Molecular Biology of the Cell
Vol. 6, 1333–1347, October 1995

Drosophila Wee1 Kinase Rescues Fission Yeast from Mitotic Catastrophe and Phosphorylates *Drosophila* Cdc2 In Vitro

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Submitted March 28, 1995; Accepted August 9, 1995
Monitoring Editor: Mitsuhiro Yanagida

Cdc2 kinase activity is required for triggering entry into mitosis in all known eukaryotes. Elaborate mechanisms have evolved for regulating Cdc2 activity so that mitosis occurs in a timely manner, when preparations for its execution are complete. In *Schizosaccharomyces pombe*, Wee1 and a related Mik1 kinase are Cdc2-inhibitory kinases that are required for preventing premature activation of the mitotic program. To identify Cdc2-inhibitory kinases in *Drosophila*, we screened for cDNA clones that rescue *S. pombe wee1*$^-$ *mik1*$^-$ mutants from lethal mitotic catastrophe. One of the genes identified in this screen, *Drosophila wee1* (*Dwee1*), encodes a new Wee1 homologue. Dwee1 kinase is closely related to human and *Xenopus* Wee1 homologues, and can inhibit Cdc2 activity by phosphorylating a critical tyrosine residue. *Dwee1* mRNA is maternally provided to embryos, and is zygotically expressed during the postblastoderm divisions of embryogenesis. Expression remains high in the proliferating cells of the central nervous system well after cells in the rest of the embryo have ceased dividing. The loss of zygotically expressed Dwee1 does not lead to mitotic catastrophe during postblastoderm cycles 14 to 16. This result may indicate that maternally provided Dwee1 is sufficient for regulating Cdc2 during embryogenesis, or it may reflect the presence of a redundant Cdc2 inhibitory kinase, as in fission yeast.

INTRODUCTION

Studies of a number of different experimental organisms have shown that activation of the conserved eukaryotic regulator Cdc2 kinase is required to trigger mitosis (King *et al.*, 1994; reviewed in Nurse, 1994). Cdc2 activity is modulated by post-translational phosphorylations and by association with cyclins and other interacting proteins, providing opportunities for an array of regulatory inputs (O'Farrell, 1992). In the fission yeast *Schizosaccharomyces pombe*, inhibitory phosphorylation of Cdc2 on tyrosine residue 15 (Tyr15) plays at least two roles in cell cycle control. The inhibitory phosphate is added by either one of two partially redundant kinases, Wee1 and Mik1, and is removed by the Cdc25 phosphatase (Russell and Nurse, 1986, 1987a; Gould and Nurse, 1989; Moreno *et al.*, 1990; Lundgren *et al.*, 1991). Activity of either Wee1 or Mik1 kinase is required during S phase to prevent premature progression of the cell cycle into mitosis and subsequent "mitotic catastrophe," where incompletely replicated chromosomes sustain catastrophic damage as they attempt to segregate (Lundgren *et al.*, 1991). The exceptionally small size of cells deficient in Wee1 suggests that it has a unique role in regulating the cell size at which mitosis normally occurs, by imposing a G2 arrest until a minimum size requirement is met (Nurse, 1975; Fantes and Nurse, 1978; Nurse and Thuriaux, 1980). Thus, in fission yeast, tyrosine phosphorylation of Cdc2 is part of a mecha-

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nism for checking advance of the cell cycle before completion of S phase, and plays a regulatory role in coupling cell division with growth.

Studies of cycling frog extracts suggested another role for inhibitory phosphorylation of Cdc2. Progress of the extracts to 'mitosis' requires cyclin synthesis, and mitotic degradation of cyclins resets the system, producing oscillations (Minshull *et al.*, 1989; Murray *et al.*, 1989). The accumulation of cyclin is gradual, but the resulting activation of Cdc2 is abrupt. This discordance can be explained in terms of the kinetics of phosphorylation and dephosphorylation of Cdc2. Phosphorylation of threonine 161 (Thr161) by Cdc2-activating kinase is required for Cdc2 to become activated, providing one level of control (Solomon *et al.*, 1992). Between pulses of kinase activation, *Xenopus* egg extracts accumulate triply phosphorylated, inactive Cdc2 (Thr14, Tyr15, and Thr161), complexed with a cyclin (Dunphy and Newport, 1989; Solomon *et al.*, 1990, 1992; Smythe and Newport, 1992). The kinase is then activated following the removal of inhibitory phosphates by Cdc25 (Gautier *et al.*, 1991; Kumagai and Dunphy, 1991). The abrupt nature of this transition is thought to result from positive feedback; once a small amount of active Cdc2 appears, it can promote further activation by inhibiting a Wee1 kinase and stimulating a Cdc25 phosphatase (Gautier *et al.*, 1989; Solomon *et al.*, 1990; Devault *et al.*, 1992; Izumi *et al.*, 1992; Izumi and Maller, 1993; Mueller *et al.*, 1995). Thus, in the frog extract system, tyrosine phosphorylation plays a role in converting a gradual signal, cyclin accumulation, into an on/off switch.

In *Drosophila* embryos, inhibitory phosphorylation of Cdc2 plays yet another role. During embryogenesis, the levels of different cell cycle regulators change, and as they change, the rate limiting step in Cdc2 activation shifts (O'Farrell, 1992). The early nuclear division cycles are run entirely by maternally contributed gene products and consist of rapid oscillations between mitosis and S phase. Constant levels of active, cytoplasmic Cdc2 kinase are detectable throughout cycles 1–8, although it is not clear if this is true in the mitotic apparatus itself (Edgar *et al.*, 1994). After cycle 9, oscillations in cyclin levels become detectable and presumably accompany the local inactivation of Cdc2, leading to a gradual slowing of the cycles (Edgar *et al.*, 1994). At the beginning of cycle 14, newly formed cells arrest in G2 before the onset of gastrulation (Foe and Alberts, 1983; Edgar and O'Farrell, 1989). Subsequent embryonic mitoses occur in an intricate pattern that is regulated both spatially and temporally (Foe and Odell, 1989; Foe *et al.*, 1993). Major cytoskeletal rearrangements are required to execute extensive cell movements during gastrulation. These morphogenetic movements are incompatible with mitosis, and it appears that the division program has evolved so as to coordinate their timing with periods of cell division (Foe *et al.*, 1993). This coordination is achieved by regulating the expression of $Cdc25^{String}$, the *Drosophila* homologue of Cdc25 phosphatase that removes inhibitory phosphates from Cdc2. Events at the beginning of cycle 14, such as destruction of maternally supplied $Cdc25^{String}$ and $Cdc25^{Twine}$ allow the accumulation of inhibitory phosphates on Cdc2, and the cells arrest in G2 once DNA replication is completed (Edgar and O'Farrell, 1989; Alphey *et al.*, 1992; Courtot, *et al.*, 1992; Edgar *et al.*, 1994). During subsequent embryonic cell cycles, transcriptionally regulated episodes of $Cdc25^{String}$ expression lead to transient loss of inhibitory phosphorylation on Cdc2 (Edgar and O'Farrell, 1990) The resulting pulses of Cdc2 kinase activity drive mitoses in the detailed patterns associated with morphogenesis (Foe and Odell, 1989). Thus, changes in inhibitory tyrosine phosphorylation of Cdc2 play a central role in coordinating cell proliferation with morphogenesis during development in *Drosophila*.

Although we understand how regulation of the $Cdc25^{String}$ phosphatase contributes to control of Cdc2 activation during *Drosophila* development, we have not yet had the means for studying the inhibitory kinase(s) responsible for this important regulatory mechanism. Here we report the isolation of *Drosophila* genes that complement *S. pombe wee1 mik1* double mutants. One of these genes encodes a Wee1-like kinase, and we show that it can function as an inhibitor of *Drosophila* Cdc2 kinase.

MATERIALS AND METHODS

Yeast Complementation Experiments

The *S. pombe* strains used in this study were a gift from Bob Booher (UCSF), and the *Drosophila* and *S. pombe* cDNA expression libraries (REP-Dm and REP-SP, respectively) were constructed by B. Edgar and C. Norbury in Paul Nurse's lab, Oxford, U.K. mRNA from 0- to 24-h *Drosophila* embryos was prepared by phenol extraction, precipitation, and then polyA+ RNA was selected by two rounds of binding to oligo-dT cellulose (New England Biolabs, Beverley, MA). The first strand cDNA was synthesized with a 1:10 mixture of avian myoblastosis virus and Moloney murine leukemia virus reverse transcriptases (Boehringer Mannheim, Indianapolis, IN), using *Bam*HI/*Not*I-polyT linker-primers. DNA polymerase1 (Stratagene, La Jolla, CA) was used to generate the second strand, in the presence of methyl-dCTP. The cDNAs were blunt-ended with T4 polymerase (New England Biolabs), and ligated to *Sal*I adapters containing a *Pac*1 site. They were then sized on Sephacryl S-400 (Pharmacia, Piscataway, NJ), cleaved with *Bam*HI and *Sal*I, and cloned into a REP3*Xho* (pMBS36Leu) *S. pombe*/*Escherichia coli* shuttle vector (Maundrell, 1993), prepared with *Sal*I and *Bam*HI. The cDNAs in this library are oriented, and can be inducibly expressed in *S. pombe* from the thiamine-repressible *nmt1* promoter (Maundrell, 1990).

