Journal of Cell Science 108, 475-486 (1995)
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The *Schizosaccharomyces pombe hus5* gene encodes a ubiquitin conjugating enzyme required for normal mitosis

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SUMMARY

Normal eukaryotic cells do not enter mitosis unless DNA is fully replicated and repaired. Controls called 'checkpoints', mediate cell cycle arrest in response to unreplicated or damaged DNA. Two independent *Schizosaccharomyces pombe* mutant screens, both of which aimed to isolate new elements involved in checkpoint controls, have identified alleles of the *hus5*$^+$ gene that are abnormally sensitive to both inhibitors of DNA synthesis and to ionizing radiation. We have cloned and sequenced the *hus5*$^+$ gene. It is a novel member of the E2 family of ubiquitin conjugating enzymes (UBCs).

To understand the role of *hus5*$^+$ in cell cycle control we have characterized the phenotypes of the *hus5* mutants and the *hus5* gene disruption. We find that, whilst the mutants are sensitive to inhibitors of DNA synthesis and to irradiation, this is not due to an inability to undergo mitotic arrest. Thus, the *hus5*$^+$ gene product is not directly involved in checkpoint control. However, in common with a large class of previously characterized checkpoint genes, it is required for efficient recovery from DNA damage or S-phase arrest and manifests a rapid death phenotype in combination with a temperature sensitive S phase and late S/G_2 phase cdc mutants. In addition, *hus5* deletion mutants are severely impaired in growth and exhibit high levels of abortive mitoses, suggesting a role for *hus5*$^+$ in chromosome segregation. We conclude that this novel UBC enzyme plays multiple roles and is virtually essential for cell proliferation.

Key words: checkpoint, feedback control, radiation sensitivity, *rad*, replication, cell cycle

INTRODUCTION

The yeast cell cycle is a sequence of interdependent events; late steps in the cell cycle are dependent on the completion of early events, and will not take place until these have been successfully completed (Pringle and Hartwell, 1981). In recent years, 'checkpoint' or 'dependency control' mutants that relieve such dependencies have been identified. Such mutants have no obvious phenotype unless the cell division cycle is blocked. When this occurs, instead of arresting cell cycle progression, these mutants attempt to continue through the cycle, resulting in abnormally high levels of cell death after only short periods of 'arrest' (the 'rapid death' phenotype). The checkpoint and dependency control mutants define a class of cell cycle controls termed 'checkpoints' or 'feedback controls' (Hartwell and Weinert, 1989; Murray, 1992). Mitotic entry checkpoint mutants that abolish mitotic arrest in response to DNA damage (Weinert and Hartwell, 1988; Al-Khodairy and Carr, 1992; Rowley et al., 1992; Al-Khodairy et al., 1994) and completion of S-phase (Weinert and Hartwell, 1988; Enoch and Nurse, 1990; Al-Khodairy and Carr, 1992; Enoch et al., 1992; Al-Khodairy et al., 1994; Weinert et al, 1994) have been identified in both budding and fission yeast. Mutants defining a checkpoint that monitors correct spindle assembly prior to anaphase have also been identified in budding yeast (Li and Murray, 1991; Hoyt et al., 1991).

Little is known about how checkpoints work at the molecular level. In the fission yeast, *Schizosaccharomyces pombe*, numerous mutants that disrupt the mitotic entry checkpoint have been identified either by examining checkpoint functions in known cell cycle control and radiation sensitive mutants (Enoch and Nurse, 1990; Al-Khodairy and Carr, 1992; Rowley et al., 1992) or by screening for novel checkpoint mutants (Enoch et al., 1992; Walworth et al., 1993; Al-Khodairy et al., 1994). Molecular analysis of many of the corresponding genes has been carried out, making the fission yeast an ideal system to begin to describe the mitotic entry checkpoints at the molecular and biochemical level (Fig. 1, and see Sheldrick and Carr, 1993 for review).

Fission yeast mitotic entry checkpoint mutants fall into three general categories. (1) Mutations that cannot arrest in S-phase. These mutants undergo lethal cell cycle progression and death

in the presence of inhibitors of DNA synthesis such as hydroxyurea (HU), or when combined with temperature sensitive mutants that block DNA replication such as *cdc22* (ribonucleotide reductase; Sarabia et al., 1993). Mutations in this category alter genes encoding the highly conserved mitotic control protein Cdc2, or the proteins that regulate its activation at G_2/M (Enoch and Nurse, 1990; Sheldrick and Carr, 1993). Another mutant, *hus6*, also falls into this category (Al-Khodairy et al., 1994), however the molecular basis of the defect in this strain has not been determined. (2) Mutations that alter G_2 arrest in response to DNA damage such as that induced by ionizing radiation. Mutations in this category include deletions of the *chk1/rad27* gene (Walworth et al., 1993; Al-Khodairy et al., 1994), which encodes a protein kinase, and deletions of the genes *rad24* or *rad25*, which are fission yeast homologs of mammalian 14-3-3 proteins (Ford et al., 1994). (3) Mutants that cannot arrest either in S-phase or in response to irradiation. These mutants are sensitive both to HU and to irradiation. This is the largest category of mutants and includes lesions in the genes *rad1, rad3, rad9, rad17, rad26* and *hus1* (Al-Khodairy and Carr, 1992; Rowley et al., 1992; Enoch et al., 1992; Al-Khodairy et al., 1994). In addition to disrupting both the radiation checkpoints and the S-phase mitotic entry dependency control, these 'checkpoint *rad*' mutants also have an additional defect as they are approximately 100-fold more sensitive to both radiation and HU compared to mutants in either categories 1 and 2 (which have equally severe checkpoint defects). To explain this observation, we have proposed that, in addition to the inhibition of mitosis, further functions are required for recovery from irradiation or S-phase inhibition (Enoch et al., 1992; Al-Khodairy et al., 1994). Nothing is known about the molecular nature of this recovery process.

