinE/CDK2, or cyclinA/CDK2 kinases releases pRB, possibly enabling E2F/DP to function as a transcriptional activator. Because the cyclin E gene itself is controlled transcriptionally by E2F/DP, a positive feedback loop ensues (Ohtani et al. 1995; Botz et al. 1996; Geng et al. 1996).

It is not clear whether in vivo E2F/DP acts primarily as a positive or a negative transcription factor or whether it plays alternate predominant roles in different types of tissues. In addition, although the importance of transcriptional control of the onset of S-phase is inferred from its correlation with DNA replication, its significance relative to post-transcriptional control mechanisms is not established. Overexpression of E2F protein in mammalian cell culture drives cells into S phase (Johnson et al. 1993; Shan and Lee 1994; Lukas et al. 1996). However, transgenic mice lacking a functional E2F-1 gene exhibited phenotypes consistent with the E2F transcription factor acting either positively or nega-
five mutations in a gene essential for PCNA regulation is crucial in controlling the G1–S transition in observations led to a model in which transcriptional thality is late in development. We find that mutations in dE2F give similar phenotypes to those in dDP; E2F/DP-dependent G1–S transcription is disrupted, but S-phase takes place and the animals survive to late larval/early pupal stages.

**Results**

Screen for genes required for S-phase transcription in the embryo

To recover genes necessary for the transcription of S-phase genes late in Drosophila embryogenesis, we used the transcription pattern as the assay in a genetic screen. The PCNA and RNR2 genes were used because they are transcribed in a pattern that mimics that observed by bromodeoxyuridine (BrdU) labeling of late Drosophila embryos (Duronio and O’Farrell 1994). The E2F/DP recognition sites in the PCNA promoter are essential for its expression (Yamaguchi et al. 1995). We devised a method for in situ hybridization to detect transcripts on large numbers of independent mutagenized lines. Embryos were collected from lines established from single, mutagenized second chromosomes, aged 8–15 hr, and hybridized in situ to a PCNA riboprobe (see Materials and Methods, Fig. 7, below). This developmental stage was chosen because between 8 and 15 hr there is a G1 phase with a regulated transcriptional program. In addition, S-phase transcription can be observed both in cells undergoing endo cycles as well as in the mitotically dividing cells of the nervous system.

From 3010 mutated second chromosome lines, seven lines were recovered that failed to express the PCNA transcript at normal levels in 8- to 15-hr embryos. In all of these lines PCNA transcript was reduced in both the mitotic and endo cycle tissues. As a secondary test, the RNR2 transcript was also affected in the mutant embryos. Complementation tests showed that the mutants fell into two complementation groups. One group, containing two alleles, showed a reduction in PCNA and RNR2 transcript levels. The second group of five mutations had a stronger reduction in PCNA or RNR2 transcripts to nearly undetectable level late in embryogenesis. This latter complementation group was subjected to further analysis.

Mutations in the Drosophila DP gene

The expression of PCNA and RNR2 in the strong complementation group was almost identical to that described previously for mutations in the dE2F gene (Fig. 1) (Duronio et al. 1995). The one distinction was that in the new complementation group there was a higher level of RNR2 transcript uniformly present throughout the epidermis. This was a weak signal that gave the appearance of a higher background in the homozygous mutant embryos (data not shown).
A homolog to the mammalian DP protein, the other subunit of the E2F transcription factor, was identified in *Drosophila* (dDP) and mapped to the second chromosome. Mutations in the dDP gene had not been identified (Hao et al. 1995). Given the similarity between the dE2F phenotype and that of our mutants, we tested whether a deficiency known to delete the dDP gene also uncovered our complementation group. All five mutations were lethal in trans to each of the three deletions that remove dDP [Df (2R)vg-B (Hao et al. 1995); Df (2R)vg-33 and Df (2R)vg-56 (R. Duronio, pers. comm.)]. Furthermore, in situ hybridization of embryos transheterozygous for the mutation and the Df(2R)vg-B deficiency gave the mutant phenotype, undetectable PCNA expression in late embryos.

To confirm that the mutations mapped to the dDP gene, we sequenced the dDP gene from two of the mutant lines. Because the mutations cause pupal lethality (see below) we were able to isolate genomic DNA from larvae transheterozygous for the mutation and a deficiency. A region of 1400 bp that encompasses the regions of the protein conserved with mammals was amplified by PCR from mutant larval genomic DNA, and the PCR product was sequenced directly. The sequenced region contains several important motifs, including the DNA-binding region, the DEF box that is predicted to be required for DP/E2F heterodimerization, and three other highly homologous regions named DP-conserved box 1 (DCB1), DCB2, and negatively charged box (NCB) (Dynlacht et al. 1994; Hao et al. 1995). To recognize polymorphisms between our strains and those used for the published sequence, we also sequenced the same region from transheterozygous adults from two unrelated lines recovered from the screen. Within the region we sequenced there are six introns ranging in size from 56 to 68 bp. There is a polymorphism in our strains, changing the histidine at position 275 to a leucine. This histidine residue is conserved between humans, mice, and *Xenopus*, and is present in the DCB2 box (Dynlacht et al. 1994; Hao et al. 1995). However, it is not essential given that strains containing a leucine at this position have a wild-type phenotype.