Yeast spheroplast transformations were done by polyethylene glycol precipitation (Moreno *et al.*, 1991) plus lipofectin at 10 μg/ml. To screen for *Drosophila* genes that complement the *wee1–50 mik::leu+* strain, spheroplast transformants were plated on Edinburgh minimal media-sorbitol media (minus leucine) and allowed a 2-day recovery period at 25°C, before shifting to 37°C (the restrictive temperature for *wee1–50*). As a negative control, cells were trans-

formed with the REP vector alone. Colonies never appeared on these controls. Colonies began to appear on the experimental plates after 5 days. These were patched onto Edinburgh minimal media, and those that regrew at 37°C were kept for further analysis. DNA was isolated from fresh patches of yeast cells by alkaline lysis, and plasmids were recovered by electroporation into an *E. coli* strain (DH5a), selecting for ampicillin resistance. Three independent colonies were tested from each sample by amplifying the cDNA inserts with REP vector primers to determine the size of the insert, and then further analyzed by restriction mapping. A number of clones recovered in the screen appear to be vector/insert concatemers, which can be resolved by subcloning for further analysis.

Molecular Analysis of Dwee1 cDNA

The complementing plasmids isolated in the yeast screen were tested for homology to *wee1* by polymerase chain reaction (PCR). Degenerate primers were made to conserved regions shared by the *S. pombe* Wee1 and Mik1 sequences and *Saccharomyces cerevisiae* SWE1 sequence corresponding to kinase subdomains VIb and IX (Russell and Nurse, 1987; Lundgren *et al.*, 1991; Booher *et al.*, 1993). A *Bam*HI site was added at the end of the 5′ primer [gct**ggatc**-cca(ct)(ct) t(acgt)ga(ct)(acgt)t(acgt)aa(ag)cc] and a *Pst*I site was added at the end of the 3′ primer [cgg**ctgcag**a(acgt)(tc)tc(acgt)g-g(acgt)gc(acgt)at(ag)ta]. The *Dwee1* complementing clone yielded a 0.3-kb PCR product that was subcloned and sequenced, determining that it represented a kinase with significant homology to Wee1 homologues in other species. The complementing plasmid, carrying a 1.7-kb fragment cDNA insert, was subcloned into a Bluescript vector to generate the plasmid pKS-Dwee1. *Exo*III deletions of pKS-Dwee1 were made to sequence the sense strand (Henikoff, 1984), and complementary oligonucleotide primers were used to sequence the antisense strand. To isolate the 5′ extension that was missing from the original complementing *Dwee1* clone, an embryonic cDNA library (Brown and Kafatos, 1988) was used to amplify a fragment by two rounds of PCR with a sense strand vector primer and two nested antisense *Dwee1* primers. An ~0.9-kb fragment was amplified, the product was subcloned, and four clones were sequenced. All four clones were N-terminal *Dwee1* clones that overlapped with previously determined sequences (3 of 4 clones began at identical positions, the fourth was missing the first 7 bp). The sequence of the full-length cDNA is shown in Figure 3.

In Vitro Kinase Assays

Drosophila cytoplasmic extracts were prepared, and in vitro translation reactions and immunoprecipitations were performed as described previously (Edgar *et al.*, 1994.). *Drosophila* nuclear extracts were a gift from Danesh Moazed and were prepared as described (Kamakaka *et al.*, 1991). The following plasmids were used for expression of Cdc2 proteins in vitro: pSF259 (HA-Dmcdc2 Thr14Ala Tyr15Phe), pSF208 (HA-Dmcdc2 Tyr15Phe), pSF191 (Ha-Dmcdc2), pSF454 (HA-DweeΔ169), and pSF564 (HA-Dwee). In pSF259, pSF208, and pSF454 the HA-tag is at the 3′ end of the coding region. In pSF454 and pSF564 the HA-tag is at the 5′ end of the coding region.

For a 55-μl reaction, 15 μl of reticulocyte lysate expressing Cdc2 protein was mixed with 15 μl of either EB-buffer, reticulocyte lysate expressing HA-Dwee, reticulocyte lysate expressing HA-DweeΔ169, and *Drosophila* cytoplasmic or nuclear extracts as indicated in Figure 5. GST-CycB (0.3 mg/ml), ATP (2 mM), and $MgCl_2$ (15 mM) were added, the volume was adjusted to 55 μl, and then the reaction mixture was incubated for 30 min at room temperature. The mixture was next immunoprecipitated using the antibody 12CA5 directed against the hemagglutinin (HA)-epitope (Babco, Berkeley, CA). Immunoprecipitates were separated by SDS-PAGE and the gel was blotted onto Immobilon-P membrane (Millipore, Bedford, MA). The membranes were autoradiographed to visualize the ^{35}S-labeled HA-Cdc2 protein and then immunoblotted with MAb4G10 antibody to detect phosphotyrosine.

Cytological and Molecular Analysis of Chromosomal Region 27C

Dwee1 was mapped cytologically to polytene chromosomes using a digoxygenin-labeled, pKS-Dwee1 cDNA fragment probe. Conditions used were as follows: 100 μg/ml probe, 50% formamide hybridization buffer, 42°C overnight, followed by washing in PBS-0.2% Tween 20 at 53°C. To detect hybrids, the slides were incubated with a 1:50 dilution of anti-digoxygenin horseradish peroxidase conjugate (Boehringer Mannheim), washed again, then developed by standard protocols using 3,3′ Diaminobenzidine as a substrate. Chromosomes were then stained briefly with Giemsa, and mounted in Permount (Fisher Scientific, Fairlawn, NJ).

Eight stocks carrying *P[ry+]* insertion mutations previously mapped to 27C were tested by genomic DNA digestion and hybridization with a *Dwee1* cDNA probe. Genomic DNA was isolated by standard methods (Bender *et al.*, 1983), digested with restriction enzymes, blotted to Hybond N (Amersham, Arlington Heights, IL), and hybridized with a digoxygenin-labeled *Dwee1* probe at a concentration of 5–10 ng/ml in 50% formamide-2× SSC. Following stringent washes (0.1× SSC-1% SDS at 65°C), hybrids were detected using Lumiphos according to the manufacturer's recommendations, with the addition of 0.2% Tween 20 to the blocking solution (Boehringer Mannheim).

The stocks analyzed were the following: 1) two *P[ry+]* inserts associated with male-sterility were obtained from Steve Wasserman (U. Texas, Southwestern Medical Center): *ms(2) ry27C* and *ms(2)ryCD* (Castrillon *et al.*, 1993); and 2) six lethal *P[ry+]* insert mutations, originally from the Spradling lab (Carnegie Institute) (Karpen and Spradling, 1992) and mapped by Todd Laverty (University of California, Berkeley, National Institutes of Health *Drosophila* Genome project and the Howard Hughes Medical Institute). These stocks were as follows: *l(2)3300*, *l(2)10280*, *l(2)2647*, *l(2)4841*, *l(2)4493*, and *l(2)10636*. Two deletions that extend into 27C were also tested (*Df(2L)JH* and *Df(2L)ade3*^{27}) (Tiong and Nash, 1990; S. Tiong, personal communication). The position of *Dwee1* relative to the *P[ry+]* insertions and to deletion breakpoints was determined by in situ hybridization to polytene chromosomes with a *Dwee1* cDNA probe (see above).

To screen for x-ray deletions of *Dwee1*, the *P[ry+]* insertion associated with *l(2)2647* was used as a dominant phenotypic marker to screen for x-ray–induced deletions. Approximately 60,000 progeny of flies that had been irradiated (1000 rad) were visually scored for ry− exceptions, which were then secondarily tested by genetic complementation against nearby lethal mutations.

Embryo in situs were performed either by using a size-reduced digoxygenin-labeled Dwee1 probe and previously described procedures (Edgar and O'Farrell, 1990) or with digoxygenin-labeled RNA probes, omitting the proteinase K digestion, with hybridization and washes performed at 70°C (Boehringer Mannheim).

RESULTS

Identifying Drosophila Genes that Rescue Mitotic Catastrophe in Fission Yeast

To isolate *Drosophila* cDNA clones encoding Wee1-like activity, we set up a complementation screen using a *S. pombe* strain that has a temperature-sensitive allele of *wee1*, and a null allele of the redundant kinase *mik1* (*wee1–50 mik1::ura*$^+$*leu*$^-$). This strain undergoes lethal mitotic catastrophe at the restrictive temperature, 37°C (Lundgren *et al.*, 1991). We transformed this strain (selecting for leucine prototrophy) with a

library of *Drosophila* embryonic cDNAs expressed from the thiamine-repressible NMT promoter (Maundrell, 1990) and screened for clones that confer viability at the restrictive temperature with the promoter on. As a positive control, we also screened a similar *S. pombe* library. Fifteen complementing colonies were isolated in the screen for *S. pombe* genes. These rescued plasmids were tested by PCR with primers specific for either *wee1* or *mik1*. By this means, we determined that three *wee1* and three *mik1* clones were among the complementors. The remaining nine clones have not been further characterized.

