Identification of Residues in Fission Yeast and Human p34^{cdc2} Required for S-M Checkpoint Control

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ABSTRACT

In fission yeast, regulation of p34^{cdc2} plays an important role in the checkpoint coupling mitosis to completion of DNA replication. The *cdc2* mutations *cdc2-3w* (C67Y) and *cdc2-4w* (C67F) abolish checkpoint control without seriously affecting normal cell proliferation. However the molecular basis of this phenotype is not known. To better understand the role of p34^{cdc2} in checkpoint control, we have screened for more mutations in *Schizosaccharomyces pombe cdc2* with this phenotype. We have isolated *cdc2-3w* and *cdc2-4w,* as well as three new *cdc2* alleles: *cdc2-6w* (N66I), *cdc2-7w* (E8V) and *cdc2-8w* (K9E). The altered residues map to two different regions on opposite faces of the protein, suggesting that the interaction between p34^{cdc2} and components of the checkpoint pathway may be complex. In contrast to *cdc2-3w* and *cdc2-4w,* the new mutations alter residues that are conserved between the fission yeast *cdc2*$^+$ and other cdks, including the human *CDC2* protein. Expression of the equivalent human *CDC2* mutants in fission yeast abolishes checkpoint control, suggesting that these residues could be involved in checkpoint-dependent regulation of other eukaryotic cdks.

CHECKPOINT controls delay or block late cell cycle events if earlier events have not been completed (HARTWELL and WEINERT 1989). A well-studied checkpoint, referred to here as the S-M checkpoint, ensures that DNA synthesis (S) has been completed before initiation of mitosis (M). Many genes involved in this checkpoint have been identified in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (SHELDRICK and CARR 1993). In *S. pombe,* one target of the S-M checkpoint pathway is likely to be p34^{cdc2}, the catalytic subunit of the major fission yeast cdk (ENOCH and NURSE 1990; ENOCH *et al.* 1991) that is required for initiation of mitosis. The mitotically active form of p34^{cdc2} is found in a complex with a B-type cyclin encoded by the *cdc13*$^+$ gene. During G2, this complex is maintained in an inactive state by phosphorylation of p34^{cdc2} on tyrosine 15 (Y15). The length of G2 is determined by the balance between the activity of inhibitory tyrosine kinases, encoded by the *wee1*+ and *mik1*+ genes, and activating tyrosine phosphatases, encoded by the *cdc25*+ and *pyp3*+ genes (NURSE 1990; LUNDGREN *et al.* 1991; MILLAR *et al.* 1992).

Mutations that completely abolish p34^{cdc2} tyrosine phosphorylation dramatically reduce the length of the fission yeast cell cycle, resulting in cells that enter mitosis at a very small size. Under these conditions mitosis is often lethal, and such an event is termed "mitotic catastrophe." Such mutants are inviable or display a greatly increased generation time compared to wild-type cells. Examples of such mutations are *cdc2-F15* (GOULD and NURSE 1989) in which the tyrosine has been changed to a nonphosphorylatable residue phenylalanine, and a *mik1 wee1* (LUNDGREN *et al.* 1991) double mutation that eliminates both inhibitory kinases. Tyrosine phosphorylation of p34^{cdc2} must also be required for cell cycle arrest in response to unreplicated DNA, since *mik1 wee1* and *cdc2-F15* mutants do not undergo cell cycle arrest when DNA replication is blocked by inhibitors such as hydroxyurea (HU) (ENOCH *et al.* 1991; LUNDGREN *et al.* 1991). Tyrosine phosphorylation of cdks also negatively regulates mitosis in many other eukaryotic cells (MALLER 1990), however it does not regulate the timing of mitosis or the S-M checkpoint in *S. cerevisiae* (AMON *et al.* 1992; SORGER and MURRAY 1992).

There are also *cdc2* mutations that alter cell cycle control in a more subtle fashion, but that still completely abolish checkpoint control. These alleles were first identified because they reduce the length of G2 and suppress loss of *cdc25*$^+$ function (FANTES 1981). Because these mutations reduce cell size at division, they were called "wee" mutants (NURSE 1975). Examples of such alleles include two dominant *cdc2* alleles, *cdc2-3w* (C67Y) or *cdc2-4w* (C67F) (ENOCH and NURSE 1990). However, other wee alleles of *cdc2*$^+$, including *cdc2-1w* (G146D), arrest normally when DNA replication is blocked (ENOCH and NURSE 1990). Thus, the checkpoint defects of *cdc2-3w* and *cdc2-4w* are not simply a consequence of the reduction of G2. In addition, genetic studies have shown that *cdc2-1w* and *cdc2-3w* respond differently to loss of *wee1*+ or *cdc25*$^+$ function (RUSSELL and NURSE 1986, 1987). These results suggest

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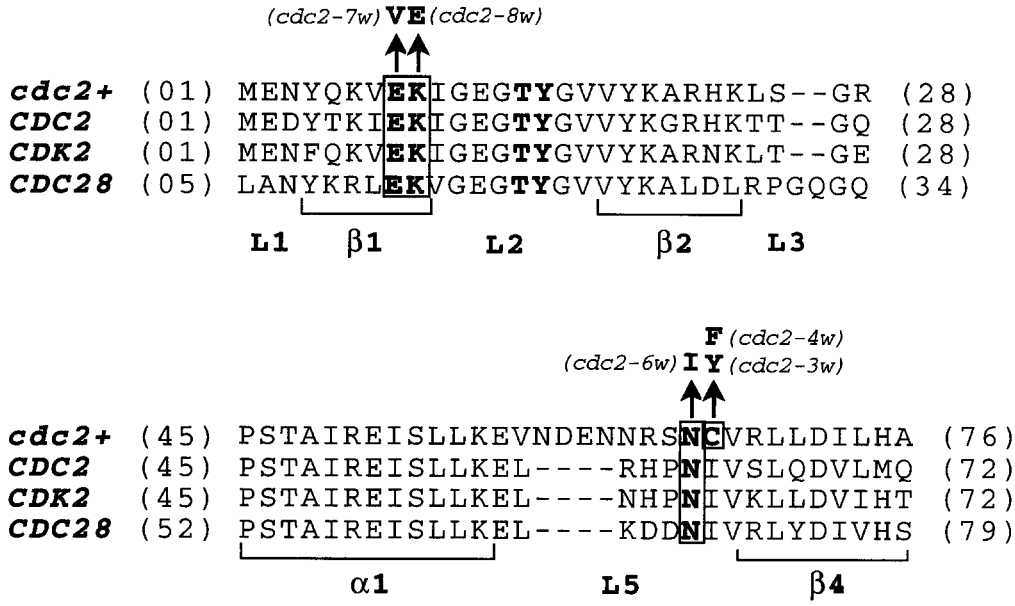

FIGURE 1.—Alignment of the sequences of the fission yeast *cdc2* protein (*cdc2+*), and the human *CDC2* and *CDK2*, and *S. cerevisiae CDC28* showing that the L5 region is structurally unique in *cdc2+*. The location of the alterations in checkpoint-deficient mutants is also shown. The secondary structure, based on the crystal structure of CDK2 (DE BONDT *et al.* 1993), is shown below the alignments. The residues altered in the mutants are in bold, and the mutant sequence is shown above the arrows. The two residues T14 and Y15 that are regulated by tyrosine-phosphorylation are also in bold.

that there are different ways to partially deregulate $p34^{cdc2}$, however the molecular basis of these phenotypes is not understood. In particular, it is not clear why mutation of C67 abolishes checkpoint control since this residue is not near tyrosine 15, and in addition levels of $p34^{cdc2}$ tyrosine 15 phosphorylation are reported to be normal in both *cdc2-3w* and *cdc2-1w* (GOULD *et al.* 1990).

It is also not known whether similar mutations would abolish checkpoint-dependent regulation of $p34^{cdc2}$ in other eukaryotes. C67 is located in a region of *S. pombe* $p34^{cdc2}$ that is structurally unique compared to other cdks (see Figure 1). The loop in which the residue is found, L5, consists of seven amino acids in other cdks, but is 11 amino acids long in $p34^{cdc2}$. Furthermore, the position equivalent to C67 (63 in human *CDC2* and *CDK2*, 70 in *S. cerevisiae CDC28*) is occupied by an isoleucine in many other cdks. It is therefore possible that the mutant phenotype in fission yeast is a consequence of the unique structural features of fission yeast protein in the L5 region. Indeed, mutation of the equivalent residue (I63) in human *CDC2* does not alter cell cycle control when the mutant alleles are expressed in fission yeast (MACNEILL and NURSE 1993a).