We found that both of the mutations were associated with codon changes within the dDP open reading frame (ORF) (Fig. 2). The first mutation is a C→T nucleotide transition that changes Arg149 to a Cys. This residue lies within the DEF box in a block of amino acids that is highly conserved between DP and E2F, and it is conserved between *Drosophila*, mice, and humans (Dynlacht et al. 1994; Hao et al. 1995). With sequence confirmation that the mutation disrupted the dDP ORF, this allele was named dDP<sup>α3</sup>. The second mutation is a G to A nucleotide transition that converts Trp<sup>173</sup> to a stop codon. The resulting truncated protein would lack part of the DEF box as well as the three highly conserved domains, DCB1, DCB2, and NCB. This allele was named dDP<sup>α2</sup>. The molecular data combined with the genetic complementation data on the five mutations confirm that this complementation group is the *Drosophila* dDP gene. Consequently, the other mutations have been designated dDP<sup>α3</sup>, dDP<sup>α4</sup>, and dDP<sup>α5</sup>.

The role of dDP and dE2F in G<sub>1</sub>–S progression

In mammalian cells, the E2F transcription factor triggers the expression of essential S-phase genes, and the transcription of these genes correlates with progression into S phase. In *Drosophila*, mutations in the dE2F gene were reported to block the G<sub>1</sub>–S transition and eliminate detectable DNA replication late in embryogenesis (Duronio et al. 1995). We tested whether the mutations in dDP also would block entry into S phase.

To determine whether dDP is required for G<sub>1</sub>–S progression, we analyzed BrdU incorporation after the first G<sub>1</sub> phase in *Drosophila* embryogenesis. Normally this G<sub>1</sub> phase is followed by polytene replication in an invariant tissue-specific pattern in the embryo (Fig. 3A) (Smith and Orr-Weaver 1991). Embryos (8–15 hr) were collected from dDP mutant lines and labeled with BrdU during a 40-min interval. To our surprise we detected BrdU incorporation in embryos homozygous for mutant alleles of...
null allele as well as the dE2F\textsuperscript{7172} mutant. The dE2F\textsuperscript{91} mutation is a stop codon early in the dE2F coding sequence (Duronio et al. 1995). The dE2F\textsuperscript{7172} mutation is also likely to be a null because dE2F protein is not detectable in homozygous embryos (Asano et al. 1996). Furthermore, the failure to observe protein in these mutant embryos implies that maternal pools of the E2F protein do not persist in late embryos. Strikingly, DNA replication was observed in embryos mutant for either of the dE2F alleles (Fig. 3C,D), although the intensity of BrdU incorporation was slightly diminished. As a control, we repeated the 40-min BrdU pulse on embryos homozygous for cyclin E\textsuperscript{PZ5}. As expected, BrdU incorporation was not detectable in the polytene tissues of cyclin E mutant embryos (Fig. 3H, arrows). We conclude that although the bursts of E2F-dependent G1–S transcription are not evident in dDP and dE2F mutant embryos, DNA replication still occurs.

Although DNA replication takes place in dDP and dE2F mutant embryos, two observations suggest that the rate of replication is slowed. First, if a 10-min pulse of BrdU labeling was used, BrdU incorporation was reduced in both polytene and neural tissues of homozygous dE2F or dDP embryos (Fig. 3F,G), whereas replication was normal with this short pulse in heterozygous controls (Fig. 3E). The previous studies that concluded DNA replication was undetectable in dE2F mutant embryos used a short pulse of BrdU labeling (Duronio et al. 1995). However, even with a 10-min BrdU pulse the level of labeling in dE2F and dDP mutants is much higher than that obtained in the endodomain with a 40-min pulse of the cyclin E mutant (Fig. 3F–H). Second, in the dE2F mutants the developmental time of the onset of the later polytene S phases appeared delayed. Replication persisted in the anterior and posterior midgut at a developmental stage when only the central portion of the midgut normally replicates. In the mutant embryos the second round of polytene replication in the central midgut was not observed (data not shown), suggesting it was delayed until after cuticle deposition when antibody detection is no longer possible.

Developmental phenotypes of dE2F and dDP

To evaluate whether the E2F/DP transcription factor functions to promote entry into S phase or suppress hyperproliferation, we determined the furthest developmental point reached by analyzing the lethal phase of both the dE2F and the dDP mutants. Because S-phase occurs during embryogenesis in dDP and dE2F mutants, we did not expect the mutations to cause embryonic lethality, although the dE2F mutations were described previously as being embryonic lethal (Duronio et al. 1995). We tested whether the dDP and dE2F mutants were embryonic lethal by scoring whether the embryos hatched as first instar larvae. For all five of the dDP alleles heterozygous mutant/+ females were crossed to Df/+ males. At least 300 eggs were collected and scored, but no embryonic lethality of the dDP alleles in trans to the deficiency was observed. In the same way, we tested

