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Drosophila wee1 Has an Essential Role in the Nuclear Divisions of Early Embryogenesis

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ABSTRACT

In Drosophila, the maternally expressed *mei-41* and *grp* genes are required for successful execution of the nuclear division cycles of early embryogenesis. In fission yeast, genes encoding similar kinases (*rad3* and *chk1*, respectively) are components of a cell cycle checkpoint that delays mitosis by inhibitory phosphorylation of Cdk1. We have identified mutations in a gene encoding a Cdk1 inhibitory kinase, *Drosophila wee1* (*Dwee1*). Like *mei-41* and *grp*, *Dwee1* is zygotically dispensable but is required maternally for completing the embryonic nuclear cycles. The arrest phenotype of *Dwee1* mutants, as well as genetic interactions between *Dwee1*, *grp*, and *mei-41* mutations, suggest that *Dwee1* is functioning in the same regulatory pathway as these genes. These findings imply that inhibitory phosphorylation of Cdk1 by Dwee1 is required for proper regulation of the early syncytial cycles of embryogenesis.

THE nuclear division cycles of early Drosophila embryogenesis provide a formidable challenge to the cell cycle regulatory machinery. Initially, these cycles consist of extraordinarily rapid oscillations between S phase (2–3 min) and mitosis (5–6 min) that utilize maternally provided protein and RNA and occur within a syncytium. Interphase length then increases progressively during cycles 10–13 prior to the midblastula (MBT) or maternal-zygotic (MZT) transition in cycle 14 that precedes the onset of gastrulation (Foe and Alberts 1983). During normal embryogenesis, the developmentally regulated lengthening of interphase during cycles 10–13 requires maternally provided *mei-41* and *grp* encoded kinases (Sibon *et al.* 1997, 1999). These genes encode homologs of the evolutionarily conserved checkpoint kinases Rad3/ATM and Chk1 (Hari *et al.* 1995; Fogarty *et al.* 1997). In fission yeast and humans, these kinases are components of a premitotic checkpoint that becomes activated in response to incompletely replicated or damaged DNA (Jimenez *et al.* 1992; Walworth *et al.* 1993; Beamish *et al.* 1996; Bentley *et al.* 1996; Walworth and Bernards 1996; Sanchez *et al.* 1997). Activation of the checkpoint is associated with inhibitory phosphorylation of Cdk1 (the central mitotic regulatory kinase) on a conserved tyrosine residue (Y15) by Wee1 kinases (O'Connell *et al.* 1997; Rhind *et al.* 1997; Rhind and Russell 1998). In response to DNA damage, Chk1 becomes activated (in a Rad3-dependent fashion) and phosphorylates Cdc25 on a residue that promotes Cdc25 interaction with members of the 14-3-3 family of proteins (Peng *et al.* 1997; Sanchez *et al.* 1997; Zeng *et al.* 1998; Chen *et al.* 1999; Lopez-Girona *et al.* 1999). This interaction prevents Cdc25 phosphatase from interacting with and activating Cdk1 by removal of Wee-catalyzed inhibitory phosphorylation. Consequently, either loss of Chk1 function or loss of Wee1 function can compromise the premitotic checkpoint.

The involvement of *mei-41* and *grp* in the slowing of the early embryonic cycles is proposed to reflect activation of a DNA replication checkpoint once maternally provided replication functions become limiting (Fogarty *et al.* 1997; Sibon *et al.* 1997, 1999). The conservation of these checkpoint genes among eukaryotes implies that the biochemical mechanism by which they function might also be conserved. If so, it suggests that Drosophila embryos employ inhibitory phosphorylation of Cdk1 by a Wee1-like kinase to coordinate this developmentally regulated checkpoint. Inhibitory phosphorylation of maternal Cdk1 is not detected in Drosophila embryo extracts during cycles 10–13 when the *mei-41/grp* pathway is required, however (Edgar *et al.* 1994). This observation may indicate that Drosophila embryos employ the *mei-41/grp* pathway in a novel mechanism that is independent of Wee1-mediated inhibitory phosphorylation of Cdk1 to lengthen the syncytial cycles. We undertook a genetic analysis of *Dwee1*, a gene that encodes a Drosophila Cdk1 inhibitory kinase, to investigate these possibilities (Campbell *et al.* 1995).

MATERIALS AND METHODS

Mutagenesis screen for *Dwee1* mutants: *cn/cn* males were mutagenized with either ethyl methanesulfonate (EMS; 25–50 mm) or diepoxybutane (DEB; 5 mm) according to standard protocols and then mated *en masse* to *Sco/CyO, cn* virgin fe-

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males. These flies were transferred onto fresh media daily for 4–5 days, whereupon mutagenized males were removed. F_1 progeny males carrying isolated mutagenized second chromosomes were collected and crossed individually to *Df(2L) Dwee1^{W05}/ CyO, cn* virgin females (the origin and characterization of this deletion is described in the results). The F_2 progeny were then scored for presence of the *cn/ Df(2L) Dwee1^{W05}* class. Absence of this class indicated recovery of a zygotic lethal mutation (lethal alleles were designated *D*L or *E*L, depending on whether DEB or EMS was the relevant mutagen). In crosses where viable hemizygous F_2 progeny were obtained, females of this class were mated to siblings to test for fertility (female-sterile alleles were designated *D*S or *E*S, depending on whether DEB or EMS was the relevant mutagen). Mutant stocks were established by mating of retained *cn/ CyO, cn* siblings. Identified mutants were then further classified by complementation crosses with known mutants in the region and with *Df(2L)spd-J2*, a deletion whose published breakpoints are 27C1-28A (Neumann and Cohen 1996). Our genetic and molecular analysis of a stock carrying this deletion suggests that the distal breakpoint of this aberration is actually 27C4-5.