We would like to understand how $p34^{cdc2}$ is regulated by the S-M checkpoint pathway in fission yeast, and to determine if checkpoint-dependent regulation works by similar mechanisms in other eukaryotes. For these reasons, we have screened for more *cdc2* alleles that abolish checkpoint control without significantly disrupting the normal cell cycle. We were able to isolate five such alleles including *cdc2-3w* and *cdc2-4w*, as well as three new alleles that we are calling *cdc2-6w*, *cdc2-7w*, and *cdc2-8w*. All the mutants are phenotypically identical by several criteria, suggesting that they disrupt checkpoint control by the same mechanism. However, the mutated residues are found in two different domains predicted to lie on opposite sides of $p34^{cdc2}$ molecule. In contrast to *cdc2-3w* and *-4w*, the new *cdc2-w* alleles alter residues that are conserved in other cdks. We constructed equivalent human *CDC2-7w* and *CDC2-8w* mutants and introduced them into fission yeast strains where human *CDC2* replaces the *S. pombe cdc2* (MACNEILL and NURSE 1993a). Expression of the mutant alleles abolished checkpoint control suggesting that these conserved residues could also be involved in checkpoint-dependent regulation of cdks in other eukaryotic cells.

MATERIALS AND METHODS

Growth of *S. pombe*: We used standard media and growth conditions (MORENO *et al.* 1991). The following strains were used: TE280 (*leu1-32 h−*), TE281 (*cdc2-L7 leu1-32 h−*), TE282 (*cdc25-22 leu1-32 h−*), TE354 (*wee1-50 leu1-32 h−*), TE460 (*cdc2::CDC2 his3-237 leu1-32 h−*). All except TE460 were generous gifts of PAUL NURSE. TE460 is the generous gift of Stuart MACNEILL. Cells were grown at 25° (TE282, TE354, TE460) or 29° (TE280, TE281) in Edinburgh Minimal Medium (EMM) containing 5 μM thiamine. For derepression of the *nmt1* promoter on REP81, cells were shifted to EMM without thiamine for 24 hr. After derepression of the promoter, hydroxyurea (HU) sensitivity was tested by growing cells in EMM containing 5 or 10 mM HU (Sigma). Growth temperatures used in particular experiments are indicated in the figure legends.

Vectors and molecular genetic techniques: General procedures used in DNA cloning and sequencing are described in

SAMBROOK *et al.* (1989). For all the experiments described in this manuscript the vector pREP81, with a weak thiamine repressible promoter, was used (MAUNDRELL 1990; BASI *et al.* 1993). cDNAs encoding either fission yeast *cdc2+* (GOULD and NURSE 1989) or human *CDC2* (DUCOMMUN *et al.* 1991) were cloned in the *Nde*I and *Bam*HI sites of pREP81. The following three oligonucleotides were used to sequence *cdc2* mutants: *nmt1-5′* (5′ATCATCAATTGAATAAGTTG) complementary to the *nmt1* promoter, *nmt1-3′* (5′CAAAATCGTAATATGCAGCTTG) complementary to the polyadenylation region of *nmt1*, and *cdc2-1* (5′TGTATCTTGTTTTTGAGT) complementary to the *cdc2+* sequence. Plasmid recovery from *S. pombe* was carried out essentially as described in HOFFMAN and WINSTON (1987), with addition of a precipitation of the DNA in 70% ethanol between the phenol extraction step and transformation in *Escherichia coli*. Transformation of *S. pombe* was carried out by the lithium acetate method (MORENO *et al.* 1991), unless otherwise indicated.

PCR mutagenesis and library construction: PCR mutagenesis of the *S. pombe cdc2* cDNA was done according to the method of LEUNG *et al.* (1989) in the absence of $MnCl_2$. The two oligonucleotides, *nmt1-5′* and *nmt1-3′* (see above), were used for the PCR, and pREP81-*cdc2+* linearized with *Pst*I was used as a template. After PCR the amplified fragment was purified by electroelution from agarose gel, digested with *Nde*I and *Bam*HI, and subcloned into *Nde*I- and *Bam*HI-digested pREP81. The ligation mix was then transformed in *E. coli* by electroporation. More than 10^5 independent colonies were obtained. The colonies were pooled and grown for 4 hr in liquid LB medium containing 100 μg/ml ampicillin. The DNA was prepared by alkaline lysis and purified with Qiagen-tips (Qiagen).

Screening for *cdc2* mutants defective in the S-M checkpoint: The conditions for screening were optimized using the temperature-sensitive TE281 (*cdc2-L7 leu1-32 h−*) strain carrying either the plasmid pREP81-*cdc2+*, or pREP81 *cdc2-F15*. The library of *cdc2* mutants was transformed in the strain TE281 by electroporation as described (PRENTICE 1991). After transformation the plates were grown at 29° for 1 day and then shifted to 36° for 3 days, to eliminate transformants expressing nonfunctional *cdc2* protein. The colonies were transferred by replica plating to EMM plates with phloxine B (Fisher) with or without 5 mM HU, and then grown for 2–3 days at 32°. Colonies unable to grow in the presence of HU were identified. More than 2×10^4 colonies were screened and 70 were isolated that were HU sensitive. Fourteen transformants that displayed a strong phenotype on HU while growing well in its absence were selected for further analysis. Plasmid was recovered from each of these by transformation of *E. coli* and used to retransform the original TE281 strain. Eleven of these plasmids conferred HU sensitivity upon retransformation. These were subjected to DNA sequence analysis. The predicted amino acid alterations of each of these *cdc2* mutants is listed in Table 1. The corresponding nucleotides mutated are as follows: mutant #6: C67F (G200T); #11: E8V (A23T), M273T (T818C); #13: E8V (A23T); #21: E8V (A23T); #28: K9E (A25G), E40G (A119G), I72V (A214G); #29: K9E (A25G), A76V (C227T); #42: C67Y (G200A), H75L (A224T), L255M (T763A); #45: C67Y (G200A); #54: E8V (A23T), S188T (T562C); #59: N66I (A197T); #67: K9E (A25G), N120D (A358G).

Flow cytometry and microscopy: Cells were fixed in 70% ethanol, stained with propidium iodide, and analyzed using a FACScan flow cytometer (Cytofluorograph II, Ortho Diagnostic Systems), as described in SAZER and SHERWOOD (1990). DAPI staining was carried out as described in BASI and DRAETTA (1995). Phase contrast and fluorescence micrographs were obtained using a Zeiss Axiophot microscope and a Photonic Microscope Image Processor C1966 (Hamamatsu).

Construction of human *CDC2-7w* and *CDC2-8w*: The mutations E8V and K9E were introduced in the human *CDC2* cDNA using two complementary oligonucleotides corresponding to the 5′ region of *CDC2*. For the E8V mutation, the oligonucleotides E8V-5′ (TAGGATCCATATGGAAGATTATACCAAAATAGTGAAAATTGGAG AAGGTAC) and E8V-3′ (CTTCTCCAATTTTCACTATTTTGGTATAAT CTTCCATCATATGGATCC) were used. For the K9E mutation, the oligos K9E-5′ (TAGGATCCATATGGAAGATTATACCAAAATAGAGGAAATTGGAG AAGGTAC) and K9E-3′ (CTTCTCCAATTTCCTCTATTTTGGTATAATCTTCCATCATATGGATCC) were used. Each pair of oligonucleotides was annealed and the fragments, which had *Nde*I- and KpnI-compatible ends, were used in a three-way ligation together with a *Kpn*I-*Bam*HI fragment of the wild-type human *CDC2* cDNA, and with the pREP81 vector cut at the *Nde*I and *Bam*HI sites. To assess the affects of these mutations on checkpoint control, *CDC2* constructs were transformed into TE460 (*cdc2::CDC2 his3-237 leu1-32 h−*), where the endogenous *S. pombe cdc2+* has been replaced by the human *CDC2* gene (MACNEILL and NURSE 1993a).

Structural analysis of *cdc2-w* mutants: The location of the residues altered in *cdc2-w* mutants was visualized on the predicted three-dimensional structure of CDK2/cyclinA using RasMol version 3.5. Crystallography coordinates were generously provided by Dr. Nikola Pavelitch.