All of the above mutants have a third phenotype in common; they are synthetically lethal with lesions in the *wee1* gene. *wee1* encodes a tyrosine kinase that negatively regulates mitosis at G_2/M by phosphorylating Cdc2 (reviewed by Nurse, 1990). *wee1* mutants have only a very short G_2. Strains that combine any of the three types of mitotic entry checkpoint mutation with a temperature sensitive allele of *wee1* display a 'mitotic catastrophe' phenotype at the restrictive temperature for *wee1*; cells are extremely small, and display multiple mitotic abnormalities. These observations suggest that checkpoint functions operate during the normal cell cycle in fission yeast, but are not essential as G_2 is long enough to allow completion of S-phase and related events, even in the absence of the checkpoints. However, in *wee1* mutants, the checkpoints become essential in order to allow the normal completion of DNA replication before mitosis takes place.

In addition to mutants with clear checkpoint phenotypes described above, genetic screens have identified numerous other mutants with weaker phenotypes that could reflect defects in checkpoint controls or related processes (Enoch et al., 1992; Al-Khodairy et al., 1994). Alleles of the *hus5* gene have been isolated in two independent screens for checkpoint mutants. The mutants, *hus5.62* and *hus5.17* are HU sensitive, radiation sensitive and also grow slowly, but do not display a clear checkpoint defect. To investigate the role of *hus5* in checkpoint control we have cloned and sequenced the corresponding gene. The *hus5*$^+$ gene encodes a novel member of the E2 family of ubiquitin conjugating enzymes (UBCs). We have used the clone to construct a strain completely lacking the *hus5* coding sequences. The deletion is virtually inviable producing microcolonies that are radiation sensitive and display multiple mitotic abnormalities. Careful analysis of the two mutant alleles suggests that *hus5* does not play a direct role in checkpoint control. Epistasis experiments suggest that *hus5* radiation and HU sensitivity may reflect a defect in the same recovery process that is deficient in the mitotic entry 'checkpoint *rad*' class of mutants.

MATERIALS AND METHODS

Strains and plasmids

The *hus5.17* and *hus5.62* alleles have been previously described (Enoch et al., 1992; Al-Khodairy et al., 1994). The isogenic strain sp011 *ura4.D18 ade6.704 leu1.32 h*$^-$ (Murray et al., 1992) was used as a *rad*$^+$ control for survival and G_2 arrest experiments. The *rad26.d* (null) allele *rad26::ura4*$^+$*ura4.D18 ade6.704 leu1.32 h*$^-$ was used as a checkpoint defective control (Al-Khodairy et al., 1994). The *S. pombe* genomic libraries used in complementation experiments and the pUD18 plasmid used in the gene deletion experiment have been previously described (Barbet et al., 1992). The cDNA library used for PCR was a kind gift from C. Norbury and B. Edgar. Bacterial strains DH5α and DH5αF′ were used for cloning and generating single stranded DNA.

Molecular and genetic techniques

Standard genetic procedures were used as described by Gutz et al.

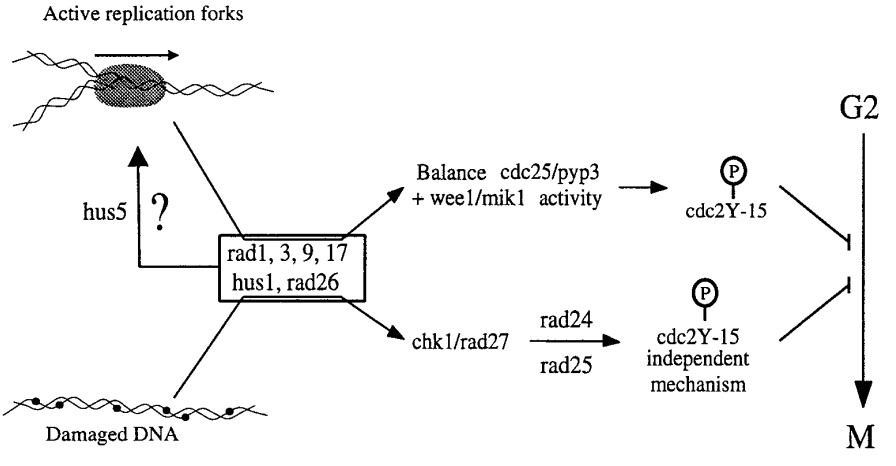


Fig. 1. Summary of the DNA damage checkpoint and DNA synthesis feedback control pathways in *S. pombe*. DNA damage and the inhibition of DNA synthesis cause independent signals to be generated which can inhibit mitosis. While the end points of these pathways remain distinct (see Sheldrick and Carr, 1993, for a review), the 'checkpoint *rad*' gene products are required in order to generate and/or transduce both the relevant signals to the mitotic machinery. In addition to mitotic delay both DNA damage and the inhibition of DNA synthesis feed signals into S phase (Enoch et al., 1992; Al-Khodairy et al., 1994), the endpoints of which are at present unclear.

(1974). *S. pombe* was transformed using the protoplasting technique described by Beach and Nurse (1981). Routine molecular biology techniques were performed as described by Sambrook et al. (1989).

Survival analysis

Two different methods were used for UV survival: (i) general survival analysis: a known density of log phase cells were plated onto YES agar plates and exposed to a dose of UV light determined either by the setting on a Stratagene 'Stratalinker' (dose ranges 50-300 Jm^{-2}) or by timed exposure under a bank of UV-C lamps with a dose rate of 12 Jm^{-2} (dose range 0-36 Jm^{-2}). (ii) Survival of plasmid containing strains: cells were treated as above but plated onto selective medium (without thiamine) and irradiated in a Stratagene 'Stratalinker'. Ionizing radiation survival was performed on growing cells in liquid culture in YE media at a density of 1×10^4 cells ml^{-1} using a Gammacell 1000 ^{137}Cs source (12 Gy min^{-1}). Plates were incubated at 29°C for 3-5 days. Colonies were counted and survival expressed as a percentage of colonies formed on unirradiated plates.

Preparation of synchronous cultures and G_2 delay measurements

Cultures of synchronous cells were prepared on a 7.5-30% lactose gradient as described by Barbet and Carr (1993) (modified from Mitchison and Carter, 1975). G_2 cells were recovered from the top of the gradient and inoculated into fresh YES media. Samples were subjected to either 0, 50, 100 or 250 Gy ionizing radiation using a Gammacell 1000 ^{137}Cs source (12 Gy min^{-1}) and incubated at 29°C. Aliquots were removed at 15 minute intervals and fixed in methanol for estimation of passage through mitosis by DAPI and calcofluor staining (Al-Khodairy et al., 1994).