Transgene rescue experiments: A heat-shock-inducible construct was made by cloning the originally described *Dwee1* cDNA into the pCaSpeR-hs vector (Campbell *et al.* 1995). A genomic DNA construct that includes all of the *Dwee1* coding region plus upstream and downstream flanking DNA was constructed by cloning a ~10-kb *Hin*dIII fragment (sequence coordinates 14,273–24,263 in the DS01321 clone shown in Figure 1) into a pUAS vector (Brand and Perrimon 1993). For the inducible *Dwee1* rescue experiments, embryos were initially collected from mated *Dwee1^{ES1}* hemizygous females carrying a hs*Dwee1* transgene for 4 days without heat shocks. No viable embryos were observed. Flies were then heat-shocked in a 37° water bath once daily as indicated, collecting embryos at ~24-hr intervals (specific conditions are available on request). Fixed embryos (37% formaldehyde:heptane for 3 min) were then stained with Hoechst 33258 for analysis. Expression of the transgene in *Dwee1^{ES1}/ hsDwee1, Df(2L)Dwee1^{W05}* adult females was confirmed by heat-shocking for 30 min (37°), with a 90-min recovery period. These flies were then homogenized in loading buffer (2.0% SDS, 60 mm Tris (pH 6.8), 0.01% bromophenol blue, 10% glycerol, and 0.1 m DTT) and the cleared extract was diluted in loading buffer prior to SDS-PAGE (8% acrylamide). Proteins were then transferred onto Hybond-P membrane and the membrane was probed with a rabbit anti-Dwee1 primary antibody (anti-DKD at 1:1600). The membrane was then reprobed with mouse anti-β-tubulin (1:500; Amersham, Buckinghamshire, UK). Secondary antibody hybridization signal was detected using the ECL+ chemiluminescence system (Amersham).

Responses of *Dwee1* mutants to hydroxyurea: A genetic cross between *Dwee1^{ES1}/ CyO* and *Df(2L)Dwee1^{W05}/ CyO* flies was done, following which 24-hr embryo collections were made. After a further 24 hr of development, 1 ml of aqueous hydroxyurea concentrate was added to the media (values in Figure 5 indicate final concentration). Distilled water (1 ml) was substituted for controls. Adult flies were scored daily once they began to eclose, to completion.

Genomic sequencing and DNA analysis: Genomic DNA was extracted by standard techniques (Campbell *et al.* 1995) from hemizygous adult flies and used as a template for direct amplification using *Pfu* polymerase. The amplified product was then cycle-sequenced on both strands using the Thermosequenase system (Amersham). Mutations were confirmed by independent sequencing reactions. The GenBank accession number for Dwee1 is U17223. Genomic DNA analysis to localize insertions and deletion breakpoints was also done as described in Campbell *et al.* (1995), using digoxygenin-labeled probes according to the manufacturer's recommendations (Boehringer Mannheim, Indianapolis).

RESULTS

A screen for lethal and sterile mutations in genes uncovered by *Df(2L)Dwee1^{WO5}*: Previous studies of the *Dwee1* locus utilized a large deletion, called *Df(2L)Dwee1;27A-28B*, that uncovers *Dwee1* as well as a number of other genes (Campbell *et al.* 1995). These studies established that loss of zygotic *Dwee1* function does not produce a detectable mitotic phenotype in embryos. To generate a smaller deletion for further genetic studies of the locus, transposase-mediated imprecise excision of a nearby *P{w+}* transposon insertion, associated with *l(2)k10413*, was used to generate w− derivative chromosomes (see Figure 1). Both *l(2)k10413* and a nearby P insertion, *l(2)02647*, have been cytologically mapped to position 27C4-5 by the Berkeley Drosophila Genome Project (BDGP) and have been described as mutant alleles of the *hrp48* gene (also called *Hrb27C*) that encodes a ribonuclear splicing factor (Matunis *et al.* 1992; Hammond *et al.* 1997). Flies that are transheterozygous for these two alleles (*l(2)k10413/ l(2)02647*) are viable but usually have small nicks in the wing margin. The w− derivatives generated by mobilization of the *P(w+)* insertion associated with *l(2)k10413* were thus initially screened by failure to complement *l(2)02647* for viability as a means of identifying potential excision events that extended toward the *Dwee1* locus. Stocks carrying noncomplementing alleles were then screened by genomic DNA analysis for molecular aberrations that would indicate the recovery of deletions uncovering the *Dwee1* coding region. A single deletion, *Df(2L)Dwee1^{WO5}*, was identified by this approach. This deletion uncovers the *Dwee1* locus and additional genes shown in Figure 1 that were identified by sequence comparisons of the completed genomic sequence of the region (P1 clone: DS01321, Berkeley Drosophila Genome Project) with the expressed sequence tag (EST) database. *Df(2L) Dwee1^{WO5}* is lethal in transheterozygous combinations with all previously characterized alleles of *hrp48* and with the single *P*-insertion allele of another gene of unknown function designated *l(2)k00213* (mapped to position 27C2-3 by BDGP). It also fails to complement *Df(2L)spd-J2* (Neumann and Cohen 1996). Homozygous *Df(2L)Dwee1^{WO5}* mutants derived from heterozygous parents die late in embryogenesis with no obvious mitotic abnormalities, consistent with previous characterization done with the larger deletion, *Df(2L)Dwee1;27A-28B.* A recombinant chromosome carrying *Df(2L)Dwee1^{WO5}* and a proximal flipase recombinase target (FRT) sequence was generated to investigate maternal requirements for *Dwee1* function (Chou and Perrimon 1996). No eggs were generated from *FLP*-expressing, ovo^D FRT/ *Df(2L)Dwee1^{WO5}*FRT transheterozygous females for analysis, however, from which we could only conclude that

