Mutations in *Drosophila DP* and *E2F* distinguish G_1–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 G_1–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 G_1–S transcription late in *Drosophila* embryogenesis and identified five mutations in the *dDP* gene. Although mutations in *dDP* or *dE2F* nearly eliminate E2F-dependent G_1–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 G_1–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 G_1 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 G_1-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 G_1. 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 G_1–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 G_1 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-

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tively in different tissues (Field et al. 1996; Yamasaki et al. 1996). In these mice some tissues atrophy, but other tissues develop tumors. The consequences of disrupting the DP genes have not been reported.

Drosophila shares many of the components of the mammalian G_1–S regulatory circuitry. *Drosophila* homologs to E2F, DP, an RB-like protein (RBF), cyclin E, CDK2, cyclin D, CDK4, and the p21 inhibitory protein (*dacapo*) have been identified (Richardson et al. 1993; Dynlacht et al. 1994; Knoblich et al. 1994; Ohtani and Nevins 1994; Hao et al. 1995; Sauer et al. 1995, 1996; de Nooij et al. 1996; Du et al. 1996a; Finley et al. 1996; Lane et al. 1996). The precise developmental control of the onset of S phase late in *Drosophila* embryogenesis makes it possible to define mutant phenotypes with high resolution and to infer the primary defect in cell cycle regulation (Smith and Orr-Weaver 1991; Smith et al. 1993). The first 13 divisions in the embryo occur in a rapid S–M cycle that is driven by maternal stockpiles of cell cycle regulators (for review, see Foe et al. 1993; Orr-Weaver 1994). After cellularization and the onset of zygotic gene expression, another three cell cycles take place with a G_2 phase during which transcription occurs, but they lack a G_1 phase. After mitosis 16, a detectable G_1 phase appears for the first time during embryogenesis, and it marks the onset of the endo cell cycle for many of the larval tissues. In these cells S phase alternates with a gap phase, but mitosis does not occur, leading to polyteny. During the latter half of embryogenesis the cells that will form the polytene larval tissues undergo S-phase in a stereotypic developmental pattern. During the same developmental period the nervous system cells continue to go through mitotic divisions.

Cyclin E is essential for S phase late in *Drosophila* embryogenesis (Knoblich et al. 1994; Sauer et al. 1995). Mutations in *cyclin E* block DNA replication after mitosis 16, coincident with the first G_1 phase. Presumably cyclin E is necessary for S phase earlier in embryogenesis but maternal stockpiles of the protein or transcripts suffice (Richardson et al. 1993). The onset of the requirement for *cyclin E* correlates with a change in its transcriptional regulation (Knoblich et al. 1994). During the postblastoderm divisions the levels of *cyclin E* transcript are constitutively high. After mitosis 16 they are downregulated in all of the tissues except the nervous system, and transcripts accumulate before S-phase in the endo cycle late in embryogenesis. The same transcriptional pattern observed for *cyclin E* also occurs for several genes encoding replication functions: proliferating cell nuclear antigen (*PCNA*), ribonucleotide reductase 1 (*RNR1*), *RNR2,* and *pol α* (Duronio and O'Farrell 1994). These observations led to a model in which transcriptional regulation is crucial in controlling the G_1–S transition in the endo cycle, with *cyclin E* being the critical target (Duronio and O'Farrell 1994, 1995; Sauer et al. 1995).

To identify regulatory genes needed for G_1–S transcription, we screened for mutants defective in the induction of *PCNA* transcription late in embryogenesis. We found five mutations in a gene essential for *PCNA* and *RNR2* transcription and demonstrated that these are alleles of the *Drosophila DP* gene. We show that the *dDP* gene is essential for viability. Despite the pronounced effect on G_1–S transcription, DNA replication still occurs and lethality is late in development. We find that mutations in *dE2F* give similar phenotypes to those in *dDP;* E2F/DP-dependent G_1–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 trancription pattern as the assay in a genetic screen. The *PCNA* and *RNR2* genes were used because they are transcribed in a pattern that mimicks 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 G_1 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 levels 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).