Approximately 10^6 leu^+ transformants from the *Drosophila* cDNA library yielded 53 transformant colonies after 5 days of growth at the restrictive temperature. Plasmids were rescued from 48 of these transformants, and restriction mapping suggested that a large number of different genes had been isolated. Rather than re-testing all of these for complementation in the original *wee1–50 mik1::ura*$^+$*leu*$^-$ background, we reasoned that a second genetic test for Wee1 function might retrieve a more restricted subset of these clones and still include any genuine Wee1 homologues. Accordingly, we transformed each of the cDNA clones into a second strain: *wee1–50 cdc2–3w leu-*, which also undergoes temperature-sensitive mitotic catastrophe at a restrictive temperature. The *cdc2–3w* allele renders entry into mitosis independent of *cdc25* expression and sensitizes the cells to reductions in Wee1 function (Fantes, 1981). Ten of the original rescuing plasmids also complemented this strain. Restriction mapping of these clones indicated that this group represented five different genes. This conclusion was confirmed by hybridization of each of the different clones to restriction-digested *Drosophila* genomic DNA, where we observed that each clone labeled distinct genomic fragments. As discussed below, one of these clones encodes a protein similar to Wee1 kinase homologues and has been named *Dwee1* (*Drosophila wee1*). The remaining four genes identified in our screen have been named *WMC* genes (*Wee-Mik-Complementing*), numbered in order of recovery.

To further characterize the *Drosophila* cDNA clones, we evaluated their rescuing activity in fission yeast. Transformants of each of the five different rescuing clones restored the growth rate (colony size) of *wee-50 cdc2–3w* cells grown at the restrictive temperature to that seen in cells transformed with the *S. pombe wee1* gene (shown for *Dwee1* and *WMC2* in Figure 1A). Additionally, *wee1–50 cdc2–3w* cells, which have a small cell defect even at the permissive temperature (the wee phenotype; Nurse, 1975), are restored toward a normal cell size by transformation with *Dwee1*, and not by transformation with a vector control plasmid (Figure 2). The cell size of the *Dwee1* transformants can be somewhat variable, presumably due to differences in copy number of

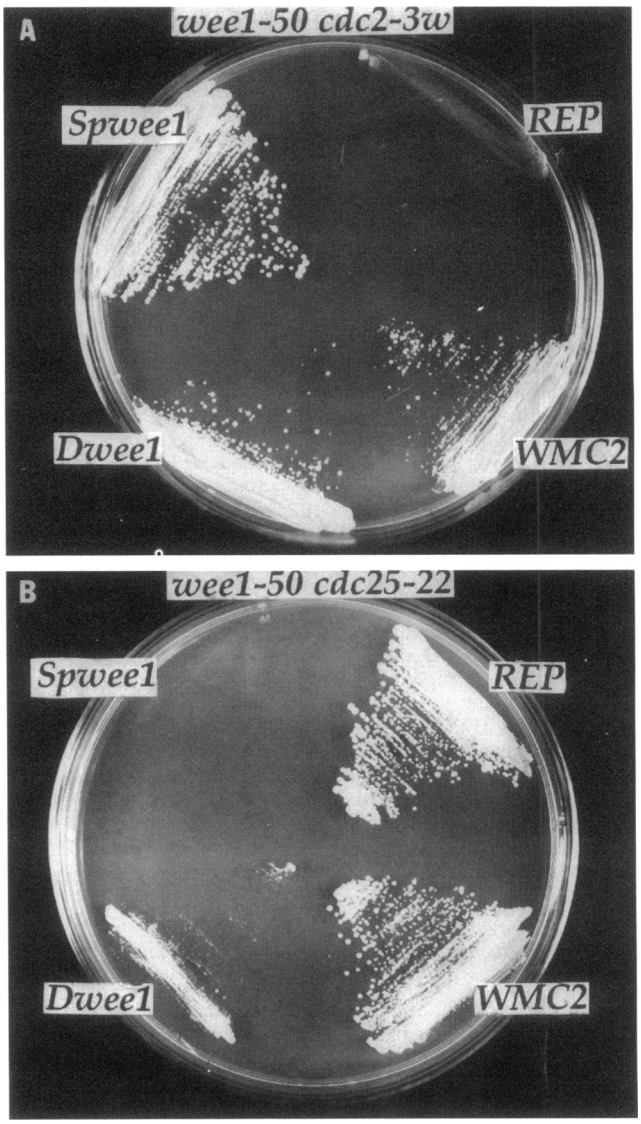

Figure 1. Complementation of two different *S. pombe* strains with *Drosophila* cDNA clones and control plasmids, all cloned into the pREP3Xho shuttle vector (Maundrell, 1993). Colonies were grown at 37°C, the restrictive temperature for the temperature-sensitive alleles *wee1–50*, *cdc2–3w*, and *cdc25–22*. (A) Cells from the *wee1–50 cdc2–3w* strain were transformed with either the REP vector clone (negative control), *S. pombe wee1* (positive control), or the *Drosophila* clones *Dwee1* (the N-terminally truncated clone Dwee1Δ169 that was originally recovered in the screen) or *WMC2*, then grown at the restrictive temperature (37°C) on media lacking leucine and thiamine, thereby selecting for transformants and allowing high level plasmid expression from the NMT promoter. The colony size of transformants for *WMC3*, *WMC4*, and *WMC5* was indistinguishable from that of *Dwee1* or *WMC2*. (B) Cells from the *wee1–50 cdc25–22* strain were transformed with the REP vector, a *pombe wee1* clone, *Dwee1*, or *WMC2* and grown at 37°C as in panel A.

the plasmid. The remaining *WMC* clones did not complement the cell size defect in this strain under identical conditions, however. Addition of thiamine represses the NMT promoter-driven expression of clones in the REP cDNA libraries by about 30-fold (Edgar, unpublished data; Maundrell, 1990). Thiamine supplementation eliminates the rescuing activity of the *Dwee1* clone in a *wee1–50 cdc2–3w* background, but not the rescuing activities of a control *S. pombe wee1* clone under the control of the same promoter. Thus, cloned *S. pombe wee1* rescues with much lower levels of expression than the *Drosophila Dwee1* kinase.

An alternative way to test *Dwee1* activity is to assay its ability to function antagonistically to Cdc25, the phosphatase that removes inhibitory phosphates from Cdc2. An otherwise lethal, temperature-sensitive *cdc25* mutant can be rescued by a temperature-sensitive mutation in *wee1* (Fantes, 1979; Russell and Nurse, 1986). When this strain (*cdc25–22 wee1–50*) is transformed with an *S. pombe wee1* clone, the cells are rendered inviable at the restrictive temperature (Figure 1B). Expression of *Dwee1* in this genetic background is not lethal but it markedly inhibits colony growth (Figure 1B). The data in both strains suggest that the *Dwee1* clone provides an activity comparable to *S. pombe wee1*, albeit at a lower level of this activity.

Dwee1 Encodes a new Member of the Wee Kinase Family

We used PCR with degenerate primers to detect *wee1* kinase homologues among the rescuing clones isolated in our screen (see MATERIALS AND