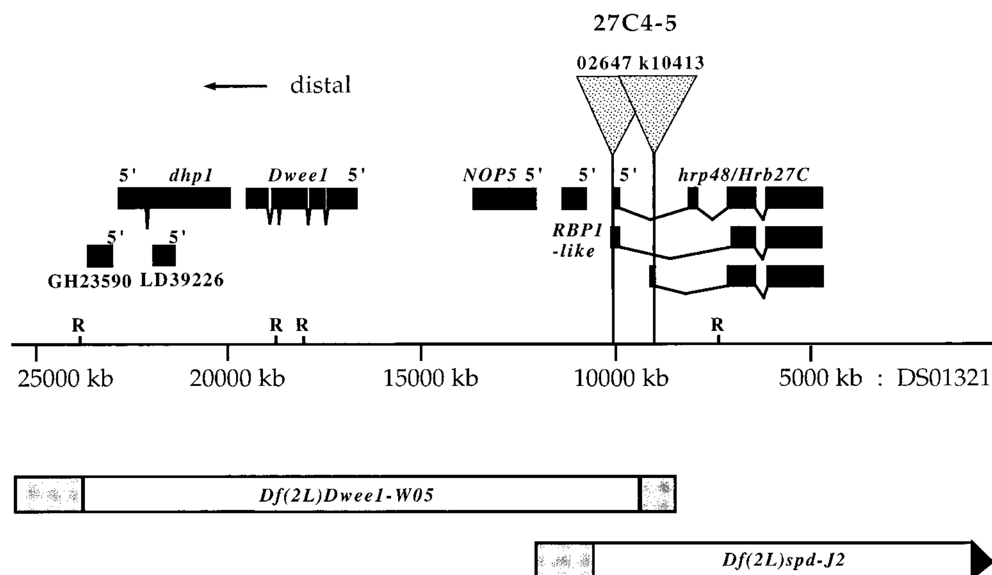

Figure 1.—Genome organization of cytological region 27C4-5 with respect to transcribed genes and the extent and position of deletions and *P*-transposon insertions described in the text. The open horizontal boxes indicate the extent of the two deletions that impinge on this region, with shaded boxes representing regions within which the deletion endpoints were molecularly mapped by genomic hybridization analysis. The numbered coordinates represent genomic DNA sequence positions within the DS01321 clone. The positions of sites for restriction enzyme *Eco*RI are indicated (R) on the horizontal line representing the genomic sequence as a further orientation guide. Triangles connected to the sequence by vertical lines represent the insertion sites of two *P*-transposon insertion alleles of the *hrp48* locus that are described in further detail in the text. BDGP determined the indicated cytological and molecular positions of these *P*-transposon insertions. Solid horizontal lines represent the exon/intron organization and orientation of transcripts identified in the region that correspond to previously sequenced ESTs in the BDGP database. ESTs with open reading frames whose translation products show high sequence similarity to proteins identified in other organisms are named with respect to those homologs. GH23590 and LD39226 represent ESTs that do not show significant homology to other known genes and may correspond to exons of the same gene. The transcript designated *dhp1* is homologous to a gene implicated in recombination and RNA metabolism called dhp1/RAT1/Dhm1 in *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, and mice, respectively (Kenna *et al.* 1993; Shobuike *et al.* 1995; Sugano *et al.* 1994). The transcript designated NOP5 is homologous to a gene of the same name implicated in nucleolar assembly in *S. cerevisiae* (Wu *et al.* 1998). The transcript designated *RBP1*-like is similar to a member of the SR protein family of splicing accessory factors required for sex-specific splicing of *doublesex* (Heinrichs and Baker 1995; Lynch and Maniatis 1996). The genes designated *Dwee1* and *hrp48* are described in further detail in the text.

one of the genes uncovered by the deletion must be essential for oogenesis (data not shown).