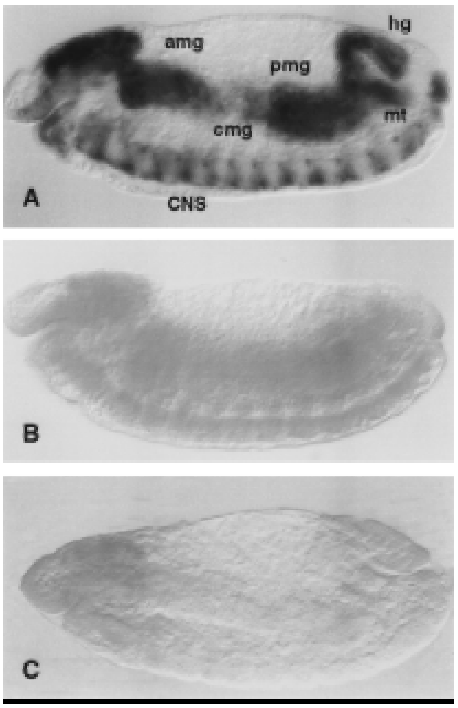

Figure 1. *PCNA* expression in *dDP* and *dE2F* mutant embryos. *PCNA* transcripts were detected by whole-mount in situ hybridization. (*A*) In wild-type 10.5-hr embryos *PCNA* is expressed in the mitotically proliferating central nervous system (CNS), in the endoreplicating anterior midgut (amg), central midgut (cmg), posterior midgut (pmg), hindgut (hg), and malpighian tubules (mt). (*B*) In 10.5-hr homozygous dDP^{a4} mutant embryos *PCNA* is expressed at greatly reduced levels in the CNS and is not detectable in the endodomains. (*C*) $dE2F^{91}$ homozygous 10.5-hr embryos show a similar phenotype. In some embryos a low level of *PCNA* expression can be seen in the CNS, but expression is absent in the endodomains.

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) (Fig. 2) (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 (Fig. 2). 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 Arg^{149} 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^{a1}. The second mutation is a G to A nucleotide transition that converts Trp^{173} 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^{a2}. 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^{a3}, dDP^{a4}, and dDP^{a5}.

The role of dDP *and* dE2F *in* G_1*–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_1–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_1–S progression, we analyzed BrdU incorporation after the first G_1 phase in *Drosophila* embryogenesis. Normally this G_1 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