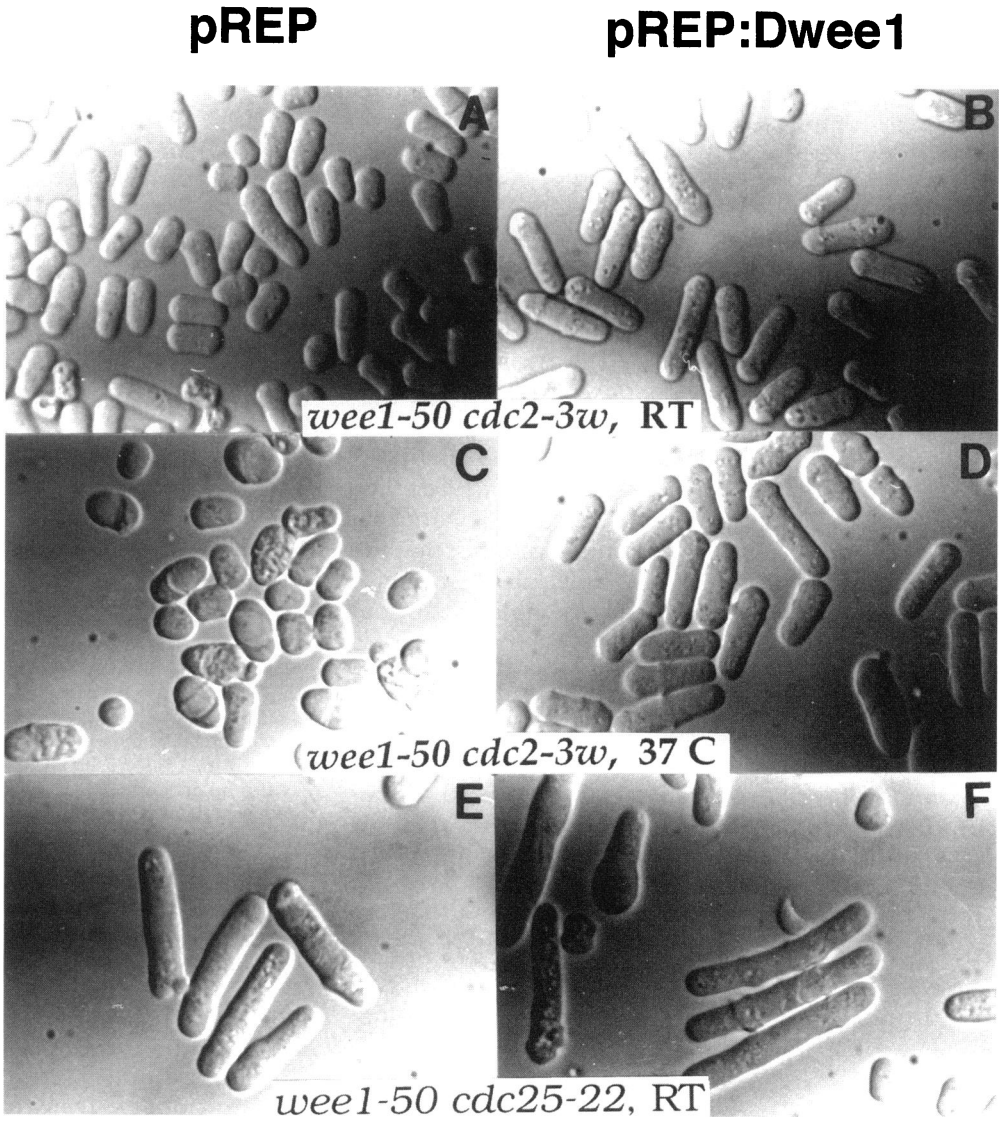

Figure 2. The cellular phenotype of *S. pombe* strains complemented with different *Drosophila* genes and control plasmids are shown. We noted considerable variability in cell size, but have chosen examples of cells that are representative of the majority in a given population. (A) At the permissive temperature (RT, 22°C), cells from a *wee1–50 cdc2–3w* strain transformed with the REP vector alone are phenotypically wee. (B) *Dwee1* transformants in a *wee1–50 cdc2–3w* background are 'less wee,' relative to the REP vector control transformants at 22°C. (C) At the restrictive temperature (37°C), REP vector transformants in a *wee1–50 cdc2–3w* background undergo lethal mitotic catastrophe. (D) Transformation of the *wee1–50 cdc2–3w* cells with *Dwee1* restores viability at the restrictive temperature, and the cells remain 'less wee.' (E) At the permissive temperature, *wee1–50 cdc25–22* cells transformed with a REP vector clone (negative control) are slightly elongated. (F) Cells from the *wee1–50 cdc25–22* strain transformed with *Dwee1* and grown at the permissive temperature are longer than the REP vector control.

METHODS; we were unable to obtain *Dwee1* clones from total genomic DNA by PCR using these primers). The *Dwee1*-complementing clone gave an ~300-bp product, encoding a protein sequence with significant homology to the Wee1 class of protein kinases. This clone was incomplete at the 5′ end, like the human Wee1 homologue that was isolated in a similar complementation screen (Igarishi *et al.*, 1991; McGowan and Russell, 1995; Watanabe *et al.*, 1995). We obtained the missing sequences by PCR from a *Drosophila* embryonic cDNA library (see MATERIALS AND METHODS). The complete cDNA sequence is shown in Figure 3. An open reading frame of 1854 bp (618 amino acids) is found that is predicted to encode a 69-kDa protein. The site at which the original complementing clone was truncated is indicated on Figure 3.

Searches of the GenBank data base indicate that the kinase domain of this protein is similar to Wee1 homologues from *S. pombe*, *S. cerevisiae*, humans, and *Xenopus* (Figure 4A). The *Dwee1* coding sequence includes the generic, conserved residues shared by all kinases, as well as the signatory 'EGD' motif previously noted for the Wee1 kinase family (Figure 4A; Hanks and Quinn, 1991; Booher *et al.*, 1993). In pair-wise comparisons, Dwee1 is most closely related to human Wee1 (Igarashi *et al.*, 1991) and a recently described *Xenopus* Wee1 homologue (Mueller *et al.*, 1995) while the three yeast kinases are more closely related to each other than to the three metazoan genes (Figure 4B). All of these Wee1-like kinases share a similar structure in that they encode a C-terminal kinase domain and a less conserved, N-terminal domain that functions in *S. pombe* as a regulatory domain (Tang *et al.*, 1993). The *Drosophila* and human Wee1 kinases also share two conserved regions in the N-terminal domain that are shown in Figure 4C. This region includes a sequence motif (NI/VNPFTPD/QS) that resembles a consensus phosphorylation site for MAP kinases, and is also found in *Xenopus* Wee1 (Thomas, 1992). In addition, eight T/SP motifs are found in the N-terminal region of Dwee1 as well as two in the C-terminal region (underlined in Figure 3). These T/SP are potential phosphorylation sites for several mitotic kinases, and multiple T/SP sites occur in the N-terminal regions of all three metazoan Wee1 homologues.

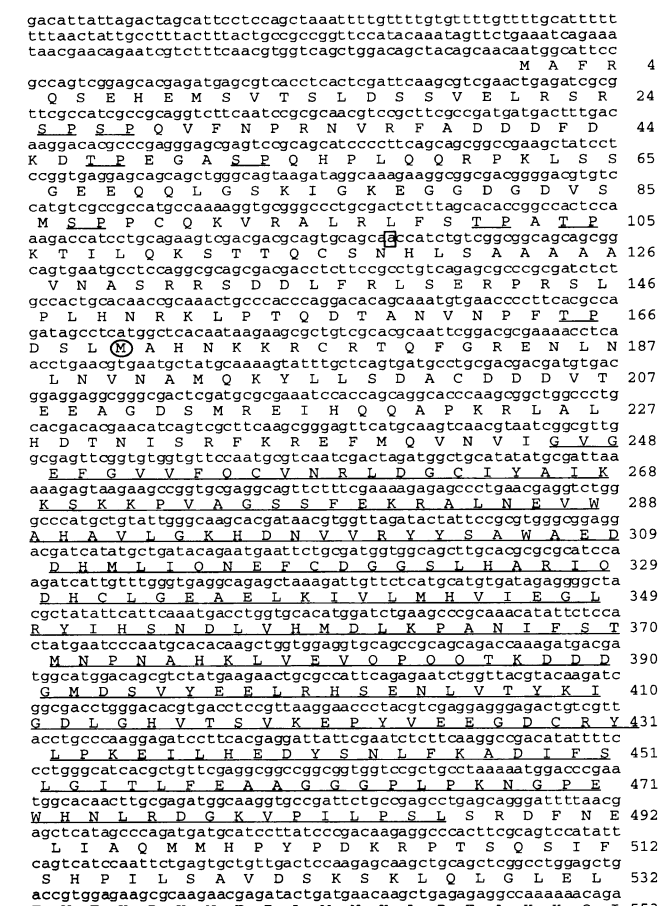

Figure 3. Sequence of a full-length 2204-bp *Dwee1* cDNA. A large open reading frame encoding a 618-amino acid protein sequence is translated. The open reading frame is preceded by stop codons in all three frames. The site at which the original complementing clone was truncated is indicated by a box in the nucleotide sequence, and the first methionine within the truncated clone has been circled. Analysis of a genomic clone that encompasses the *Dwee1* coding region indicated the presence of 4 short introns, at positions 677 (155 bp intron), 1014 (56 bp intron), 1717 (~150 bp intron), and 1848 (~100 bp intron) in the cDNA sequence. The kinase domain is underlined, as are TP and SP motifs (8/10 of which are in the N-terminal domain) that could serve as regulatory phosphorylation sites for a number of mitotic kinases (Mueller *et al.*, 1995). The GenBank accession number for this sequence is U17223.

Drosophila Cdc2 Is Phosphorylated on Tyr15 in the Presence of Dwee1 or Drosophila Nuclear Extracts

The sequence conservation of Dwee1 strongly suggests that this protein acts as a Cdc2 inhibitory kinase, and we have examined this premise directly in vitro. We first developed assays for Cdc2 phosphorylation, using HA epitope-tagged *Drosophila* Cdc2 produced by reticulocyte lysate translation, and GST-Dm cyclin B expressed in *E. coli*. Nuclear and cytoplasmic extracts were prepared from 0- to 12-h *Drosophila* embryos, and tested for enzymatic modification of HA-DmCdc2/cyclin B complexes. Nu-

clear extracts contained an activity that tyrosine phosphorylated HA-DmCdc2 as detected by probing Western blots with an antibody to phosphotryosine (Figure 5). This phosphorylation was strongly stimulated by Dm cyclin B. Phosphorylation of DmCdc2 alters its electrophoretic mobility. S^{35} labeling of the in vitro–translated DmCdc2 allows detection of total DmCdc2, and assessment of the efficiency with which input DmCdc2 is modified. The shift in the mobility of the protein indicates that the majority of the protein is modified (Figure 5, lane 5). Although a number of new forms are seen, only two bands containing phosphotyrosine are detected by the antibody.

Like the nuclear extract, in vitro–translated Dwee1 stimulates tryosine phosphorylation of DmCdc2, but the reaction is more complete. Only the lower mobility phosphotyrosine-containing band is produced, and strong phosphorylation is seen in the absence of cyclin B (Figure 5, lanes 7 and 8). The more rapidly migrating phosphotryosine-containing band is seen if cytoplasmic extract is included during the reaction, suggesting that it is produced by secondary modification by a separate activity (Figure 5, lane 9). Indeed, even though the cytoplasmic extract on its own does not cause tryosine phosphorylation, it does modify DmCdc2 to produce a more rapidly migrating form (Figure 5, lane 3). Based on previous characterization, this more rapidly migrating form is phosphorylated on Thr161 (Edgar *et al.*, 1994). The Thr161-modifying activity appears to present in both cytoplasmic and nuclear extracts, but not in reticulocyte-translated protein extracts. Mutation of Tyr15 of DmCdc2 to Phe15 (Y15 to F15) blocks tryosine phosphorylation, suggesting that this is the residue that is modified by Dwee1 (Figure 5, lane 14). Furthermore, the effect of this mutation on the migration of the higher mobility form of DmCdc2 suggests that efficient phosphorylation of both Tyr15 and Thr161 occurs when both in vitro–translated Dwee1 and cytoplasmic extract are present (Figure 5, compare lanes 9 and 15).