Figure 2. Amino acid changes in the dDP\textsuperscript{91} and dDP\textsuperscript{92} mutant lines. The wild-type Drosophila dDP sequence from a 1400-bp region PCR amplified from genomic DNA is compared with the human DP-1 sequence. The amino acid numbering of the Drosophila sequence is that of Dynlacht et al. (1994). Amino acid changes in dDP\textsuperscript{91} and dDP\textsuperscript{92} are indicated, and both of these changes occur in the DEF box (outlined). The region of genomic DNA sequenced contains six small introns: the first, in amino acid 28, is 57 bp; the second, in amino acid 71, is 63 bp; the third, after amino acid 182, is 68 bp; the fourth, in amino acid 261, is 60 bp; the fifth, in amino acid 289, is 63 bp; and the sixth, in amino acid 317, is 56 bp. The sequence shown does not include the first 14 amino acids of the amino terminus reported by Dynlacht et al. (1994). The polymorphism present between our sequence and that of Dynlacht et al., is shown in boldface type.
It took between 288 and 432 hr for the larval life, we observed a dramatic delay in larval growth. The larvae to pupate, compared to 120 hr for heterozygous larvae that initiated pupation, numerous additional small pseudotumors formed.

We compared the lethal phenotype of the dDP alleles over a deficiency to that of the dE2F mutations (Table 1). Unlike the dE2F mutants, the larval growth of the dDP

![Figure 3](https://example.com/figure3.png)

Figure 3. S-phase occurs in dDP and dE2F mutants. DNA replication was assayed by BrdU and shown for 10.5-hr embryos labeled for either 40 min (A–C,H), 20 min (D), or 10 min (E–G) followed by antibody staining during the pulse. The central nervous system (CNS), anterior midgut (amg), central midgut (cmg), posterior midgut (pmg), malpighian tubules (mt), and hindgut (hg) incorporate BrdU. The characteristic replication pattern can be seen for the 40-min BrdU pulse for dE2F7172/+ embryos (A) which have a wild-type phenotype, homozygous mutant dDPa4 (B) and dE2F91 (C) embryos, for the 20-min pulse in the homozygous dE2F7172 (D), and for the 10-min BrdU pulse for dE2F7172/+ (E) embryos. The 10-min BrdU pulse for the dDPa4 and dE2F91 mutants shows reduced labeling in both the mitotic (CN S) and polytene cells (F,G), but replication is still detected. (H) There is no detectable replication in the endodomains (arrows) of a cyclin E25 homozygous mutant after a 40-min BrdU pulse.

The dE2F91 and dE2F7172 alleles in trans to a deficiency that uncovers dE2F. The alleles were also examined in trans to each other. In each experiment 500 eggs were collected and scored, and there was no embryonic lethality. We tested dE2F91 homozygous embryos and found the homozygous chromosome to cause embryonic lethality. However, this is clearly the result of other mutations on the chromosome, as both the dE2F91 and dE2F7172 in trans to a deficiency or each other are not embryonic lethal.

We examined the role of dE2F in larval and pupal development to determine whether the gene was essential and whether the dE2F mutations caused any defects in polytenization or cell proliferation. In Drosophila most of the larval cells do not undergo mitosis after completion of embryogenesis but grow from polytene replication. However, the imaginal cells that will form the adult body remain diploid. Thus, defects in the end cell cycle producing polytene cells would result in small larvae, whereas defects in proliferation would be manifest after pupation when the imaginal cells differentiate to produce the adult body. Larvae and pupae mutant for dE2F were identified by the absence of a dominant marker (see Materials and Methods). Approximately 240 total larvae were examined, and 63% of the dE2F91/dE2F7172 survived to third instar. Of the third instar larvae, 54% initiated pupation. Because the mutant animals die as late larvae or early pupae, dE2F is clearly essential for development. The late lethality of the dE2F mutant is consistent with the high levels of dE2F protein observed in wild-type second and third instar larval extracts (Brook et al. 1996).

Although the dE2F mutant animals survive through larval life, we observed a dramatic delay in larval growth. It took between 288 and 432 hr for the dE2F mutant larvae to pupate, compared to 120 hr for heterozygous sibling controls. Five days after egg laying (AEL) the dE2F mutant larvae were very sluggish and much smaller in size than their wild-type counterparts (Fig. 4A,B). The polytene salivary gland and diploid imaginal discs could not be identified in the 5-day-old dE2F mutant larvae, presumably because they were so small. The brains were also greatly reduced in size as compared to wild type (Fig. 4E,F). The size of the dE2F mutant larval increases over time (Fig. 4B,C), and the internal tissues approached wild-type size (Fig. 4F,G; data not shown). Therefore, DNA replication can occur during this larval period, but it is slow. Replication in the absence of dE2F is further evidenced by the formation of banded polytene salivary gland chromosomes in some of the 12- to 18-day larvae (Fig. 4H). Although the polytene chromosomes from the dE2F mutant larvae were smaller and more fragile than normal they were clearly visible. Thus, we conclude that S phase occurs in the absence of dE2F, but dE2F is necessary for timely replication and growth.