RESULTS

Isolation of checkpoint-deficient *cdc2* mutants: We developed a method for screening plasmid-born copies of *cdc2+* for mutations that abolish the S-M checkpoint. Wild-type *cdc2+* and the *cdc2-F15* mutants were subcloned into the REP81 vector that bears a mutated version of the thiamine-repressible *nmt1* promoter (MACNEILL and NURSE 1993a). Upon induction this promoter directs expression of physiological levels of p34*cdc2* (our unpublished data; LABIB *et al.* 1995). Plasmids bearing wild-type and mutant alleles of *cdc2* were introduced into TE281 (*cdc2-L7 leu1-32 h−*), which has a temperature-sensitive allele of *cdc2+*, called *cdc2-L7* as well as a *leu1* marker that can be used for selection of transformants. Colonies were initially grown in the absence of thiamine at 36.5°, the restrictive temperature for *cdc2-L7*, to establish that plasmid-born copies of *cdc2* were functional. Checkpoint function was assayed by examining the phenotypes of transformants on HU at 32°, as HU arrest is more efficient at this temperature. Cells transformed with wild-type *cdc2+* initially undergo cell cycle arrest under these conditions, and then resume the cell cycle with a greatly extended S-phase. The result is a slow growing colony of highly elongated cells. In contrast, cells transformed with *cdc2-F15* fail to form colonies as cells die attempting to undergo mitosis in the absence of DNA replication (data not shown).

To identify new checkpoint-defective *cdc2* mutants, a *cdc2* mutant library was constructed in the vector REP81 (see MATERIALS AND METHODS). The library was transformed into TE281 strain and was initially grown at 36° under inducing conditions for the *nmt1* promoter. This

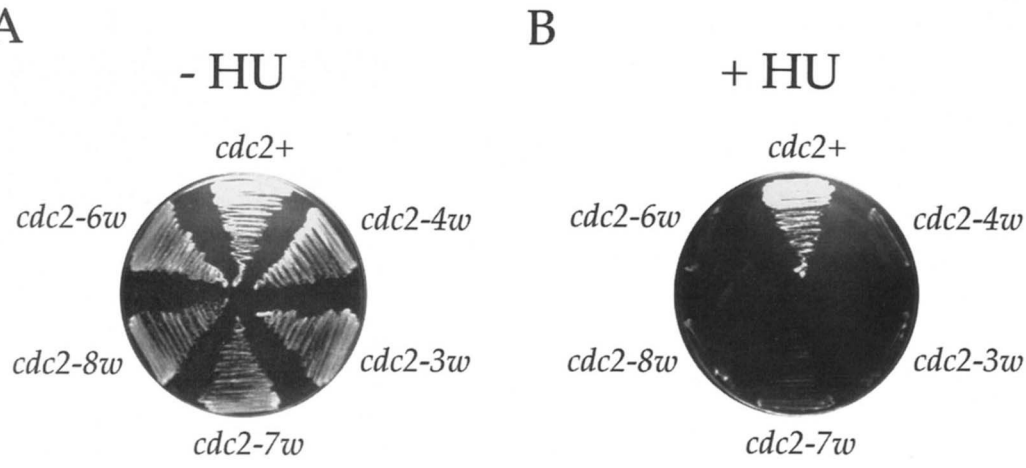

FIGURE 2.—HU sensitivity of the *cdc2* mutants on HU plates. Derivatives of TE281 (*cdc2-L7 leu1-32 h−*) transformed with REP81 plasmids containing the indicated *cdc2* alleles were grown at 32° in the absence of thiamine on EMM in the absence (A) or presence (B) of 10 mM HU.

was done to eliminate nonfunctional *cdc2* molecules. Approximately 70% of the colonies contained plasmids that allowed growth of the cells at the restrictive temperature. To identify checkpoint-defective mutants, 20,000 transformants capable of growth at 36° were replica-plated to plates with and without HU, and transformants that were unable to form colonies on HU were identified. From an initial set of 70 HU sensitive transformants, 14 that showed the strongest phenotypes were selected for further study.

Plasmid DNA was isolated from each HU-sensitive transformant, recovered in *E. coli*, and then retested in TE281 for the ability to confer HU sensitivity. Eleven of the plasmids conferred HU sensitivity upon retransformation. Typical results from cells that were transformed with five of the plasmids that were ultimately selected for further analysis (see below) are shown in Figure 2. Panel A shows cells transformed with *cdc2+* or each of five checkpoint-deficient *cdc2* mutants on standard plates at 32°. As can be seen all six transformants grow well. Panel B shows the same transformants growing at 32° on plates containing HU. Only the *cdc2+* transformant is capable of forming colonies in the presence of HU.

All 11 of the plasmids bearing checkpoint-deficient *cdc2* alleles were subjected to DNA sequence analysis. The results of this study are summarized in Table 1, which lists the mutant and the predicted amino acid alterations. Many of the mutants were found to have more than one amino acid alteration. However each plasmid with multiple mutations was found to have only one alteration in common with other plasmids. From this data we were able to determine five amino acid alterations of *cdc2* that can abolish checkpoint control. Two of these alterations, C67Y (found singly or in combination with other changes in mutants 42, 45, and 58) and C67F (found in mutant 6) are identical to the alterations found in the two previously identified *cdc2* checkpoint mutants *cdc2-3w* and *cdc2-4w* (CARR *et al.* 1989). The multiple isolates of these mutants in our collection confirms the effectiveness of our screening procedure.

In addition to confirming the importance of C67 in checkpoint control, our collection identifies three new $p34^{cdc2}$ residues that are also likely to be involved. The alteration N66I is found in mutant 59 and changes a residue that is next to C67, confirming the importance of this region of the protein in checkpoint control. The other seven mutants contain either one of the two mutations E8V or K9E (Figure 1). These two adjacent residues, glutamic acid in position 8 and lysine in position 9, identify a new region of $p34^{cdc2}$ that is required for checkpoint control. Three mutants were found to carry the mutation E8V, either as a single mutant (mutant 13) or in combination with other mutations (mutant 11 and mutant 54). Three mutants (mutant 28, mutant 29, and mutant 67) were found to carry the mutation K9E in combination with an additional mutation. The mutants N66I (mutant 59), E8V (mutant 13), and K9E/A76V (mutant 29) were chosen for further studies. A76V is a conservative substitution in a residue that is not conserved between cdks. Since expression of these *cdc2* mutant alleles confers a wee phenotype to

TABLE 1

Predicted amino acid alterations in checkpoint-defective *cdc2* alleles

Mutant name	Mutation 1	Mutation 2	Mutation 3	Isolate number
cdc-3w	C67Y			45
	C67Y	K221R		58
	C67Y	H75L	L255M	42
cdc2-4w	C67F			6
cdc2-6w	N66I			59
cdc2-7w	E8V			13
	E8V	S188T		54
	E8V	M273T		11
cdc2-8w	K9E	A76V		29
	K9E	N120D		67
	K9E	E40G	I72V	28

Mutants are classified according to allele name (left column). Mutations found in the corresponding cDNAs together with our original isolate number are listed.

the cells (see below), the alleles were named *cdc2-6w* (N66I), *cdc2-7w* (E8V), and *cdc2-8w* (K9E/A76V).

Transformation with *cdc2-6w, cdc2-7w* and *cdc2-8w* alleles abolishes the S-M checkpoint: We wished to establish that the HU sensitivity conferred by the new *cdc2-w* alleles was due to loss of checkpoint control. When checkpoint-deficient cells are exposed to hydroxyurea, mitosis is initiated, and cells die because the septum cleaves the single nucleus or divides the cell such that one daughter is anucleate. This phenotype resembles that of *cut* mutants, which undergo cytokinesis in the absence of chromosome segregation under restrictive conditions (HIRANO *et al.* 1986). Therefore, the induction of "cut" cells by treatment with HU indicates that the cells are checkpoint defective, and the kinetics and extent of cut formation can be used to assess the severity of the checkpoint defect (ENOCH *et al.* 1992). To assess the checkpoint defect conferred by the new *cdc2-w* alleles, HU was added to mid-log cultures of TE281 cells transformed with different mutant alleles growing at 32°. At various times after HU addition, cells were fixed and stained with DNA specific dye, DAPI. Representative photomicrographs for each strain are shown in Figure 3. The left panels show cultures before the addition of HU; the right panels show cells after 4 hr of incubation in HU. All the transformants are morphologically normal in the absence of HU (Figure 3, a, c, e and g). HU induces cell cycle arrest in *cdc2+* transformants, so the cells are elongated, unseptated and have a single nucleus (Figure 3b, compare to a). In contrast many of the cells in the HU treated *cdc2-w* transformants are septated, however none of the septated cells are binucleate. Instead the septum bisects the nucleus, or divides the cell into nucleate and anucleate compartments (Figure 3, d, f and g; examples of cut cells in each panel are indicated by arrows). We conclude that all the new *cdc2-w* transformants initiate mitosis when DNA replication is blocked with HU and must therefore be deficient in the S-M checkpoint. Similar results were obtained when *cdc2-w* plasmids were introduced into TE280 (*leu1-32h−*), which is wild type for *cdc2+*, indicating that these mutants, like *cdc2-3w* and *cdc2-4w,* are dominant (data not shown).