Transcription and in vitro translation of the N-terminally truncated clone of Dwee1 originally isolated in our complementation screen (DweeΔ169) gave lower levels of tyrosine kinase activity (Figure 5, lanes 10–12). The efficiency of phosphorylation in the reactions shown in Figure 5 should not be related to the specific activity of the kinase, because we have not measured or normalized for the amount of the kinase present.

We conclude that Dwee1 encodes a tyosine kinase that adds inhibitory phosphates to DmCdc2 and that there is similar kinase activity in a nuclear extract. Thus, the catalytic activity of *Drosophila* Wee1 is consistent with that expected for a Cdc2 inhibitory kinase and with its ability to compensate for loss of *wee1* and *mik1* functions in fission yeast.

Genomic Characterization of Dwee1 and Isolation of a Chromosomal Deletion that Uncovers It

Hybridization of a Dwee1 cDNA clone to polytene chromosomes localized the gene to the left arm of chromosome II at cytological region 27C, just distal to two deficiencies that encroach on 27C: *Df(2L)JH*, 27C2–9;28B3–4 and *Df(2L)ade-3*^{27},27C6;28A1 (Tiong and Nash, 1990; Tiong, personal communication). We collected strains carrying P-element inserts previously mapped to 27C, and analyzed these by genomic blots hybridized with digoxygenin-labeled *Dwee1* cDNA (see MATERIALS AND METHODS for a list of the stocks tested). Restriction map alterations were associated with two *P[ry+]* insertions: *l(2)2647* and *l(2)10280* (Karpen and Spradling, 1992; Figure 6A). Further restriction mapping indicated that the insertions were ~7.5 kb and ~9 kb upstream of the 5′ end of the Dwee1 cDNA sequence. These *P[ry+]* insertion mutations are allelic and associated with embryonic lethality. No obvious mitotic (or other) defect is seen in collections of embryos from the balanced *l(2)2647* stock, suggesting that lethality is not manifested until late in embryogenesis. In situ hybridizations to embryos from stocks segregating the *l(2)2647* chromosome detected normal levels and distributions of *Dwee1* mRNA. Thus, we have no reason to think that the lethal phenotype associated with these insertions results from disruption of essential *Dwee1* regulatory sequences; we think it is more likely due to mutation of an adjacent vital gene.

To construct a molecular map of the *Dwee1* genomic region, a genomic cosmid clone was isolated from a wild-type library (J. Tamkun, University of California, Santa Cruz) by hybridization with a *Dwee1* cDNA probe. Restriction mapping, limited sequencing, and PCR analysis of this clone has established that the *Dwee1* gene has four small introns whose locations are indicated in the legend for Figure 3. The structure of the gene and its position relative to the nearby P insertions is shown in Figure 6B.

To isolate deletions in the region of *Dwee1*, we screened for x-ray–induced loss of the *P[ry+]* insertion associated with *l(2)2647* (see MATERIALS AND METHODS). Complementation tests of ry− exceptions recovered in a screen of ~60,000 F1 progeny identified a single large deletion, designated *Df(2L)-Dwee1*. The chromosome carrying *Df(2L)Dwee1* fails to complement *Df(2L)JH* and *Df(2L)ade3*^{27}, as well as all available *P[ry+]* lethal mutations in 27C. A polytene chromosome preparation hybridized with a *Dwee1* probe from a larva that was heterozygous for *Df(2L)Dwee1* and a wild-type chromosome is shown in Figure 6C, demonstrating that *Dwee1* lies within the region uncovered by this deletion, which extends from 27A to 28B (indicated by a bracket in Figure 6C).

A

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                          ● ●  I ●               II ●                         III
Sp Wee1 (573-786)   GSGEFSEVFQVEDPVEKTL-KYAVKKLKVKFSGP--KERNRL--LQEVS-   616
Sp Mik1 (296-510)   HESDFSFVYHVSSINPPTETVYVVKMLKKNAAKFTGKERH----LQEVS-   340
SWE1    (451-674)   GKGQFSTVYQVTFAQTNKK--YAIKAIKPNKYNSLKRILLEIKILNEVTN   498
D Wee1  (246-486)   GVGEFGVVFQCVHRLDGLI--YAIKKSKKPVAGSS-FEKR---ALNEVW-   288
Hu Wee1 (306-537)   GSGEFGSVFKCVKRLDGCI--YAIKRSKKPLAGSV-DEQN---ALREVY-   348
X Wee1  (217-448)   GAGEFGSVFKCVKRLDGCF--YAIKRSKKPLAGST-DEQL---ALREVY-   259
Consensus           g.geFg.Vfqcv.rldg..--YaiK.sKkp.ags.-.e..---.LnEV.-   50

                         IV                              V
Sp Wee              -IQRALKGHDHIVELMDSWEHGGFLYMQVELCENGSLDRFLEEQGQLS--   663
Sp Mik1             ILQRLQ-ACPFVVNLVNVWSYNDNIFLQLDYCENGDLSLFLSELGLLQ--   387
SWE1                QITMDQEGKEYIIDYISSWKFQNSYYIMTELCENGNLDGFLQEQVIAKKK   548
D Wee1              -AHAVLGKHDNVVRYYSAWAEDDHMLIQNEFCDGGSLHARIQDH------   331
Hu Wee1             -AHAVLGQHSHVVRYFSAWAEDDHMLIQNEYCNGGSLADAISEN--YRIM   395
X Wee1              -AHAVLGHHPHVVRYYSAWAEDDHMIIQNEYCNGGSLQDLIVDNNKEG--   307
Consensus           -ahavlg.h..vvry.saWaeddhm.iqnE.CegGsL..fi.e.------   100

                                  VIa          VIb ● ● ●
Sp Wee1             RLDEFRVWKILV-EVALGLQFIHHK-NYVHLDLKPANVMITFEG------   705
Sp Mik1             VMDPFRVWKMLF-QLTQALNFIHLL-EFVHLDVKPSNVLITRDG------   429
SWE1                RLEDWRIWKIIV-ELSLALRFIHDSCHIVHLDLKPANVMITFEG------   591
D Wee1              -CLGEAELKIVLMHVIEGLRYIHSN-DLVHMDLKPANIFSTMNPNAHKLV   379
Hu Wee1             SYFKEAELKDLLLQVGRGLRYIHSM-SLVHMDIKPSNIFISRTSIPNAAS   444
X Wee1              QFVLEQELKEILLQVSMGLKYIHGS-GLVHMDIKPSNIFI-----CRKQT   350
Consensus           ....erelKill-qv..gLryiH..-.lVHmDlKPsNifit..g------   150

                                                VII ●                *
Sp Wee1             ---------------------------TLKIGDFGMAS-VWPVPRGMERE   727
Sp Mik1             ---------------------------NLKLGDFGLAT-SLPVSSMVDLE   451
SWE1                ---------------------------NLKLGDFGMATHLPLEDKSFENE   614
D Wee1              EVQPQQTKDDDGMDSVYEELRHSENLVTYKIGDLGHVT---SVKEPYVEE   426
Hu Wee1             EEGD----EDD----------WASNKVMFKIGDLGHVT---RISSPQVEE   477
X Wee1              LGQEESDGEDD----------LSSGSVLYKIGDLGHVT---SILNPQVEE   388
Consensus           ---------dd---------.....v.lKiGDlGhvt---.v....veE   200

                    ** VIII ●            ● IX ●              X
Sp Wee1             GDCEYIAPEVLANHLYD-KPADIFSLGITVFEAAANIVLPDNGQSWQKLR   776
Sp Mik1             GDRVYIAPEILASHNYG-KPADVYSLGLSMIEAATNVVLPENGVEWQRLR   500
SWE1                GDREYIAPEIISDCTYD-YKADIFSLGLMIVEIAANVVLPDNGNAWHKLR   663
D Wee1              GDCRYLPKEILHEDYSNLFKADIFSLGITLFEAAGGGPLPKNGPEWHNLR   476
Hu Wee1             GDSRFLANEVLQENYTHLPKADIFALALTVVCAAGAEPLPRNGDQWHEIR   527
X Wee1              GDSRFLANEILQEDYSQLPKADIFALGLTIALAAGAAPLPCNEDSWHHIR   438
Consensus           GD.rylapEil.e.y.-l.kADifsLglt..eaAgn.pLP.Ng..Wh.lR   250