In addition to the growth delay, the dE2F mutant larvae had another striking phenotype, melanotic pseudotumors were formed (Fig. 4C,D). Melanotic tumors are groups of cells within the larvae that are recognized by the immune system and encapsulated in melanized cuticle (Sparrow 1978; Watson et al. 1991). We refer to them as pseudotumors to emphasize that they are not necessarily the consequence of hyperproliferation but can be abnormal cells recognized by the immune cells. Small pseudotumors were first observed in the dE2F mutants 7 days AEL, and these early pseudotumors grew and darkened as the larvae aged. In the dE2F mutants that initiated pupation, numerous additional small pseudotumors formed.

We computed the lethal phenotype of the dDP alleles over a deficiency to that of the dE2F mutations (Table 1). Unlike the dE2F mutants, the larval growth of the dDP
mutants was not delayed dramatically. The lethality was largely pupal, and some melanotic pseudotumors were observed in the early pupae. The dDP alleles ranged in the severity of the phenotype they produced (Table 1). The strongest phenotype was observed with dDPa5, which resulted in late larval lethality when in trans to a deficiency. The dDPa2 and dDPa4 mutants in trans to a deficiency survived to the pupal stage. We think that the pupal stage is the lethal phase, and that the dDPa5 mutant is unusual. The dDPa5 larval lethality is either attributable to another mutation on the chromosome that enhances the phenotype, or because this allele is antimorphic. The phenotype of the other alleles in trans to dDPa5 was stronger than in trans to the deficiency.

Approximately half of the dDPa2/Df pupae reached adulthood in the pupal case (Table 1). These adults struggled to eclose but ultimately died. Organisms dissected from the pupal case had essentially normal heads and thoraxes. However, their abdominal defects were severe. This is informative as the head and thorax are derived from imaginal discs, whereas the abdomen arises from the abdominal histoblast nests. The imaginal discs proliferate during larval stages, but the abdominal histoblast nests proliferate during pupal development. Thus, pupal lethality may result from a defect in abdomen formation that occurs during pupal development.

The weakest allele, dDPa1, was semilethal in trans to a deficiency (Table 1). The recovered adults had rough eyes and wing vein defects. These phenotypes are diagnostic of compromised mitotic proliferation. They also had thin and short bristles that indicate a defect in polytene replication, because the cells that give rise to the bristle shaft and socket endoreplicate (Lees and Waddington 1942). We conclude that dDP, like dE2F, provides an essential function for the development of the organism. The eye and bristle defects indicate that dDP is required for normal development in both mitotic and endo cycle cells.

Genetic interaction between dDP and dE2F
To demonstrate that the late lethality and developmental phenotypes associated with the dDP mutants were attributable to loss of dDP function rather than a syn-

### Table 1. Developmental phenotypes of dDP mutants

| Genotype          | Lethal phase | Phenotype                                                                 |
|-------------------|--------------|---------------------------------------------------------------------------|
| dDPa1/Df(2R)vg-B  | adult flies 11%–20%; adults in pupal case 80%–89% | rough eyes, incomplete wing vein, thin and short bristles, etched tergites, more female flies than males, females and males sterile, females lay eggs with thin choriors |
| dDPa3/Df(2R)vg-B  | adults in pupal case | kidney-shaped rough eyes, thin and short bristles on thorax and head, severe abdominal defects |
| dDPa2/Df(2R)vg-B  | adults in pupal case 50%; pupae 50% |                                   |
| dDPa4/Df(2R)vg-B  | adults in pupal case 50%; pupae 50% |                                   |
| dDPa5/Df(2R)vg-B  | late larval lethal |                                   |

aLethal phase is the furthest developmental stage reached by the dDP mutants.
thetic effect with other loci, we tested whether the lethality could be rescued by expressing the wild-type dDP gene under a heat shock promoter (hsp). We introduced an hsp70-dDP transgene into the dDP<sup>a1</sup> background (Table 2) (Duronio et al. 1996). At 25°C we observed a partial rescue of the dDP<sup>a1</sup>/Df lethality attributable to basal expression of dDP from the hsp70 promoter. The recovered dDP<sup>a1</sup>/Df; hs–dDP/+ males were fertile. The recovered adults, however, did have rough eyes, wing vein, and bristle defects and were female sterile. The effect of induced dDP expression (37°C heat treatment) on the dDP mutants was even more dramatic (Table 2). The lethality of dDP<sup>a1</sup>/Df was completely rescued, and all the developmental phenotypes were suppressed. The rescue confirms that the pupal lethality, male and female sterility, rough eye, wing vein, and bristle defects are all attributable to loss of dDP function.

Because ectopic expression of dE2F and dDP in the Drosophila eye results in excess cell proliferation (Asano et al. 1996; Du et al. 1996b), we examined the effect of dDP mutations on eye development in more detail. We used scanning electron microscopy (SEM) to analyze the eyes of dDP<sup>a1</sup>/dDP<sup>a2</sup>; hs–dDP/+ with and without heat shock. The eyes of dDP mutant flies carrying the uninduced hs–dDP transgene were rough (Fig. 5B). The reduced size of the eye, missing, and disorganized ommatidia, as well as stunted, missing, and disorganized bristles suggested a proliferation defect in the dDP mutant (Fig. 5B). Strikingly, flies of the same genotype that underwent daily heat shock treatments had normal eyes (Fig. 5A,C). dDP<sup>a1</sup> in trans to a deficiency resulted in the same rough eye phenotype as observed for dDP<sup>a1</sup>/dDP<sup>a2</sup> flies at 25°C, and the rough eye was rescued by induced ectopic dDP expression (data not shown). Because the dDP<sup>a2</sup> allele exhibits the same strength phenotype as a deficiency, it behaves genetically as a null allele.