To further investigate the function of *Dwee1*, a chemical mutagenesis screen was devised to identify point mutations in genes within the region delimited by *Df(2L)Dwee1*^{W05}. A standard F_2 screening protocol was followed as described in materials and methods. Hemizygous F_2 progeny were tested for zygotic lethality and those that were viable were then tested for female sterility. By this approach we identified four lethal and three female-sterile mutants from progeny representing ~4500 individual candidate chromosomes.

Characterization of lethal alleles recovered in the screen: Complementation tests established that the first lethal mutation recovered in our screen (*l(2)EL1*) is an allele of the gene thought to be associated with the *P*-transposon insertion designated *l(2)k00213* (Torok *et al.* 1993). We have not yet determined which gene within or overlapping *Df(2L)Dwee*^{W05} is affected by these mutations, but there are clearly several potential candidates (Figure 1).

Df(2L)spd-J2, previously characterized as uncovering cytological interval 27C1-28A, was crossed to all of the mutations recovered in our screen as well as to known *P*-element mutations in the region (Neumann and Cohen 1996). This deletion failed to complement lethal *P*-insertion alleles of the *hrp48* locus [*l(2)02647* and *l(2)k10413*] as well as our new lethal mutation called *l(2)EL2*, but does complement all other mutations described in this study except *Df(2L)Dwee1*^{W05}. These observations prompted us to determine the distal breakpoint of this deletion using molecular techniques. We found it within an interval just distal to *hrp48*, as indicated in Figure 1. The complementation patterns of two other lethal alleles recovered in our screen, *l(2)EL3* and *l(2)DL1*, are complex (see Table 1). Both complement the two *P*-insertion alleles of *hrp48* as well as *Df(2L)spd-J2*, suggesting that these mutations have not disrupted the *hrp48* locus. However, our new *hrp48* allele *l(2)EL2* failed to complement *l(2)EL3.* Furthermore, while *l(2)DL1* complemented all the *hrp48* alleles we tested, it failed to complement *l(2)EL3.* We are not able to resolve the genetic basis for these peculiar genetic interactions at this time. One possibility is that our data reflect interallelic complementation among different alleles of a complex genetic locus. Various cDNA clones characterized by the BDGP from the *hrp48* locus fall into three distinct classes of splice products, possibly accounting for the observed genetic complexity (see Figure 1).

Characterization of *Dwee1* female-sterile mutations recovered in the screen: Complementation tests showed

TABLE 1

Summary of complementation at the *hrp48/Hrb27C* locus

Allele	*l(2)02647*	*l(2)k10413*	*l(2)EL2*	*l(2)EL3*	*l(2)DL1*
l(2)02647	Lethal				
l(2)k10413	Semilethal	Lethal			
l(2)EL2	Lethal	Lethal	ND[a]		
l(2)EL3	Viable	Viable	Lethal	ND	
l(2)DL1	Viable	Viable	Viable	Lethal	ND
Df(2L)spd-J2	Lethal	Lethal	Lethal	Viable	Viable
Df(2L)Dwee1^{W05}	Lethal	Lethal	Lethal	Lethal	Lethal

[a] Allele combinations marked "ND" are lethal but are treated as uninformative since it is likely these chromosomes harbor second-site lethal mutations.

that all three female-sterile mutations recovered in our screen (*Dwee1*^{ES1}, *Dwee1*^{ES2}, and *Dwee1*^{DS1}) are alleles of the same gene, and data described later in this section establish that this gene corresponds to *Dwee1.* We undertook a detailed phenotypic analysis of one of the mutant alleles, *Dwee1*^{ES1}. Hemizygous *Dwee1*^{ES1} mutant females are viable but completely sterile and show no paternal rescue effect (hemizygous males are fertile, however). Hemizygous females lay abundant eggs of normal appearance that proceed through the early syncytial nuclear cycles without incident. During cycles 11 and 12, however, nuclei in mutant-derived embryos fail to separate at the end of mitosis and remain fused (Figure 2). This phenotype and the subsequent clumping and fragmentation of nuclei that we observe (Figure 3C) is identical to what is seen in embryos collected from *grp* or *mei-41* mutant females (Fogarty *et al.* 1994; Sibon *et al.* 1999). This observation suggests a possible role for *Dwee1* in the same developmental checkpoint as *mei-41* and *grp.*

We undertook two different approaches to demonstrate that the complementation group represented by the three female-sterile mutations does in fact correspond to *Dwee1.* First, we sequenced genomic DNA isolated from adults hemizygous for each of the alleles (*Dwee1*^{ES1}, *Dwee1*^{ES2}, and *Dwee1*^{DS1}), covering the entire transcribed region and ~150 bp of flanking DNA at each end. For comparison, we sequenced genomic DNA from the stock used to generate the mutants. With respect to their maternal phenotype, *Dwee1*^{ES1} and *Dwee1*^{ES2} behave as classical amorphic alleles (Muller 1932). Genomic DNA isolated from each of these mutants contains a DNA lesion within the kinase domain of *Dwee1* that is expected to either abolish or severely disrupt the function of the gene (Figure 4). *Dwee1*^{ES1} contains an 8-bp deletion causing a frameshift followed by a stop codon, truncating the protein in kinase domain IV. *Dwee1*^{ES2} contains a missense mutation that changes a glutamate residue that is conserved among Wee1-like kinases to a lysine at position 308 in the protein (E308K). *Dwee1*^{DS1} behaves as a classical hypomorphic allele in that the phenotype of embryos derived from homozygous females is much less severe (many cellularize and some even develop to adulthood) than that of embryos derived from hemizygous females (which rarely cellularize and never hatch). Sequence analysis