Sp Wee1             SGDLSDAPRL                                           786
Sp Mik1             SGDYSNLPNL                                           510
SWE1                SGDLSDAGRL                                           674
D Wee1              DGKVPILPSL                                           486
Hu Wee1             QGRLPRIPQV                                           537
X Wee1              KGNLPHVPQL                                           448
Consensus           sGdlp..p.l                                           323
```

B

	Sp Wee1	Sp Mik1	SWE1	Hu Wee1	D Wee1	X Wee1
Sp Wee1		46%	50%	30%	32%	34%
Sp Mik1	64%		37%	26%	26%	32%
SWE1	65%	61%		25%	26%	31%
Hu Wee1	49%	47%	44%		54%	71%
D Wee1	52%	49%	48%	67%		55%
X Wee1	49%	48%	46%	78%	69%	

Figure 4.

Phenotypic analysis of *Df(2L)Dwee1;27A-28B* has been hampered by dominant female sterility in the balanced heterozygous stock. Male heterozygotes carrying the deletion are fertile, however. The female sterility phenotype is complemented in transheterozygotes carrying a duplication that covers cytological region 26A-28E (*Dp(2;2)C619*; Thomas and Roberts, 1966), suggesting that the observed dominant female sterility is a consequence of haploinsufficiency for one of the genes uncovered by the deletion. We took advantage of the fact that females heterozygous for *Df(2L)Dwee1* and *Dp(2;2)C619* are fertile, to examine whether a cell-cycle defect is associated with homozygous *Df(2L)Dwee1* embryos. Homozygous *Df(2L)Dwee1* embryos should comprise 1/4 of the embryos from a cross of heterozygous parents. We stained collections of embryos with anti-β tubulin to visualize microtubules and with Hoescht 33258 to visualize DNA during cycles 14–16, a period when zygotically regulated proliferation occurs. If Wee1-like kinase activity became limiting during embryogenesis, an observable consequence might be cell lethality due to mitotic catastrophe. We were unable to detect any obvious mitotic defect during this developmental period, however. We did not pursue this analysis at later stages of development, because *Df(2L)Dwee1* uncovers many genes whose loss is expected to contribute to the terminal embryonic phenotype of *Df(2L)Dwee1* homozygotes.

Dwee1 Is Expressed in Dividing Cells during Embryogenesis

Digoxygenin-labeled Dwee1 probes were hybridized to *Drosophila* embryos to study expression of the gene during early development. Abundant maternal *Dwee1* mRNA is seen throughout the embryo during the early rapid nuclear cycles (Figure 7A, upper embryo). This mRNA is degraded early in interphase 14 (Figure 7A, lower embryo), at a time when many maternal mRNAs (including $Cdc25^{String}$) are degraded (Edgar and O'Farrell, 1989). Maternal *Dwee1* mRNA persists in the pole cells, however (Figure 7B), and shows a perinuclear, granular signal similar to that seen with perduring maternal cyclin B mRNA in pole cells during the same stage (Lehner and O'Farrell, 1990; Raff *et al.*, 1990). The 3′-untranslated region sequence of *Dwee1* was searched for motifs associated with translational control of cyclin B expression in the pole cells (Dalby and Glover, 1993), but no similar signal sequences were found.

Low levels of *Dwee1* mRNA reaccumulate throughout the embryo during gastrulation, indicating that zygotic transcription of the gene is activated at this time (Figure 7B). This zygotic expression is fairly uniform (although consistently stronger in the mesoderm; see Figure 7C) and has no discernible pattern related to cell cycle progression during the three major post-blastoderm divisions. After germ band retraction, *Dwee1* expression is observed in the mitotically active nervous system, as well as in hindgut, foregut, and midgut tissues that have withdrawn from proliferative cell cycles and are beginning to endoreplicate (Figure 7D; Edgar and O'Farrell, 1990; Smith and Orr-Weaver, 1991). Still later in embryogenesis, the proliferating cells of the central nervous system express high levels of *Dwee1* mRNA, while expression is substantially lower in the rest of the embryo (Figure 7E; Edgar and O'Farrell, 1990). Strong *Dwee1* expression is also seen in the brain of a first instar larva (Figure 7F).

DISCUSSION

During embryogenesis in *Drosophila*, distinct molecular mechanisms appear that regulate cell cycle timing

C

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Hu Wee1(174-247)  PPHKTFRKLRLFDTPHTPKSLLSKARGIDSSSVKL
D  Wee1 (89-173)  PCQKV-RALRLFSTPATPKTILQKSTTQCSNHLSA

Consensus:        P..K..R.LRLF.TP.TPK  L K.....S.....


---------RGSSLFMDTEKSGKREFDVRQTP---QVNINPFTPDSLLLHS
AAAAVNASRRSDDLFRLSERPRSLPLHNRKLPTQDTANVNPFTPDSLMAHN

.........R...LF...E.........R..P.....N.NPFTPDSL..H.
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Figure 4 (cont). (A) An alignment of the *Dwee1* kinase domain with *S. pombe* Wee1 and Mik1, *S. cerevisiae* SWE1, human Wee1, and *Xenopus* Wee1 homologues. Amino acids that are identical in at least three of the five proteins are in bold, and a consensus sequence showing absolutely conserved (upper case) and partially conserved (lower case) residues is indicated. In cases where the yeast homologues share a consensus that is different than that shared by the metazoan homologues, the metazoan consensus is indicated. The genes that encode the Wee1 homologues included in the alignment are as follows: Sp Wee = *wee1* from *S. pombe* (Russell and Nurse, 1987); Sp Mik = *mik1* from *S. pombe* (Lundgren *et al.*, 1991); SWE1 = *SWE1* from *S. cerevisiae* (Booher *et al.*, 1993); Hu Wee = *Huwee1* from humans (Igarashi *et al.*, 1991); and X Wee1 = *Xenopus* Wee1-like kinase (Mueller, *et al.*, 1995). Roman numerals above the alignment indicate kinase subdomains, and residues conserved among all kinases are indicated with superscript dots (Hanks and Quinn, 1991). The conserved 'EGD' motif is indicated by superscript asterisks. (B) Sequence identity (above diagonal) and similarity (below diagonal, in italics) in pairwise comparisons of all five *wee1* homologue kinase domains. Groups of amino acids considered to be similar are the following: A, L, V, I, and M; K and R; D and E; S and T; N and Q; and Y and F. (C) An alignment of a region of the N-terminal domain of human and *Drosophila* Wee1 homologues that shows sequence conservation.

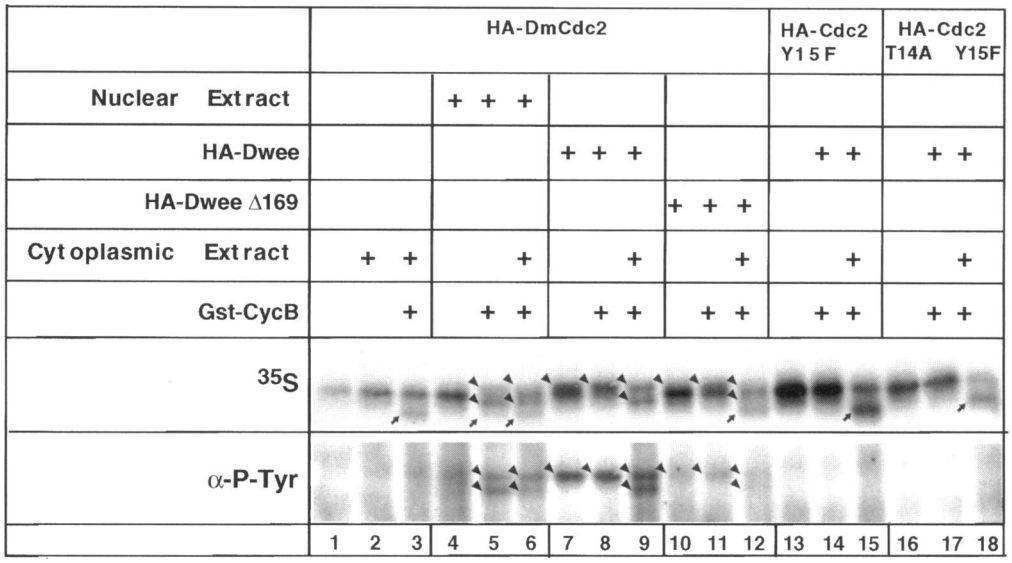