Having shown that heat shock dDP rescues the dDP mutants we defined the developmental period during which ectopic dDP expression is capable of rescuing the lethality of the dDP mutants. Females heterozygous for dDP<sup>a2</sup> were mated to heterozygous dDP<sup>a1</sup> males carrying the hs–dDP transgene. The heat shock regimen began immediately after the 24-hr egg collection was completed or 5–6 days later (late larval/early pupal life). Both treatments yielded the same results, 100% rescue of dDP<sup>a1</sup>/dDP<sup>a2</sup> mutant animals. Thus, the late lethality of dDP mutants is not a manifestation of a defect in the early development of the organism, but rather it stems from defects in larval/pupal life.

We tested whether the lethality of dDP mutants was a result of disrupting the E2F/DP heterodimer by asking whether overexpression of dE2F could suppress the dDP mutant phenotype. The dE2F transgene under the inducible heat shock promoter was crossed into the dDP<sup>a1</sup> and dDP<sup>a2</sup> background. The dDP<sup>a2</sup> allele was chosen because this mutation changes a single conserved residue in the E2F/DP heterodimerization domain (Girling et al. 1993; Hao et al. 1995). The dDP<sup>a2</sup> mutation truncates the protein and is predicted to delete regions necessary for E2F binding, providing a useful comparison for the dDP<sup>a1</sup> mutation. Mild overexpression of dE2F at 25°C resulted in significant rescue of the dDP<sup>a1</sup>/Df mutant phenotype (Table 2). At 37°C the rescue conferred by the ectopic expression of dE2F is dampened (Table 2). This may result either from high levels of ectopic dE2F expression being detrimental (Asano et al. 1996), or it may be attributable to fewer dDP<sup>a1</sup>/Df adult escapers arising at 37°C. In contrast to the effect on dDP<sup>a1</sup>, overexpression of dE2F provided no rescue of the dDP<sup>a2</sup> mutants (data not shown). The ability of ectopic dE2F to rescue phenotypes in the dDP<sup>a1</sup> but not the dDP<sup>a2</sup> mutants suggests that despite the alteration in the DEF box of the dDP<sup>a2</sup> allele, the mutant DP protein is still capable of binding E2F. This observation also indicates that the dDP phenotypes are a result of a failed E2F/DP transcription factor activity.

Table 2. Rescue of dDP mutants by overexpression of dDP and dE2F

| Genotype of recovered adults | 25°C | 37°C |
|-----------------------------|------|------|
| dDP<sup>a1</sup>/Df<sup>+</sup> | 11%<sup>a</sup> (males and females sterile) | 0%<sup>d</sup> |
| dDP<sup>a1</sup>/hs–dDP/Df<sup>+</sup> | 42%<sup>b</sup> (males fertile; females sterile) | 100%<sup>e</sup> |
| dDP<sup>a1</sup>/hs–dE2F/Df<sup>+</sup> | 37%<sup>c</sup> (males and females sterile) | 18%<sup>f</sup> (males and females sterile) |

<sup>w: Df(2R)vg-56/CyO females were mated to w; dDP<sup>a</sup>/CyO; P[w;hs–dDP]/+ or w; dDP<sup>a</sup>/CyO; P[w;hs–dE2F]/+ males at 25°C. Eggs collected from the crosses were either allowed to develop at 25°C or received heat shock treatments at 37°C. The recovered adults of the indicated genotype were expected to be one-quarter of the CyO progeny according to Mendelian ratios (see Materials and Methods): 99/324; 1034/324; 202/218; 20/184; 51/184; 52/266. The numerator is the number of recovered adults of the indicated genotype. The denominator is the number of the CyO progeny. To calculate the percentage, the ratios were multiplied by 4.</sup>
Genetic interactions between the E2F/DP heterodimer and cyclin E

We investigated whether dDP is necessary for cyclin E expression. In embryos that were dDPa1/Df, cyclin E expression was not detectable in the endodomains but present in the central nervous system (CNS) (Fig. 6A,B), a pattern comparable to that seen previously in homozygous dE2F91 mutant embryos (Fig. 6C) (Duronio and O'Farrell 1995). Because the levels of cyclin E transcripts are so much higher in the nervous system than in the endodomains, it is difficult to assess whether the transcript levels are reduced in the mutants in the CNS. The cyclin E mutant phenotype is more severe than that of the dE2F and dDP mutants, although cyclin E transcription requires the E2F/DP transcription factor. In contrast to the near-normal pattern of replication seen in dE2F and dDP mutant embryos, DNA replication was not detected in the endodomains of embryos homozygous for cyclin EPZ5 (see Fig. 3H). In this cyclin E allele S-phase continued in the CNS in late embryos (Fig. 1H). No embryonic lethality resulted from the cross of cyclin E725 heterozygous mothers to fathers heterozygous for a cyclin E deficiency, rather the cyclin E725/Df transheterozygotes died as early first instar larvae. Because BrdU incorporation is not seen in cyclin E mutant embryos in the endodomains, this shows that the embryonic endocycles are not required for hatching.