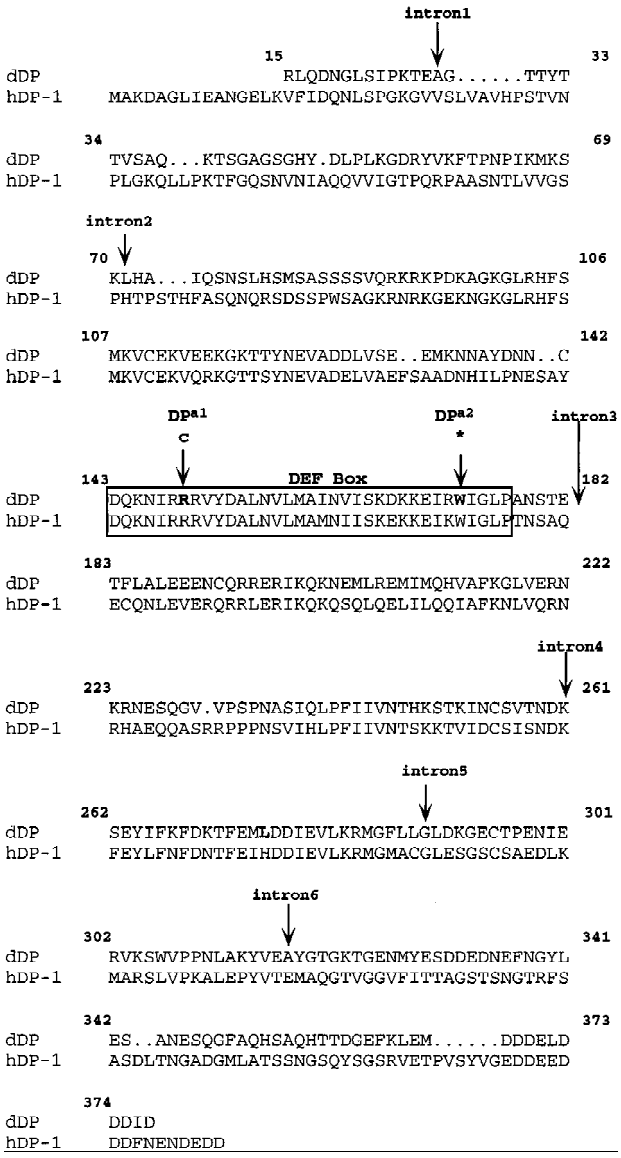

Figure 2. Amino acid changes in the dDP^{a1} and dDP^{a2} mutant lines. The wild-type *Drosophila dDP* sequence from a 1400-bp region PCR amplified from genomic DNA is compared with the human DP-1 sequence. The amino acid numbering of the *Drosophila* sequence is that of Dynlacht et al. (1994). Amino acid changes in dDP^{a1} and dDP^{a2} 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.

dDP, and the spatiotemporal pattern of BrdU incorporation in these embryos was normal (Fig. 3B).

The normal replication observed in the *dDP* mutants prompted us to examine DNA synthesis in the $dE2F^{91}$ null allele as well as the $dE2F^{7172}$ mutant. The $dE2F^{91}$ mutation is a stop codon early in the *dE2F* coding sequence (Duronio et al. 1995). The $dE2F^{7172}$ 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 $cyclin\ E^{PZ5}$. 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 G_1–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 endodomains 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

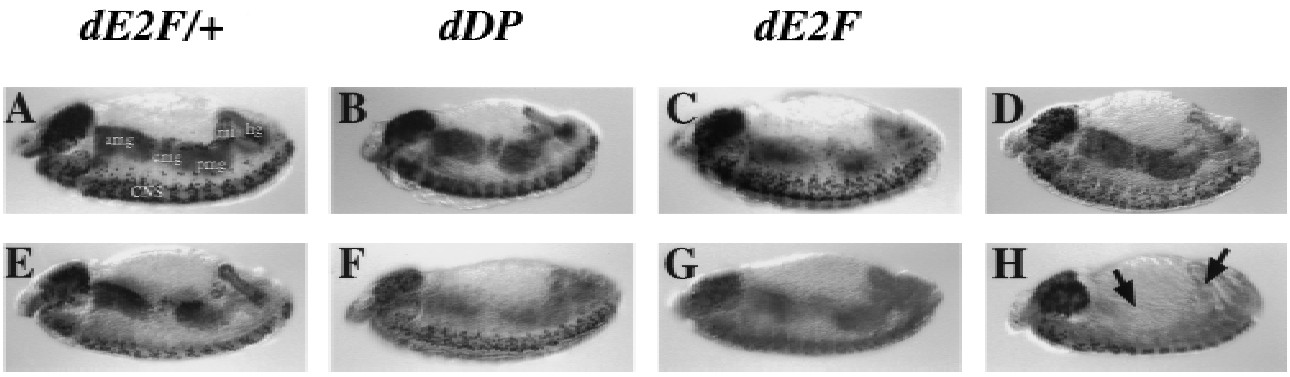

Figure 3. S-phase occurs in *dDP* and *dE2F* mutants. DNA replication was assayed by BrdU and shown for 10.5-hr embryos labeled for either 40 min (*A–C,H*), 20 min (*D*), or 10 min (*E–G*) followed by antibody staining detected by the horseradish peroxidase (HRP) reaction. The darkly staining cells are those that incorporated BrdU 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 $dE2F^{7172}$/+ embryos (*A*) which have a wild-type phenotype, homozygous mutant dDP^{a4} (*B*) and $dE2F^{91}$ (*C*) embryos, for the 20-min pulse in the homozygous $dE2F^{7172}$ (*D*), and for the 10-min BrdU pulse for $dE2F^{7172}$/+ (*E*) embryos. The 10-min BrdU pulse for the dDP^{a4} and $dE2F^{91}$ mutants shows reduced labeling in both the mitotic (CNS) and polytene cells (*F,G*), but replication is still detected. (*H*) There is no detectable replication in the endodomains (arrows) of a *cyclin* E^{PZ5} homozygous mutant after a 40-min BrdU pulse.