The new *cdc2-w* mutants are as checkpoint-deficient as *cdc2-3w:* Previous work has established that although *cdc2-3w* cells grow almost normally under most conditions, they are completely deficient in the S-M checkpoint (ENOCH and NURSE 1990). To determine the extent of the checkpoint defect or the new *cdc2-w* mutants, we quantitatively compared the HU responses of all the *cdc2-w* transformants. To do this, we determined the percentage of cells initiating mitosis in HU (cuts) in cultures of each transformant. Figure 4A shows that HU-treated cultures *cdc2-6w, cdc2-7w,* and *cdc2-8w* transformants contain about the same proportion of cuts as similarly treated *cdc2-3w* cultures indicating that all four mutants are equally deficient in the checkpoint response. As another measure of checkpoint deficiency, we measured cell death in HU for each transformant by determining the percentage of viable cells in each culture after 2, 4 and 6 hr of HU treatments (Figure 4B). Aliquots of cells were taken at different time points after addition of HU to asynchronous cultures and plated on minimal media. Plates were incubated for 2–3 days and the number of viable cells was determined by counting colonies. As shown in Figure 4B, loss of viability in *cdc2-7w* and *cdc2-8w* cultures is kinetically and quantitatively similar to loss of viability in the *cdc2-3w* culture, while *cdc2-6w* is somewhat less sensitive. In these experiments, the *cdc2-3w* transformant may be slightly less checkpoint-defective than strains bearing the *cdc2-3w* allele at the *cdc2* locus. Since *cdc2-3w* is completely deficient in the S-M checkpoint (ENOCH and NURSE 1990), it seems likely that the *cdc2-7w* and *cdc2-8w* mutations also completely abolish the S-M checkpoint.

Despite its affects on the S-M checkpoint, the *cdc2-3w* mutation has no affect on the checkpoint required

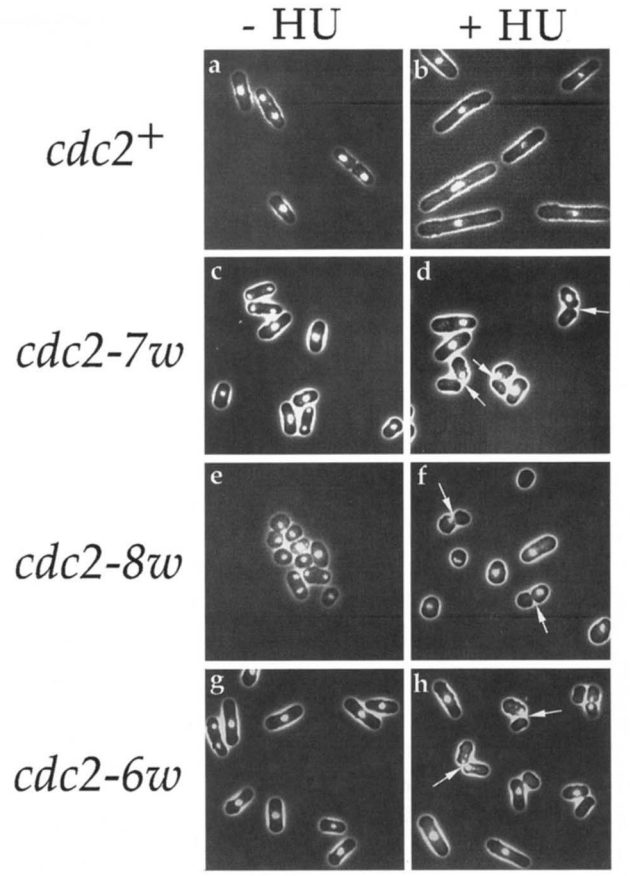

FIGURE 3.—*cdc2-6w, cdc2-7w* and *cdc2-8w* mutants enter mitosis in HU. Derivatives of TE281 (*cdc2-L7 leu1-32 h−*) transformed with REP81 plasmids bearing wild-type or mutant *cdc2* alleles were grown in liquid culture at 32°, in the absence of thiamine without (−HU) or with (HU) 10 mM HU. After 6 hr, cells were fixed and stained with the DNA binding dye, DAPI. Resulting fluorescence micrographs are shown. (a and b) *cdc2+*, (c and d) *cdc2-7w,* (e and f) *cdc2-8w,* (g and h) *cdc2-6w.* Arrows indicate cells initiating mitosis in the absence of DNA replication resulting in cut morphology.

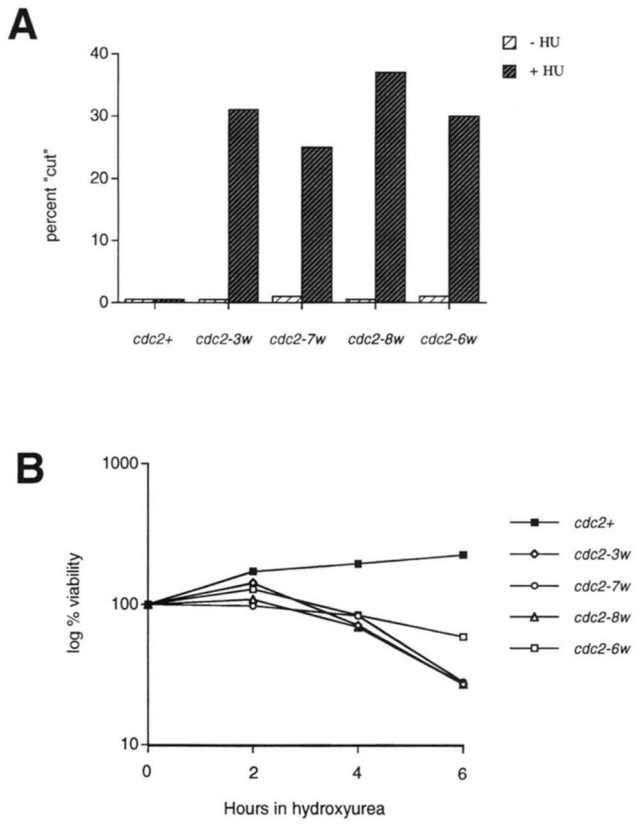

FIGURE 4.—Quantitative analysis of viability and cut phenotype of the *cdc2-w* mutants in response to HU. Derivatives of TE281 (*cdc2-L7 leu1-32 h−*) carrying plasmids containing wild-type or mutant *cdc2* were grown at 32° in EMM. After derepression of the promoter 10 mM HU was added to the medium. (A) The number of cells entering mitosis without completing DNA replication was determined by counting the number of cut cells as determined by fluorescence microscopy as shown in Figure 3. Measurements were made at time 0 (−HU) or 6 hr after the addition of HU to the medium (+HU). One hundred cells were counted for each data point. (B) Viability of derivatives of TE281 (*cdc2-L7 leu1-32 h−*) cells transformed with wild type of mutant *cdc2* alleles after incubation in HU. Aliquots of cells were removed at the indicated times and plated on EMM plates lacking thiamine. Plates were incubated at 32° and the number of viable colonies was determined after 2—3 days. The 100% data point consisted of 100—300 cells for each experiment.

for G2 arrest in response to DNA damage induced by UV or ionizing radiation. Thus, *cdc2-3w* mutants show wild-type levels of resistance to radiation in contrast to other checkpoint mutants that are deficient in both the S-M and DNA damage checkpoints (SHELDRICK and CARR 1993). Similarly, none of our new *cdc2-w* mutations affect resistance to UV irradiation, indicating that they also do not affect the DNA damage checkpoint (data not shown).

