

Mutations of the *Drosophila* *dDP*, *dE2F*, and *cyclin E* Genes Reveal Distinct Roles for the E2F-DP Transcription Factor and Cyclin E during the G₁-S Transition

ROBERT J. DURONIO,^{1,2,3*} PETER C. BONNETTE,^{1,2} AND PATRICK H. O'FARRELL⁴

Department of Biology,¹ Lineberger Comprehensive Cancer Center,² and Program in Molecular Biology and Biotechnology,³ University of North Carolina, Chapel Hill, North Carolina 27599, and Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143⁴

Received 13 June 1997/Returned for modification 28 July 1997/Accepted 27 October 1997

Activation of heterodimeric E2F-DP transcription factors can drive the G₁-S transition. Mutation of the *Drosophila melanogaster* *dE2F* gene eliminates transcriptional activation of several replication factors at the G₁-S transition and compromises DNA replication. Here we describe a mutation in the *Drosophila* *dDP* gene. As expected for a defect in the *dE2F* partner, this mutation blocks G₁-S transcription of *DmRNR2* and *cyclin E* as previously described for mutations of *dE2F*. Mutation of *dDP* also causes an incomplete block of DNA replication. When S phase is compromised by reducing the activity of *dE2F*-*dDP* by either a *dE2F* or *dDP* mutation, the first phenotype detected is a reduction in the intensity of BrdU incorporation and a prolongation of the labeling. Notably, in many cells, there was no detected delay in entry into this compromised S phase. In contrast, when cyclin E function was reduced by a hypomorphic allele combination, BrdU incorporation was robust but the timing of S-phase entry was delayed. We suggest that *dE2F*-*dDP* contributes to the expression of two classes of gene products: replication factors, whose abundance has a graded effect on replication, and cyclin E, which triggers an all-or-nothing transition from G₁ to S phase.

One of the key points of cell cycle regulation is the transition from G₁ into S phase (50, 58). Despite the identification of a number of the regulators that influence this transition, we do not fully understand how it is controlled. Detailed analyses of the phenotypes of mutations in the genes that regulate the transition might provide more insight into the nature of the decisive events that trigger the onset of DNA replication. Such analyses of *Saccharomyces cerevisiae* have demonstrated two types of phenotypes when entry into S phase is partially compromised (40, 55). Defects in some genes do not delay the transition into S phase but prolong S phase, apparently by reducing replication efficiency. Defects in other genes appear to have an all-or-nothing effect on a decisive event that is presumed to represent a switch from the G₁ to the S-phase states. Analysis of the behavior of cells in other organisms (26) and of nuclei replicating in *Xenopus laevis* in vitro extracts (39) suggests that an all-or-nothing switch is generally associated with the G₁-to-S transition.

Several findings argue for the importance of the activation of transcription of specific genes that accompanies the G₁-S transition. Chief among these is the finding that activation of specific transcription factors appears to occur immediately upstream of the decisive transition into S phase in yeast (1, 5), *Drosophila melanogaster* (15, 17), and mammalian cells (37, 44). These factors stimulate a program of expression of genes whose products are required for DNA replication. However, because at least some of the required products are stable and are present as a result of earlier expression, the activation of this gene expression does not necessarily provide direct coupling between replication factor transcription and entry into S phase. The role of triggering entry into S phase may instead be

mediated by a second type of gene product induced as part of the S-phase transcription program. In yeast (43, 45), *Drosophila* (16), and vertebrates (4, 23, 47), G₁ cyclins are part of this transcription program (e.g., *CLN1* and *CLN2* in *S. cerevisiae* and *cyclin E* in metazoans). It is not entirely clear what roles each of these two classes of induced gene products, replication factors and G₁ cyclins, plays in the G₁-to-S transition.

In mammals and *Drosophila*, the G₁-S-phase transcription program is controlled by the E2F-DP family of heterodimeric transcription factors (17, 37, 44, 59). Five related E2F genes and two related DP genes have been identified in mammals (59). Mice containing a homozygous knockout mutation of the *E2F-1* gene develop normally and are viable, indicating that *E2F-1* is not essential for cell cycle progression (22, 72). While *E2F-1* knockout mice do display several abnormal phenotypes, most strikingly the eventual acquisition of a variety of tumors in older individuals, the absence of a more dramatic phenotype due to loss of *E2F-1* function could be explained by redundancies among the five known E2Fs. In *Drosophila*, only a single E2F gene (*dE2F*) and DP gene (*dDP*) have yet been reported (18, 27, 48), although an *E2F*-related sequence has recently been identified as an EST from a *Drosophila* embryonic cDNA library (28). As in mice, therefore, there exists a potential for functional redundancy among *Drosophila* E2F genes. Nevertheless, mutations of *dE2F* cause lethality (6, 17). In embryos, *dE2F* mutations block the activation of transcription that usually occurs at the G₁-to-S transition and inhibit DNA replication (17). Thus, *dE2F* plays an essential role, likely as a requirement for normal cell cycle progression.

Here we explore further the role of the activation of G₁-S phase transcription by identifying mutations of the *dDP* gene and comparing embryonic phenotypes caused by genetic reductions in the function of *dDP*, *dE2F*, or cyclin E, a downstream target of *dE2F*-*dDP* that is required for S phase (16, 33, 51). We find that both *dDP* and *dE2F* are required for the activation of transcription of the same genes at the G₁-to-S

* Corresponding author. Mailing address: Department of Biology, Campus Box 3280, University of North Carolina, Chapel Hill, NC 27599. Phone: (919) 962-7749. Fax: (919) 962-1625. E-mail: duronio@med.unc.edu.

transition, consistent with dE2F and dDP acting as a heterodimer *in vivo*. If cyclin E and dE2F-dDP are jointly required to trigger a common step needed for the initiation of DNA replication, then reductions in the functions of these gene products should lead to similar phenotypes. In contrast to this, we find distinct behaviors. Reducing dE2F-dDP function first diminishes the rate of DNA replication, and only with more severe reductions in functions do we detect delays or defects in the transition into S phase. Reduction of cyclin E function leads to a stochastic increase in the length of G₁ without compromising replication in those cells that initiate DNA synthesis. We discuss these results in terms of two distinct roles for dE2F-dDP and cyclin E in the pulse of transcription at the G₁-to-S transition: one role is a direct and dose-dependent participation in DNA replication and the other is a regulatory role that triggers an all-or-nothing change required for S phase.

MATERIALS AND METHODS

Genetics and fly stocks. The overlapping *Df(2R)vg⁵⁶* and *Df(2R)vg³³* deficiencies (breakpoints 49D;49F and 49D;50A, respectively) and the recessive-lethal EMS-induced mutations from the 49F region were isolated in a previously described mutagenesis screen (36). Single mutant stocks representing a subset of the 18 complementation groups were screened with the *DmRNR2* probe (vr-3, -4, -6, -8, -9, -10, -11, -13, -15, -17, -18, and -19). These were graciously provided to us by Ting Wu of Harvard University. The *dDP^{vr10}* mutation we refer to throughout the text was originally designated vr10-13 (36) and contained the recessive markers *al*, *b*, *c*, and *sp*. We separated three other lethal mutations from the original *dDP^{vr10}* chromosome by two successive meiotic recombinations with a wild-type Sevelen second chromosome. The possibility that this new recombinant chromosome (designated *dDP^{vr10}-5D*) only contains the *dDP^{vr10}* semilethal allele is supported by the rare (<2%) appearance of *dDP^{vr10}/dDP^{vr10}* homozygous flies in the stock. The presence of these three additional lethal mutations did not significantly affect the phenotype in comparisons of *DmRNR2* staining of *dDP^{vr10}/dDP^{vr10}*, *dDP^{vr10}/Df(2R)vg⁵⁶*, *dDP^{vr10}-5D/dDP^{vr10}-5D*, and *dDP^{vr10}-5D/Df(2R)vg⁵⁶* mutant embryos. The *cyclin E^{P28}* and *cyclin E⁰⁵²⁰⁶* alleles are described in references 2 and 32 and 33, respectively. The *dE2F¹⁶⁴* P-element allele is described in reference 56; all other *dE2F* alleles are described in reference 17. All second chromosome mutant stocks were maintained with a CyO P[w⁺; *wg-lacZ*] balancer, allowing unambiguous identification of mutant progeny embryos, which fail to express *lacZ*.

Molecular analysis of the dDP locus and dDP cDNA. Polytene chromosome hybridizations were performed with digoxigenin-labeled *dDP* cDNA (encoding amino acids 76 to 322) exactly as described previously (15). To identify the *dDP^{vr10}* mutation, *dDP* genomic DNA was PCR amplified in three overlapping regions with primers designed from the sequence of the full-length cDNA (see below). Template DNA was prepared from single adult *dDP^{vr10}/Df(2R)vg⁵⁶* or Canton S control flies as described previously (25). For each genotype, products from two independent PCR amplifications with *Thermococcus litoralis* DNA polymerase (New England Biolabs) were subcloned into pBluescript and sequenced. The missense mutation was found in both *dDP^{vr10}* PCR amplifications but not in the wild-type amplifications. Several pieces of evidence suggested that our original *dDP* cDNA (18) was not full length. First, immunoblot analysis revealed that heat shock expression of this cDNA produces a polypeptide that by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis migrates with an apparent molecular mass ~8 kDa smaller than that of the endogenous *dDP* (14). Second, Hao et al. (27) reported the amino acid sequence of a *Drosophila dDP* cDNA that was nearly identical to the previously reported *dDP* cDNA translation product (18) from residues 8 to 377 but contained an amino-terminal extension of 74 amino acids. Third, the Dynlacht et al. (18) sequence does not contain an in-frame stop codon upstream of the assigned initiator Met. We PCR amplified the 5' region from a probable full-length *dDP* cDNA with nested primers and template DNA prepared from a 0- to 4-h embryonic cDNA library (7). In the first PCR, a vector primer that hybridizes 5' to the cloning site and contains a *HindIII* recognition sequence was paired with a primer (5' ACAGT TGTTGTCGTACGCAT) that hybridizes at the unique *BsiWI* site in the *dDP* cDNA. A 1:1,000 dilution of the first reaction mixture was used as the template in a second PCR that paired the same 5' vector primer with a *dDP* primer (5' TTGCCCGATGCCGCTAGCAC) that hybridizes at the unique *NheI* site just upstream of the initiator Met assigned by Dynlacht et al. (18). The single product from this reaction was cut with *HindIII/NheI* and subcloned in frame with the Dynlacht et al. (18) cDNA contained in pBluescript SK.