the $dE2F^{91}$ and $dE2F^{7172}$ 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 $dE2F^{91}$ 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 $dE2F^{91}$ and $dE2F^{7172}$ 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 endo 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 $dE2F^{91}$/$dE2F^{7172}$ 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. 4 E,F). The size of the *dE2F* mutant larvae 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 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*

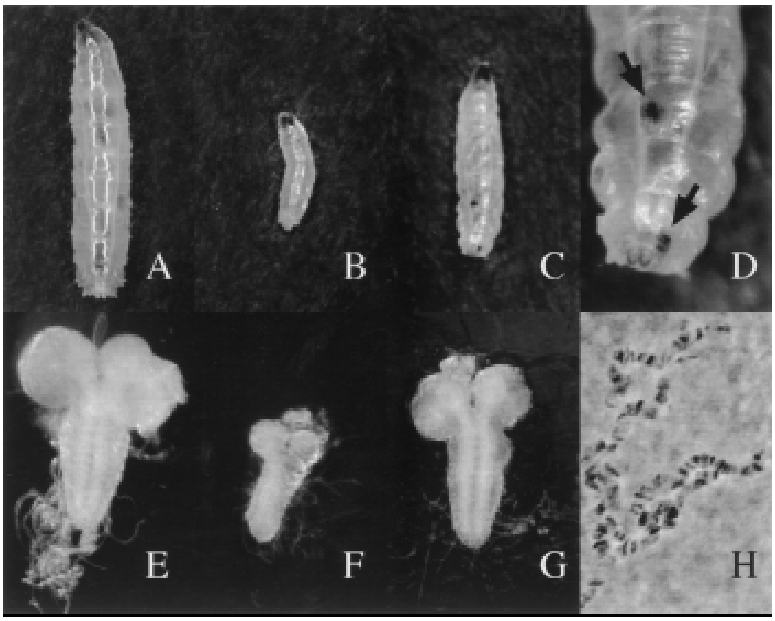

Figure 4. *dE2F* is required for wild-type growth in mitotic and polytene tissues. (*A–C*) All the larvae are at the same magnification. (*A*) $dE2F^{7172}$/+ larva at 5 days AEL. (*B*) $dE2F^{91}/dE2F^{7172}$ mutant larva 5 days AEL. (*C*) $dE2F^{91}/dE2F^{7172}$ mutant larva 13 days AEL with two posterior melanotic pseudotumors. (*D*) A magnification of posterior pseudotumors (arrows) shown in *C*. (*E–G*) All the brains are at the same magnification. (*E*) Brain of a $dE2F^{7172}$/+ larva at 5 days AEL. (*F*) Brain of a $dE2F^{91}/dE2F^{7172}$ mutant larva at 5 days AEL. (*G*) Brain of a $dE2F^{91}/dE2F^{7172}$ mutant larva at 9 days AEL. (*H*) Polytene chromosomes from the salivary glands of a $dE2F^{91}/dE2F^{7172}$ mutant larva at 13 days AEL.

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 dDP^{a5}, which resulted in late larval lethality when in *trans* to a deficiency. The dDP^{a2} and dDP^{a4} mutants in *trans* to a deficiency survived to the pupal stage. We think that the pupal stage is the lethal phase, and that the dDP^{a5} mutant is unusual. The dDP^{a5} 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 dDP^{a5} was stronger than in *trans* to the deficiency.