The length of G2 is reduced in *cdc2-w* transformants: Cells expressing the new *cdc2-w* mutants had the same generation time as cells expressing *cdc2+*, but showed a reduced size at cell division (Figure 3, compare the lengths of binucleate cells in a to binucleates in c, e, and g). In *S. pombe* this *wee* phenotype has been shown to be due to a shortening of G2, which represents 80% of the cell cycle in logarithmically growing wild-type cells. To determine if our *cdc2-w* transformants were also advanced into mitosis, we analyzed the cell cycle distribution of asynchronous cultures using FACS analysis of DNA content. As shown in Figure 5A, the *cdc2+* transformant population is made up almost entirely of cells with a 2C DNA content. In these cells, S-phase is initiated immediately after anaphase, even before cytokinesis. Thus, by the time the cells complete cell division they already have a 2C DNA content. In contrast a 1C population can readily be detected in *cdc2-3w* transformants (Figure 5B) and in the cells transformed with the new *cdc2-w* alleles (Figure 5, C–E). This is because *wee* cells enter mitosis prematurely and are below the size threshold for initiation of S-phase upon completion of anaphase (NURSE 1975). These cells undergo cytokinesis first and then after growing for an interval, initiate S-phase. Therefore the proportion of a population in G1 is a measure of the degree to which cells are advanced into mitosis. In cells expressing the *cdc2-3w* allele, 38% of the population was in G1 (Figure 5A). For all the other mutants a significant proportion of the cells was in G1, confirming that the mutants were advanced in mitosis. The percentage of cells in G1 ranged from 26% in *cdc2-6w* to 43% in *cdc2-8w*. These values correlate well with the observation that the length of the cells was shortest in *cdc2-8w* and longest in *cdc2-6w* (see Figure 3).

***cdc2-7w* and *cdc2-8w* transformants are independent of *cdc25+* and very sensitive to the absence of *wee1+*:** Many wee mutants have been identified in fission yeast, and genetic studies have established that they must be disrupting cell cycle control by different mechanisms. For example, the *cdc2-1w* allele reduces the length of G2, but in contrast to *cdc2-3w* displays normal checkpoint control. There are also differences in the way wee mutants respond to loss of *cdc25+* or *wee1+*. The *cdc2-3w* and *cdc2-4w* mutations completely suppress loss of *cdc25* function, while the *cdc2-1w* suppresses *cdc25* mutations poorly. The combination of *cdc2-3w* with loss of *wee1* is synthetically lethal, while the phenotype of the *cdc2-1w wee1* double mutant is indistinguishable from either single mutant (RUSSELL and NURSE 1986, 1987). Since the *cdc2-7w* and *-8w* mutations define a new region of $p34^{cdc2}$ required for cell cycle control, we wanted to determine if these mutants behave more like *cdc2-3w* or *cdc2-1w* with respect to their interactions with *cdc25* and *wee1* mutations.

To test interactions with *wee1,* plasmids bearing *cdc2+*, *cdc2-3w*, *cdc2-7w* and *cdc2-8w* were introduced into TE354 (*wee1-50 leu1-32 h−*), which has *wee1-50,* a temperature-sensitive allele of *wee1.* Transformants were then tested for growth at the permissive (25°) and nonpermissive (36°) temperatures for *wee1-50.* As shown in Figure 6A, all the transformants grew at 25°.

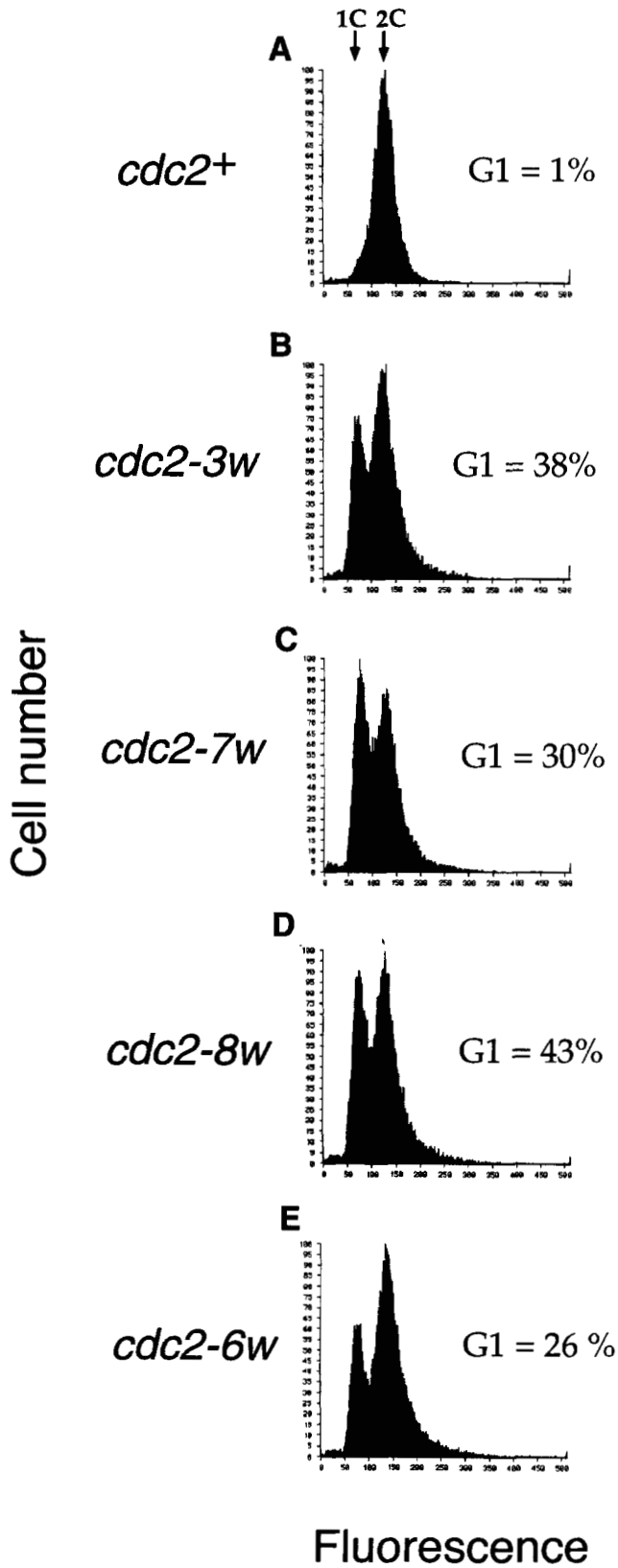

FIGURE 5.—Cells carrying the newly isolated *cdc2* mutants are advanced in mitosis. Flow cytometric analysis of distribution of DNA content of derivatives of TE281 (*cdc2-L7 leu1-32 h*−) carrying REP81 plasmids bearing wild-type or mutant alleles of fission yeast *cdc2*. Cells were grown 24 hr in the absence of thiamine at 32°. The estimated percentage of G1cells was determined using HU arrested cells as a standard and bracketing the corresponding region in experimental samples in the FACS output.

However, as shown in Figure 6B, only the *cdc2+* transformants formed colonies at 36°. Microscopic analysis revealed that the *cdc2-3w, -7w* and *-8w* transformants all appeared to be undergoing mitotic catastrophe (data not shown). We also examined the ability of our new mutants to suppress loss of *cdc25* function. *cdc2+* and *cdc2-w* plasmids were introduced into TE282 (*cdc25-22 leu1-32 h*−), a strain that carries a temperature-sensitive allele of *cdc25*. Transformants were grown to early mid-log phase and then equal numbers of cells were plated at 25° (the permissive temperature) and 36° (the restrictive temperature). After 2–3 days the number of colonies on each plate was determined; percentage of survival was calculated by dividing the number of colonies on the 36° plate by the number of colonies on the 25° plate. The results are plotted in Figure 7. As expected, the *cdc2+* transformants could not form colonies at 36°. However transformation with any of the *cdc2-w* mutants rescued *cdc25-22*. The extent of colony formation differed between the mutants, being the lowest for *cdc2-6w* (12%), highest for *cdc2-8w* (75%) and intermediate for *cdc2-3w* (39%) and *cdc2-7w* (28%). The *cdc2-3w* allele at the *cdc2+* locus efficiently complements *cdc25-22* and a complete deletion of *cdc25* (RUSSELL and NURSE 1986). We conclude that *cdc2-7w* and *cdc2-8w* are, like *cdc2-3w*, largely independent of *cdc25+* function. *cdc2-6w* appears to be somewhat less able to function in the absence of *cdc25+*. This correlates with its relatively greater viability in HU (Figure 4B) and longer G2 (Figure 5). We conclude that the checkpoint-deficient *cdc2* alleles we have isolated have many characteristics in common with *cdc2-3w*, even though we did not deliberately select for these characteristics in our screen.

The human *CDC2-K9E* and *CDC2-E8V* alleles are checkpoint-deficient in fission yeast: To understand the importance of mitotic regulation in multicellular organisms, it would be valuable to have *wee* alleles of the human *CDC2*. Human *CDC2* alleles equivalent to *cdc2-3w* and *-4w* do not show any abnormalities in cell cycle control (MACNEILL and NURSE 1993a), perhaps because this region of the protein is not well conserved between fission yeast and humans (see Introduction). However, the E8 and K9 residues, which are altered in *cdc2-7w* and *cdc2-8w*, are conserved between the fission yeast *cdc2* and the human *CDC2* proteins. We therefore investigated whether phenotypically similar human *CDC2 wee* alleles could be created by introducing the E8V and K9E alterations in the human protein.