Embryo in situ hybridization and BrdU labeling. Digoxigenin-labeled RNA probes from *DmRNR2* genomic DNA (15) and *cyclin E* type I cDNA (51) were used for embryo in situ hybridizations as described previously (15, 38, 65). Embryos were pulse labeled for 15 min in Schneider's medium containing 1 mg of BrdU/ml as described previously (19, 54). Incorporated BrdU was detected

with anti-BrdU mouse monoclonal primary antibody (Becton Dickinson) and rhodamine-conjugated goat anti-mouse secondary antibody (Jackson). For heat shock experiments, eggs from *dDP^{vr10}/CyO P[w⁺; wg-lacZ]*; *hsp70-dDP/hsp70-dDP* flies were collected on grape juice agar plates, aged at 18°C, and heat shocked by floating the collection plate on the surface of a 37°C water bath for 30 min. After a 70-min recovery at room temperature, the embryos were fixed and stained for *DmRNR2* expression. The *hsp70-dDP* P-element construct is described in reference 14.

Immunoblot analysis. Eggs were collected for 1 h at room temperature from *w; Df(2R)vg⁵⁶/CyO P[w⁺; wg-lacZ]* flies and aged 23 h at 17°C. The eggs were dechorionated in 2.5% sodium hypochlorite, and the embryos were devitelinated and fixed by shaking in a 1:1 mixture of methanol and heptane. The embryos were washed twice with MeOH, transferred to an aqueous buffer containing protease inhibitors (10 mM Tris [pH 7.8], 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamide, 0.05% Tween 20), and stained with the DNA binding dye Hoechst 33258. Ten *Df(2R)vg⁵⁶/Df(2R)vg⁵⁶* or 10 *Df(2R)vg⁵⁶/CyO P[w⁺; wg-lacZ]* methanol-fixed embryos were hand selected under UV light with a fluorescence microscope. The three types of progeny from this stock [*Df(2R)vg⁵⁶/Df(2R)vg⁵⁶*, *Df(2R)vg⁵⁶/CyO P[w⁺; wg-lacZ]*, and *CyO P[w⁺; wg-lacZ]/CyO P[w⁺; wg-lacZ]*] could easily be distinguished from each other at germ band-retracted stages due to differences between the aberrant morphology of the deficiency homozygotes and the CyO/CyO balancer homozygotes, which have a *wg* mutant phenotype. Selected embryos were dissociated by boiling them for 3 min in sample buffer (62.5 mM Tris [pH 6.8], 2% SDS, 5% glycerol, 5% β-mercaptoethanol) and subjected to SDS-polyacrylamide gel electrophoresis. Separated proteins were electrophoretically transferred to nitrocellulose, and dDP polypeptides were detected with a 1:10 dilution of monoclonal antibody supernatant Yun1 (13).

Nucleotide sequence accession number. The additional *dDP* cDNA sequence discussed above has been submitted to GenBank (accession no. AF031700).

RESULTS

Identification of a dDP mutation. *Drosophila dDP* cDNA was cloned previously and shown to encode a functional binding partner for dE2F (18, 27). We took a reverse genetic approach to identify mutations of *dDP* in order to test its role in transcription and cell cycle control. Hybridizations of a *dDP* cDNA probe to salivary gland polytene chromosomes (Fig. 1A) allowed us to map the *dDP* locus to position 49F on the right arm of the second chromosome (not shown). A series of overlapping deletion mutations that uncover region 49F were generated by Lasko and Pardue (36). In situ analysis of *Df(2R)/+* polytene chromosomes revealed that the *Df(2R)vg⁵⁶* deletion chromosome failed to hybridize with our *dDP* cDNA probe (Fig. 1A). We conclude from this result that *dDP* coding sequences are missing in the *Df(2R)vg⁵⁶* deletion. Hao et al. previously reported that *dDP* mapped outside this particular deficiency (27). While we cannot readily explain this discrepancy, two other observations confirm our mapping of *dDP* within the *Df(2R)vg⁵⁶* deletion. First, in embryos that had progressed to cellularization or beyond, in situ hybridization detected zygotic *dDP* expression in control but not in *Df(2R)vg⁵⁶/Df(2R)vg⁵⁶* embryos, consistent with the elimination of maternal *dDP* RNA by cell cycle 14 (data not shown). Second, Western blotting (see Materials and Methods) of total protein extracted from germ band-retracted *Df(2R)vg⁵⁶/Df(2R)vg⁵⁶* embryos with anti-dDP monoclonal antibodies indicated a severe reduction in the amount of dDP protein compared to that of *Df(2R)vg⁵⁶/+* sibling embryos (Fig. 1B). Taken together these data indicate that the *Df(2R)vg⁵⁶* chromosome lacks a functional *dDP* gene.

Mutations of *dE2F* reduce the zygotic expression of several genes involved in DNA replication, including those encoding the small subunit of ribonucleotide reductase (*DmRNR2*) and *PCNA* (17). This phenotype is first apparent in the central nervous system (CNS) of germ band-extended embryos during late stage 11. In germ band-extended *Df(2R)vg⁵⁶/Df(2R)vg⁵⁶* embryos the amount of *DmRNR2* and *PCNA* mRNA in the CNS was significantly reduced relative to that of the wild type (Fig. 1C). An essentially identical gene expression defect was observed in embryos homozygous for *Df(2R)vg³³* (not shown),

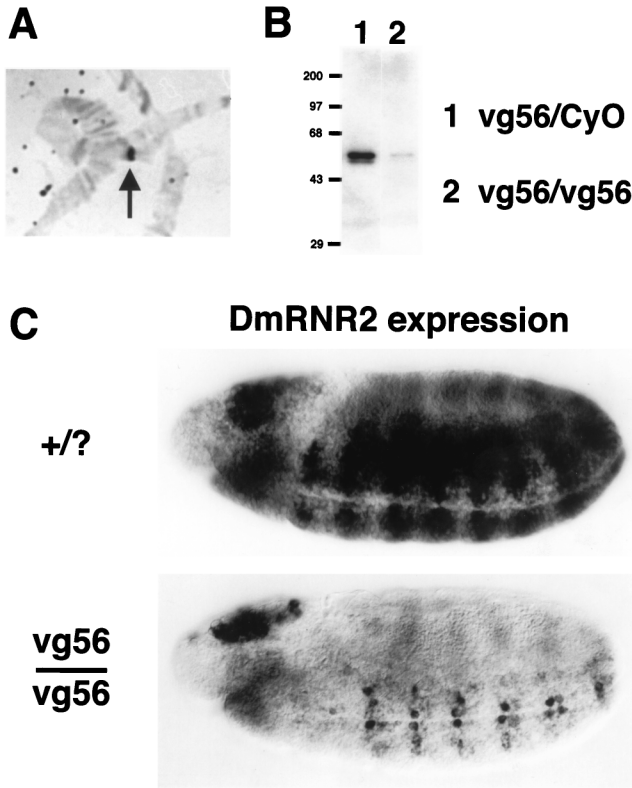


FIG. 1. Identification of *dDP* mutations. (A) Hybridization of a *Df(2R)vg⁵⁶/+* salivary gland polytene chromosome with a *dDP* cDNA probe. The hybridization signal (arrow) is lost in the *Df(2R)vg⁵⁶* deletion and consequently appears over half the width of the heterozygous polytene chromosome. (B) Western blot of extract of germ band-retracted embryos collected from the *Df(2R)vg⁵⁶/CyO* stock probed with anti-dDP monoclonal antibody. Since *Df(2R)vg⁵⁶* deletes the *dDP* gene, any dDP protein present in *Df(2R)vg⁵⁶/Df(2R)vg⁵⁶* embryos must be of maternal origin. (C) Germ band-extended control (+/?) and *Df(2R)vg⁵⁶/Df(2R)vg⁵⁶* mutant stage 11 embryos subjected to in situ hybridization with a digoxigenin-labeled probe derived from the *DmRNR2* gene. *DmRNR2* expression is failing in the developing ventral (toward the bottom) nerve cord of the mutant embryo. (D) Intron and exon structure of the transcribed region of the *dDP* gene. Exons are represented by shaded boxes, and introns are represented by a straight line. ATG and TGA indicate the beginning and end of the *dDP* open reading frame, respectively. The Arg-to-His missense mutation in exon 5 of the *dDP^{vr10}* allele is indicated. (E) Alignment of a highly conserved region of the heterodimerization domain of DPs from *Drosophila* (this paper), human (hum) (30, 70), mouse (mus) (30, 49), and *Xenopus* (xen) (24). The numbers indicate the position in each primary amino acid sequence. The position of the Arg-to-His change in the *dDP^{vr10}* allele is indicated at the top.

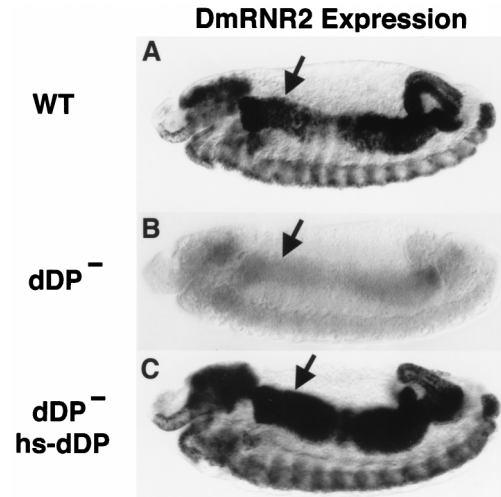


FIG. 2. *dDP* is required for expression of *DmRNR2*. Each panel shows a stage 13 embryo (anterior at the left and dorsal at the top) subjected to in situ hybridization with a digoxigenin-labeled probe derived from the *DmRNR2* gene. (A) Wild type (WT). The pattern of *DmRNR2* expression is coincident with the pattern of DNA replication (Fig. 5A). This includes internal endoreplicating cells (e.g., midgut cells) and proliferating cells in the CNS. (B) *dDP^{vr10}/dDP^{vr10}* (*dDP⁻*). *DmRNR2* expression is not activated in the *dDP* mutant. (C) A *dDP^{vr10}/dDP^{vr10}; hsp70-dDP/hsp70-dDP* (*dDP⁻ hs-dDP*) embryo subjected to a 30-min 37°C heat shock followed by a 70-min recovery at room temperature. Expression of *dDP* cDNA in the mutant restores the normal pattern of *DmRNR2* expression. Each of the images has an internal focal plane in order to observe the anterior midgut (arrows).

another deletion mutation isolated by Lasko and Pardue that removes polytene region 49F (36). Therefore, loss of the *dDP* locus is associated with a phenotype that closely resembles the phenotype associated with the loss of *dE2F* function.