Approximately half of the dDP^{a2}/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, dDP^{a1}, 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[a]	Phenotype
$dDP^{a1}/Df(2R)vg\text{-}B$	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 chorions
$dDP^{a3}/Df(2R)vg\text{-}B$	adults in pupal case	rough eyes, etched tergites, thin and short bristles
$dDP^{a2}/Df(2R)vg\text{-}B$	adults in pupal case 50%; pupae 50%	kidney-shaped rough eyes, thin and short bristles on thorax and head, severe abdominal defects
$dDP^{a4}/Df(2R)vg\text{-}B$	adults in pupal case 50%; pupae 50%	
$dDP^{a5}/Df(2R)vg\text{-}B$	late larval lethal	

[a]Lethal 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^{a1} background (Table 2) (Duronio et al. 1996). At 25°C we observed a partial rescue of the dDP^{a1}/Df lethality attributable to basal expression of *dDP* from the *hsp70* promoter. The recovered dDP^{a1}/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^{a1}/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^{a1}/dDP^{a2}; *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^{a1} in *trans* to a deficiency resulted in the same rough eye phenotype as observed for dDP^{a1}/dDP^{a2} flies at 25°C, and the rough eye was rescued by induced ectopic *dDP* expression (data not shown). Because the dDP^{a2} 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^{a2} were mated to heterozygous dDP^{a1} 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^{a1}/dDP^{a2} 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^{a1} and dDP^{a2} background. The dDP^{a1} allele was chosen because this mutation changes a single conserved residue in the E2F/DP heterdimerization domain (Girling et al. 1993; Hao et al. 1995). The dDP^{a2} mutation truncates the protein and is predicted to delete regions necessary for E2F binding, providing a useful comparison for the dDP^{a1} mutation. Mild overexpression of *dE2F* at 25°C resulted in significant rescue of the dDP^{a1}/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^{a1}/Df adult escapers arising at 37°C. In contrast to the effect on dDP^{a1}, overexpression of *dE2F* provided no rescue of the dDP^{a2} mutants (data not shown). The ability of ectopic *dE2F* to rescue phenotypes in the dDP^{a1} but not the dDP^{a2} mutants suggests that despite the alteration in the DEF box of the dDP^{a1} 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	Recovered/expected	
	25°C	37°C
$\frac{dDP^{a1}}{Df}; \frac{+}{+}$	11%[a] (males and females sterile)	0%[d]
$\frac{dDP^{a1}}{Df}; \frac{hs–dDP}{+}$	42%[b] (males fertile; females sterile)	100%[e] (males fertile; females semifertile)
$\frac{dDP^{a1}}{Df}; \frac{hs–dE2F}{+}$	37%[c] (males and females sterile)	18%[f] (males and females sterile)

w; Df(2R)vg-56/CyO females were mated to *w; dDP^{a1}/CyO; P[w^+;hs–dDP]/+* or *w; dDP^{a1}/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): [a]9/324; [b]34/324; [c]20/218; [d]0/184; [e]51/184; [f]12/266. 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.

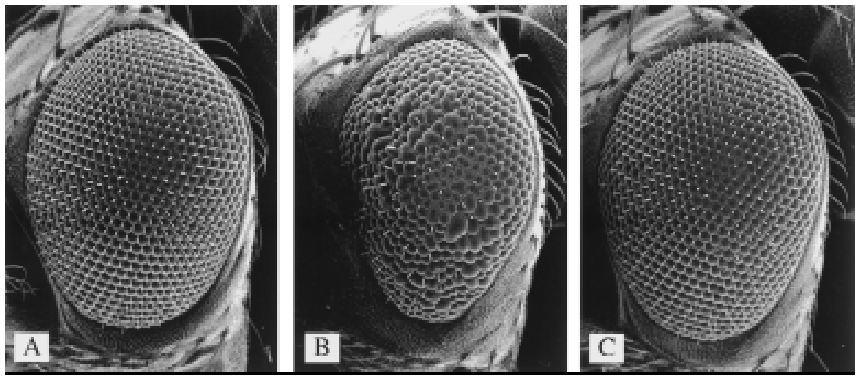

Figure 5. Overexpression of *dDP* rescues the eye phenotype of *dDP* mutants. Shown are SEMs of *Drosophila* compound eyes at 200× magnification. The genotypes are: (*A*) *Df(2R)vg-56*/+ . (*B*) dDP^{a2}/dDP^{a1} with a *hs–dDP* transgene raised at 25°C. The reduced size of the eye and aberrant morphology are indicative of a proliferation defect in the *dDP* mutant. (*C*) dDP^{a2}/dDP^{a1} with a *hs–dDP* transgene raised at 25°C with daily heat shocks at 37°C. Overexpression of *dDP* completely rescues the size and morphology of the *dDP* mutant eye.