The E8V and K9E mutations were introduced by site-directed mutagenesis into a human *CDC2* cDNA under the control of the REP81 promoter (see MATERIALS AND METHODS). These new alleles were designated *CDC2-7w*

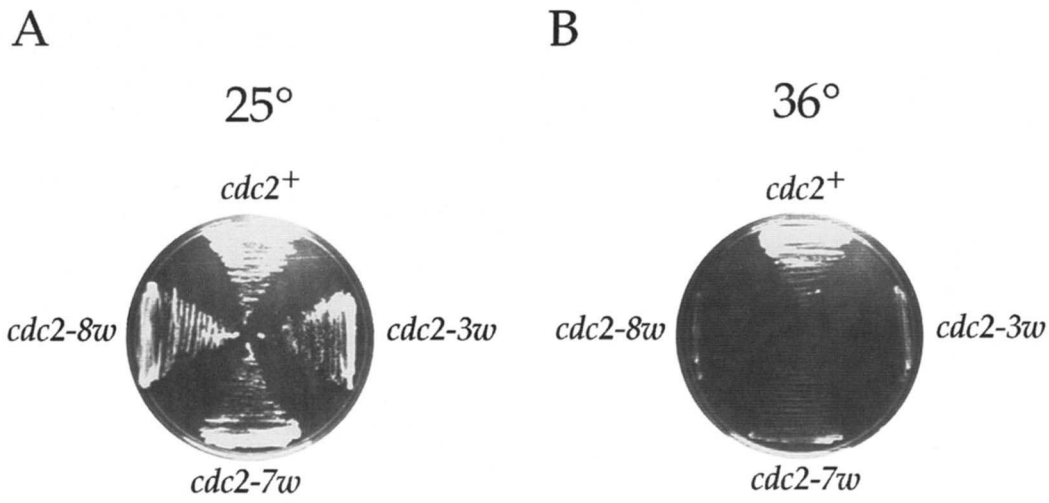

FIGURE 6.—Lethality of *cdc2* mutants with *wee1-50*. (A) REP81 plasmids bearing wild-type or mutant *cdc2* alleles were introduced into TE354 (*wee1-50 leu1-32 h−*), which contains temperature-sensitive allele of *wee1*. The indicated transformants were on plates lacking thiamine to derepress the REP81 promoter, restreaked onto plates without thiamine and incubated at either 25° (A) or the restrictive 36.5° (B).

and *CDC2-8w* (by convention the uppercase letters are used to designate the human gene and lowercase letters are used for the fission yeast gene). To study the new alleles, we transformed mutant and wild-type plasmids into TE460 (*cdc2::CDC2 his3-27 leu1-32 h−*) an *S. pombe CDC2* strain, in which the fission yeast *cdc2+* gene has been replaced by the human *CDC2* gene (MACNEILL and NURSE 1993a).

To examine checkpoint control in these transformants, we investigated their ability to grow in the presence of HU. *CDC2* cells transformed with the REP81 vector alone or with wild-type *CDC2* were able to grow on plates containing HU. In contrast cells transformed with either *CDC2-7w* or *CDC2-8W* were inviable in the presence of HU although they were able to form colonies on standard plates, suggesting that they are checkpoint defective, like the equivalent *S. pombe* mutants (data not shown). To assess whether the mutants were checkpoint deficient, we examined their morphology before and after exposure to HU for 6 hr. Representative photomicrographs are shown in Figure 8. Cells transformed with wild-type *CDC2* undergo cell cycle arrest in HU and are elongated (Figure 8b) compared to untreated cells (Figure 8a). However cells transformed with *CDC2-7w* or *CDC2-8w* fail to elongate, and as with

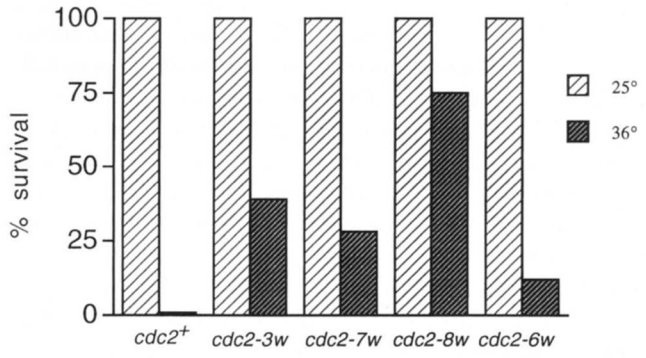

FIGURE 7.—*cdc2-6w, cdc2-7w,* and *cdc2-8w* complement *cdc25-22*. Derivatives of TE282 (*cdc25-22 leu1-32 h−*), a strain carrying a temperature-sensitive allele of *cdc25,* were transformed with REP81 plasmids bearing wild-type or mutant *cdc2* alleles and grown to early mid-log phase in EMM without thiamine. Aliquots of cells were removed and plated on EMM plates that were incubated at 25° or 36° for 3 days before counting colonies. Data represent the number of colonies grown at 36° expressed as a percentage of the colonies grown at 25° (100%). The 100% data point corresponds to between 100 and 300 colonies depending on the experiment.

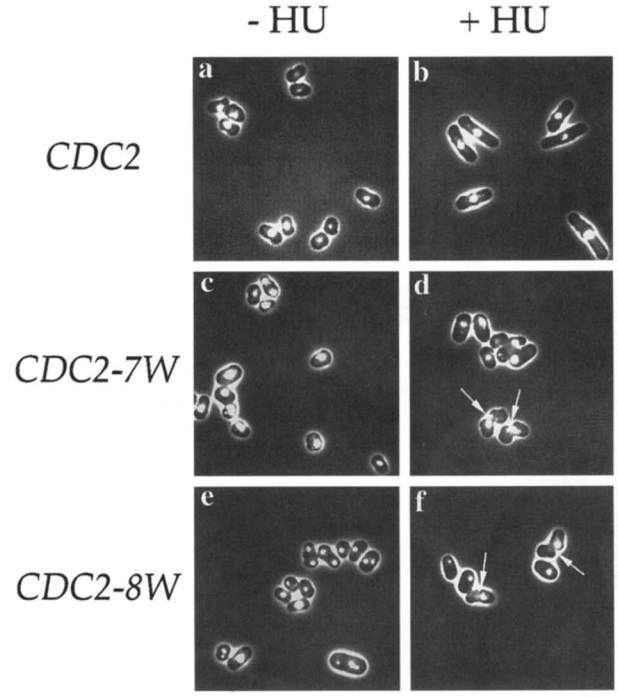

FIGURE 8.—Human CDC2-7w and CDC2-8w alleles enter mitosis without completing DNA replication. TE460 (*cdc2::CDC2 his 3-27 leu1-32 h−*), a human *CDC2* yeast strain in which the yeast *cdc2* has been replaced with the human *CDC2* (MACNEILL and NURSE 1993a), was transformed with REP81 plasmids bearing wild-type or mutant human *CDC2* alleles. Cells were grown to early mid-log phase in the absence of thiamine to derepress the *nmt1* promoter and then HU was added to 10 mM to half of each culture. Cells were incubated for 6 hr and then fixed and stained with DAPI as described in Figure 3. Arrows indicate cuts. (a and b) *CDC2,* (c and d) *CDC2-7w,* (e and f) *CDC2-8w.*