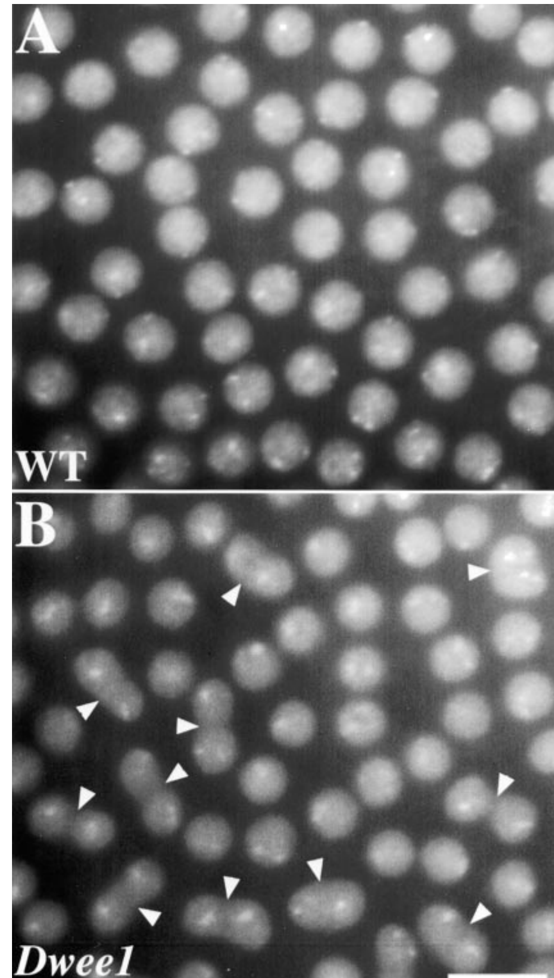

Figure 2.—Comparison of nuclei from equivalently staged wild-type and *Dwee1* mutant embryos. (A) Nuclei from a wild-type embryo in interphase of cycle 13. (B) Nuclei from a *Dwee1*-derived embryo in interphase of cycle 13. The arrowheads indicate nuclei that have failed to divide in mitosis 12. Bar, 12 μm.