Figure 5. Characterization of DWee1 kinase activity in vitro. Autoradiograms (showing ^{35}S-HA-Cdc2) and Western blots (immunoblotted with αP-Tyr) of immunoprecipitated HA-Cdc2 after SDS-PAGE are shown. HA-tagged Cdc2 was translated in reticulocyte lysates containing [^{S}35]methionine and incubated in the presence (+) or absence of GST-DmcycB, extracts of *Drosophila* embryos (0- to 12-h nuclear extracts or 0- to 12-h cytoplasmic extracts), and HA-tagged DWee1 that was translated in reticulocyte lysates (HA-DWee1: full length DWee1; HA-DweeΔ169: N-terminal truncated Dwee1 lacking the first 169 amino acids). ATP (2 mM) and $MgCl_2$ (10 mM) was added and the reaction was incubated for 30 min at room temperature. These reactions were immunoprecipitated with antibody to the HA tag, the immunoprecipitates were electophoresed on SDS gels, blotted, and proteins were detected either by autoradiography (S^{35}) or probing with antibody to phosphotryosine (αP-Tyr). An activity that is present in nuclear extracts but essentially absent in cytoplasmic extracts can phosphorylate HA-Cdc2 on tyrosine as is seen by detection with antibody to phosphotryosine. Incubation of HA-Cdc2 with cytoplasmic extract in the presence of GST-DmcycB results in phosphorylation of Cdc2 on Thr161 (arrows; Edgar *et al.*, 1994). Incubation with nuclear extract in the presence of GST-CycB or with reticulocyte–produced HA-Dwee can phosphorylate HA-Cdc2 on tyrosine (arrowheads). Mutants in DmCdc2 that lack Tyr15 (HA-DmCdc2-Y15F and HA-DmCdc2-T14A Y15F) are not phosphorylated by Dwee1. The arrows point to the Thr161-phosphorylated form of Cdc2; the arrowheads point to tyrosine-phosphorylated forms.

at successive stages of development (Edgar *et al.*, 1994). The regulation of Cdc2 activity by inhibitory phosphorylation plays an essential role in coordinating cell cycle progression with morphogenetic movements during gastrulation and subsequent postblastoderm development. The expression of Cdc25^{String} (a Cdc25-related phosphatase that removes inhibitory phosphates from Cdc2) is required for these postblastoderm divisions, and the timing of zygotic Cdc25^{String} expression determines the timing of mitosis (Edgar and O'Farrell, 1989, 1990). Our goal in the present work is to further examine the roles of Cdc2 inhibitory phosphorylation at different stages of development.

To define the molecular mechanisms regulating inhibitory phosphorylation of Cdc2 in *Drosophila*, we sought the relevant kinase(s). *Drosophila* cDNA clones encoding potential Cdc2 inhibitors were selected according to their ability to rescue fission yeast *wee1 mik1* double mutants from mitotic catastrophe. One of the clones identified in this screen encodes a *Drosophila* homologue of *wee1* that we have named *Dwee1*. In addition to *Dwee1*, rescuing cDNA clones representing four other *Drosophila* genes were recovered that can also complement two different mutant fission yeast strains for a mitotic catastrophe defect. Previous complementation screens using strains similar to those used here have shown that a number of cell cycle genes unrelated to Wee1 kinases can bypass a requirement for Wee1 kinase. Genes identified this way include *S. cerevisiae CDC6* (Bueno and Russell, 1992), *S. pombe suc1* (Lundgren *et al.*, 1991), a *Xenopus* cDNA of unknown function (Su and Maller, 1994), and a novel *Drosophila* RNA helicase (Warbrick and Glover, 1994). These examples illustrate that additional criteria are needed to define the function of clones recovered in complementation screens of this sort.

This report demonstrates that we have identified a *Drosophila* Cdc2 inhibitory kinase Dwee1, by the following criteria: complementation of fission yeast mutations, sequence homology to a family of Wee1-like kinases, and biochemical demonstration of Cdc2 tyrosine kinase activity.

We examined the expression of the *Dwee1* complementing clone in different genetic backgrounds in fission yeast to determine the extent to which the clone is able to substitute for *S. pombe wee1*. When expressed at high levels, the rescuing *Dwee1* clone complements both the cell size defect of *wee1* mutants and the via bility requirement for *wee1* or *mik1*. Expression of the *Dwee1* clone is also able to confer a growth defect in a temperature-sensitive *wee1 cdc25* mutant background, although not so severe as that produced by re-transformation with *pombe wee1*. These functions were supplied by an N-terminally truncated Dwee1 clone Dwee1Δ169, which encodes the kinase domain but is missing a putative regulatory region.

The kinase domain of Dwee1 is closely related to human and *Xenopus* Wee1 homologues and, like them,

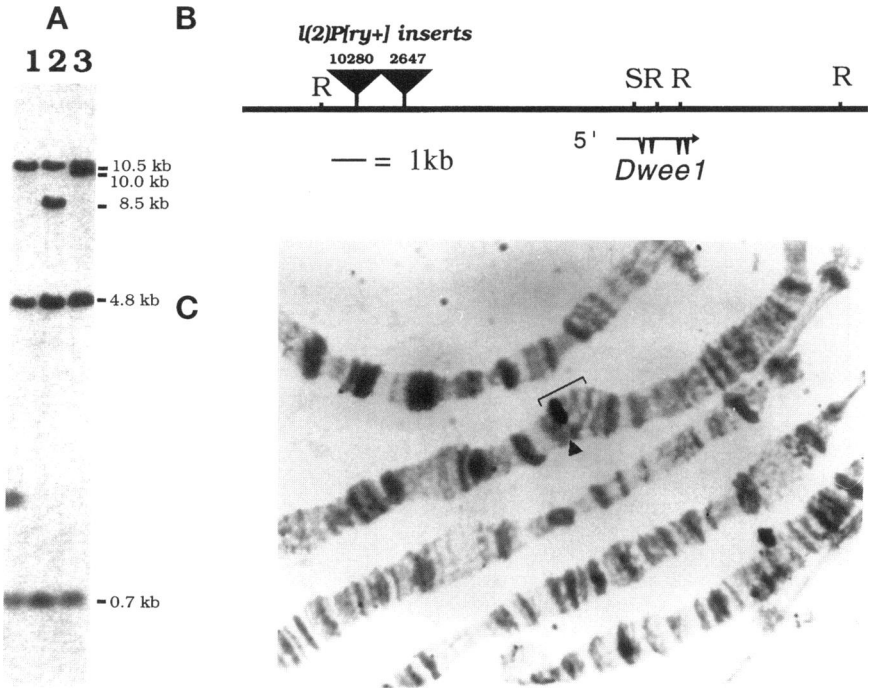

Figure 6. (A) The *l(2)10280* and *l(2)2647 P[ry+]* insertion mutations are associated with DNA rearrangements that are detectable with a digoxygenin-labeled *Dwee1* probe. A genomic blot of DNA isolated from mutant flies (heterozygous with a balancer chromosome) and wild-type (Sevelen) adult flies, each digested with *Eco*RI is shown. Lane 1 is wild type, lane 2 is the *l(2)2647 P[ry+]* heterozygous stock, and lane 3 is the *l(2)10280* heterozygous stock. The novel bands seen in the *l(2)10280* and *l(2)2647* digests indicate DNA rearrangements in the *Dwee1* 5′ flanking region. (B) A schematic drawing of the *Dwee1* genomic region, showing the intron/exon structure of the Dwee1 cDNA and its position relative to the *P[ry+]* insertions associated with *l(2)10280* and *l(2)2647*. (C) Cytological localization of *Dwee1* to polytene region 27C and characterization of *Df(2L)Dwee1*. A chromosome spread from a *Df(2L)Dwee1*/+ heterozygote hybridized with a Dwee1 cDNA probe is shown. The arrowhead shows where the probe hybridizes at cytological position 27C, within the limits of the breakpoint of this deficiency (27A-28B; as indicated by a square bracket).

the protein encodes multiple potential sites for phosphorylation in its N-terminal domain (Igarashi *et al.*, 1991; McGowan and Russell, 1995; Mueller *et al.*, 1995; Watanabe, *et al.*, 1995). We have examined the biochemical activity associated with reticulocyte-translated HA-tagged Dwee1 and our data indicates that it phosphorylates *Drosophila* Cdc2 on tyrosine 15, as expected for a Cdc2 inhibitory kinase. Addition of cyclin B stimulates this phosphorylation (slight stimulation can be seen in Figure 5, and more dramatic stimulation was seen in other experiments), however, almost complete phosphorylation of DmCdc2 can be achieved in the absence of cyclin. This indicates that both DmCdc2 and a complex of DmCdc2/Dm cyclin B are acceptable substrates, and that the complex is a preferred substrate. Because a large fraction of substrate has been phosphorylated in our assays and we do not have initial rate measures, we cannot assess the degree of the preference, but superficially our results suggest a minor preference in comparison to results reported for the *Xenopus* Wee1 (Tang, *et al.*, 1993; Mueller, *et al.*, 1995). In these analyses of *Xenopus* Wee1, phosphorylation of Cdc2 showed a complete dependence on cyclin addition.

A similar kinase activity is seen in *Drosophila* nuclear extracts but not in cytoplasmic extracts, suggesting that Dwee1 kinase activity is localized to the nucleus. Antibodies specific for Dwee1 are being developed that will allow us to determine the localization of the protein. Nuclear localization of Dwee1 would be consistent with the finding that a myc-tagged clone of human Wee1 kinase also localizes to the nucleus in a mammalian cell line (Heald *et al.*, 1993).