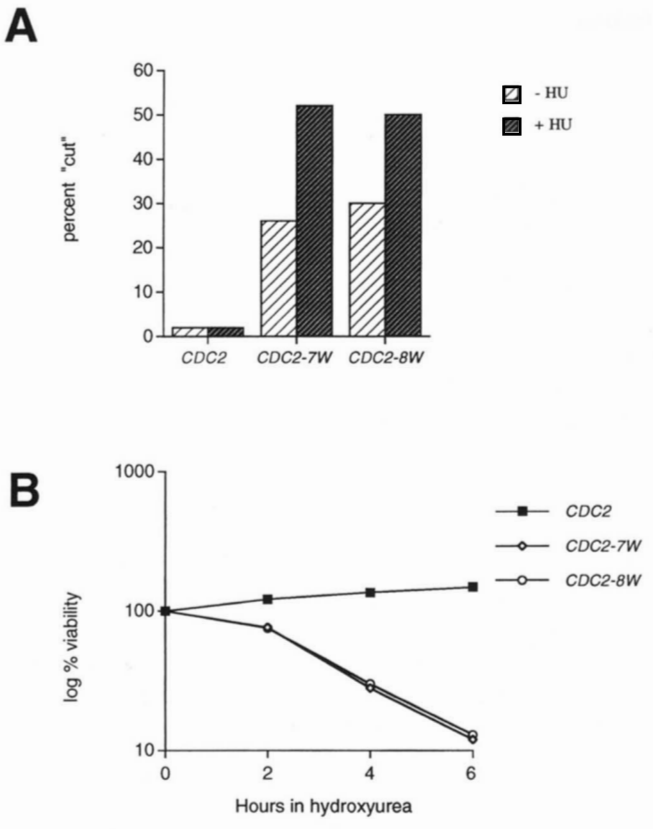

FIGURE 9.—Quantitative analysis of checkpoint defect of *CDC2-7w* and *CDC2-8w* alleles in fission yeast. The checkpoint defect of human *CDC2* yeast strains transformed with wild-type and mutant alleles of *CDC2* was measured quantitatively. (A) Percentage of cut formation. The percentage of cells entering mitosis in the absence of DNA replication (cuts) was determined in samples prepared as described in Figure 8. (B) Loss of viability in cultures of CDC2 strains transformed with mutant or wild-type *CDC2* alleles was determined as described in Figure 4.

the *S. pombe* mutants, a high proportion of cells initiate mitosis and septate with only one nucleus (Figure 8, d and f). These results are presented quantitatively in Figure 9A where the percentage of cuts before and after HU treatment is shown. Even in the absence of HU, cells transformed with the *CDC2-7w* or *-8w* were extremely short, with high number of binucleate cells and some cuts (Figure 8, c and e; Figure 9A), although the proportion of cuts was increased by treatment with HU. We also quantitatively measured loss of viability of the transformants in HU by plating aliquots of the cultures after various lengths of time in HU. As shown in Figure 9B, wild-type *CDC2* transformants remain viable after 6 hr of incubation in HU whereas 90% of *CDC2-7w* and *CDC2-8w* mutants die.

When the human *CDC2-7w* and *CDC2-8w* mutants were expressed in TE461 (*cdc2::CDC2 cdc25-22 leu1-32 his3-27 h−*), a *CDC2* strain with the temperature-sensitive *cdc25-22* allele, cells were wee at 29°. In contrast, cells transformed with wild-type human *CDC2* were somewhat elongated, suggesting that, like the equivalent fission yeast mutants, *CDC2-7w* and *-8w* mutations render cells independent of *cdc25*. However, we could not test their ability to rescue *cdc25-22* at 36°, because expression of the mutants at that temperature was lethal even in a strain containing wild-type *cdc25+* (data not shown).

These results establish that the E8V or K9E mutations in both human CDC2 and fission yeast *cdc2+* creates dominant, checkpoint-deficient alleles. It therefore seems likely that the introduction of *CDC2-7w* or *-8w* into human cells will also disrupt checkpoint control.

DISCUSSION

Mutation of conserved residues of p34^{cdc2} can abolish checkpoint control without disrupting normal cell division: To better understand checkpoint-dependent regulation of p34^{cdc2}, we have screened a library of *cdc2* mutants for novel checkpoint-deficient alleles that permit normal cell division under standard conditions. Using this approach we have reisolated both the known checkpoint-deficient *cdc2* alleles *cdc2-3w* (C67Y) and *cdc2-4w* (C67F), which confirms the effectiveness of our screen. In addition we have identified three novel checkpoint-deficient alleles, *cdc2-6w* (N661), *cdc2-7w* (E8V) and *cdc2-8w* (K9E). Most of the mutations were isolated more than once independently suggesting that the screen is close to saturation (Table 1). We did not expect to identify *cdc2-F15* mutants, as these are defective for proliferation under standard conditions, and we limited our analysis to mutants that grew normally in the absence of HU.

In addition to being checkpoint-defective, the new mutants share several other distinctive phenotypes with *cdc2-3w*. All of the mutants are small (or *wee*), and this is due to a reduction in the G2 phase of the cell cycle (Figure 5). In addition, the new mutants render cells hypersensitive to loss of *wee1+* activity (Figure 6) and independent of *cdc25+* (Figure 7). These genetic interactions are not a consequence of the shortening of G2, as *cdc2-1w*, another wee allele of *cdc2^{+}*, does not interact with *wee1+* or *cdc25+* in this manner (RUSSELL and NURSE 1987). The similarities between *cdc2-3w, cdc2-4w, cdc2-6w, cdc2-7w* and *cdc2-8w* suggests that all of the mutations alter checkpoint-dependent regulation of p34^{cdc2} by the same mechanism.

In contrast to C67, the residues altered in the new mutants are conserved in other members of the cdk family. N661, the *cdc2-6w* mutation, alters a residue that is conserved in many kinases (HANKS and QUINN 1991). Another *cdc2* mutant altered at this residue, *cdc2-N66D*, has previously been described (MACNEILL and NURSE 1993b). However unlike N66I, this mutation does not alter regulation of p34^{cdc2} by *cdc25*. The checkpoint phenotype of this mutant is not reported. E8V and K9E, the *cdc2-7w* and *cdc2-8w* mutations, alter residues that are unique to a subfamily of cdks that includes *cdc2+*, *CDC28*, human *CDC2* and *CDK2* (Figure 1), as well as

CDK3, CDK5 and other members of the PSTAIRE family. The residues are not conserved in *CDK4,* or in general in other kinases (HANKS and QUINN 1991). Since the altered residues are located near the phosphorylated tyrosine 15, they may be part of a phosphorylation consensus sequence for a cdk tyrosine kinase. This would explain why these residues are mostly unique to cdks, as these are the only kinases known to be regulated by tyrosine 15 phosphorylation. However tyrosine 15 phosphorylation of $p34^{cdc2}$ is reportedly normal in *cdc2-3w* (GOULD *et al.* 1990), instead of reduced, as this model would predict.

Alternatively, the mutations may prevent $p34^{cdc2}$ from interacting with unidentified components of the S-M checkpoint machinery. In this regard, it is interesting that the *Aspergillus nidulans nimA* kinase is one of the few non-cdk kinases that does have E and K at positions 8 and 9. However in contrast to the cdks, position 15 in *nimA* is occupied by a nonphosphorylatable residue, phenylalanine. Like *cdc2+, nimA* kinase is a rate-limiting regulator of entry into mitosis (OSMANI *et al.* 1988) and may therefore also interact with the S-M checkpoint machinery, even though it is not regulated by tyrosine kinases. If E8 and K9 residues are required for checkpoint control but not for control of tyrosine phosphorylation, *nimA-E8V* and *nimA-K9E* mutations would be predicted to alter S-M checkpoint control in Aspergillus, a hypothesis that is readily testable.

It is also interesting that E8 and K9 are not conserved in *CDK4,* which is also regulated by tyrosine phosphorylation (TERADA *et al.* 1995). If E8 and K9 are required for interactions with tyrosine kinases, this may allow *CDK4* to be regulated independently of *CDK2* and *CDC2* by different tyrosine kinases. Alternatively, if E8 and K9 are required for interactions with the S-M checkpoint machinery, they may not be necessary for *CDK4* regulation, since *CDK4* is believed to function during G1 instead of G2.

Residues mutated in *cdc2-w* mutants are in two spatially separated regions on the three dimensional CDK2/cyclin A structure: As discussed above, it seems likely that the new mutations cause the same molecular defect as C67Y or C67F, the alterations found in *cdc2-3w* and *cdc2-4w.* To understand the molecular basis of the mutant phenotype, we examined the location of all the altered residues on the predicted three-dimensional structure of CDK2/cyclin A. Figure 10 is a schematic representation of the location of each mutation on a space-filling model of cyclin A/CDK2 derived from the crystallographic coordinates (JEFFREY *et al.* 1995). In Figure 10A, the structure is oriented with the CDK2 subunit (colored aqua) on the left and the cyclin A subunit (colored gray) on the right. The molecule is positioned so that we are looking into the ATP-binding cleft (ATP is dark green), with the PSTAIRE helix (dark blue) located along an axis that is perpendicular to the page. The T-loop is colored yellow. [This is approximately the same orientation as the ribbon structure shown in JEFFREY *et al.* (1995), Figure 2a]. As can be seen in Figure 10A, E8 and K9 (shown in red) are solvent-accessible residues at the end of $\beta 1$. They are linked to the phosphorylated tyrosine, Y15 (orange) by the L2 loop (shown in white). C67 and N66 (shown in red in