Cloning and sequencing

The *hus5*$^+$ gene was cloned by transforming 10 micrograms each of pURSP1 and pURSP2 library DNA (Barbet et al., 1992) into 10^8 *hus5.62* protoplasts using the method described by Beach and Nurse, 1981. Both transformations were plated on 6 plates, colonies were allowed to form and were then scraped off into water, re-plated at a density of 10^6 cells per plate and irradiated with 100 Jm^{-2} UV radiation. Following colony formation this treatment was repeated twice, and then individual colonies were analyzed for radiation resistance and co-instability of the resistant phenotype and the *ura*$^+$ phenotype encoded by the plasmid. A single complementing plasmid was isolated from each library. Plasmid was rescued from appropriate strains directly into DH5α. The two plasmids, pUR-62A and pUR-62B had identical inserts. The complementing activity was localized to the 3.3 kb *Hin*dIII fragment following subcloning of this region into pUR19N (Barbet et al., 1992) and reintroduction into *hus5.62*. This fragment was then cloned into pGEM3$^+$ in both orientations and

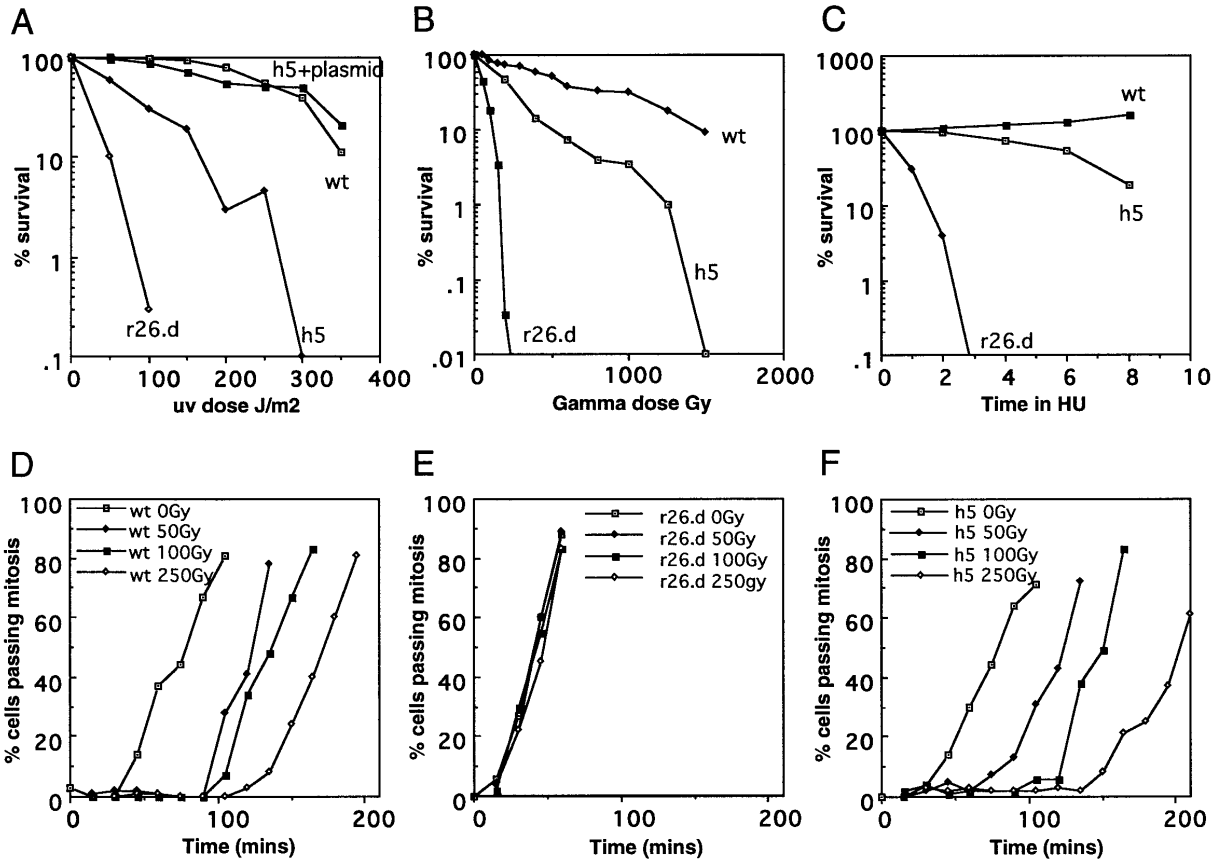


Fig. 2. Radiation survival and G_2 arrest of *hus5.62* cells. (A) UV radiation, (B) Gamma radiation and (C) hydroxyurea sensitivity of exponentially growing *hus5.62* (h5), wild-type (wt) and *rad26* (r26.d) null mutant cells. *hus5.62* cells show a radiation and hydroxyurea sensitivity intermediate between those for wild-type and *rad26.d* ('checkpoint *rad*') cells. (D-F) The G_2 arrest profiles of wild-type (wt), *rad26.d* (r26.d) null mutant and *hus5.62*, (h5) cells. Cultures were synchronized in G_2 phase, split into 4 samples and irradiated with 0, 50, 100 or 250 Grays of ionizing radiation. Samples were taken at 15 minute intervals and fixed in methanol for subsequent microscopic examination. The percentage of cells passing through mitosis was scored by comparing the proportions of mononucleate (G_2) cells with mitotic, binucleate (post mitotic) and septated cells. *hus5.62* cells show a delay profile essentially identical to wild-type cells, whereas the *rad26.d* checkpoint deficient cells are completely lacking the delay to mitosis following irradiation.

sequenced in both directions using a sequenase kit and the *Exo*III deletion procedure (Henikoff, 1984). Gaps in the sequence were determined by using synthetic deoxyribonucleotide primers. Computer analysis revealed several short open reading frames. Taking into account the consensus sequences for *S. pombe* introns (Prabhala et al., 1992), a single ORF could be created which encoded a novel member of the ubiquitin conjugating family of E2 enzymes. PCR using an *S. pombe* cDNA library as template with primers h5Nde (AATCATATGTCATCTCTTTGTAAA) and h5Bam (AAGGATCCAGTCAGTTTATGGAGC) was used to isolate a cDNA clone which could complement both the *hus5.62* and *hus5.17* alleles to wild-type levels of radiation resistance. Sequence analysis of this cDNA confirmed the existence of the five postulated introns.

