6. The modified pT7-7 expression plasmid for the production of full-length CF2-II, carries a 2-bp deletion in the region between the initiation codon and the Shine-Dalgarno sequence, reducing but not eliminating the level of translational initiation. The deletion appears to prevent a basal level of CF2 transcription in the absence of T7 RNA polymerase, which is deleterious and prevents cloning of full-length CF2 in the wild-type vector. The expressed protein formed inclusion bodies that we purified according to F. A. Marston [in *DNA Cloning: A Practical Approach*, D. M. Glover, Ed. (IRL Press, Oxford, 1987), pp. 65 and 80], with slight modifications.
Of the selected sequences, 15 (10/90 from the fourth cycle and 5/75 from the fifth cycle) bore no similarity to the consensus and did not bind CF2 significantly, as verified by gel shift assays. These were not included in the alignment; their occurrence was ascribed to either streaking of the free probe during electrophoresis or to nonspecific binding.
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11. Although finger 6 is clearly important for binding, finger 3 has unusual properties suggesting that either it is structurally variant and unable to interact with DNA, or it recognizes a triplet that is noncontiguous and at a variable distance from the rest. Another possibility is that this finger is not accessible to DNA because of tertiary protein structure.
12. Asymmetric footprints extending 3′ to the consensus were also observed with the maltose binding protein–form I COOH-terminal domain fusion interacting with the CF2 promoter (*1*).
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14. In a control experiment, the same low-degeneracy oligonucleotide was subjected to three cycles of selection and amplification with a partial CF2-II protein containing only the COOH-terminal zinc finger domain. Twenty amplified products of the third selection cycle were cloned and sequenced; in 15 of them the degenerate positions were ATA, with selection being 95% A in the first position, 100% T in the second, and 75% A in the third (Fig. 4B).
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16. Repeated selection cycles, especially with decreasing protein concentration, underestimate the permissiveness of binding, because they emphasize the highest affinity sequences. The constrained permissiveness was most clearly demonstrated experimentally by means of a simplified selection protocol, with direct DNA sequencing after only one selection cycle, without PCR amplification and cloning. With this approach, we have previously reported [J. A. Gogos *et al.*, *Nucleic Acids Res.* **19**, 1449 (1991)] that an A → T transversion is highly favored at position 8 of the recognized strand, as are T → A/G transversions at position 3. Using two additional low degeneracy oligonucleotides, we further showed that, after only one cycle of selection, T is highly favored over A at position 2, whereas at positions 1 and 7 either purine is acceptable (J. A. Gogos, unpublished data).
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March 26, 1992; accepted July 28, 1992.

Inactivation of the p34^{cdc2}–Cyclin B Complex by the Human WEE1 Tyrosine Kinase

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Entry into mitosis in *Schizosaccharomyces pombe* is negatively regulated by the *wee1*$^+$ gene, which encodes a protein kinase with serine-, theonine-, and tyrosine-phosphorylating activities. The *wee1*$^+$ kinase negatively regulates mitosis by phosphorylating p34^{cdc2} on tyrosine 15, thereby inactivating the p34^{cdc2}–cyclin B complex. The human homolog of the *wee1*$^+$ gene (*WEE1Hu*) was overproduced in bacteria and assayed in an in vitro system. Unlike its fission yeast homolog, the product of the *WEE1Hu* gene encoded a tyrosine-specific protein kinase. The human WEE1 kinase phosphorylated the p34^{cdc2}–cyclin B complex on tyrosine 15 but not on threonine 14 in vitro and inactivated the p34^{cdc2}–cyclin B kinase. This inhibition was reversed by the human Cdc25C protein, which catalyzed the dephosphorylation of p34^{cdc2}. These results indicate that the product of the *WEE1Hu* gene directly regulates the p34^{cdc2}–cyclin B complex in human cells and that a kinase other than that encoded by *WEE1Hu* phosphorylates p34^{cdc2} on threonine 14.