To study the function of *Dwee1* during development we have initiated a genetic analysis of the locus, and examined the expression of the gene during embryogenesis. Two closely linked *P[ry+]* transposon insertion mutants were identified upstream of the *Dwee1* coding region. Because no existing deletion was available that uncovered the locus, one of these insertions was used as a dominant marker in a screen for an x-ray–induced deletion of the region. The stock carrying this deletion, *Df(2L)Dwee1;27A-28B*, has a dominant female sterile phenotype that can be complemented by a duplication that covers the region, suggesting that maternal haploinsufficiency for one of the genes uncovered by the deletion is responsible. We did not detect any obvious mitotic defects during cycles 14–16 in embryos that are homozygous for *Df(2L)Dwee1*, implying that zygotic Dwee1 expression is not required during this period of development. This result may reflect the perdurance of maternally provided Dwee1 function. For example, maternally provided *Drosophila* Cdc2 is sufficient for embryogenesis and larval development, and DmCdc2 null mutants are pupal lethals (Stern *et al.*, 1993). In addition, there may be redundant Wee1-like kinases in *Drosophila* that can provide Cdc2 inhibitory functions during embryogenesis, analogous to Mik1 in *S. pombe* (Lundgren *et al.*, 1991).

In situ hybridization studies have shown that *Dwee1* mRNA is maternally supplied and present in all di-

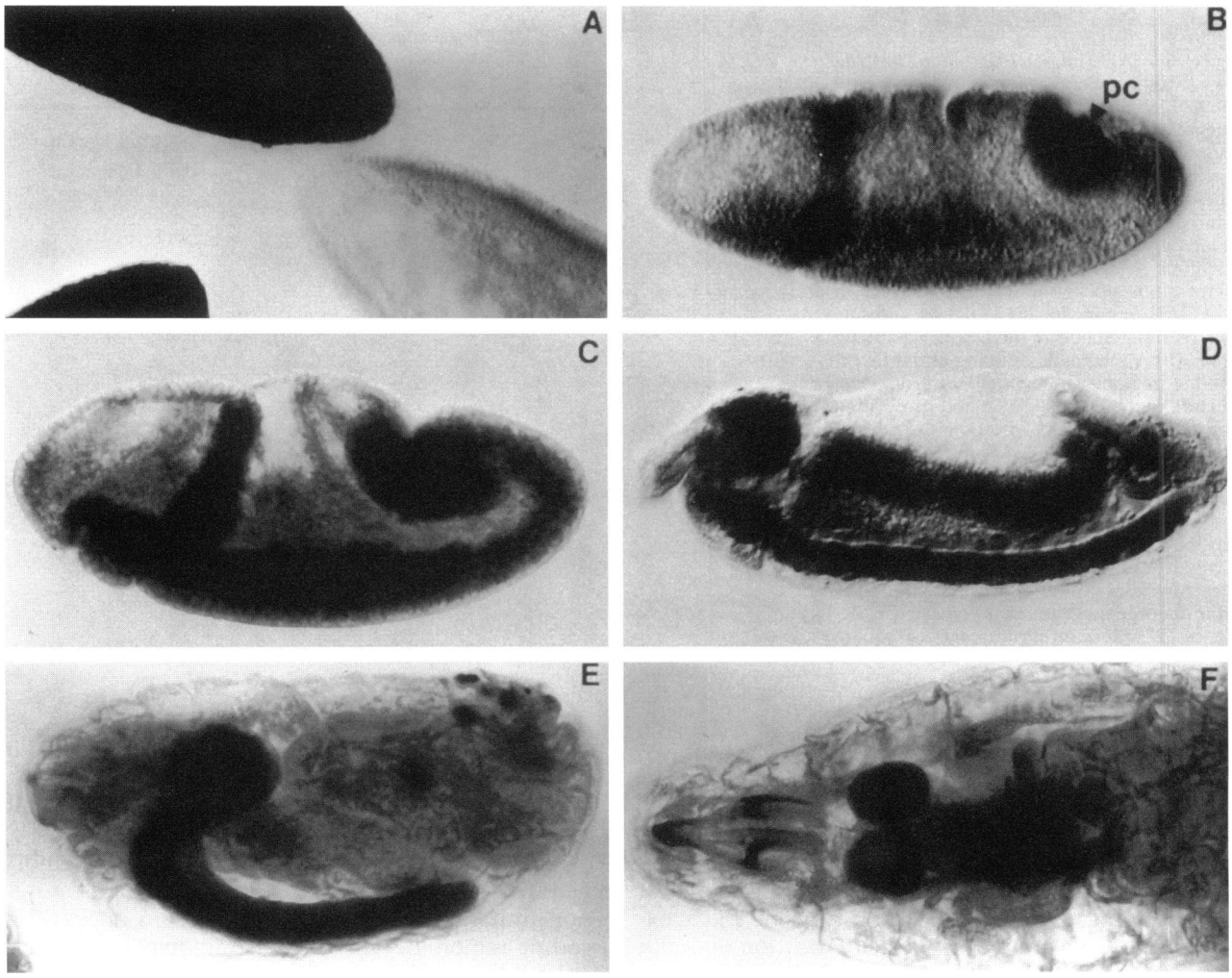

Figure 7. In situ localization of *Dwee1* mRNA to whole mount embryo and larval preparations. Except in panel A, all embryos were mounted with anterior to the left and dorsal up. Staging was by reference to Campos-Ortega and Hartenstein, 1985. (A) Maternal *Dwee1* is degraded in early cycle 14. Shown are a pre-cellular blastoderm embryo (upper, darkly stained) and an embryo in early cycle 14, stage 5 (lower, lightly stained). (B) A gastrulating embryo (stage 6), showing low levels of staining throughout most of the embryo. Maternal *Dwee1* transcripts are not degraded in the pole cells (pc) as they are in the remainder of the embryo (arrowhead). (C) A slightly older gastrulating embryo (stage 7), with the plane of focus set below the epidermis, showing darker staining in the mesoderm. (D) After germ band retraction (stage 14), staining is seen throughout the gut and the central nervous system. (E) A stage 17 embryo is shown where staining has declined throughout most of the embryo except in the central nervous system. (F) The head from a first instar larva is shown, with strong staining in the larval brain (the optic lobes are the round structures, and the ventral ganglion is at a slightly lower plane of focus).

viding cells during embryogenesis, but expression is not modulated transcriptionally during the cell cycle. Expression of *Dwee1* remains high in the central nervous system late in embryogenesis, which is the only tissue that is still proliferating at this time. We were surprised to see abundant maternal *Dwee1* mRNA in the early embryo, because there is no detectable tyrosine phosphorylation of Cdc2 during the early cleavage divisions (Edgar *et al.*, 1994). Frog Cdc2 shows a similar lack of inhibitory phosphorylation during the cleavage divisions (Ferrell *et al.*, 1991). There are several explanations that could satisfy this apparent paradox. Maternally supplied *Dwee1* mRNA may not be translated, or it may be translated but inactive due to post-translational modifications. Regarding this second possibility, negative and positive regulators of Wee1 kinase homologues have been identified in fission yeast, frogs, and humans that act by regulating their phosphorylation states (Russell and Nurse, 1987a; Feilotter *et al.*, 1991; Millar *et al.*, 1992; Coleman *et al.*, 1993; Parker *et al.*, 1993; Tang *et al.*, 1993; Wu and Russell, 1993; McGowan and Russell, 1995; Mueller *et al.*, 1995; Watanabe *et al.*, 1995). Alternatively, it is also possible that Dwee1 kinase is

present and active but incapable of modifying a significant fraction of the Cdc2 kinase because Dwee1 is localized to nuclei, whereas the bulk of the Cdc2 is dispersed in the cytoplasm of the egg and preblastoderm embryo. If this latter explanation is correct, we may find that Dwee1-catalyzed phosphorylation of nuclear Cdc2 does play an important functional role during early embryogenesis, even though this phosphorylation is not presently detectable. In mammalian cells, inactivation of nuclear Cdc2 by human Wee1 is sufficient for rescue from mitotic catastrophe, and rescue is not hindered by the presence of active cytoplasmic Cdc2 kinase (Heald *et al.*, 1993). By analogy, there could be a local nuclear cycle of Cdc2 modification where phosphorylation by Dwee1 kinase inhibits Cdc2 during interphase, and dephosphorylation by maternally provided $Cdc25^{String}$ and the related phosphatase $Cdc25^{Twine}$ locally activates Cdc2 in early embryos as required (Alphey *et al.*, 1992; Courtot *et al.*, 1992; Edgar *et al.*, 1994). We hope to be able to resolve which of these explanations has merit by biochemical experiments with Dwee1 antibodies, and by genetic approaches to identifying and studying *Dwee1* mutants.

ACKNOWLEDGMENTS

Bob Booher is owed many thanks for his advice on *pombe* genetics. Paul Russell (Scripps Institute, San Diego, CA) alerted us to the possibility that our original Dwee1 clone was N-terminally truncated. We thank Susan Younger and Ed Grell for assisting with the x-ray machine. Charles Girdham helped with the polytene in situs. Nick Brown and John Tamkun provided cDNA libraries and a genomic library, respectively. Recombinant GST-DmCyclinB (Doug Kellogg), mutant DmCdc2 clones used to make HA-tagged versions (Pierre Leopold and Lynn Connolly), and nuclear extracts (Danesh Moazed) are gratefully acknowledged. Deborah Morrison (National Cancer Institute, Frederick, MD) provided the 4G10 antibody. Stimulating conversations with members of the UCSF Cell Cycle Club, Ira Herskowitz, Paul Russell, and our excellent colleagues in the O'Farrell lab are gratefully acknowledged. Fly stocks used in these studies were provided by the following: Kathy Matthews (Indiana *Drosophila* Stock Center, Bloomington); Glenn Dougherty and Todd Laverty (NIH *Drosophila* Genome Project and Howard Hughes Medical Institute, Berkeley); David Nash and Stanley Tiong (University of Alberta); and Stephen Wasserman (University of Texas, Southwestern Medical Center). Funding for this work was provided by a grant from the National Institutes of Health to P.O'F. (R01-GM57193). S.D.C. has been funded by a Postdoctoral Fellowship and a Centennial Scholarship from the Medical Research Council of Canada. B.A.E. is a Scholar of the Lucille P. Markey Charitable Trust. Funding for F.S. comes from the Human Frontiers in Sciences Program.

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