Genetic interactions between the E2F/DP heterodimer and cyclin E

We investigated whether *dDP* is necessary for *cyclin E* expression. In embryos that were dDP^{a1}/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 $dE2F^{91}$ 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* E^{PZ5} (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* E^{PZ5} heterozygous mothers to fathers heterozygous for a *cyclin E* deficiency, rather the *cyclin* E^{PZ5}/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 endo cycles 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 $dE2F^{91}/dE2F^{7172}$, 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* $E^{PZ5}/+;dE2F^{91}/dE2F^{7172}$ 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-

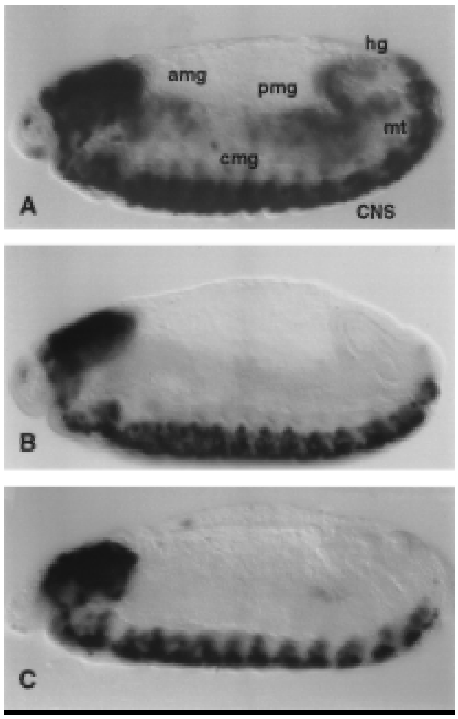

Figure 6. *cyclin E* expression in *dDP* and *dE2F* mutant embryos. *cyclin E* transcripts were detected by whole-mount in situ hybridization. (*A*) In wild-type 10.5-hr embryos *cyclin E* is expressed at high levels in the mitotically proliferating CNS and at low levels in the endodomains. (The endodomains are labeled as in Fig. 1). (*B*) In dDP^{a1}/Df 10.5-hr mutant embryos *cyclin E* is expressed at high levels in the CNS but is undetectable in the endodomains. (*C*) $dE2F^{91}$ homozygous mutant 10.5-hr embryos show the same phenotype. *cyclin E* is expressed at high levels in the CNS but is undetectable in the endodomains.

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; Heibert 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 G_1–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 G_1 (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 pupariate, 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 G_1–S transition. Because the imaginal discs are present in *dE2F* mutants yet fail 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 $dE2F^{91}$ mutation is a truncation after amino acid 31 (Duronio et al. 1995), and dDP^{a2} 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, dDP^{a2} 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 (Poodry 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 G_1–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, was 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 G_1–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 G_1–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 G_1–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* E^{PZ5} allele and the *cyclin E* deficiencies *Df(2L)TE116(R)GW1* and *Df(2L)TE116(R)GW3* were acquired from J. Roote (University of Cambridge, UK).

The $dE2F^{91}$ and $dE2F^{7172}$ 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 Bell 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 ethylmethane 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.