Gene deletion

The *Sac*I-*Pst*I insert from the pUR-62A clone was transferred to pUC18 and cleaved with *Mlu*I and *Spe*I to remove a fragment from the insert which encodes all but 7 amino acids of the *hus5*$^{+}$ gene. The large linear fragment was recircularised in the presence of a *Mlu*I/*Spe*I to *Not*I adapter (created by mixing oligos Spe-Not CGCGGCGGC-

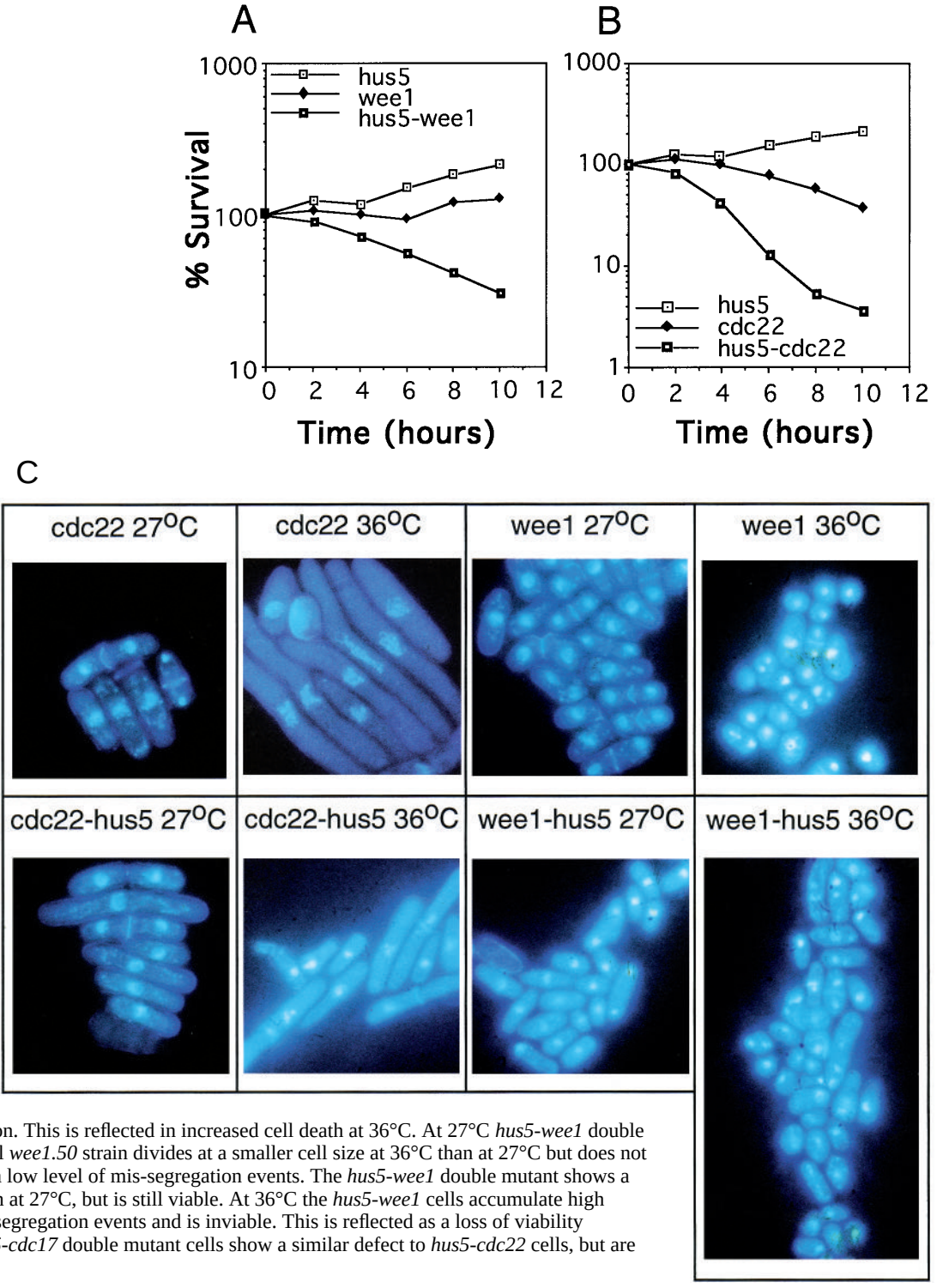


Fig. 3. Phenotypes of *hus5.62-cdc22* and *hus5.62-wee1.50* double mutants. (A) The loss of viability associated with *hus5.62-wee1.50* double mutants at the restrictive temperature. (B) The rapid cell death phenotype associated with *hus5.62-cdc22* double mutants at the restrictive temperature. Cells were propagated in exponential phase and shifted from 27°C to 36°C at time zero. Survival was measured by plating samples of a known dilution onto YE agar plates and counting colonies after 4 days incubation at 27°C. (C) Associated cell and nuclear morphologies before and 8 hours after temperature shift to the restrictive temperatures for *wee1.50* and *cdc22*. At 27°C *hus5-cdc22* double mutants are viable and show the phenotypes of *hus5* cells. While the control *cdc22* strain elongates normally at 36°C, the *hus5-cdc22* cells accumulate evidence of 'cut' nuclei and mis-segregation. This is reflected in increased cell death at 36°C. At 27°C *hus5-wee1* double mutants are viable. The control *wee1.50* strain divides at a smaller cell size at 36°C than at 27°C but does not lose viability and only shows a low level of mis-segregation events. The *hus5-wee1* double mutant shows a higher level of mis-segregation at 27°C, but is still viable. At 36°C the *hus5-wee1* cells accumulate high levels of 'cut' nuclei and mis-segregation events and is inviable. This is reflected as a loss of viability following a shift to 36°C. *hus5-cdc17* double mutant cells show a similar defect to *hus5-cdc22* cells, but are very slow growing at 27°C.