The mechanisms that regulate progression through the eukaryotic cell cycle are highly conserved. The G_2-M phase transition is universally regulated by p34^{cdc2}, a Ser-Thr protein kinase. The activity of the p34^{cdc2}–cyclin B complex is required for progression of cells into the M phase (*1*). In fission yeast, several mitotic regulators have been identified that are thought to directly regulate the p34^{cdc2}–cyclin B complex. One of these regulators, *wee1*$^+$, encodes a kinase (p107^{wee1}) that has been classified as a dual-specificity kinase on the basis of its ability to autophosphorylate on Ser and Tyr residues (*2–4*). p107^{wee1} phosphorylates the p34^{cdc2}–cyclin B complex on Tyr^{15}, thereby rendering p34^{cdc2} inactive (*2*). A second mitotic regulator, *cdc25*$^+$, encodes a protein phosphatase that dephosphorylates Tyr^{15} and activates the p34^{cdc2}–cyclin B complex (*5–10*). In higher eukaryotes, p34^{cdc2} is negatively regulated by phosphorylation on both Thr^{14} and Tyr^{15} (*11, 12*). The human kinases responsible for these phosphorylations are unknown, although the *cdc25*$^+$ gene product has been implicated in both Thr^{14} and Tyr^{15} dephosphorylation (*6, 8, 10*). On the basis of the conservation of structure and function demonstrated for cell cycle regulators throughout evolution, it was predicted that the human homolog of *wee1*$^+$ would also encode a dual-specificity kinase that would negatively regulate p34^{cdc2} by phosphorylation of Tyr^{15} and Thr^{14} (*13*). A gene (*WEE1Hu*) from a human foreskin fibroblast cDNA library has been cloned by its ability to rescue *wee1*$^+$ mutants in *Schizosaccharomyces pombe* (*14*). *WEE1Hu* shares 29% sequence identity within the kinase domain of *wee1*$^+$ and is predicted to encode a protein kinase of ~49 kD.

To analyze the biochemical activities associated with the human *WEE1* gene product, we expressed it in bacteria as a fusion protein with glutathione-S-transferase (GST) (*15*). A bacterial expression system was chosen on the basis of the apparent lack of endogenous tyrosine kinase activity in bacteria and the success of this expression system for the characterization

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of other dual specificity kinases (*13*). The resulting fusion protein (GST-p49^{WEE1Hu}) migrated on SDS-polyacrylamide gels with an apparent molecular size of 76 kD (Fig. 1A). Cleavage of the fusion protein with thrombin generated a 49-kD protein denoted p49^{WEE1Hu}. The GST-p49^{WEE1Hu} protein was phosphorylated both in vitro (Fig. 1B) and in vivo (Fig. 1D) (*16*). In both cases, phosphoamino acid analysis revealed only phosphotyrosine (Fig. 1, C and E). Similar results were obtained with p49^{WEE1Hu} from which GST had been removed (*17*). These results are in contrast to those seen with p107^{wee1} from *S. pombe*, where autophosphorylation occurs primarily on Ser, Tyr, and, to a lesser extent, Thr residues (*2–4*).