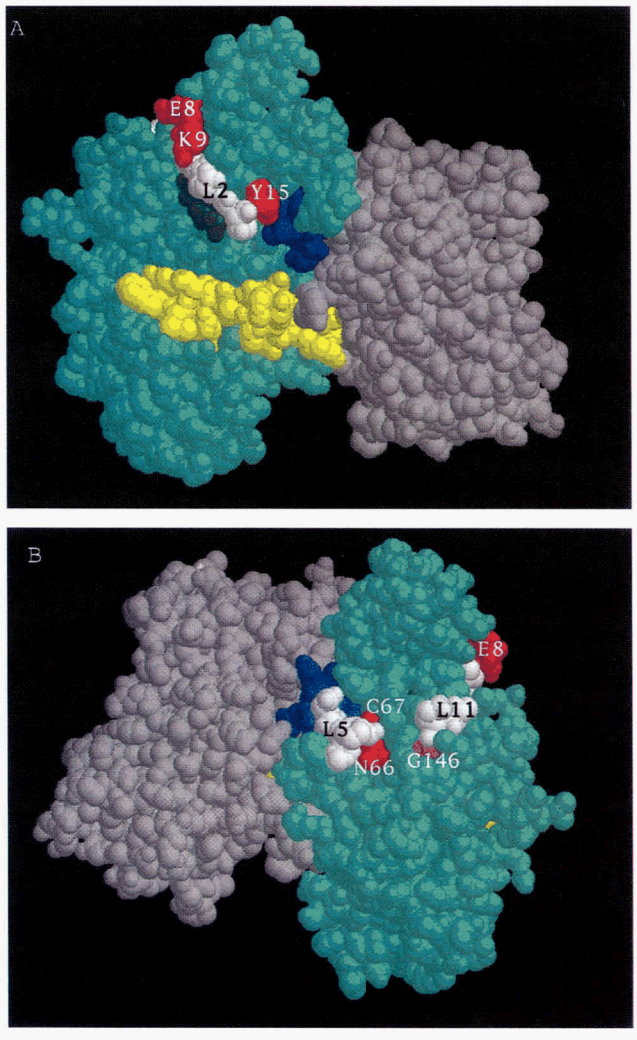

FIGURE 10.—Predicted location of altered residues in *cdc2-w* mutants on the three-dimensional structure of CDK2/cyclinA. Crystallography coordinates for the CDK2/cyclinA structure (JEFFREY *et al.* 1995) were generously provided by Dr. NIKOLA PAVELITCH of Memorial Sloan-Kettering Cancer Center. RasMol version 3.5 software was used to generate a space-filling model of the complex, and the residues equivalent to those mutated in *cdc2-w* alleles were highlighted with distinctive colors. The CDK2 subunit is colored aqua; the cyclin A subunit is gray. The PSTAIRE helix (residues 43–57) is colored dark blue; the T-loop (residues 152–170) is colored yellow. ATP is dark green. The fission yeast numbering system has been used to indicate the altered residues in the figure. The actual residues in CDK2 are N62 (N66), I63(C67) and G139 (G146). The loops L2 (residues 10–14) L5 (residues 58–61) and L11 (residues 136–138) are colored white. (A) The molecule looking into the ATP-binding cleft. This is approximately the same orientation shown in Figure 2a of JEFFREY *et al.* (1995). (B) The molecule rotated ~180° about the axis perpendicular to the page.

Figure 10B) are located at the base of L5, (shown in white) a loop that protrudes out of the surface of the protein. Possibly the mutations alter the configuration of L5, thus changing the shape of this surface of cdk. This may be particularly significant in *S. pombe,* where L5 is unusually large (see Figure 1).

It is clear from Figure 10 that the mutated residues map to two separate domains on opposite faces of the protein (in Figure 10B the structure is rotated about the axis perpendicular to the page compared to A). However, our genetic analysis argues that all four residues are required for the same molecular process. It is difficult to imagine an interaction with a single molecule involving such widely separated residues and also to understand why no residues between these regions are required. Perhaps tyrosine kinases or components of the S-M checkpoint machinery wrap around the cdk subunit making contacts with residues on both sides of the molecule. Alternatively, *in vivo* two or more cdk/cyclin complexes may be physically associated. Perhaps in such a multimeric structure N66 and C67 on one cdk molecule are adjacent to E8 and K9 on a second molecule.

There could also be more trivial explanations for these findings. As has been mentioned, the *S. pombe* $p34^{cdc2}$ protein structure is unique in the L5 region. Furthermore, when $p34^{cdc2}$ interacts with tyrosine kinases, it is found in a complex with $p56^{cdc13}$ a B-type cyclin (HAYLES and NURSE 1995). Thus there may be aspects of the $p34^{cdc2}/p56^{cdc13}$ structure that cannot be adequately modeled using the CDK2/cyclin A coordinates. It is also possible that, despite the phenotypic similarities, the two classes of mutants are disrupting cell cycle control by different molecular mechanisms. For example, alteration of N66 and C67, which are near the PSTAIRE loop, could be affecting the cyclin/cdk interaction directly and thus somehow indirectly reducing the interaction with tyrosine kinases or the S-M checkpoint machinery while the E8 and K9 mutations could be directly disrupting interactions with tyrosine kinases.

We also examined the location of the residue altered in *cdc2-1w* (G146 in $cdc2^+$, G139 in CDK2). Although the *cdc2-1w* mutation does not alter checkpoint control, it must interact abnormally with some cell cycle regulators since the length of G2 is also reduced in this mutant. As shown in Figure 10B, like C67 and N66, G146 (shown in magenta in Figure 10B) is located at the base of a solvent-accessible loop, in this case L11 (shown in white). Interestingly, the altered residue is fairly near C67 and N66 again suggesting that this surface of the protein may be involved in interactions with G2/M cell cycle regulators.

Construction of checkpoint-defective alleles of human *CDC2*: Because the E8V and K9E mutations alter conserved residues of $p34^{cdc2}$, we were able to construct equivalent human *CDC2* mutants, which we call *CDC2-7w* and *CDC2-8w*. Since human *CDC2* can fully replace *cdc2+* in fission yeast (MACNEILL and NURSE 1993a) we were able to introduce plasmid-born copies of the mutant alleles into *CDC2* yeast and show that the mutant alleles disrupt the S-M checkpoint in a dominant fashion (Figure 8 and 9). MACNEILL and NURSE have previously attempted to generate checkpoint-deficient alleles of human *CDC2* by constructing alleles that were equivalent to *cdc2-3w* and *cdc2-4w* (*CDC2-I63Y* and *CDC2-I63F*). However, expression of these mutants did not alter cell cycle control when they were introduced into *CDC2* yeast, perhaps because, as previously discussed, the entire L5 regions is not well conserved between the yeast and human proteins (MACNEILL and NURSE 1993a).

In contrast to the fission yeast *cdc2-w* mutants, the *CDC2-7w* and *CDC2-8w* disrupt normal cell division to some extent in the absence of HU. At 29° the cells are very small, there are many binucleate cells and a significant proportion of cuts (Figure 8, c and e; Figure 9A). At 36° the transformants are inviable and appear to be going through mitotic catastrophe. This phenotype resembles the phenotypes of other fission yeast strains such as *cdc2-F15, cdc2-3w wee 1-50* or *mik1 wee1-50.* Replacement of the fission yeast *cdc2+* gene with wild-type *CDC2* has been shown to render fission yeast wee, probably because fission yeast regulators interact less efficiently with the human protein (MACNEILL and NURSE 1993a). The checkpoint defect in *CDC2-w* mutants, combined with this intrinsic tendency of the human protein to be deregulated in fission yeast, may explain the relatively severe phenotype of these mutants. It seems likely that expression of *CDC2-7w* and *CDC2-8w* will have a less significant effect on cell division in human cells, since the proteins will come under the control of their natural regulators.

The role of *CDC2* in checkpoint control in other eukaryotic cells has previously been studied by introducing plasmids bearing *CDC2-F15* mutants into cultured cells. Transfected cells undergo abnormal mitoses that cannot be blocked by inhibitors of DNA replication, indicating that this mutation completely disrupts cell cycle control, much like the *S. pombe cdc2-F15* mutant (KREK and NIGG 1991; HEALD *et al.* 1993). It could be useful to have *CDC2* mutants that disrupt checkpoint control more specifically. We have demonstrated that plasmid-born *CDC2-7w* or *CDC2-8w* alleles disrupt checkpoint control in a yeast carrying a chromosomal copy of wild-type human *CDC2.* It therefore seems likely that expression of these mutant alleles will also disrupt checkpoint control in other eukaryotic cells.

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