CGC and Mlu-Not CTAGGCGGCCGC) to mark the deletion boundary with a *Not*I site. This site was then used to introduce a 1.7 kb fragment containing the *ura4*$^+$ gene using the partial fill in methodology described by Barbet et al. (1992). The *Sac*I-*Pst*I fragment was then excised, gel purified, GeneCleaned and transformed into a diploid *ura4.D18*/*ura4.D18 leu1.32*/*leu1.32 ade6.704*/*ade6.704* h^+/h^+ strain. Four stable *ura4*$^+$ transformants were identified and subjected to Southern blot analysis. Two strains (62d-3 and 62d-4) contained a single disruption event in one chromosome at the *hus5* locus and two (62d-1 and 62d-2) contained multiple integration events at this locus. h^+/h^{90} derivatives of 62d-3 and 62d-4 were isolated and sporulated, and the spores plated. *ura*$^+$ colonies deleted for the *hus5* gene were radiation sensitive and grew extremely slowly with misshapen elongated cells.

The diploid *hus5::ura4*/*hus5*$^+$ *leu1.32*/*leu1.32 ura4.D18*/*ura4.D18 ade6.704*/*ade6.704* h^+/h^+ was transformed with a leucine replicating vector containing the *hus5* cDNA and with an empty vector control. h^{90}/h^+ derivatives were obtained, sporulated and spores plated directly onto plates lacking leucine and uracil. The slow growth phenotype, aberrant cell morphology and the radiation sensitivity of the null mutant could be complemented to approximately wild-type levels by the *hus5* cDNA construct, but not by the empty vector. This confirms that the phenotype of the null mutant is caused solely by the disruption of the *hus5* gene.

In situ antibody staining

For analysis of null mutants, several micro-colonies of *hus5* deleted cells were pooled and grown for 3 days on YES media to 5×10^6 cells ml^{-1}. For analysis of *hus5.62* and *hus5.17* mutants, cells were grown in exponential culture in YES media and the temperature of the culture shifted from 25 to 36°C for 3 hours. Samples were taken every hour after the shift for processing for immunofluorescence using the formaldehyde/glutaraldehyde fixation technique described by Hagan and Hyams (1988). Tubulin was detected with TAT antibody (a gift from K. Gull) and the spindle pole bodies with anti-*sad1* antibody (I. Hagan and M. Yanagida, unpublished observations; Funabiki et al., 1993).

RESULTS

Radiation survival and G_2 delay

The *hus5.62* mutant was tested for survival following exposure to UV radiation, ionizing radiation or hydroxyurea (Fig. 2A-C).