To test whether the human p34^{cdc2}–cyclin B complex is a substrate for the human WEE1 kinase, we performed phosphorylation reactions in vitro (*2*). The p34^{cdc2}–cyclin B complex was isolated from insect cells that had been co-infected with recombinant viruses encoding GST–cyclin B and a mutant of p34^{cdc2} encoding Arg for Lys^{33} [p34^{cdc2}(Arg^{33})]. Mutation of Lys^{33} renders the p34^{cdc2}-cyclin complex inactive (*2, 12, 18*). GST–cyclin B and p34^{cdc2}(Arg^{33}) were used in this experiment because large amounts of p34^{cdc2}–cyclin B complex were easily isolated with glutathione agarose as an affinity reagent (Fig. 2A), and background phosphorylation that was a result of an active p34^{cdc2}-GST–cyclin B complex was eliminated (*2*). Both GST-p49^{WEE1Hu} (Fig. 1A) and GST (Fig. 1A) were produced in bacteria, isolated on glutathione agarose, and then eluted with excess glutathione. Kinase assays were then performed in vitro with purified proteins (*19*). The p34^{cdc2} (Arg^{33}) protein was not detectably phosphorylated when kinase assays were performed in vitro in the absence of GST-p49^{WEE1Hu} but was phosphorylated in the presence of GST-p49^{WEE1Hu} (Fig. 2B). Phosphoamino acid analysis of p34^{cdc2} revealed only phosphotyrosine (Fig. 2C). A single phosphopeptide was detected upon two-dimensional phosphotryptic mapping, and Tyr^{15} was identified as the site of phosphorylation (Fig. 2D). The human WEE1 kinase did not catalyze the phosphorylation of p34^{cdc2}(Arg^{33}) on Thr^{14}. Similar results were obtained with the human WEE1 kinase produced in insect cells (*17*). The catalytic domain of the human Cdc25C phosphatase dephosphorylated p34^{cdc2}(Arg^{33}) on Tyr^{15}, and sodium orthovanadate blocked the dephosphorylation of Tyr^{15} by the Cdc25C phosphatase (Fig. 2B) (*20*). The human WEE1 kinase was also tested for its ability to phosphorylate enolase, casein, histone H1, monomeric p34^{cdc2}, and a peptide derived from p34^{cdc2} containing Thr^{14} and Tyr^{15}. Only monomeric p34^{cdc2} and the peptide were phosphorylated by the WEE1Hu kinase, and the phosphorylation was on Tyr^{15} in both cases (*17*).

To determine the effect of Tyr^{15} phosphorylation on the kinase activity of the p34^{cdc2}–cyclin B complex, we performed histone H1 kinase assays (Fig. 3) (*21*). When the p34^{cdc2}-GST–cyclin B complex was incubated either alone or with purified GST, the complex functioned efficiently as a histone H1 kinase. However, phos-

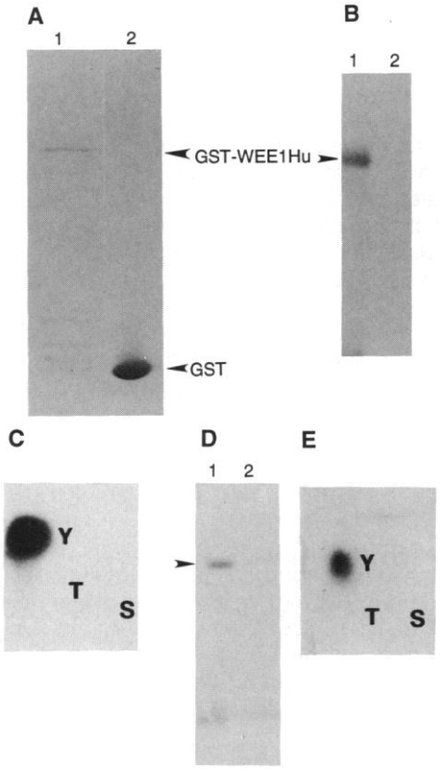

Fig. 1. Expression and phosphorylation of the human WEE1 kinase in bacteria. (**A**) GST-p49^{WEE1Hu} (lane 1) and GST (lane 2) were precipitated from bacterial lysates with glutathione agarose. Proteins were resolved by SDS-PAGE and visualized by staining with Coomassie blue. (**B**) GST-p49^{WEE1Hu} (lane 1) and GST (lane 2) were precipitated from bacterial lysates with glutathione agarose, and kinase assays were performed in vitro. Proteins were resolved by SDS-PAGE and visualized by autoradiography. (**C**) Two-dimensional phosphoamino acid analysis of GST-p49^{WEE1Hu} labeled in vitro. (**D**) Bacteria that expressed either GST-p49^{WEE1Hu} (lane 1) or GST (lane 2) were incubated with [^{32}P]orthophosphate. Proteins were precipitated with glutathione agarose, resolved by SDS-PAGE, and visualized by autoradiography. Arrowhead indicates GST-WEE1Hu. (**E**) Two-dimensional phosphoamino acid analysis of GST-p49^{WEE1Hu} labeled in vivo; Y, phosphotyrosine; S, phosphoserine; T, phosphothreonine.