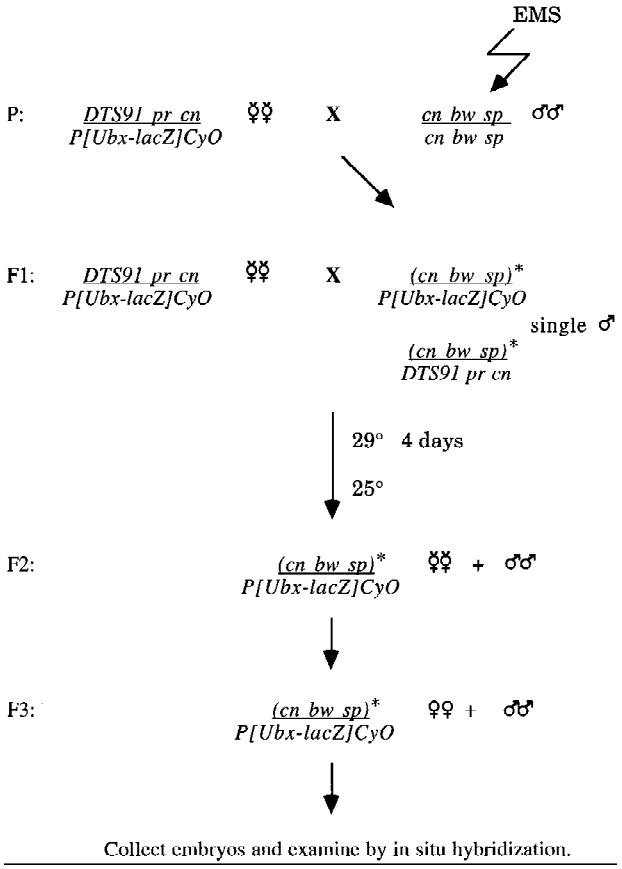

Figure 7. Screen for mutations that disrupt G_1–S transcription. Isogenized *cn bw sp* flies were mutagenized with EMS. The *DTS91* chromosome was used to select for flies bearing the mutagenized chromosome over a balancer expressing *lacZ*. From the F_3 adults, 8- to 15-hr embryos were collected and hybridized in situ with *PCNA* and *lacZ* riboprobes. Embryos that did not express *lacZ* were homozygous for the mutagenized second chromosome. F_3 lines that gave rise to progeny with aberrant *PCNA* expression were maintained.

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. Digoxygenin-labeled antisense RNA probes were made as described in the Boehringer Mannheim kit. The *RNR2* clone used to make probe was obtained by PCR amplification of Oregon R genomic DNA using degenerate primers (Duronio and O'Farrell 1994). The PCR products were cloned into Bluescript KS. *cyclin E* 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 pC4β-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 $dDP/T(2;3)TSTL^{14}$ and $Df(2R)vg\text{-}B/T(2;3)TSTL^{14}$ adults by selecting non-Tb larvae. $T(2;3)TSTL^{14}$ 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′-CTTTAGTCAGATGGGCAGTCAAG-3′ and 5′-CTGTAACTAACTCGACTACCAC-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).

Lethal phase

Embryonic lethality was assessed by mating *dDP/+* females to *Df(2R)vg-B/CyO* males. The parents were allowed to mate at 25°C for several days to reduce the number of unfertilized eggs in the collections. The eggs were collected on apple juice plates at 25°C and counted. The number of eggs that hatched was recorded 24 hr later. The same procedure was carried out for alleles of *dE2F*. The furthest developmental stage reached by the *dDP* alleles in *trans* to each other or in *trans* to *Df(2R)vg-B* was determined by generating stocks with the *dDP* alleles and the deficiency over $T(2;3)TSTL^{14}$ (Gatti and Goldberg 1991). The *dE2F* alleles were balanced over *TM6B* to determine the lethal phase.

To test for the effect of reducing the dosage of *cyclin E* on the viability of *dE2F* mutants two reciprocal crosses were done. The effect of zygotic expression alone was examined by crossing $dE2F^{7172}/TM6B$ females to $cycE^{PZ5}$; $dE2F^{91}/TSTL$ males. The effect of the maternal plus the zygotic contribution was tested by the reciprocal cross.

Heat shock rescue experiments

w; Df(2R)vg-56/CyO females were mated to $w; dDP^{a1}/CyO; P[w^+;hs\text{–}dDP]/+$ or $w; dDP^{a1}/CyO; P[w^+;hs\text{–}dE2F]/+$ 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 dDP^{a1}/dDP^{a2} transheterozygotes, dDP^{a2}/CyO females were crossed to $w; dDP^{a1}/CyO; P[w^+; hs\text{–}dDP]/TM3, Sb$ males at 25°C. No dDP^{a1}/dDP^{a2} 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 treament 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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