Embryos homozygous for the *Df(2R)vg⁵⁶* and *Df(2R)vg³³* deletions deviate dramatically from wild-type morphology during and after germ band retraction, presumably because of the loss of multiple gene functions. This precluded an accurate analysis of gene expression patterns in the deficiencies at stages when we usually analyze the G₁-S transcription program in wild-type embryos. We therefore examined existing mutations that mapped to 49F for perturbations in embryonic *DmRNR2* expression. Mutations causing defective *DmRNR2* expression would be candidates for alleles of *dDP*. This approach had proven successful in identifying *dE2F* mutations (17). A collection of EMS-induced mutations representing 18 lethal complementation groups that fail to complement *Df(2R)vg⁵⁶*, *Df(2R)vg³³*, and other deletions in the 49F region were generated by Lasko and Pardue (36). Embryos were collected from extant stocks carrying representative alleles from most of these complementation groups (see Materials and Methods) and probed for *DmRNR2* expression by in situ hybridization. In one lethal complementation group, designated *dDP^{vr10}*, *DmRNR2* expression was greatly reduced compared to that of wild-type embryos (for germ band-retracted stages, cf. Fig. 2A and B). No obvious change in the expression of *DmRNR2* was observed in any of the other mutants tested. At germ band-extended stages, the *dDP^{vr10}* *DmRNR2* expression phenotype was similar to that caused by *Df(2R)vg⁵⁶* and *Df(2R)vg³³* (not shown), suggesting that the *dDP^{vr10}* chromosome contained a mutation in the relevant gene deleted in these deficiencies.

The original complementation mapping by Lasko and Pardue (36) placed the *dDP^{vr10}* lethal mutation outside of the *Df(2R)vg⁵⁶* deletion; i.e., *dDP^{vr10}* and *Df(2R)vg⁵⁶* were scored

TABLE 1. Results of *Df(2R)/dDP^{vr10}* complementation tests

Cross	No. of adult progeny when reared at ^a :	
	25°C	29°C
<i>Df(2R)vg⁵⁶/CyO wg-lacZ</i> × <i>dDP^{vr10}/CyO wg-lacZ</i>	12/683 (2%)	0/487 (0%)
<i>Df(2R)vg⁵⁶/CyO wg-lacZ</i> × <i>dDP^{vr10}/CyO wg-lacZ; hsp70-dDP/hsp70-dDP</i>	20/265 (8%)	46/475 (10%)
<i>Df(2R)vg³³/CyO wg-lacZ</i> × <i>dDP^{vr10}/CyO wg-lacZ</i>	5/531 (1%)	0/467 (0%)
<i>Df(2R)vg³³/CyO wg-lacZ</i> × <i>dDP^{vr10}/CyO wg-lacZ; hsp70-dDP/hsp70-dDP</i>	21/388 (5%)	43/468 (9%)

^a Data are expressed as the number of *Df(2R)/dDP^{vr10}* adult progeny/total number of adult progeny. Full complementation is 33% (*CyO wg-lacZ/CyO wg-lacZ* progeny do not survive until adulthood).

as complementing mutations in their test crosses. Since this was inconsistent with our interpretation of the *DmRNR2* expression phenotype, we repeated the complementation tests with our *dDP^{vr10}* stock (Table 1). When crosses with *Df(2R)vg⁵⁶* flies were reared at 25°C, less than 2% of the adult progeny ($n = 683$) were of the *dDP^{vr10}/Df(2R)vg⁵⁶* class (complementing mutations should comprise 33% of adult progeny). Similarly, in crosses with *Df(2R)vg³³* flies less than 1% of the adult progeny ($n = 531$) were of the *dDP^{vr10}/Df(2R)vg³³* class. These rare “escapers” were not phenotypically normal: many flies had notched wings, some had rough eyes, and both males and females were sterile (the females failed to lay eggs). Thus, the *dDP^{vr10}* chromosome is semilethal at 25°C over both deficiencies. Because this semilethality gives occasional survivors, it could be scored as complementation when smaller numbers of progeny are scored: presumably this explains the mapping results of Lasko and Pardue. When we reared our crosses at 29°C, no *Df(2R)/dDP^{vr10}* flies eclosed (Table 1). A single copy of a *hsp70-dDP* construct contained in a P-element vector was sufficient to induce *dDP^{vr10}/Df(2R)vg⁵⁶* or *dDP^{vr10}/Df(2R)vg³³* flies to eclose at 29°C in the absence of a 37°C heat shock treatment (Table 1). We conclude that the *dDP^{vr10}* mutation fails to complement deficiencies that delete the *dDP* locus and that low-level expression of a *dDP* cDNA can rescue *dDP^{vr10}/Df(2R)* lethality. Additionally, the incomplete lethality of the *dDP^{vr10}/Df(2R)* heteroallelic combination at lower temperatures indicates either that *dDP* is not absolutely essential or that *dDP^{vr10}* is not null for the locus.

These data suggested that the *dDP^{vr10}* chromosome contained a mutation in the *dDP* gene. To confirm this, we molecularly characterized the *dDP* locus of wild-type and *dDP^{vr10}* chromosomes. The full-length *dDP* transcription unit spans approximately 4 kb of genomic DNA and contains eight introns (Fig. 1D). Our analysis predicts that the wild-type *dDP* translation product consists of 446 residues with a predicted molecular mass of 49,718 Da. This is in contrast to previously published reports (see Materials and Methods). Southern blotting indicated that there was no gross rearrangement of the *dDP* locus in the *dDP^{vr10}* chromosome (data not shown). PCR amplification and sequencing of the *dDP* locus from individual *dDP^{vr10}/Df(2R)vg⁵⁶* or *dDP^{vr10}/dDP^{vr10}* adult flies revealed a single nucleotide change encoding an Arg-to-His substitution at residue 217 (Fig. 1E). This nucleotide change destroys a unique *AatII* restriction site present in our *dDP* cDNA. This consequently provided a means of readily assessing whether the change is simply a wild-type polymorphism. PCR amplification products of *dDP* genomic DNA isolated from *dDP^{vr10}/CyO* flies could be partially digested by *AatII*, indicating that the *CyO* balancer chromosome contains the *AatII* site (not shown). In contrast, similar *dDP* genomic PCR products derived from a stock (*vr18/CyO*) containing a mutant chromosome (*vr18*) independently isolated in the original Lasko and Pardue (36) mutagenesis screen could be completely digested

by *AatII*, strongly suggesting that the *vr18* chromosome contains the *AatII* site (not shown). Furthermore, *vr18* complements *dDP^{vr10}* and does not cause defects in *DmRNR2* expression (not shown). We conclude from this data that the Arg-to-His amino acid change present in *dDP^{vr10}* is not a polymorphism that was present in the flies originally mutagenized by Lasko and Pardue (36).

Arg 217 is in a region of the dDP protein that is highly conserved among *Xenopus*, mouse, human, and *Drosophila* DPs (Fig. 1E). This region of mammalian DP-1 has been shown to be required for dimerization with E2F-1 (3, 30, 34, 69). Thus, the Arg-to-His mutation may reduce the ability of the *dDP^{vr10}* polypeptide to bind to dE2F. Dimerization of dDP with dE2F is required for efficient DNA binding activity in vitro (18). Moreover, coexpression of *dDP* and *dE2F* is needed for effective transactivation of promoters containing dE2F-dDP binding sites in cultured cells (13, 18) and for stimulating transcription of endogenous target genes in embryos (14). We conclude that the *dDP^{vr10}* chromosome contains an alteration of the *dDP* gene and suggest that this change impairs the ability of dDP protein to act as a transcription factor, perhaps due to a reduced capacity to dimerize with dE2F. Royzman et al. (52) have also recently reported the characterization of a *dDP* mutant allele (*a1*) encoding an Arg-to-Cys change at residue 217.

Since *dDP^{vr10}* encodes a point mutation of dDP, we cannot be sure that the phenotypes we describe below represent the null situation. Moreover, we could still detect a small amount of maternal dDP protein in germ band-retracted *Df(2R)vg⁵⁶/Df(2R)vg⁵⁶* embryos (Fig. 1B). Nevertheless, we did not observe obvious differences in the transcription and DNA replication phenotypes in *dDP^{vr10}/dDP^{vr10}* and *dDP^{vr10}/Df(2R)vg⁵⁶* embryos. This indicates that a twofold reduction in the zygotic dose of *dDP^{vr10}* does not detectably enhance the phenotypes we are scoring and suggests that *dDP^{vr10}* retains very little function.

***dDP* is required for the expression of replication genes at the G₁-S transition.** The first 16 cell cycles of *Drosophila* embryogenesis lack a G₁ phase. Following the introduction of a G₁ phase in cell cycle 17, subsequent entry into S phase is accompanied by coordinate *dE2F*-dependent transcriptional activation of replication factor genes (15). We used probes derived from *DmRNR2* or *cyclin E* as representatives of this group of genes (cf. Fig. 2A and 4A). *DmRNR2* transcripts are present continuously and nearly uniformly prior to cycle 17 in *dDP^{vr10}* mutants, as they are in the wild type (not shown). By cycle 17, *DmRNR2* is not expressed in its usual stereotypic pattern in endoreplicating tissues in the mutants (cf. Fig. 2A and B). The *dDP^{vr10}* mutation also dramatically reduces *DmRNR2* expression in proliferating cells of the developing CNS (brain lobes and ventral nerve cord). Heat shock expression of a *dDP* cDNA contained in a P-element transgene restored the wild-type pattern of *DmRNR2* expression (Fig. 2C). We conclude that *dDP* function is required for the activation of