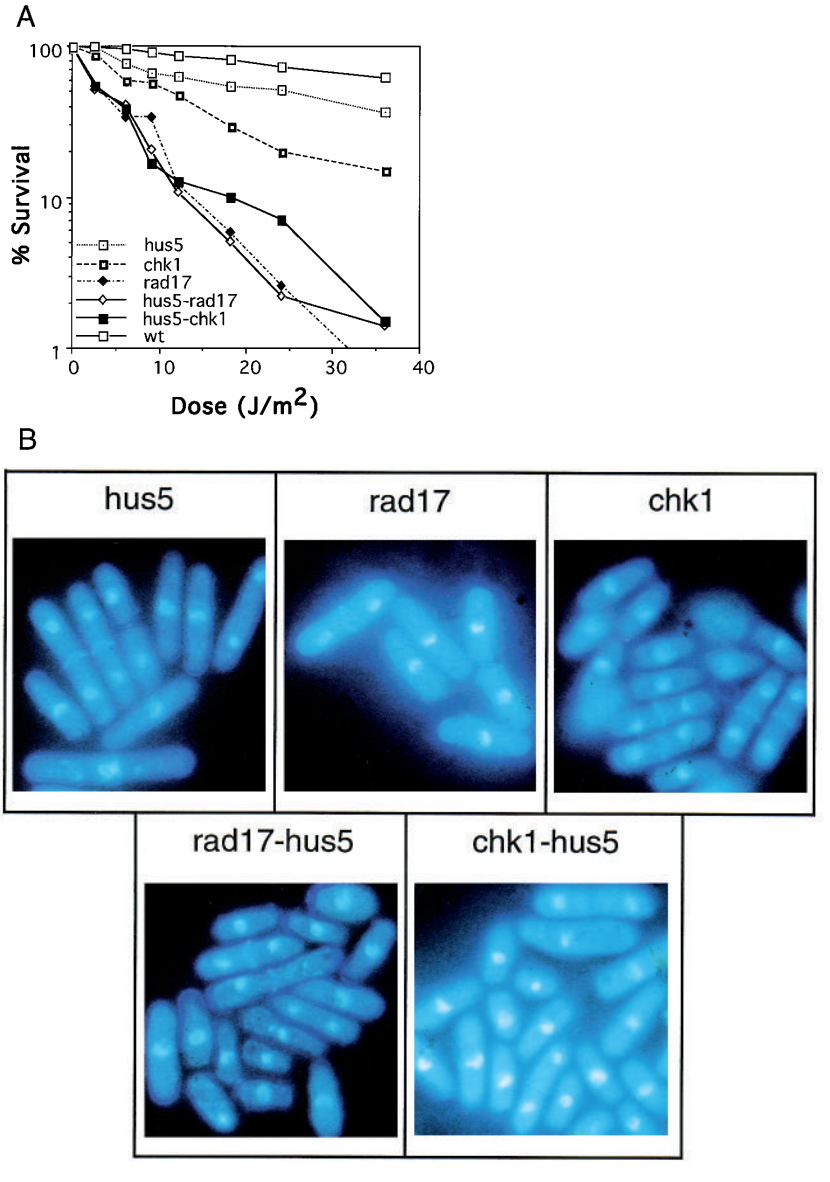


Fig. 4. Epistasis analysis. (A) Radiation survival in *hus5.62*, *chk1.r27d* and *hus5.62-chk1.r27d* double mutants, *rad17.d* and *hus5.62-rad17.d* double mutants. The radiation sensitivities of the *chk1* and *hus5* single mutants are less than the 'checkpoint *rad*' representative *rad17*. The *hus5-chk1* double mutant is more sensitive than either single mutant and has a sensitivity which is similar to the 'checkpoint *rad*' mutants. A *hus5-rad17* double mutant is no more sensitive than the *rad17* single mutant, indicating that the *hus5* radiation response defect is equivalent to one of the defects previously identified in the 'checkpoint *rad*' class of mutant. (B) DAPI and calcofluor staining of exponentially growing *hus5.62*, *chk1.r27d*, *rad17.d* single mutants and *hus5.62-chk1.r27d* and *hus5.62-rad17.d* double mutants. *hus5* cells divide at a slightly larger cell size than wild type (17.4 μm), whereas *chk1* and *rad17* mutants divide at approximately wild-type cell size (13.1, 14.5 and 14.4 μm, respectively). *hus5-chk1* and *hus5-rad17* double mutants divide at a similar size to the *chk1* and *rad17* single mutants (14.1 and 14.1, respectively), suggesting that the elongation seen in *hus5* cells is dependent on the activation of one or more of the G_2 checkpoints. The size of septated cells is an average of 10 independent measurements. Both double mutants grow more slowly than the *hus5.62* single mutant.

Table 1. Yeast strains and their phenotypes

Genotype	Phenotype and reference
Strains used	
rad$^+$ *ura4.D18 leu1.32 ade6.704*	Wild type (Grimm et al., 1988)
hus5.17 ura4.D18	See text (Enoch et al., 1992)
hus5.62 ura4.D18 leu1.32 ade6.704	See text (Al-Khodairy et al., 1994)
cdc17.K42 (DNA ligase)	Elongates (*cdc*) (Nasmyth and Nurse, 1981)
cdc22.M45 (Ribonucleotide reductase)	Elongates (*cdc*) (Nasmyth and Nurse, 1981)
wee1.50 ura4.D18 leu1.32 ade6.704	Small size at division (Nurse, 1975)
chk1.r27d ura4.D18 leu1.32 ade6.704	uv$^-$ hu$^+$ (Al-Khodairy et al., 1994)
rad17.d ura4.D18 leu1.32 ade6.704 (Checkpoint *rad*)	uv$^-$ hu$^-$ (N. Barbet and A.M.C., unpublished)
rad26.d ura4.D18 leu1.32 ade6.704 (Checkpoint *rad*)	uv$^-$ hu$^-$ (Al-Khodairy et al., 1994)
Strains created	
hus5.62 wee1.50 (27°C)	*hus5.62* phenotype
hus5.62 wee1.50 (36°C)	Mitotic catastrophe, inviable
hus5.62 cdc17.K12 (27°C)	Slow growth
hus5.62 cdc17.K12 (36°C)	Rapid death, 'cut' phenotype
hus5.62 cdc22.M45 (27°C)	*hus5.62* phenotype
hus5.62 cdc22.M45 (36°C)	Rapid death, 'cut' phenotype
hus5.62 chk1.r27d	*rad17* like uv survival
hus5.62 rad17.d	*rad17* like uv survival

In common with *hus5.17*, the *hus5.62* mutant manifests a significant sensitivity following all of these treatments. In order to determine if the sensitivity of the *hus5.62* allele to ionization radiation was due to an inability to arrest mitosis following DNA damage, we compared the extent of the G_2 delay of *hus5.62* cells with wild-type cells (*rad*$^+$) and with a radiation checkpoint defective mutant (*rad26.d*). Following several different doses of ionizing radiation (Fig. 2D-F), the *rad26.d* mutant showed no radiation induced delay to mitosis. In contrast, the *hus5.62* cells arrest prior to mitosis with a similar profile to that seen in the *rad*$^+$cells. Thus the radiation checkpoint is intact in *hus5.62* cells and the radiation sensitivity of the *hus5.62* mutant is not due to a defect in arresting mitosis following DNA damage.

Genetic interactions

S. pombe mutants defective in the feedback controls which establish the dependency relationships within the cell cycle are characterized by a rapid death phenotype when combined with most conditional cell division control (*cdc*) mutants. In addition, they demonstrate a synthetic lethality in a *wee1*$^-$ genetic background. We have crossed the *hus5.62* mutant with the representative *cdc* mutants *cdc17* (DNA ligase) and *cdc22* (ribonucleotide reductase) and with *wee1.50*, a conditional *wee1* loss of function mutation. Following a temperature shift in a *cdc17* or *cdc22* genetic background, *hus5.62* cells show rapid death kinetics (similar to that reported for *rad9* DNA ligase (cdc9) double mutants in *Saccharomyces cerevisiae* by Weinert and Hartwell, 1988) and a partial mitotic catastrophe phenotype reminiscent of those seen with *cdc2.3w*, *rad1.1*, *rad3.136*, *rad9.d*, *rad17.d*, *rad26.d*, *rad26.T12* and *hus1.14* mutants (Fig. 3 and Table 1) (Enoch and Nurse, 1990; Al-Khodairy and Carr, 1992; Al-Khodairy et al., 1994). In addition, *hus5.62* mutants show a mitotic catastrophe phenotype following loss of *wee1.50* function at 36°C (Fig. 3 and Table 1). This phenotype is similar to that seen with the above mutants and has previously been reported for the *hus5.17* allele (Enoch et al., 1992).