We tested whether persistent maternal levels or low levels of zygotic expression of cyclin E transcript accounted for the viability of dE2F mutant larvae. In contrast to the late larval/early pupal lethality normally seen with dE2F91/dE2F2712, reducing the maternal dosage of cyclin E dramatically enhanced the lethality. This resulted in early larval lethality, with only 5% of the dE2F mutant larvae surviving until the second to small third instar larval stage. Reducing the dosage of the cyclin E gene in the father also enhanced the dE2F lethality, but to a lesser extent. In this cross 30% of the cyclin E725/dE2F91/dE2F2712 progeny survived until the second to third instar larval stage. The pronounced effect of reducing the gene dosage in the mother indicates that persistent maternal pools of cyclin E transcript or protein permit viability of dE2F mutant animals. In addition, zygotically provided transcripts also contribute, but we cannot distinguish whether this is attributable to persistence of constitutively expressed transcripts or cyclic transcription below our detection limit.

Discussion

From a genetic screen to identify mutants that fail to undergo the S-phase transcriptional program during em-
bryogenesis we recovered five mutations in the Drosophila DP gene. The mutant phenotype reveals that dDP, like dE2F, is required for viability in Drosophila and that the gene has an essential role in vivo. The mutant phenotypes of dDP and dE2F provide insight into the functions these genes provide in controlling the cell cycle during development.

The E2F/DP transcription factor promotes S phase in vivo

Analysis of the activity of E2F in mammalian cell culture showed that the transcription factor was capable of driving cells into S-phase when overexpressed and that this could lead to apoptosis (Johnson et al. 1993; Shan and Lee 1994; Hebert et al. 1995; Lukas et al. 1996). This observation was consistent with results demonstrating that E2F could activate the transcription of a number of genes needed for the G1-S transition and the onset of S-phase. However, it was also shown that when complexed with pRB the E2F transcription factor could repress the transcription of some of these genes in G1 (Weintraub et al. 1992, 1995; Zwicker et al. 1996). In transgenic mice in which the E2F-1 gene was disrupted tumors developed (Field et al. 1996; Yamasaki et al. 1996). These latter two observations raised the possibility that in some tissues the E2F transcription factor could act primarily as a tumor suppressor to repress progression of the cell cycle.

The phenotypes of the Drosophila E2F and DP mutants reveal that the E2F transcription factor indeed plays a significant role as an activator of S phase in vivo. It was surprising that mutations in either gene have almost no effect on BrdU incorporation during embryogenesis. However, the rate of nucleotide incorporation and DNA replication appears to be slower in both mutants, and the slow larval growth rate in dE2F mutants is particularly striking. Because larval growth occurs by increasing ploidy of the larval tissues by the G-S endo cycle, these phenotypes reflect a compromised ability to undergo S phase in the mutants.

In addition to the effects on polytene tissues, mitotically proliferating cells are affected dramatically by mutations in both dE2F and dDP. In the dE2F mutants that puplicate, either a very small adult body is formed inside the pupal case or an adult body is not formed at all. This is consistent with a defect in proliferation that reduces the number of imaginal disc cells and abdominal histoblasts that give rise to adult structures. Previously, it was observed that mutations in dE2F reduce the rate of cell growth in clones in the eye (Brook et al. 1996). The interpretation that mutations in the dE2F and dDP genes disrupt the diploid cell cycle in the imaginal cells is supported by experiments demonstrating that overexpressing dE2F and dDP induced S-phase and excess cell proliferation in the eye (Asano et al. 1996; Du et al. 1996b).

The defects in diploid larval cells most likely arise from aberrant cell proliferation caused by the dE2F and dDP mutations. Although we do not know the cell cycle stage affected, the demonstrated effects on polytene larval cells imply that it is the G1-S transition. Because the imaginal discs are present in dE2F mutants yet fall to differentiate to produce adult tissues, it is also likely that E2F has a role in post-mitotic differentiation. Such a role was demonstrated previously by clonal analysis in the eye disc (Brook et al. 1996). A failure in differentiation of the imaginal cells could be a contributing factor in the dDP mutant phenotypes as well.