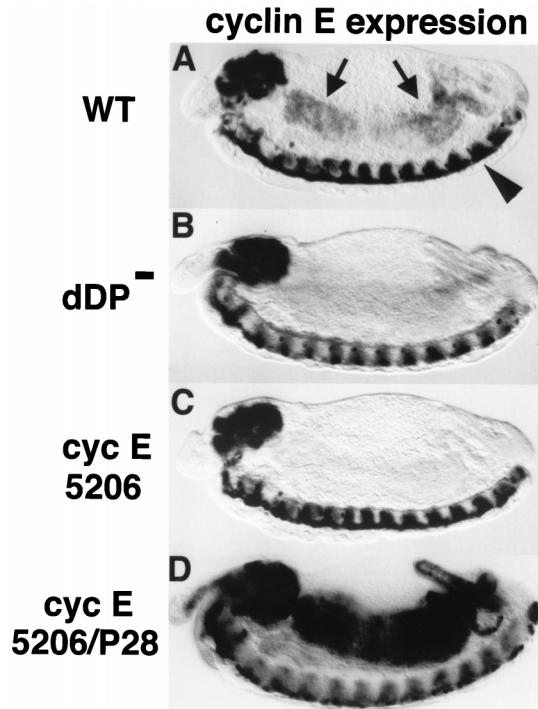


FIG. 3. *dDP* is required for *cyclin E* expression at the G₁-S transition in endoreduplicating cells. Each panel shows a stage 13 embryo (anterior at the left and dorsal at the top) subjected to in situ hybridization with a digoxigenin-labeled probe derived from *cyclin E* cDNA. (A) Wild type (WT). The arrows indicate the midgut. The arrowhead indicates the ventral nerve cord. (B) *dDP^{vr10}/Df(2R)vg⁵⁶* (*dDP*⁻). The cells of the midgut normally express *cyclin E* at this stage but fail to do so in the *dDP* mutant. CNS expression of *cyclin E* is normal in the *dDP* mutant. (C) *cyclin E⁵²⁰⁶/cyclin E⁵²⁰⁶* mutant embryo. This allele contains a P-element insertion into the upstream regulatory region of the *cyclin E* gene (33). *cyclin E* is not expressed in the midgut or other cells that normally endoreduplicate but is expressed in the CNS. (D) *cyclin E⁵²⁰⁶/cyclin E^{P28}* transheterozygote. Expression of *cyclin E*, presumably from the *cyclin E^{P28}* homolog, initiates at the correct developmental time in the midgut. However, transcription is not down regulated as usual, leading to persistently high levels of *cyclin E* mRNA.

DmRNR2 transcription in both endoreduplicating and proliferating cells.

The pattern of *cyclin E* expression resembles that of genes encoding other replication factors during cycle 17 (16, 33). However, while genes encoding other factors (e.g., *DmRNR2*) require *dE2F* function in all domains of their expression (16), *cyclin E* expression relies on *dE2F* in all domains except the CNS (16). Similarly, expression of *cyclin E* relies on *dDP* in all domains but the CNS (cf. Fig. 3A and B). The factors that control *cyclin E* expression in the CNS have not yet been identified.

Our results indicate that the *dDP* mutant phenotype is very similar to that of *dE2F* mutants with respect to activation of transcription of *DmRNR2* and *cyclin E*. This is consistent with a requirement for *dE2F*-*dDP* heterodimers in the transcription of replication genes in vivo. However, if *dE2F* and *dDP* perform any functions independent of each other, then we would also expect to observe differences in their respective phenotypes. This possibility is further suggested by the presence of at least one other *E2F*-like gene, and therefore at least one other potential *dDP* binding partner, in *Drosophila* (28). We detected a phenotypic difference in the embryonic CNS with a probe derived from the *Drosophila dMCM3* gene (22a, 64). *dMCM3* is a member of the MCM family of proteins, which act

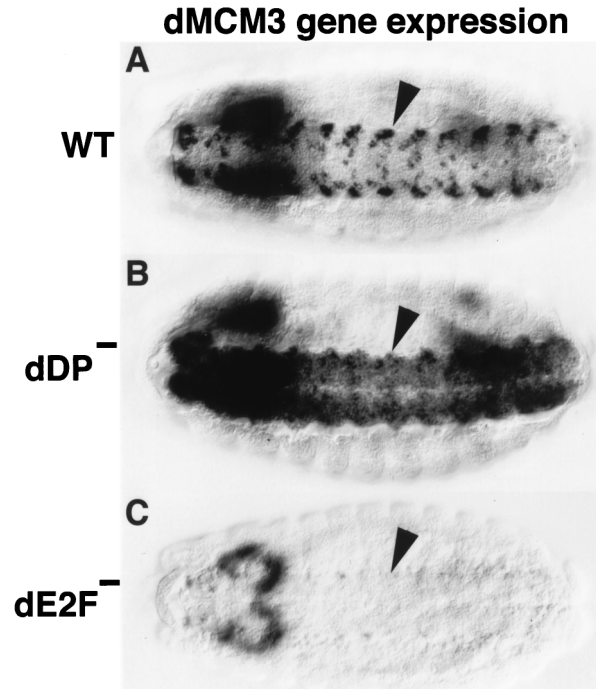


FIG. 4. Mutation of *dDP* and *dE2F* causes distinct phenotypes. Each panel shows a stage 14 embryo (anterior at the left) subjected to in situ hybridization with a digoxigenin-labeled probe derived from *dMCM3* cDNA. The perspective is ventral to view the nerve cord (VNC) of the CNS (arrowheads). (A) Wild type (WT). Expression occurs coincident with replication, generating the observed pattern. (B) *dDP^{vr10}/dDP^{vr10}* mutant. *dMCM3* expression continues in the VNC, but occurs in an expanded domain of cells. This suggests that *dDP* functions to prevent *dMCM3* from becoming derepressed in this tissue. (C) *dE2F⁹¹/dE2F⁹¹* null mutant. Very little if any *dMCM3* expression can be detected in the VNC, indicating that *dE2F* is required for normal *dMCM3* expression. No ectopic *dMCM3* expression is observed, perhaps because *dE2F* does not perform an essential repression function. Residual *dMCM3* expression in the brain accounts for the out-of-focus staining at the left.

at origins of replication and are required for S phase (8, 61, 67). *dMCM* genes are expressed in replicating cells in a pattern similar to that of genes like *DmRNR2* (21, 66). In *dE2F* mutant embryos, there is substantially less *dMCM3* RNA in the CNS than in the wild-type CNS, indicating that *dE2F* is required for *dMCM3* expression in this tissue (cf. Fig. 4A and C). The *dDP^{vr10}* mutation also alters *dMCM3* expression, but in very different way. The intense staining of particular cells seen in wild-type embryos is absent in the mutant, suggesting that this high-level expression requires *dDP* (Fig. 4B). However, other cells of the CNS that normally have extremely low levels of *dMCM3* RNA exhibit increased staining (Fig. 4B). This derepression of *dMCM3* as a result of mutation of *dDP* suggests that *dDP* normally plays a role in the repression of this gene, at least in some cells of the CNS.

Mutation of *dDP* attenuates DNA replication and lengthens S phase. Replicating nuclei can be readily observed in situ by pulse labeling live embryos with BrdU and subsequently detecting BrdU incorporation immunologically after fixation. The wild-type profile of endocycle S phases during embryonic stages 12 to 15 in the midgut has been well described (15, 60). The first of these S phases begins from G₁ of cycle 17 (Fig. 5A). *dDP^{vr10}/dDP^{vr10}* and *dDP^{vr10}/Df(2R)vg⁵⁶* mutant embryos incorporated much less BrdU in the midgut during a 15-min pulse labeling than did wild-type embryos (for *dDP^{vr10}/dDP^{vr10}*, cf. Fig. 5A and B). Reduced BrdU incorporation is also seen in

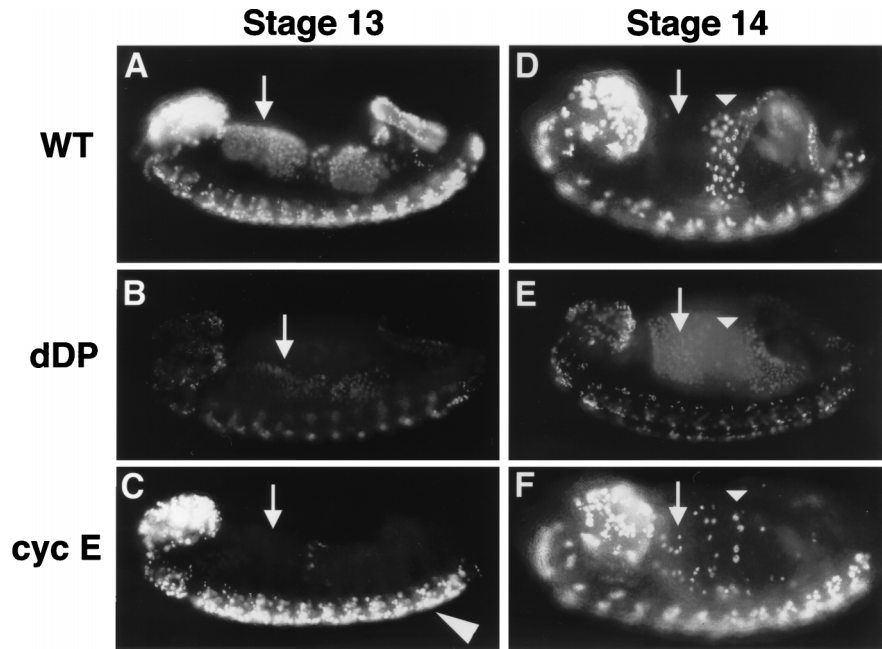


FIG. 5. Different cell cycle roles for cyclin E and dDP. Embryos were collected and aged until stage 13 (A to C) or stage 14 (D to F), pulse labeled for 15 min with BrdU, and then immediately fixed. BrdU that incorporated into replicating nuclei during the pulse was subsequently detected by indirect immunofluorescence. The orientation of each embryo is the same as in Fig. 2. (A and D) Wild type (WT). (B and E) dDP^{vr10}/dDP^{vr10} (dDP). (C and F) $cyclin E^{E28}/cyclin E^{05206}$ (cyc E). The arrows indicate the cells of the anterior midgut (AMG). These cells begin S phase 17 during stage 12, continue replication into stage 13 (A), and exit S phase by stage 14 (D). In the dDP mutant, all or most of the AMG cells enter S phase at the correct time but incorporate much less BrdU (B). By stage 14 these cells are still replicating (E), indicating that S phase is longer than it is in the wild type. In the hypomorphic cyclin E mutant, the AMG cells do not begin S phase 17 on schedule (C) but eventually enter S phase in a random fashion beginning in stage 14 (F). Thus, G_1 of cycle 17 is longer than usual in these cells. The actual length of G_1 depends upon when the cells reach the critical level of cyclin E activity that triggers S phase. Also note that replication in the CNS appears normal, consistent with wild-type cyclin E expression in the CNS of $cyclin E^{05206}$ mutants.

other endocycling tissues, such as the hindgut. This decrease in BrdU incorporation was not restricted to endocycles, as mitotic cells in the CNS also labeled poorly relative to the wild type (cf. Fig. 5A and B). We conclude that dDP function is required for wild-type levels of DNA synthesis in endocycling and dividing cells.