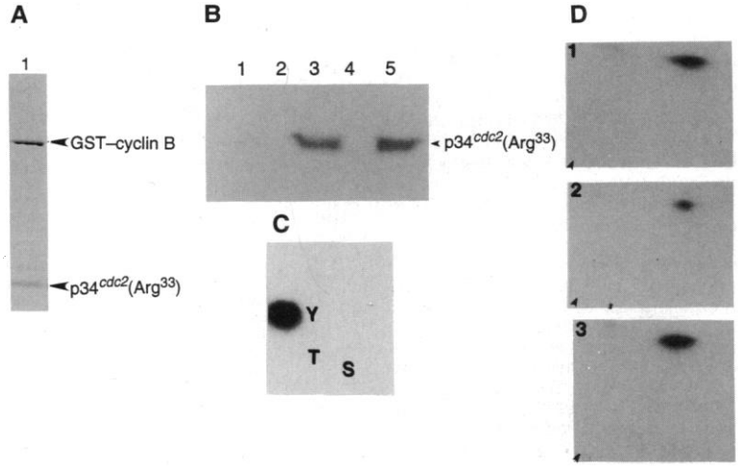

Fig. 2. Regulation of Tyr^{15} phosphorylation by the human WEE1 kinase and the Cdc25C phosphatase in vitro. (**A**) Insect cells were co-infected with recombinant viruses encoding p34^{cdc2}(Arg^{33}) and GST–cyclin B. Lysates were prepared, and the p34^{cdc2}(Arg^{33})–cyclin B complex was isolated on glutathione agarose beads. The complex was resolved by SDS-PAGE and visualized by staining with Coomassie blue. (**B**) The p34^{cdc2}(Arg^{33})–cyclin B complex was isolated as described in (A). Kinase assays were performed in vitro in the presence of the p34^{cdc2}(Arg^{33})–cyclin B complex alone (lane 1) or in the presence of the p34^{cdc2}(Arg^{33})–cyclin B complex with either soluble GST (lane 2) or with soluble GST-p49^{WEE1Hu} (lanes 3 through 5). Kinase reactions were washed, and phosphatase assays were performed in the presence of phosphatase buffer alone (lanes 1 through 3) or phosphatase buffer containing GST-C215 protein in the absence (lane 4) or in the presence (lane 5) of 2 mM vanadate. Reactions were stopped by boiling in SDS–sample buffer; proteins were resolved by SDS-PAGE and visualized by autoradiography. (**C**) Two-dimensional phosphoamino acid analysis of p34^{cdc2}(Arg^{33}) phosphorylated by GST-p49^{WEE1Hu}; Y, phosphotyrosine; S, phosphoserine; T, phosphothreonine. (**D**) Two-dimensional phosphotryptic maps of (1) p34^{cdc2}(Arg^{33}), (2) the Thr^{14}- and Tyr^{15}-containing peptide phosphorylated by pp60$^{v\text{-}src}$ in vitro, and (3) a mixture of (1) and (2). Arrowheads indicate the origin.

phorylation of the p34^{cdc2}–cyclin B complex by GST-p49^{WEE1Hu} ablated the histone H1 kinase activity (Fig. 3). The activity was partially restored upon addition of the catalytic domain of the Cdc25C phosphatase, and sodium orthovanadate blocked the action of the Cdc25C phosphatase (Fig. 3). The ability of the Cdc25C phosphatase to reactivate the complex varied between experiments; on average, 50% of the activity was restored. This may reflect either a loss of complex during the experimental manipulations or some variability in the ability of the Cdc25C phosphatase to completely dephosphorylate the phosphorylated form of the complex under these conditions. Alternatively, during the course of the experiment p34^{cdc2} may become partially dephosphorylated on Thr^{161}, which would prevent reactivation of the p34^{cdc2}–cyclin B kinase (*12*, *22*).