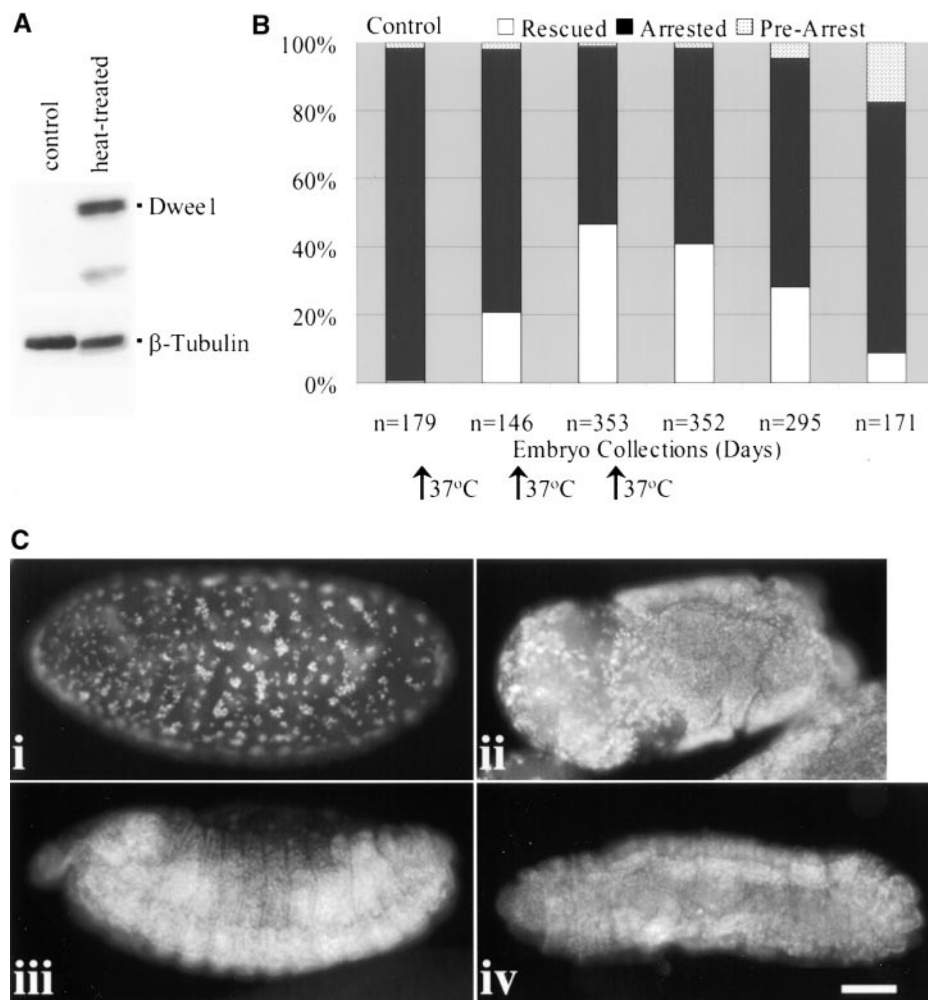

Figure 3.—Expression of a *hsDwee1* transgene can rescue the maternal lethal phenotype of *Dwee1* mutant-derived embryos. (A) Immunoblot showing induction of Dwee1 protein by heat shock of $Dwee1^{ES1}$/*hsDwee1*, $Df(2L)Dwee1^{W05}$ adult female flies. (B) Classes of embryos produced by $Dwee1^{ES1}$/*hsDwee1*, $Df(2L)Dwee1^{W05}$ mothers. Numbers of embryos counted for each collection are given at the bottom of the graph. The bar marked "control" represents embryos collected prior to induction of the transgene. Embryos that were completely syncytial and displayed the typical nuclear defects described for *Dwee1* mutants were scored as "arrested." Embryos were scored as rescued if they were able to complete syncytial development and cellularize (at least partially). Embryos that were developmentally too early to score as being either arrested or rescued were scored as "pre-arrest." (C) Variation in embryonic phenotypes observed in the heat-shock rescue experiment. (i) Syncytial arrest phenotype typical of *Dwee1*-derived embryos. (ii) Mosaic embryo with both cellularized (right) and arrested (left) domains. (iii and iv) Apparently normal late embryo and early larva, respectively. Bar, 62 μm.

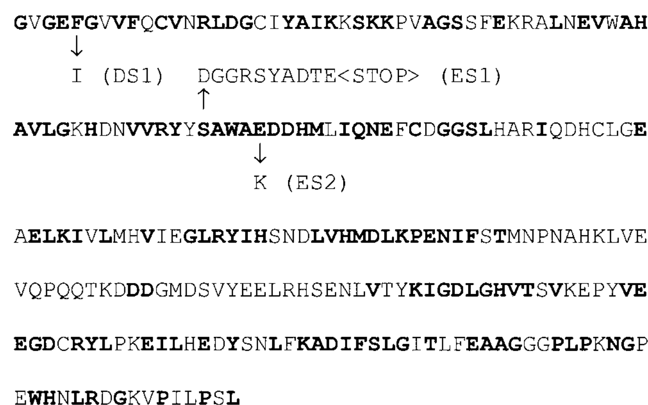

Figure 4.—Location of molecular lesions identified in genomic DNA of the *Dwee1* mutants identified in this study. The sequence shown represents amino acid residues 246 to 486 of the *Dwee1* coding region and the letters denote amino acids in the standard single-letter designation. Letters in bold signify residues that are conserved in other Wee1 kinases. The arrows indicate the position of lesions identified in the $Dwee1^{ES1}$ (ES1) and $Dwee1^{ES2}$ (ES2) EMS-induced and $Dwee1^{DS1}$ (DS1) DEB-induced mutant alleles, respectively, and point to the altered residues that the mutant alleles are predicted to encode.

of this allele showed that it contains a missense mutation changing a conserved phenylalanine residue to isoleucine at amino acid residue 250 within the ATP-binding site of the protein (F250I). Presumably this lesion is still compatible with low-level function of the protein. The $Dwee1^{ES1}$ allele shows an antimorphic interaction with the $Dwee1^{DS1}$ allele in that the phenotype of embryos derived from $Dwee1^{DS1}$/$Dwee1^{ES1}$ transheterozygous mothers is more severe (embryos never cellularize) than seen in $Dwee1^{DS1}$/$Df(2L)Dwee1^{W05}$ hemizygotes. Conceivably, this reflects titration of positive regulatory factors by the truncated $Dwee1^{ES1}$ protein, thus lowering the effective levels of $Dwee1^{DS1}$ function.

We were also able to partially rescue the phenotype of mutant embryos with a heat-inducible *Dwee1* cDNA transgene. Maternal $Dwee1^{ES1}$ hemizygous flies carrying this transgene were briefly heat-shocked to induce expression as confirmed by immunoblot analysis (Figure 3A). Rescue was scored as development at least to the cellularization stage (cycle 14), which mutant-derived embryos otherwise never reach. By this measure, ∼50% of the embryos could be rescued by maternal expression of the transgene. Cessation of heatshocks produced a decline in numbers of rescued embryos (Figure 3B).