Analysis of BrdU incorporation in an embryo allows one to score the timing of entry into S phase and the duration of S phase in addition to scoring the level of labeled nucleotide incorporated during the pulse. As described above, the dDP^{vr10} mutant embryos exhibit an obvious reduction in BrdU incorporation, but the pattern of BrdU incorporation reveals that the programming of the G_1 -to-S transition still operates. The tissues that enter S phase 17 relatively early in the wild type, such as the anterior midgut, posterior midgut, and hindgut, appear to begin their compromised S phase at a similar embryonic stage in the dDP mutant (Fig. 5B). This demonstrated that reduction of dDP function first affects the level of incorporation rather than the timing of the transition from G_1 to S phase. Analysis of the incorporation pattern in successively older embryos, whose age is determined both by time from egg deposition and by staging according to morphological criteria, defines the duration of S phase. Whereas the tissues that begin S phase 17 early in wild-type embryos discontinue incorporation by stage 14, in the mutant incorporation continues at this stage. This changes the pattern of BrdU incorporation such that cells which no longer label in the wild type are still labeled in the mutant (e.g., anterior midgut cells [Fig. 5D and E]). The prolongation of S phase combined with decreased BrdU incorporation suggests that the replication rate is reduced. Since the dDP^{vr10} mutant compromises the expression of genes such as

DmRNR2, we suggest that the mutant exhibits a reduction in DNA synthesis as a result of decreases in the levels of replication functions.

Our original analysis of the replication phenotype in *dE2F* mutant embryos led us to the conclusion that the mutation blocked S phase (17), whereas the analysis of the dDP mutation suggests a more quantitative defect. We thus conducted more detailed analyses of *dE2F* mutants. The results suggest that reductions in the function of *dE2F* also have a quantitative effect on replication. *dE2F¹⁶⁴* is a homozygous, viable, hypomorphic P-element-induced allele of *dE2F* that expresses nearly undetectable levels of *dE2F* mRNA (56). Nonetheless, *dE2F¹⁶⁴* fully complements a deletion [*Df(3R)e^{D7}*] of the *dE2F* gene: adult flies are viable and fertile. Despite the absence of an effect on viability, we detected a change in the pattern of BrdU incorporation in *Df(3R)e^{D7}/dE2F¹⁶⁴* mutant embryos. As with the dDP^{vr10} mutant, this pattern is consistent with a prolongation of S phase 17 in the midgut (cf. Fig. 5E and 6A). This suggests that the requirement for *dE2F* can be satisfied by relatively low levels of activity and that a reduction in the efficiency of S phase is not lethal. We therefore reexamined embryos homozygous for lethal null alleles of *dE2F*. We could detect a low level of BrdU incorporation in the midgut, as well as in the CNS (Fig. 6B). This incorporation can be more readily detected when longer BrdU-labeling periods are used (e.g., 40 vs. 15 min). Again, the pattern of BrdU incorporation was altered in a way that suggests the prolongation of S phase 17 in the midgut (cf. Fig. 5E and 6B). We conclude that reductions in function of *dE2F/dDP* activity over a large range result in a graded reduction in S phase.

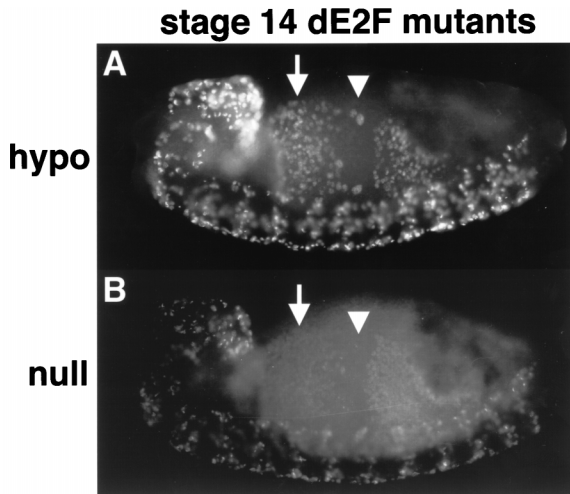


FIG. 6. Prolonged S phase in *dE2F* mutants. Stage 14 *dE2F¹⁶⁴/Def(3R)^{eD7}* (hypo) (A) and *dE2F⁷¹⁷²/dE2F⁷¹⁷²* (null) (B) embryos pulse labeled for 15 min with BrdU. S phase 17 continues in the anterior midgut (arrows) when in the wild type it has already finished (Fig. 5D). This is an indication that S phase is prolonged in the mutants. In the central portion of the midgut (arrowheads) S phase 18 has not started on schedule for most of the cells (Fig. 5D). This indicates that *dE2F* also contributes to correct timing of the G₁-S transition.

Cyclin E triggers S phase in an all-or-nothing manner. During G₁ phase, eukaryotic cells are thought to respond to growth-promoting signals by making a commitment to enter the cell cycle and begin S phase. A typical feature of the process of commitment is that it is an all-or-nothing step. Reductions in function of a component contributing to such an all-or-nothing step ought to exhibit a sharp threshold, with reductions below a critical level blocking the ability to progress beyond the decisive step. As described above, mutations that compromise the activity of dE2F-dDP suggest that this transcription factor makes a graded contribution to the efficiency of S phase, the antithesis of the expectation for a regulator of a decisive all-or-nothing transition.

Cyclin E mutations on the other hand show the behavior expected for a contribution to an all-or-nothing transition. Cyclin E null mutations complete embryonic cell cycle 16 and cause an arrest in G₁ of cell cycle 17 (33). Our immunofluorescent methods cannot detect any BrdU incorporation in these mutants. Thus, in this instance the cyclin E threshold is never reached. To test the consequence to cell cycle progression of reduced but significant levels of cyclin E function, we used two hypomorphic mutations of cyclin E: *cyclin E⁰⁵²⁰⁶*, a P-element insertion mutation that eliminates cyclin E expression in endocycling cells but not in the CNS (Fig. 3C), and *cyclin E^{P28}*, an EMS-induced mutation. We examined BrdU incorporation in the anterior midgut cells of *cyclin E⁰⁵²⁰⁶/cyclin E^{P28}* transheterozygous mutant embryos. At stage 13, no BrdU incorporation was detected (Fig. 5C). These cells are therefore still in G₁ of cycle 17 at a time when wild-type cells have already begun S phase 17 (Fig. 5A). However, at stage 14 we could detect BrdU incorporation in some of the anterior midgut cells (Fig. 5F). The number of BrdU-positive cells varied from embryo to embryo, and the distribution of cells within the tissue was random. In sharp contrast to the results with the *dDP* and *dE2F* mutations, the level of BrdU staining in those nuclei that had made the transition to S phase was just as intense as in the wild type. We interpret this phenotype as a delay of the G₁-S transition, or a reduction of the probability of undergoing this transition, and suggest that it reflects a partial

defect in the execution of an all-or-nothing event leading to S phase (Fig. 7).

Strikingly, a small number of *cyclin E⁰⁵²⁰⁶/cyclin E^{P28}* progeny eclosed as adult flies (12 out of 317 total progeny [3.7%]). These flies were sterile, had rough eyes, and had incomplete formation of the fifth wing vein. Therefore, this combination of *cyclin E* alleles is semilethal. This result indicates that dramatic alterations to the timing of the G₁-S transition at embryonic stages is still compatible with a substantial amount of development.

Drosophila cyclin E inhibits its own expression in endocycles by one or more negative feedback loops (16, 41, 53), and mammalian cyclin E has been shown to promote its own destruction (9, 68). As a result, cyclin E is ordinarily expressed only transiently at the G₁-S transition. We examined the behavior of *cyclin E* expression in *cyclin E⁰⁵²⁰⁶/cyclin E^{P28}* embryos and found that these embryos activate *cyclin E* transcription (presumably from the mutant *cyclin E^{P28}* chromosome) at the correct time in cycle 17 in the midgut but fail to terminate transcription at the normal time (Fig. 3D). This, and similar results with *cyclin E* null mutants (16, 53), suggests that the normal down regulation of cyclin E synthesis involves negative feedback. There appear to be multiple components to this negative feedback. Cyclin E acts particularly effectively as a repressor of its own expression (53), and in a separate step it plays a role in the down regulation of dE2F-dDP-dependent transcription in general (16). This mode of regulation buffers the effect of mutation on cyclin E function in that mutant alleles of *cyclin E* are expressed for a longer time and mRNA (and presumably the mutant protein it encodes) can accumulate to higher levels (Fig. 3D). Therefore, we propose that part of the delay in progress to S phase in the *cyclin E⁰⁵²⁰⁶/cyclin E^{P28}* mutant is due to an extension in the time required for mutant cyclin E protein to achieve a critical threshold of cyclin E function (e.g., activating *cdk2*).

dE2F/dDP contributes to the all-or-nothing transition into S phase. Based on the characteristic defects seen in hypomorphic conditions for *cyclin E* and *dE2F*, and in the *dDP^{vr10}* mutant, it appears that cyclin E has important inputs into an all-or-nothing event required for transition to S phase while dE2F-dDP makes a quantitative contribution to functions required for S phase. However, our work has demonstrated that cyclin E expression requires *dE2F* and *dDP* at the G₁-S transition of cycle 17. We consequently expected that dE2F-dDP should also make a contribution to the G₁-S phase trigger. Such a contribution might be masked if a relatively high level of dE2F-dDP activity were required to fully satisfy the quantitative contribution to S phase while a very low level of activity of dE2F-dDP provided sufficient cyclin E to satisfy the threshold of the all-or-nothing event. Indeed, further characterization of the *dDP* and *dE2F* mutant phenotypes suggests that this is the case.