Epistasis analysis with *rad17* and *chk1*

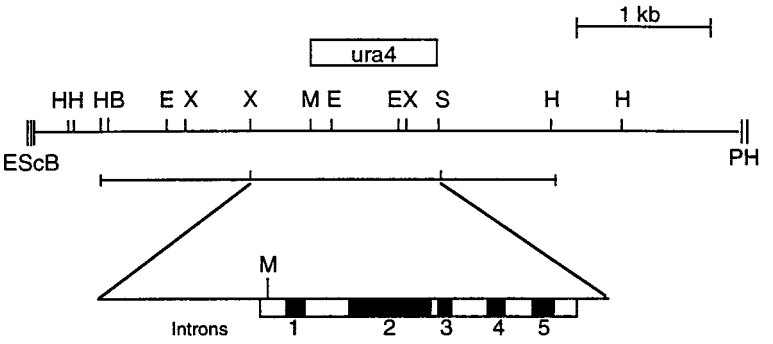
A large class of checkpoint mutants are highly sensitive to both DNA damage, and to inhibition of DNA synthesis by hydroxyurea. Not all of this sensitivity is attributable to the inability to prevent mitosis following treatment with DNA damaging agents (Al-Khodairy et al., 1994) or hydroxyurea (Enoch et al., 1992). The additional sensitivity is evident during S phase of the cell cycle, and it has been proposed that, in addition to the inability to arrest mitosis, these mutant cells are defective in a function which regulates S phase following DNA damage or a block to DNA synthesis (Al-Khodairy et al., 1994). In order to determine if *hus5* mutants are defective in this G_2 arrest independent pathway, we have analyzed the epistatic relationships between *rad17.d*, *chk1.r27d* and *hus5.62*. *rad17.d* is believed to lack both the checkpoint and recovery functions, while *chk1.r27d* is only defective in checkpoint control (Al-Khodairy et al., 1994). The *hus5.62-rad17.d* double mutant has the same sensitivity as the *rad17.d* single mutant, suggesting the *hus5* and *rad17* have defects in the same radiation response pathway. When the *hus5.62* mutation is combined with the *chk1.r27d* null mutant (which is defective only in the radiation checkpoint) the double *hus5.62-chk1.r27d* mutant is considerably more sensitive than either *chk1.r27d* or *hus5.62* alone, and has a sensitivity similar to that seen in the *rad17.d* single and *hus5.62-rad17.d* double mutants (Fig. 4). These results indicate that *hus5*$^+$ may be required for the checkpoint independent recovery process that is absent in *rad17* and many of the other 'checkpoint *rad*' mutants, but not in the *chk1* mutants.

Cloning of the hus5 gene

Both of the isolated *hus5* alleles grow slowly compared with the parental strain, both appear to increase average cell length at septation (see Fig. 3) and the variability of cell length at septation is greater. In order to ascertain the nature of the defect in *hus5* mutant cells, the *hus5* locus has been cloned by complementation of the radiation sensitivity of *hus5.62* cells with the pURSP1 and pURSP2 genomic libraries. Two independently isolated clones contained identical 5.2 kb inserts (Fig. 5A).

The *hus5* complementing clone was integrated into the genome using the *ura4*$^+$ marker. Two independent integrants