The results presented here suggest that the human gene *WEE1Hu* encodes a Tyr-specific protein kinase (p49^{WEE1Hu}) unlike its fission yeast homolog, which encodes a dual specificity kinase. Both kinases phosphorylate p34^{cdc2} on Tyr^{15}. Coincident with the Tyr^{15} phosphorylation of p34^{cdc2} by p49^{WEE1Hu} was an inactivation of the histone H1 kinase activity of the human p34^{cdc2}–cyclin B complex. This inhibition was reversed upon Tyr^{15} dephosphorylation in vitro, which was catalyzed by the Cdc25C phosphatase. These results demonstrate that p49^{WEE1Hu} and the Cdc25C phosphatase possess antagonistic activities that are capable of regulating the activity of the p34^{cdc2}–cyclin B complex in vitro. This is not to say that p49^{WEE1Hu} regulates the activity of the p34^{cdc2}–cyclin B complex in vivo. The ability to rescue mutants in yeast is not a definitive test of functional homology; for example, human G_2 cyclins rescue CLN (G_1 cyclins) function in *Saccharomyces cerevisiae* (*23*). Multiple human homologs of both *cdc25*$^{+}$ and the cyclins have been identified (*5*, *23*, *24*). Thus, p49^{WEE1Hu} may not be the only member of its family.

p34^{cdc2} in higher eukaryotes is phosphorylated on both Thr^{14} and Tyr^{15}. Phosphorylation of either residue inactivates the p34^{cdc2}–cyclin B complex (*11*, *12*). Neither *S. pombe* p107^{wee1} nor human p49^{WEE1Hu} phosphorylated p34^{cdc2} on Thr^{14} in vitro. This suggests that a kinase other than that encoded by *WEE1Hu* phosphorylates p34^{cdc2} on Thr^{14}. Thus, more than one signal transduction pathway in higher eukaryotes could result in the inactivation of p34^{cdc2}.

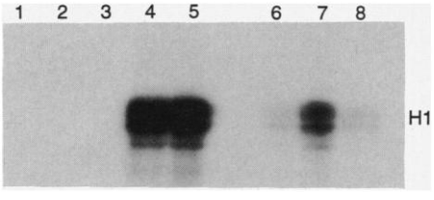

Fig. 3. Regulation of p34^{cdc2}–cyclin B kinase activity by the human WEE1 kinase and the Cdc25C phosphatase in vitro. The human WEE1 kinase and the Cdc25C phosphatase were tested for their ability to regulate the histone H1 kinase activity of the p34^{cdc2}–cyclin B complex in vitro. Insect cells were co-infected with recombinant viruses encoding wild-type p34^{cdc2} and GST–human cyclin B. The p34^{cdc2}–cyclin B complex was isolated on glutathione beads, and kinase assays were performed in kinase buffer alone (lane 4) or in kinase buffer containing either soluble GST (lane 5) or soluble GST-p49^{WEE1Hu} (lanes 6 through 8). Kinase reactions were washed, and then phosphatase assays were performed in the presence of either phosphatase buffer alone (lanes 4 to 6) or phosphatase buffer containing Cdc25 protein in the absence (lane 7) or in the presence (lane 8) of sodium orthovanadate (2 mM). Reactions were washed, and histone H1 kinase assays were performed. Recombinant Cdc25(C215) (lane 1), GST-p49^{WEE1Hu} (lane 2), and GST (lane 3) were also assayed for histone H1 kinase activity.