As we described above, cells that enter S phase 17 relatively early (e.g., anterior midgut cells) in the wild type do so at approximately the normal time in the *dDP^{vr10}* mutant. However, the ensuing S phase is prolonged in the mutant and displays a reduced rate of BrdU incorporation. If a low level of maternal dDP persists until the time that tissues begin to enter S phase 17, one might expect that, as a result of continued decay, cells which enter S phase early would benefit from a higher level of this maternal dDP than cells which enter S phase at a later stage. Consistent with this, S phase 18 in the central midgut (Fig. 5D), which begins about 2 h after the beginning of S phase 17 in the anterior midgut, seems to be more dramatically affected in the *dDP^{vr10}* mutant embryos (Fig. 5E). Importantly, the timing of entry into S phase 18 is

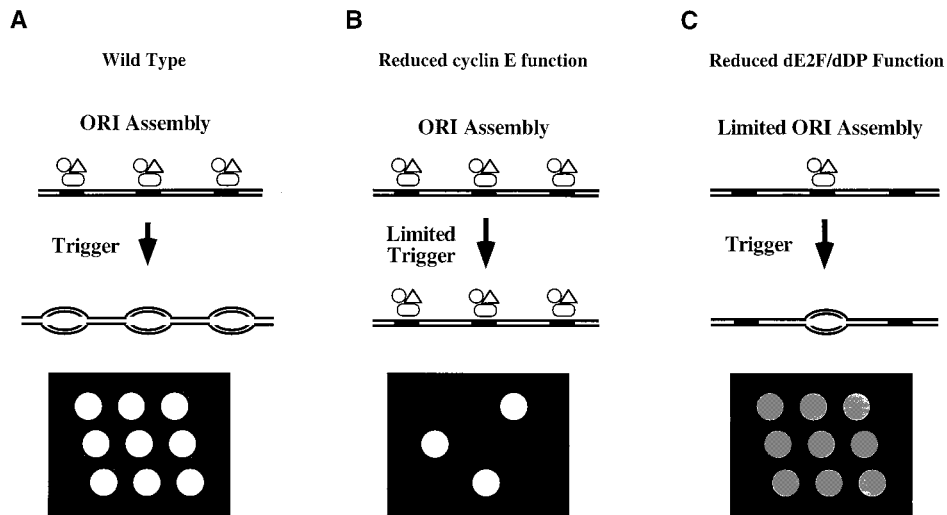


FIG. 7. Model of dE2F-dDP and cyclin E function at the G_1 -S transition and schematic representation of the G_1 -S transition and BrdU incorporation in the embryonic midgut. (A) Wild type. Components of the prereplicative complex (open symbols) assemble on chromosomes (parallel lines) at origins of DNA replication (solid rectangles). Since E2F-DP is required for the transcription of genes encoding known components of prereplicative complexes like dMCM3 in *Drosophila* and HsOrcl in humans (46), we propose that dE2F-dDP contributes to prereplicative complex assembly. Cells remain in G_1 until they are induced to progress through the cell cycle by a developmental signal. DNA synthesis is then triggered at the beginning of S phase via cyclin E action, and bidirectional replication forks appear in the chromosome (ovals). After BrdU pulse labeling and in situ immunofluorescent detection of incorporated BrdU, the nuclei in a field of cells that synchronously entered S phase stain brightly and uniformly (nine open circles on the black background). (B) Reduced cyclin E function. Prereplicative complexes form normally at a frequency identical to that of the wild type, because dE2F-dDP is activated independently of cyclin E (16). If cyclin E function is eliminated, replication is not triggered and cells remain in G_1 . If the cyclin E function is reduced but not eliminated, S phase will still be triggered provided cyclin E function can eventually achieve the critical threshold. In the latter situation, G_1 is prolonged and DNA replication is normal once S phase begins. In a field of cells of this type, the length of G_1 might vary stochastically as cells approach the S phase threshold. The pattern of BrdU-labeled nuclei would appear random, and each nucleus would have the wild-type intensity of staining (three open circles instead of nine on the black background). (C) Reduced dE2F-dDP function. Decreased provision of components causes limited assembly of prereplicative complexes. If those that assemble are triggered at the usual time in development, the length of G_1 does not change. However, fewer prereplicative complexes initiate fewer bidirectional replication forks. Consequently, S phase is prolonged and BrdU incorporation during pulse labeling is significantly reduced. A field of cells of this type appears uniformly and weakly labeled (nine shaded circles on the black background). (Note that the symbols designating components of the prereplicative complex are not shown on the DNA after bidirectional DNA synthesis has begun for the purpose of clarity. Some components of this complex [e.g., ORC] are thought to remain bound to the DNA throughout the entire cell cycle [11].)

affected as well as the efficiency of replication. In stage 14, only a few cells in the mutant's central midgut have begun to incorporate BrdU (Fig. 5E) compared to the number in wild-type embryos (Fig. 5D). These cells have either been arrested or they initiate S phase at a much later time than the wild type (i.e., beyond the stages we have examined). Moreover, the few cells that do incorporate BrdU are stained weakly, again consistent with a reduced rate of DNA replication once S phase is initiated. A very similar phenotype is seen in the same cells of *dE2F* mutant embryos (Fig. 6). These data suggest that dE2F-dDP contributes both to the correct timing of the G_1 -S transition and to the efficiency of S phase.

DISCUSSION

In this report we describe the identification and characterization of mutations of the *Drosophila* *dDP* gene, which encodes a component of the E2F-DP transcription factor. *dDP* is required for activation of expression of replication genes at the G_1 -S transition in embryonic cells, and mutants have an attenuated and sometimes delayed S phase. Characterization of BrdU incorporation in different mutant backgrounds and at different embryonic stages suggests that reductions in dE2F-dDP over a large range results in graded attenuation of DNA replication and that only severe reductions in dE2F-dDP function delay or block entry into S phase. In contrast, partial loss of function of cyclin E results in a delay of S-phase entry without detectable diminution of BrdU incorporation. We suggest that dE2F-dDP makes two different kinds of contributions to DNA replication. It drives the expression of numerous rep-

lication functions, some of which are limiting for the rate of DNA replication. Additionally, it drives the expression of at least one product, cyclin E, that is required to trigger an all-or-nothing transition from G_1 into S phase.

dE2F and dDP collaborate to activate G_1 -S transcription. A substantial amount of biochemical evidence indicates that E2F-DP heterodimers function as a DNA binding transcription factor that contributes to the activation of transcription at the G_1 -S transition (reviewed in references 37 and 59). Our previous genetic analysis had demonstrated that the *Drosophila* *dE2F* gene is essential for transcriptional activation of several replication genes at the G_1 -S transition in embryos. The present work demonstrates that mutation of the *Drosophila* *dDP* gene also eliminates this activation of expression for the *DmRNR2* and *cyclin E* genes. While this article was undergoing review, Royzman et al. reported similar transcriptional phenotypes due to mutation of *dDP* (52). The parallel defects in the transcription of several genes are consistent with the joint action of dE2F and dDP in this aspect of their function. Several experiments imply that transcriptional activation is likely a result of a direct interaction between dE2F-dDP and *cis*-acting elements in the promoters of replication genes. Expression of the *dE2F* cDNA stimulates the DNA polymerase α promoter in cultured *Drosophila* cells (48). Furthermore, dE2F-dDP binding sites found upstream of the *PCNA* transcriptional start site are required for reporter gene expression in cell culture and β -galactosidase activity in embryo extracts prepared from flies carrying *PCNA*-promoter-*lacZ* fusion transgenes (71).

Is normal regulation of S phase essential? The *vr10* allele of *dDP* produced some adult flies when it was heterozygous to a

deficiency for the locus. While it is possible that this result is in part due to residual function of this mutant allele, it is clear that this same allele reduces G₁-S transcription of various replication genes to levels below detection and substantially compromises replication in the embryonic S phases that we have examined. Similarly, a *dE2F* hypomorphic allele that alters embryonic S phases is viable, and a combination of *cyclin E* alleles that greatly altered the embryonic S-phase program (Fig. 5F) nonetheless gave viable flies. Based on these observations, we suggest that most aspects of patterning, growth, and differentiation are regulated in ways that are not easily disturbed by alterations in the G₁-S program. Note that adult *dDP^{v710}* mutant flies were rare, defective, and infertile. Hence the activity of the gene and presumably the control of S phase is clearly of some relevance to development, but it is nonetheless surprising that an intact fly can be produced when defects in S phase can be scored in midembryogenesis.

Is dE2F-dDP activity essential to S phase? We previously reported that the compromised embryonic S phases in a *dE2F* mutant indicated that dE2F function was required for the G₁-S transition (17). However, here we have shown that the available *dE2F* and *dDP* mutations have their primary effect on the efficiency of S phase. If dE2F-dDP is an integral part of the normal cascade of signaling that triggers S phase, we might expect that the mutations would result in an absolute block to S phase rather than a reduction in its efficiency. However, we find that many cells enter a compromised S phase 17 according to a normal schedule. Nonetheless, we cannot conclude that dE2F-dDP function is dispensable for the G₁-S transition, because it is possible that the threshold of dE2F-dDP activity required to drive the transition is lower than the levels of dE2F-dDP required for maximal efficiency of DNA replication.

There are three possible sources of dE2F-dDP activity in the mutant embryos. First, it appears that a small amount of maternally expressed protein persists to late embryonic stages (Fig. 1B). Second, the mutations might not remove all activity of the gene. Third, there may be homologs that provide a comparable activity. Indeed, sequences of random cDNAs (ESTs) by the Berkeley *Drosophila* Genome Project uncovered the existence of a second dE2F homolog (28). This and perhaps other undetected homologs of dE2F and/or dDP might contribute to dE2F-dDP activity in the mutant backgrounds. Hence, while our experiments allow us to say that mutations that reduce dE2F-dDP activity affect S phase, it is more difficult to address whether DNA replication seen in these mutant backgrounds depends on a low level of residual dE2F-dDP activity from one or more of these sources.

One important observation leads us to suggest that dE2F-dDP contributes to the transition from G₁ to S as well as to the efficiency of DNA replication. Analysis of a later S phase (S phase 18 in the central midgut), when maternal functions have further decayed, shows that entry into S phase is delayed and variable. Additionally, an involvement of dE2F-dDP in the G₁-S transition is also suggested by the involvement of the dE2F-dDP-stimulated gene *cyclin E* in triggering the G₁-S transition (see below) and by the extraordinary correlation between the schedule of activation of dE2F-dDP and the developmental program of S phases. We consequently favor the interpretation that dE2F-dDP drives expression of *cyclin E* at the G₁-S transition and that cyclin E is essential for this transition.

Our favored interpretation might seem strained because it requires that undetectable levels of *cyclin E* RNA trigger S phase. However, the genetics indicates that zygotic expression of *cyclin E* is absolutely required for S phase 17 (33). Hence,

regardless of our failure to detect *cyclin E* RNA in the *dE2F* and *dDP* mutant embryos, sufficient cyclin E protein must accumulate to allow S phase. How can undetectable levels of *cyclin E* mRNA produce sufficient cyclin E protein to trigger S phase? We suggest that particular aspects of cyclin E regulation might allow gradual accumulation of cyclin E protein at very low levels of mRNA. Cyclin E inhibits its own dE2F-dDP-mediated transcription in embryos (16, 53). Moreover, mammalian cyclin E protein appears to drive its own destruction through ubiquitin-mediated proteolysis (9, 68). Hence, once expression begins cyclin E will accumulate until it reaches levels that activate these negative feedback loops. As the level of *cyclin E* RNA is reduced, the rate of accumulation of cyclin E protein will decline, but it will accumulate over a longer period and ultimately reach its threshold levels. Accordingly, we suggest that severe reductions in dE2F-dDP activity would be required before one could detect a substantial defect in the G₁-S transition. In addition, it is also possible that in the complete absence of dE2F-dDP, low basal expression of *cyclin E* might allow accumulation of cyclin E protein over a very long time. In this case, entry into S phase would not be timed properly, but could still occur. Such dE2F-dDP-independent expression of *cyclin E* might sustain the growth of *dE2F* and *dDP* mutant larvae, as reported by Royzman et al. (52).