Although the mutant phenotypes suggest that the predominant function of the E2F transcription factor in Drosophila is to promote progression of the cell cycle, two observations raise the possibility that it may have an inhibitory role in some tissues. First, in dDP mutant embryos a low level of RNR2 transcripts is present in the epidermis, and it may result from a failure to repress transcription. Second, melanotic pseudotumors develop in the dE2F and dDP mutants. We refer to these as pseudotumors because they arise in Drosophila larvae when the lamellocytes of the immune system recognize aberrant cells, surround them, and secrete a cuticle that melanizes and becomes black (Sparrow 1978). Thus, the clearest cause of melanotic tumors is an alteration of the cell surface. In the dE2F and dDP mutants this could result from perturbation of the differentiation program or apoptosis. There is evidence in Drosophila suggesting that overproliferation may contribute to melanotic tumor formation (Watson et al. 1991, 1992, 1994; Bryant et al. 1993). Although it is possible that the melanotic pseudotumors in the dE2F and dDP mutants result from hyperproliferation, we did not observe overproliferation in the larval imaginal tissues. Apoptosis may occur in some tissues in the dE2F and dDP mutants, leading to melanotic tumors. Further analysis will be required to determine whether E2F represses the cell cycle in some developmental contexts and to distinguish between these potential mechanisms for the formation of melanotic pseudotumors in the mutants.

Does dE2F have a function that is independent of dDP?

Although both the dDP and dE2F mutants show late lethality, there are differences in the phenotypes resulting from mutations in the two genes. The dE2F mutant animals have slower larval growth than dDP mutants, and the dDP mutants develop further as pupae. We think that the difference in strength of the phenotypes reflects the biological system rather than strength of the dE2F and dDP alleles, because we analyzed null alleles of both genes. The dE2F91 mutation is a truncation after amino acid 31 (Duronio et al. 1995), and dDP92 is a truncation at the end of the DEF box. In mammalian cells truncation of DP after the DEF box abolishes both the E2F and DNA-binding ability (Wu et al. 1996). Given the high degree of conservation between the mammalian and Drosophila proteins, dDP92 is likely to ablate DP activity.

One explanation for the less severe phenotype of the dDP mutations is that there is a redundant activity for dDP but not for dE2F. This could be because there are
additional family members in the Drosophila genome, and these may complement the dDP defect better than that of the dE2F mutants. Alternatively, the maternal stockpiles of the dDP gene may persist longer than those of dE2F. DP protein is more easily detected in embryo extracts than E2-F and may be present at higher levels (N. Dyson, pers. comm.).

Another explanation for the more severe effects exhibited by the dE2F mutants is that dE2F plays biological roles independent of dDP. Possibly the dE2F protein can act as a homodimer. The rate of larval growth is influenced greatly by nutritional signals (Poody and Woods 1990). One possibility is that dE2F links the endo and mitotic cell cycles to nutritional input, and it does so without requiring dDP function.

Significance of E2F/DP-dependent G1–S transcription

There is a clear correlation between the E2F/DP-dependent transcriptional activation of genes whose products are necessary for DNA replication and the onset of S phase. The implication was that this relationship was causal and that the cyclic transcription of these genes, some of which like cyclin E are known to be key regulatory genes, is necessary for normal S phase. The striking observation from the Drosophila dDP and dE2F mutants is that although cyclic transcription of cyclin E, PCNA, and RNR2 is not detectable, S phase still occurs. Although we cannot exclude the possibility that cyclic transcription of these genes occurs at a low level driven by maternal pools of dDP and dE2F, the bursts of transcription that normally precede S phase are not essential for the G1–S transition. In these mutants the cell cycle may be driven by basal levels of transcripts and post-transcriptional regulation. The maternal pools of components of the replication machinery can persist until late in development, as evidenced by the fact that mutations in PCNA and MCM2 cause late larval lethality (Henderson et al. 1994; Treisman et al. 1995).

The precise developmental control exercised over the cell cycle in Drosophila permits the in vivo role of cell cycle regulators to be evaluated. The ability to visualize the G1–S transcriptional program during embryogenesis enabled us to recover mutations in dDP. Although the mutant phenotypes reveal that dE2F and dDP promote progression of the cell cycle, they reveal a distinction between the effect on E2F/DP-dependent G1–S transcription and the onset of S phase.

Materials and methods

Fly strains

The cn bw sp and DTS 91 strains were provided by R. Lehmann (Skirball Institute, New York University Medical Center, New York, NY). The Ubx–lacZ CyO balancer chromosome has been described previously (McCall et al. 1994). The cyclin E2F91 and the cyclin E deficiencies Df(2L)TE116(R)GW1 and Df(2L)TE116(R)GW3 were acquired from J. Rooto (University of Cambridge, UK).

The dE2F91 and dE2F7172 alleles have been described previously (Duronio et al. 1995). The P[w+, hsp70–dE2F] and P[w+, hsp70–dDP] strains were provided by N. Dyson (Duronio et al. 1996). The deficiencies uncovering the dDP genes Df(2R)vg-33, Df(2R)vg-56, and Df(2R)vg-B have been described previously (Morgan et al. 1938; Lasko and Pardue 1988; Williams and Bel 1988) and were provided by R. Duronio and the Bloomington stock center. The deficiency uncovering dE2F, Df(3R)e-BS2 was obtained from the Bloomington Stock Center.