We observed wide phenotypic variation in the extent of phenotypic rescue, presumably reflecting variations in the amount and timing of Dwee1 protein and mRNA deposited into individual eggs. These ranged from mosaic embryos containing both cellularized and syncytial sectors to apparently normal late embryos and first instar larvae that were nonetheless unable to complete development (Figure 3C). In contrast, heat-shock treatment of *Dwee1*^{ES1} hemizygous females lacking the transgene produced no cellularized embryos, eliminating the possibility that our mutants were being rescued by the experimental protocol alone. Furthermore, a single transgene copy of a genomic DNA construct that contains *Dwee1* coding sequences plus flanking DNA (and includes the adjacent *dhp1*-like gene) can completely rescue the maternal lethal phenotype. These two lines of evidence demonstrate that molecular lesions consistent with loss of function in *Dwee1* are found in the female-sterile mutants and also show that *Dwee1* expression is both necessary and sufficient to rescue the maternal lethal phenotype. We conclude from this evidence that we have identified mutant alleles of *Dwee1.* The striking similarity between the phenotype of *Dwee1* mutant-derived embryos and embryos derived from *grp* or *mei-41* mutants provides a strong argument that maternally provided *Dwee1* plays an essential role in the same developmental process as *grp* and *mei-41.*

Additional evidence in favor of this hypothesis is afforded by providing extra maternal copies of the genomic *Dwee1* transgene in a *mei-41*^{D3} mutant background. Females homozygous for the *mei-41*^{D3} allele produce cellularized embryos at a very low frequency (2%, $N = 106$). The frequency of cellularized embryos is dramatically increased by adding an extra maternal copy of a *Dwee1* genomic transgene (20%, $N = 109$). The *mei-41*^{D3} mutant embryos are further rescued by addition of two *Dwee1* transgenes (50%, $N = 72$), to the extent that some *mei-41*^{D3}-derived embryos were able to develop to adulthood. In contrast, parallel experiments in a *grp*^{1} background did not produce any rescue of the mutant phenotype with either one or two extra copies of *Dwee1.* The simplest interpretation we can offer for why the results differ between *grp* and *mei-41* mutants in these experiments is that the *mei-41*^{D3} is not a complete loss-of-function allele, and consequently *mei-41*^{D3} mutants are more sensitive to increased dosage of *Dwee1* than *grp*^{1} mutants. Alternatively, *grp* may respond to two different signaling pathways whereas *mei-41* may respond to only one of the two. Dwee1 overproduction could be sufficient to rescue the common function but not the *grp*-specific one according to this model. Another test for functional interactions among these genes was to assess the effect of lowering the maternal dosage of *mei-41*+ or *grp*+ in a homozygous *Dwee1*^{DS1} maternal background. The incompletely penetrant syncytial arrest phenotype of homozygous *Dwee1*^{DS1}-derived embryos (54% cellularized, $N = 107$) was enhanced by subtracting a maternal copy of *mei-41*+ (39%, $N = 141$). Removal of one maternal copy of *grp*+ produced an even greater enhancement of the mutant phenotype of *Dwee1*^{DS1} embryos (29% cellularized, $N = 127$).

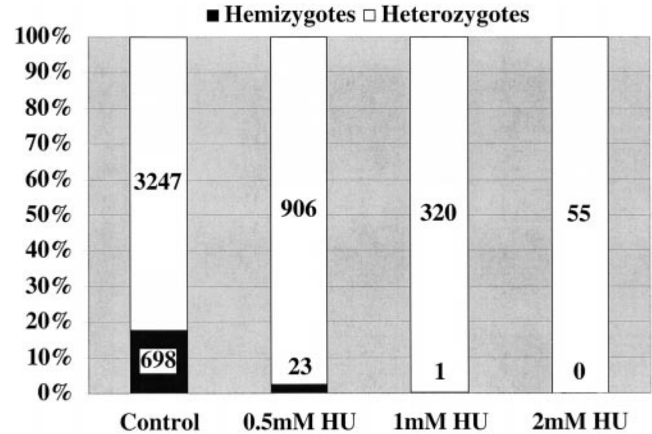

Figure 5.—*Dwee1* mutants are defective for a zygotic DNA replication checkpoint. The graph shows populations of progeny from *Dwee1*^{ES1}/*CyO* flies crossed with *Df(2L)Dwee1*^{W05}/*CyO* flies that were grown in vials containing the indicated concentration of hydroxyurea. The visible adult marker *Cy* carried on the second chromosome balancer in the heterozygotes was used to distinguish the genotypes of hemizygous and heterozygous flies.

We wanted to assess whether *Dwee1* hemizygous flies derived from heterozygous parents were capable of mounting an effective response to delays in DNA replication, since the slowing of the late syncytial cycles has been proposed to reflect activation of a DNA replication checkpoint (Sibon *et al.* 1999). For this experiment, we assessed the sensitivity of *Dwee1*^{ES1} hemizygous larvae to treatment with hydroxyurea (HU), a drug that inhibits DNA replication. In fission yeast, the “checkpoint rad” group of mutants as well as *wee1* mutants are all extremely sensitive to HU. In Drosophila, *mei-41* and *grp* mutant larvae also exhibit this response (Sibon *et al.* 1999). Genetic crosses between balanced heterozygous stocks carrying either the *Dwee1*^{ES1} mutant chromosome or the *Df(2L)Dwee1*^{W05} chromosome generate both heterozygous and hemizygous viable adult progeny. Exposure to 1 or 2 mm HU eliminated the hemizygous *Dwee1*^{ES1} class of progeny, indicating that *Dwee1* mutant larvae are indeed highly sensitive to HU, presumably reflecting a requirement for *Dwee1* activity in a fully functional DNA replication checkpoint (Figure 5).