The dE2F-dDP contribution to the efficiency of DNA replication. In *S. cerevisiae*, limitation of components that bind replication origins (e.g., ORC and MCM proteins) causes a reduced rate of replication that is associated with reduced origin utilization (reviewed in reference 11). In *Drosophila*, reduction in the function of dMCM2 results in a prolongation of S phase consistent with such a reduction in origin firing (66). We suggest an analogous interpretation of the phenotype caused by reduction of dE2F-dDP function (Fig. 7). In both the *dDP* and *dE2F* mutant embryos the first defect in DNA replication that appears is a reduction in the intensity of BrdU incorporation. An increase in the length of S phase 17 observed in the midgut argues that this decreased incorporation is associated with a reduced rate of DNA replication. Although we have not identified a specific factor whose expression is rate limiting for these embryonic S phases, we have demonstrated that *dE2F* and *dDP* are required for the expression of several genes encoding replication factors. We propose that one or more replication factors become limiting when dE2F-dDP function is compromised and that the consequence is a reduction in the number of forks that successfully initiate replication.

Cyclin E provides an S-phase threshold. Evidence from many different systems indicates that cyclin E-cdk2 activity is required for cell cycle progression (reviewed in reference 29). In what way is cyclin E-cdk2 activity required for S phase? Here we have shown that *Drosophila* cyclin E provides a threshold for entry into S phase. By reducing but not eliminating cyclin E function by a hypomorphic combination of alleles, we showed that endoreduplicating embryonic midgut cells fail to execute a developmentally controlled G₁-S transition on schedule in cell cycle 17. But rather than remaining in G₁ arrest indefinitely, these cells subsequently enter S phase. In this delayed S phase, BrdU incorporation appears normal, probably because dE2F-dDP is independently activated in these cells and the replication machinery other than cyclin E is not limiting (17). The midgut cells do not all enter S phase at the same time, however, but rather at random times. We interpret this stochastic entry into S phase in cycle 17 as evidence for the participation of cyclin E in a molecular threshold. That is, when cyclin E, and by extension cyclin E-cdk2 activity, reaches a critical level S phase is triggered in an all-or-nothing

manner. This will only be true if cyclin E function can continue to accumulate during the extended G_1 period. Consistent with this, the mutant cyclin E gene is activated at the normal developmental time and continues to be expressed during the extended G_1 period due to a failure to activate its own down regulation (see above).

The molecular mechanism behind such an all-or-nothing threshold is not clear. Positive feedback amplification has been a popular proposal for controlling all-or-nothing cell cycle transitions. The biological activities of mammalian and *Drosophila* cyclin E and E2F-DP can formally be placed into a positive feedback loop: overexpression of cyclin E activates E2F-DP, probably due to cyclin E-cdk2 phosphorylation of pRB, and E2F-DP activates *cyclin E* transcription. This type of positive feedback loop makes an attractive model for control of the restriction point (10, 23, 57), the time during G_1 that mammalian cells commit to S-phase entry (50). Similar types of interactions between the *S. cerevisiae* Swi4/6 transcription factor and G_1 cyclin genes (*CLNs*) can be demonstrated (43, 45). However, during START, the yeast equivalent of the restriction point, careful genetic experiments have ruled out positive feedback amplification between CLN1 or CLN2 and the Swi4/6 transcription factor (12, 63). Similarly, in *Drosophila* endocycling cells the genetic evidence indicates that there is no positive feedback amplification between cyclin E and dE2F-dDP at the G_1 -S transition, although each can induce the activity of the other when overexpressed (14, 16). The lack of positive feedback appears to be the consequence of negative feedback mechanisms involving cyclin E, as described above (16, 41, 53). These overcome any contribution of cyclin E to positive feedback. Thus, because cyclin E does not make a positive contribution to its own expression in our experimental context, cyclin E provides a threshold for entry into S phase by a mechanism that does not require positive feedback between cyclin E and dE2F-dDP.

What then is the function of cyclin E? After formation of prereplicative complexes during G_1 , and a commitment to progress into S phase, a poorly described mechanism exists that triggers the initiation of DNA replication. In vitro experiments suggest that cdk activity could comprise part of the trigger mechanism. In frog and human extracts, cyclin E-cdk2 and cyclin A-cdk2 are required for DNA synthesis (20, 31, 35, 62). These kinases presumably have substrates that must be phosphorylated in order for replication origins to fire. Moreover, cyclin E has an essential S-phase function that is independent of E2F-DP-mediated transcription (14, 42). Whatever the critical substrates are, our data suggest that cyclin E-cdk2 must be part of a mechanism that in vivo has an all-or-nothing response. Cyclin E could activate an amplifying kinase cascade, trigger changes in protein degradation, or affect DNA unwinding and initiation directly at origins of replication.

ACKNOWLEDGMENTS

We thank Nick Dyson for *dDP* cDNAs and anti-dDP monoclonal antibodies, Ting Wu for mutant stocks from the 49F region, Carole Seum and Pierre Spierer for the *dE2F¹⁶⁴* mutant stock, Shelagh Campbell and Tin Tin Su for library cDNA and primers, Peter Follette for *dMCM3* cDNA, Terry Orr-Weaver for communicating results prior to publication, and Mark Peifer and Jenny Adam for comments on the manuscript.

This work was initiated while R.J.D. was supported by a fellowship from the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation (DRG-1161). This work was funded in part by a grant from the National Institutes of Health to P.H.O. (GM37193).

REFERENCES

- Andrews, B. J., and I. Herskowitz. 1990. Regulation of cell cycle-dependent gene expression in yeast. *J. Biol. Chem.* **265**:14057-14060.
- Ashburner, M., P. Thompson, J. Roote, P. F. Lasko, Y. Grau, M. El Messal, S. Roth, and P. Simpson. 1990. The genetics of a small autosomal region of *Drosophila melanogaster* containing the structural gene for alcohol dehydrogenase. VII. Characterization of the region around the *snail* and *cactus* loci. *Genetics* **126**:679-694.
- Bandara, L. R., V. M. Buck, M. Zamanian, L. H. Johnston, and T. N. La. 1993. Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F. *EMBO J.* **12**:4317-4324.
- Botz, J., K. Zerfass-Thome, D. Spitkovsky, H. Delius, B. Vogt, M. Eilers, A. Hatzigeorgiou, and P. Jansen-Durr. 1996. Cell cycle regulation of the murine cyclin E gene depends on an E2F binding site in the promoter. *Mol. Cell. Biol.* **16**:3401-3409.
- Breeden, L. 1996. Start-specific transcription in yeast. *Curr. Top. Microbiol. Immunol.* **188**:95-127.
- Brook, A., J. E. Xie, W. Du, and N. Dyson. 1996. Requirements for dE2F function in proliferating cells and in post-mitotic differentiating cells. *EMBO J.* **15**:3676-3683.
- Brown, N. H., and F. C. Kafatos. 1988. Functional cDNA libraries from *Drosophila* embryos. *J. Mol. Biol.* **203**:425-437.
- Chong, J. P., P. Thommes, and J. J. Blow. 1996. The role of MCM/P1 proteins in the licensing of DNA replication. *Trends Biochem. Sci.* **21**:102-106.
- Clurman, B. E., R. J. Sheaff, K. Thress, M. Groudine, and J. M. Roberts. 1996. Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by cdk2 binding and cyclin phosphorylation. *Genes Dev.* **10**:1979-1990.
- DeGregori, J., T. Kowalik, and J. R. Nevins. 1995. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G_1 /S-regulatory genes. *Mol. Cell. Biol.* **15**:4215-4224.
- Diffley, J. F. 1996. Once and only once upon a time: specifying and regulating origins of DNA replication in eukaryotic cells. *Genes Dev.* **10**:2819-2830.
- Dirick, L., T. Bohm, and K. Nasmyth. 1995. Roles and regulation of Cln-Cdc28 kinases at the start of the cell cycle of *Saccharomyces cerevisiae*. *EMBO J.* **14**:4803-4813.
- Du, W., M. Vidal, J. E. Xie, and N. Dyson. 1996. RBF, a novel RB-related gene that regulates E2F activity and interacts with cyclin E in *Drosophila*. *Genes Dev.* **10**:1206-1218.
- Duronio, R. J., A. Brook, N. Dyson, and P. H. O'Farrell. 1996. E2F-induced S phase requires cyclin E. *Genes Dev.* **10**:2505-2513.
- Duronio, R. J., and P. H. O'Farrell. 1994. Developmental control of a G_1 -S transcriptional program in *Drosophila*. *Development* **120**:1503-1515.
- Duronio, R. J., and P. H. O'Farrell. 1995. Developmental control of the G_1 to S transition in *Drosophila*: cyclin E is a limiting downstream target of E2F. *Genes Dev.* **9**:1456-1468.
- Duronio, R. J., P. H. O'Farrell, J. E. Xie, A. Brook, and N. Dyson. 1995. The transcription factor E2F is required for S phase during *Drosophila* embryogenesis. *Genes Dev.* **9**:1445-1455.
- Dynlacht, B. D., A. Brook, M. Dembski, L. Yenush, and N. Dyson. 1994. DNA-binding and trans-activation properties of *Drosophila* E2F and DP proteins. *Proc. Natl. Acad. Sci. USA* **91**:6359-6363.
- Edgar, B. A., and P. H. O'Farrell. 1990. The three postblastoderm cell cycles of *Drosophila* embryogenesis are regulated in G_2 by string. *Cell* **62**:469-480.
- Fang, F., and J. W. Newport. 1991. Evidence that the G_1 -S and G_2 -M transitions are controlled by different cdc2 proteins in higher eukaryotes. *Cell* **66**:731-742.
- Feger, G., H. Vaessin, T. T. Su, E. Wolff, L. Y. Jan, and Y. N. Jan. 1995. dpa, a member of the MCM family, is required for mitotic DNA replication but not endoreplication in *Drosophila*. *EMBO J.* **14**:5387-5398.
- Field, S. J., F. Y. Tsai, F. Kuo, A. M. Zubiaga, W. G. Kaelin, Jr., D. M. Livingston, S. H. Orkin, and M. E. Greenberg. 1996. E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell* **85**:549-561.
- Follette, P., and P. H. O'Farrell. Unpublished data.
- Geng, Y., E. N. Eaton, M. Picon, J. M. Roberts, A. S. Lundberg, A. Gifford, C. Sardet, and R. A. Weinberg. 1996. Regulation of cyclin E transcription by E2Fs and retinoblastoma protein. *Oncogene* **12**:1173-1180.
- Girling, R., L. R. Bandara, E. Ormondroyd, E. W. Lam, S. Kotecha, T. Mohun, and N. B. La Thangue. 1994. Molecular characterization of *Xenopus laevis* DP proteins. *Mol. Biol. Cell* **5**:1081-1092.
- Gloor, G. B., C. R. Preston, D. M. Johnson-Schlitz, N. A. Nassif, R. W. Phillis, W. K. Benz, H. M. Robertson, and W. R. Engels. 1993. Type I repressors of P element mobility. *Genetics* **135**:81-95.
- Gong, J., F. Traganos, and Z. Darzynkiewicz. 1995. Threshold expression of cyclin E but not D type cyclins characterizes normal and tumour cells entering S phase. *Cell Prolif.* **28**:337-346.
- Hao, X. F., L. Alphey, L. R. Bandara, E. W. Lam, D. Glover, and N. B. La Thangue. 1995. Functional conservation of the cell cycle-regulating transcription factor DRTF1/E2F and its pathway of control in *Drosophila melanogaster*. *J. Cell Sci.* **108**:2945-2954.
- Harvey, D., L. Hong, M. Evans-Holm, J. Pendleton, C. Su, P. Brokstein, S. Lewis, and G. M. Rubin. 1997. Clone #LD02934. Berkeley *Drosophila* Genome Project, Berkeley, Calif.
- Heichman, K. A., and J. M. Roberts. 1994. Rules to replicate by. *Cell* **79**:557-562.