Genetic screen

Isogenized cn bw sp homozygous males were fed 35 mM ethyl-methane sulfonate (EMS) in a 1% sucrose solution for a 24-hr period (Fig. 7). Immediately after feeding, these males were crossed to virgin females heterozygous for a chromosome carrying a dominant temperature-sensitive mutation DTS91 pr cn and a CyO balancer carrying a lacZ reporter gene under the control of the Ubx promoter P[Ubx–lacZ] CyO. Single male progeny from this cross with the genotypes cn bw sp/P[Ubx–lacZ] CyO and cn bw sp/DTS91 pr cn were collected and mated individually to DTS91 pr cn/P[Ubx–lacZ] CyO virgin females.
To facilitate collection of the desired cn bw sp /P[Ubx-lacZ]--CyO virgin females and male progeny, the vials were placed at the restrictive temperature for the DTS91 mutation, 29°C, for 3 days. The adults were removed, and the vials were incubated at 29°C for an additional day before being moved to 25°C. cn bw sp/P[Ubx-lacZ]--CyO virgin female and male progeny from this cross were collected and mated to establish mutant lines. Embryos (8-15 hr) were collected from these lines and hybridized in situ with a PCNA riboprobe. To distinguish between embryos homozygous for the cn bw sp mutagenized chromosome and their heterozygous cn bw sp/Ubx-lacZ siblings, the embryos were hybridized simultaneously with a lacZ riboprobe.

In situ hybridization

In situ hybridization was carried out essentially as described previously (Tautz and Pfeifle 1989). Multiwell baskets were used to perform in situ hybridization on large numbers of independent samples. Hybridization was carried out at 65°C. Digoxigenin-labeled antisense RNA probes were made as described in the Boehringer Mannheim kit. The LRNR2 clone used to make probe was obtained by PCR amplification of Oregon R genomic DNA using degenerate primers (Duronio and O’Farrell 1994). The genomic probes were cloned into Bluescript KS. cDNA probes were made from the E41-1 clone obtained from H. Richardson (Richardson et al. 1993). The PCNA probe was made from a full-length cDNA isolated in this laboratory by J. M. Axton from the library generated by N. Brown (Brown and Kafatos 1988). lacZ probes were made from the Pc4α-galactosidase plasmid (Thummel et al. 1988).

Sequence analysis of the dDP alleles

Trans-heterozygous dDP/Df(2R)vg-B third instar larvae were collected from a cross of dDP/T(2;3)TSL14 and Df(2R)vg-B/T(2;3)TSL14 adults by selecting non-Tb larvae. T(2;3)TSL14 is a translocation between the balancers SM5 and TM6B that carries the dominant larval/pupal marker Tubby (Tb) (Gatti and Goldberg 1991). To recognize polymorphisms between our strains and the published sequence, we isolated DNA from adults that were transheterozygous for two unrelated lines created in our screen. Genomic DNA was isolated from these larvae and adults and a 1400-bp region encompassing approximately amino acids 15-377 (Dynlacht et al. 1994) was amplified by PCR. Because two different 5′ ends have been described for the dDP gene (Dynlacht et al. 1994; Hao et al. 1995), the amplified region contained the conserved domains but not the 5′ end of the gene. The amino acid numbering is that of Dynlacht et al. (1994). Primer sequences were 5'-CTTTAGTCA-GATGGGCGATCAAG-3' and 5'-CTGTAACCTACTCGACTACAC-3'. Ten to fifteen separate PCR reactions were pooled and sequenced directly in the Whitehead sequencing facility using fluorescence automated sequencing. Primers for sequencing were spaced at ~300-bp intervals.

BrdU labeling

Embryos (8-15 hr) were labeled with BrdU according to a protocol obtained from Rolf Bodmer, a modified version of Bodmer et al. (1989). After permeabilization, the embryos were incubated with BrdU for either 10, 20, or 40 min at room temperature. The fixed embryos were hydrolyzed in 2 N HCl for 70 min. The 70-min acid treatment is the key difference from the published protocol and provides better detection of the label. The balancers used to maintain the dDP and dE2F mutant lines were marked with a Ubx-lacZ transgene. An anti-β-galactosidase antibody (Promega) was used in conjunction with the anti-BrdU antibody to distinguish the 25% homozygous mutant embryos from their siblings. The antibody staining was detected using the horseradish peroxidase histochemical reaction (Bodmer et al. 1989).

Heat shock rescue experiments

w; Df(2R)vg56/CyO females were mated to w; dDPα1/CyO; P[w+;hs-dDP]/+ or w; dDPα1/CyO; P[w+;hs-dE2F]/+ males at 25°C (Duronio et al. 1996). All the progeny from the cross were counted and placed into genotypic classes. To test for rescue of dDPα1/dDPα2 transheterozygotes, dDPα2/CyO females were crossed to w; dDPα1/CyO; P[w+;hs-dDP]/TM3, Sb males at 25°C. No dDPα1/dDPα2 adults were observed that lacked the hsdDP gene.

For the heat shock treatments, embryos were collected (from the crosses shown above) for 24 hr at 25°C in vials. The heat shock treatment was administered by placing the vials in a 37°C air incubator for 1 hr and then the vials were returned to 25°C. The heat shocks were initiated either immediately after the completion of a 24-hr collection or after the eggs were allowed to develop at 25°C for 5-6 days. In either case, subsequent heat shocks were delivered two times daily.

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Note added in proof

The GenBank accession no. for the sequence reported here is AF011362.

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