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15. The plasmid pGEX-2TWEE1Hu was created as follows: the *WEE1Hu* gene of pCD2-*WEE1Hu* (*14*) was amplified by polymerase chain reaction with the following primers: 5′-CCGCTAGCGGATCCATGGATACAGAAAAATC-3′ and 5′-GGGCTAGCGAATTCGGAGTAGCTCAGTATATAG-3′. The amplified DNA was digested with Bam HI and Eco RI and inserted into the Bam HI–Eco RI site of pGEX-2T (Pharmacia). For the induction of GST and GST-p49^{WEE1Hu}, overnight cultures (100 ml) of JM109 transformed with pGEX-2TWEE1Hu or pGEX-2T were diluted 1:10 in rich media and incubated at 37°C for 1 hour. Cultures were then adjusted to 1 mM isopropyl-1-thio-β-D-galactoside (IPTG) and incubated for 3 hours at 30°C. Cells were sonicated in 5 ml of lysis buffer [50 mM tris (pH 7.4), 0.25 M $NaCl_2$, 50 mM NaF, 10 mM NaPPi, 0.1% NP-40, 2 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, 2 mM sodium orthovanadate, and 20 μM leupeptin]. Lysates were centrifuged for 10 min at 10,000*g*, and clarified lysates were incubated with a 1:1 slurry of glutathione agarose beads (Sigma) in phosphate-buffered saline (100 μl). Bound proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE).
16. Labeling in vitro: Bacteria were grown and proteins were induced as described (*15*). After the addition of IPTG, cells were incubated for 3 hours at 30°C and lysed as described (*15*). GST and GST-p49^{WEE1Hu} were precipitated with glutathione agarose beads (100 μl) as described (*15*). The precipitates were washed twice in lysis buffer and twice in incomplete kinase buffer, and kinase assays were performed as described (*5–10*). Labeling in vivo: Bacteria were grown and induced as described (*15*). After the addition of IPTG, cells were incubated at 30°C for 1 hour and washed in labeling buffer [50 mM tris (pH 7.4), 100 mM NaCl, and 10 mM $MgCl_2$]. Washed cellular pellets were suspended in labeling buffer (1 ml) that contained 2 mCi [^{32}P]orthophosphate and incubated at 37°C for 15 min. Cells were lysed by the addition of lysozyme (1 mg/ml) and were centrifuged at 10,000*g* for 10 min. GST and GST-p49^{WEE1Hu} were precipitated from the supernatant with glutathione agarose (100 μl), and precipitates were resolved by SDS-PAGE. Two-dimensional phosphoamino acid analysis was performed as described (*3*).
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19. Procedures relating to viral propagation, culturing of insect cells, isolation of the p34^{cdc2}–cyclin B complex, kinase assays, phosphoamino acid analysis, tryptic phosphopeptide mapping, and phosphorylation of the Tyr^{15}-containing peptide by pp60$^{v\text{-}src}$ have been described (*2*, *3*) [H. Piwnica-Worms, in *Current Protocols in Molecular Biology*, F. Ausubel *et al.*, Eds. (Greene, New York, 1990), sections 16.8.1 to 16.11.6]. Bacterially expressed GST-p49^{WEE1Hu} and GST were induced and isolated as described (*15*). GST and p49^{WEE1Hu} were eluted by incubation of glutathione agarose beads with an equal volume of elution buffer [20 mM glutathione in 50 mM tris (pH 7.4) and 100 mM NaCl] for 15 min at 4°C.
20. The COOH-terminal catalytic domain of human Cdc25C (C215) was purified from bacteria as a fusion protein with GST as follows: frozen bacterial pellets from cultures (50 ml) of induced JM109 cells were suspended in 5 ml of NETN [20 mM tris (pH 8.0), 100 mM, NaCl, 1 mM EDTA, and 0.5% NP-40] (*8*), lysed by sonication, and spun at 10,000*g* for 15 min. Glutathione agarose beads were added to clarified lysates, and reactions were incubated at 4°C for 30 min (1 to 5 ml of supernatant per 25 μl of packed beads). Pelleted beads were washed twice with NETN and three times with either phosphatase buffer [50 mM tris, (pH 7 4), 10 mM $MgCl_2$, and 1 mM dithiothreitol] or phosphatase buffer containing 2 mM sodium orthovanadate. C215 bound to beads was then used to dephosphorylate p34^{cdc2}.
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25. We thank H. Okayama and M. Igarashi for pCD2-WEE1Hu. Supported by NIH and an NIH postdoctoral fellowship (L.L.P.). H.P.-W. is a Pew Scholar in the Biomedical Sciences.

13 April 1992; accepted 27 July 1992