30. Helin, K., C. L. Wu, A. R. Fattaey, J. A. Lees, B. D. Dynlacht, C. Ngwu, and E. Harlow. 1993. Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative trans-activation. *Genes Dev.* 7:1850-1861.
31. Jackson, P. K., S. Chevalier, M. Philippe, and M. W. Kirschner. 1995. Early events in DNA replication require cyclin E and are blocked by p21CIP1. *J. Cell Biol.* 130:755-769.
32. Karpen, G. H., and A. C. Spradling. 1992. Analysis of subtelomeric heterochromatin in the *Drosophila* minichromosome DP1187 by single P-element insertional mutagenesis. *Genetics* 132:737-753.
33. Knoblich, J. A., K. Sauer, L. Jones, H. Richardson, R. Saint, and C. F. Lehner. 1994. Cyclin E controls S phase progression and its down-regulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation. *Cell* 77:107-120.
34. Krek, W., D. M. Livingston, and S. Shirodkar. 1993. Binding to DNA and the retinoblastoma gene product promoted by complex formation of different E2F family members. *Science* 262:1557-1560.
35. Krude, T., M. Jackman, J. Pines, and R. A. Laskey. 1997. Cyclin/Cdk-dependent initiation of DNA replication in a human cell-free system. *Cell* 88:109-119.
36. Lasko, P. F., and M. L. Pardue. 1988. Studies of the genetic organization of the vestigial microregion of *Drosophila melanogaster*. *Genetics* 120:495-502.
37. La Thangue, N. B. 1994. DP and E2F proteins: components of a heterodimeric transcription factor implicated in cell cycle control. *Curr. Opin. Cell Biol.* 6:443-450.
38. Lehner, C. F., and P. H. O'Farrell. 1990. The roles of *Drosophila* cyclins A and B in mitotic control. *Cell* 61:535-547.
39. Leno, G. H., and R. A. Laskey. 1991. The nuclear membrane determines the timing of DNA replication in *Xenopus* egg extracts. *J. Cell Biol.* 112:557-566.
40. Liang, C., M. Weinreich, and B. Stillman. 1995. ORC and Cdc6p interact and determine the frequency of initiation of DNA replication in the genome. *Cell* 81:667-676.
41. Lilly, M. A., and A. C. Spradling. 1996. The *Drosophila* endocycle is controlled by Cyclin E and lacks a checkpoint ensuring S-phase completion. *Genes Dev.* 10:2514-2526.
42. Lukas, J., T. Herzinger, K. Hansen, M. C. Moroni, D. Resnitzky, K. Helin, S. I. Reed, and J. Bartek. 1997. Cyclin E-induced S phase without activation of the pRb/E2F pathway. *Genes Dev.* 11:1479-1492.
43. Nasmyth, K., and L. Dirick. 1991. The role of SW14 and SW16 in the activity of G1 cyclins in yeast. *Cell* 66:995-1013.
44. Nevins, J. R. 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science* 258:424-429.
45. Ogas, J., B. J. Andrews, and I. Herskowitz. 1991. Transcriptional activation of CLN1, CLN2, and a putative new G1 cyclin (HCS26) by SW14, a positive regulator of G1-specific transcription. *Cell* 66:1015-1026.
46. Ohtani, K., J. DeGregori, G. Leone, D. R. Herendeen, T. J. Kelly, and J. R. Nevins. 1996. Expression of the *HsOrc1* gene, a human *ORC1* homolog, is regulated by cell proliferation via the E2F transcription factor. *Mol. Cell Biol.* 16:6977-6984.
47. Ohtani, K., J. DeGregori, and J. R. Nevins. 1995. Regulation of the cyclin E gene by transcription factor E2F1. *Proc. Natl. Acad. Sci. USA* 92:12146-12150.
48. Ohtani, K., and J. R. Nevins. 1994. Functional properties of a *Drosophila* homolog of the E2F1 gene. *Mol. Cell Biol.* 14:1603-1612.
49. Ormondroyd, E., S. de la Luna, and N. B. La Thangue. 1995. A new member of the DP family, DP-3, with distinct protein products suggests a regulatory role for alternative splicing in the cell cycle transcription factor DRTF1/E2F. *Oncogene* 11:1437-1446.
50. Pardee, A. B. 1989. G1 events and regulation of cell proliferation. *Science* 246:603-608.
51. Richardson, H. E., L. V. O'Keefe, S. I. Reed, and R. Saint. 1993. A *Drosophila* G1-specific cyclin E homolog exhibits different modes of expression during embryogenesis. *Development* 119:673-690.
52. Royzman, I., A. J. Whittaker, and T. L. Orr-Weaver. 1997. Mutations in *Drosophila* DP and E2F distinguish G1-S progression from an associated transcriptional program. *Genes Dev.* 11:1999-2011.
53. Sauer, K., J. A. Knoblich, H. Richardson, and C. F. Lehner. 1995. Distinct modes of cyclin E/cdc2c kinase regulation and S-phase control in mitotic and endoreplication cycles of *Drosophila* embryogenesis. *Genes Dev.* 9:1327-1339.
54. Schubiger, M., and J. Palka. 1987. Changing spatial patterns of DNA replication in the developing wing of *Drosophila*. *Dev. Biol.* 123:145-153.
55. Schwob, E., and K. Nasmyth. 1993. CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in *Saccharomyces cerevisiae*. *Genes Dev.* 7:1160-1175.
56. Seum, C., A. Spierer, D. Pauli, J. Szidonya, G. Reuter, and P. Spierer. 1996. Position-effect variegation in *Drosophila* depends on dose of the gene encoding the E2F transcriptional activator and cell cycle regulator. *Development* 122:1949-1956.
57. Sherr, C. J. 1996. Cancer cell cycles. *Science* 274:1672-1677.
58. Sherr, C. J. 1994. G1 phase progression: cycling on cue. *Cell* 79:551-555.
59. Slansky, J. E., and P. J. Farnham. 1996. Introduction to the E2F family: protein structure and gene regulation. *Curr. Top. Microbiol. Immunol.* 208:1-30.
60. Smith, A. V., and T. L. Orr-Weaver. 1991. The regulation of the cell cycle during *Drosophila* embryogenesis: the transition to polyteny. *Development* 112:997-1008.
61. Stillman, B. 1996. Cell cycle control of DNA replication. *Science* 274:1659-1664.
62. Strausfeld, U. P., M. Howell, P. Descombes, S. Chevalier, R. E. Rempel, J. Adamczewski, J. L. Maller, T. Hunt, and J. J. Blow. 1996. Both cyclin A and cyclin E have S-phase promoting (SPF) activity in *Xenopus* egg extracts. *J. Cell Sci.* 109:1555-1563.
63. Stuart, D., and C. Wittenberg. 1995. CLN3, not positive feedback, determines the timing of CLN2 transcription in cycling cells. *Genes Dev.* 9:2780-2794.
64. Su, T. T., N. Yakubovich, and P. H. O'Farrell. 1997. Cloning of *Drosophila* MCM homologs and analysis of their requirement during embryogenesis. *Gene* 192:283-289.
65. Tautz, D., and C. Pfeifle. 1989. A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene hunchback. *Chromosoma* 98:81-85.
66. Treisman, J. E., P. J. Follette, P. H. O'Farrell, and G. M. Rubin. 1995. Cell proliferation and DNA replication defects in a *Drosophila* MCM2 mutant. *Genes Dev.* 9:1709-1715.
67. Tye, B.-K. 1994. The MCM2-3-5 proteins: are they replication licensing factors? *Trends Cell Biol.* 4:160-166.
68. Won, K. A., and S. I. Reed. 1996. Activation of cyclin E/CDK2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. *EMBO J.* 15:4182-4193.
69. Wu, C.-L., M. Classon, N. Dyson, and E. Harlow. 1996. Expression of dominant-negative mutant DP-1 blocks cell cycle progression in G₁. *Mol. Cell Biol.* 16:3698-3706.
70. Wu, C.-L., L. R. Zukerberg, C. Ngwu, E. Harlow, and J. A. Lees. 1995. In vivo association of E2F and DP family proteins. *Mol. Cell Biol.* 15:2536-2546.
71. Yamaguchi, M., Y. Hayashi, and A. Matsukage. 1995. Essential role of E2F recognition sites in regulation of the proliferating cell nuclear antigen gene promoter during *Drosophila* development. *J. Biol. Chem.* 270:25159-25165.
72. Yamasaki, L., T. Jacks, R. Bronson, E. Goillot, E. Harlow, and N. J. Dyson. 1996. Tumor induction and tissue atrophy in mice lacking E2F-1. *Cell* 85:537-548.