DISCUSSION

These studies establish that *Dwee1* has an essential maternal function during the nuclear division cycles of embryogenesis and also implicate zygotic *Dwee1* function in a cell cycle checkpoint that responds to inhibition of DNA replication. The demonstration that *Dwee1* has a role during the early syncytial nuclear cycles calls

into question a previous assumption that inhibitory phosphorylation does not control these cycles. Analyses of the state of phosphorylation during the early cycles had failed to detect inhibitory phosphorylation of Cdk1 prior to cycle 13 (Edgar *et al.* 1994). Furthermore, because reduction in the gene dose of cyclin A and cyclin B slowed the late nuclear cycles, it was suggested that progress of these cycles is regulated by accumulation of cyclins to a threshold level. The finding that *Dwee1* is required for completing the nuclear division cycles suggests that inhibitory phosphorylation does play a role in their regulation after all. The failure to detect inhibitory phosphorylation during these cycles can be explained if only a small pool of Cdk1 is subject to this modification. Wee1-type kinases are predominantly nuclear in Drosophila and other organisms and nuclear Wee1 activity is sufficient to block entry into mitosis even in the presence of high cytoplasmic Cdk1 activity (Heald *et al.* 1993; Campbell *et al.* 1995). Hence, we suggest that inhibitory phosphorylation of a small nuclear pool of Cdk1 contributes importantly to the control of the syncytial cycles. The proposal that inhibitory phosphorylation regulates syncytial cycles was an implicit component of a recently proposed model for the mechanism by which *mei-41* and *grp* regulate the progressive lengthening of these cycles (Sibon *et al.* 1997, 1999). In response to incompletely replicated DNA, the recognized activities of these conserved checkpoint kinases arrest the cell cycle by preventing the removal of inhibitory phosphates from Cdk1. While this model appears to be at odds with the lack of detectable inhibitory phosphorylation of Cdk1 during the syncytial cycles (Edgar *et al.* 1994), our findings that *Dwee1* is required for the early nuclear division cycles supports this proposal. Indeed, the apparent parallels in the phenotypes of *mei-41*, *grp*, and *Dwee1* maternal mutants suggest that these genes operate by a similar mechanism. Because our results implicate this pathway without defining precisely how it is induced, it remains possible that the same pathway could be used in a unique regulatory circuit, as was recently suggested (Su *et al.* 1998). In either case, the lesson seems to be that the remarkable conservation of the eukaryotic cell cycle regulatory machinery is coupled with an equally remarkable flexibility in how that machinery can be deployed, depending on the particular developmental constraints of each organism. In early Drosophila embryos, a regulatory pathway that usually serves a surveillance function plays an essential cell cycle role.

It was unexpected that zygotic *Dwee1* function would be dispensable under normal growth conditions, since Cdk1 inhibitory phosphorylation appears to play an important role in cell cycle regulation at many stages of development in Drosophila. Following the last syncytial division during interphase of cycle 14, Cdk1 becomes quantitatively inhibited by phosphorylation (Edgar *et al.* 1994). This dramatic regulatory transition could result from delocalization of Dwee1, activation of a cytoplasmically localized Cdk1 inhibitory kinase, inhibition of cytoplasmic Cdc25, or more active exchange of Cdk1 between the nucleus and cytoplasm during cycle 14. We are currently investigating these possibilities. It has been demonstrated that entry into mitosis 14 depends on zygotic expression of $Cdc25^{stg}$ phosphatase and removal of inhibitory phosphate from Cdk1 (Edgar and O'Farrell 1989, 1990). Furthermore, $Cdc25^{stg}$ activity is also required during the following postblastoderm mitoses of embryogenesis and during imaginal disc development (Edgar and O'Farrell 1989, 1990; Milan *et al.* 1996; Johnston and Edgar 1998). $Cdc25^{twe}$ activity is also required during meiosis (Alphey *et al.* 1992; Courtot *et al.* 1992). These requirements for $Cdc25^{stg}$ imply that inhibitory phosphorylation is normally significant at all of these stages of development. In fission yeast, loss of Wee1 kinase can suppress requirements for the Cdc25 phosphatase. In Drosophila, however, loss of zygotic *Dwee1* function does not bypass the requirement for $Cdc25^{stg}$ activity (Campbell *et al.* 1995). The continued requirement for $Cdc25^{stg}$ activity might be due to maternal perdurance of *Dwee1* function. Alternatively, there might be other Wee1 kinases that can function either redundantly with *Dwee1* or independently. We have recently cloned the gene encoding a Drosophila homolog of Myt1, a Wee1-related kinase, which may contribute to some of these activities (S. Campbell, unpublished data; Mueller *et al.* 1995).

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