A



B

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                                                                    HindIII
                                                                    AAGCTTA
-1500  GATGTTAGTGAATAAACGACCGGTTTAGGAAACTTGCTTTGAGCTAAAAAAAAAATTGCAAAAGGATCCAGTTAC
-1425  TTGAAAGTAGTGTATATTTTATTTAATGAGAGTTCGTATAATGTAGACGTAAATATTAGTTAATTATTCGATTAA
-1350  TTAACAGTCAGTGCTTCTTTACTGGAAAACACTTTACAGCGTATAAGTAAATGGACGCTTATAACTATAAACGAC
-1275  ATGTAATTCTTAAAATGAGTTAGTCGAAAAACAAAATCGGTCGTTGTATGTGTAAGAATTGGTTATATGGAACAG
-1200  CATCTGCTTTTCATCAATTATGCAGCATAAATTAAAAAAATAGTTGCGCGGCCAGGTTTCGATCCTGGGACCTAC
-1125  GGGTTATGAGCCCGTCGGAGTTCCACTCTCCTACCGCGCACAAATACCGTCACCAGCAGGATTTGAACCTGCGCG
-1050  GGCGAAGCATTAGATGCCACGAATGTAGGAATTCGAGTCTAACTCCTTAACCACTCGGACATAGTGACTTATCTG
 -975  ACACTCATTGTAAATTAATATATATATAGGCATTTTGTTTAGTTAAAGGTACTTAAGTAATTAGTATAAACGAAC
 -900  CAATTTTATAATCAGGAAGTTAAGTGAATGGTAGCACATGTCGTAAAAATTGTGAATTTTTATTGAATAATATTT
 -825  TAAATACAAGCCTTTCTAGACTAGGTATACTCATAAACATATGAGCAAAAGGATAGAGGAGATTACATTGCATCT
 -750  TCTACAAATTTATTTATTGCCCTTTACTGAAAAATTAAATAATGAGTACTAAATGATAAAAAGCGCTCAGTACAA
 -675  GAAAGATTGCAAAAATATTGCATTCTTCATGAATTAAAGTTGCATATAAGGCATATTGAAAGTAATAGTACTAAA
 -600  ACAGCAGTTAGCGAAAATTAATAGAATTATATTCGCAAGACAATTGTGACATTAAATTAAAAAATTGTAGAATTT
 -525  TTACTATCCTCTTTAACGCCATGAGCCTTTATAAAAAGGTTAAATTAGTTTTAACATTCTTTTTTTGAGTAAGAG
 -450  TTTACAATTTATCAAAACCTGTGTTATTATATTCATTAGTTTCAATTTATTAGCATCTAGAGAAAAATCAATTGG
 -375  CAGTTACATTGTTGGAATTTATGAAGAAAAAGAATCTACAACGGAGAATAAGTTGCTGATCGCTTTCCCTAAAAT
 -300  TGTATATTTTGCTGAGCTTATTTTGACATTTCGTTGAAGTTTTCTCTAATTCGCATTCATTTTAAGTAAAACAAT
 -225  GAGAAATAAAATTACAAAAAATACAAATTAAAATACAATTTTTAGCTATAATTATAGACGATGCCCTTGTATCCC
 -150  ATTCTGTCTCGCTTGCCCCTACTTTTTATCTTTTATATACCATAATGAACGCTGCCGCTACTAACCATACCCCGA
  -75  TTTTACATTTTACATTTCGGACTCCCAAGGACGTACAAAATAGAAAACTATAGAAAAAAATAATCAGAAAATAGC
       ATGTCATCTCTTTGTAAAACGCGTTTGCAAGAAGAAAGGAAACAATGGAGAAGAGATCATCCATTTgtatgtaaa 75
       M  S  S  L  C  K  T  R  L  Q  E  E  R  K  Q  W  R  R  D  H  P  F
       ttttagtaaacttgaagaaatcactaacaacttctcttacttagGGATTCTATGCAAAACCTTGTAAATCATCTG 150
                                                    G  F  Y  A  K  P  C  K  S  S  D
       ATGGAGGACTCGATTTAATGAATTGGAAGGTTGGAATTCCCGGAAAACCGAAAACGTCTTGGGAAGGCGGTTTAT 225
         G  G  L  D  L  M  N  W  K  V  G  I  P  G  K  P  K  T  S  W  E  G  G  L  Y
       ACAAACTCACAATGGCTTTTCCTGAAGgtatgtattccaaatgaatatcagatatttttcttcaaagcctttaat 300
         K  L  T  M  A  F  P  E  E
       ttgcgctccttagcttggaattttgtttagacattcactacttttgttcaaactgaattttagctttatcctca 375
       cgataaaacatgtcgatatgcatttccttttagctaatcaaaagttcacggcttttcgttttattatttacaaaa 450
       catttactaactgcaatcAcagAATATCCAACTCGTCCCCCCAAATgtaagcatataactacgatttttgctaa 525
                              Y  P  T  R  P  P  K  C
       tgcttttgtacagGTAGGTTTACTCCACCGTTATTTCATCCAAACGTTTATCCTTCTGGCACCGTTTGTCTCTCA 600
                     R  F  T  P  P  L  F  H  P  N  V  Y  P  S  G  T  V  C  L  S
       ATACTTAATGAAGAAGAAGGCTGGAAACCGGCAATTACAATTAAGCAGgtaagaattcaaatcaatttcttaatc 675
       I  L  N  E  E  E  G  W  K  P  A  I  T  I  K  Q
       tccaggttttaaccatttctagATTTTGCTGGGTATTCAGGATCTTTTAGATGATCCTAATATTGCCTCTCCCGC 750
                           I  L  L  G  I  Q  D  L  L  D  D  P  N  I  A  S  P  A
       TCAAACAGAAGCATATACAATGTTCAAgtaagtatcataactactgtttccacaaccatcaaatataattaacct 825
        Q  T  E  A  Y  T  M  F  K
       cgtttagAAAGGATAAAGTCGAGTACGAAAAACGAGTTCGTGCTCAGGCTCGTGAAAATGCTCCATAAACTGACT 900
              K  D  K  V  E  Y  E  K  R  V  R  A  Q  A  R  E  N  A  P  STOP
       GGTACTTTTTCTTATTTTTTATTGTTTAATATTATTTTTCCTGATGTGTATATTTCTTTGCTTGACTAGTTGAGA 975
       TAAAATCTCAATCTTATTAGCGGTTTCACAAGTCGGTTGCTTTTTGTTTGTTAGGATAAAAGATTGATACAAGAT 1050
       AATACCACTAATTATTTAAATATGTTTTTTTTAAAAAGTTCATTAAATCAACCACGTCTTAAGTGATAGAATTTA 1125
       CCTCGACAAATTCCTTTAAATCAATTAGAAAGTAAACTATAATTTAATAATTGTATGCCCTCAAAAAATGGTCAC 1200
       TATTTAAATATAGTGTTTTTTTTTTTACAACTAAGACAGTAGAATGTACTTTAATAAGCACAATCAAGGTTTCAGA 1275
       ACATTAAGCAACCAAAAATAGTATTTTAATGATCAACAATAAACATTCTTTTTAGTAATGCTCAAACGCTATCTA 1350
       CTGTGGGTGCGATTGATCACCGCTGACACCATGCATTTGAGCATAAATTTGCTGTTGATAATATTGTACATACCC 1425
       ATTGTATCCTCCATAACTGCGTACGGATCTACCCTGGAGTAGTTAAACCAGGCGTACCAGTCCTGGTGCTTCTGT 1500
       ACCAGGAGCACTAACTCCAGGAGCACCAAACATAGTAGGCATACCCTGTAAAGCAGTAGAAGAAGTCGTCGGTAC 1575
       TGCAATAGATGAAGTGACACCAGGAGGCATTGCATAGCTTCCATTCCTGGAACAGCTGGAATAGATGGTACGGGT 1650
       AACGATGGTTGTTACTAATGGTGCAGGGGAAGCATTAGTTGGTTGAGTTTTTTCTTGTCGTGAGTCTCGTGCGGA 1725
       GAATGCCTGATTCCGGTTTGGTGGATTGATGGCACAGTTTCCTTGATTTTGATCACGATCTTTTGGTTGTGACTT 1800
       CCAAGCTT                                                                    1807
         HindIII
```

Fig. 5. Cloning and sequencing of *hus5*. (A) The pUR-62A and pUR-62B clones contain 5.2 kb of DNA. A 3.3 kb subclone (shown below) was tested and complemented the radiation sensitivity of *hus5.62*. The region deleted and replaced with the *ura4*$^+$ gene is indicated above. The ORFs are shown in the expanded region as open boxes and the five introns as filled boxes. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; M, *Mlu*I; P, *Pst*I; S, *Spe*I; Sc, *Sac*I; X, *Xba*I. Bold vertical bars indicate vector derived sites. (B) The complete nucleotide sequence of the 3.3 kb complementing *Hin*dIII fragment. The ORFs of the *hus5* gene are shown in uppercase with the predicted translation products indicated below. Introns are shown in lowercase. This sequence has been deposited in the EMBL database under accession number X81846.

```
              10v       20v       30v       40v       50v
HUS5     MSSLCKTRLQEERKQWRRDHPFGFYAKPCKSSDGGLDLMNWKVGIPGKPKT
         MS:  : RL  : K: ::D P G  A P      : ::M W:  I G : T
RHP6     MSTTARRRLMRDFKRMQQDPPAGVSASP-----VSDNVMLWNAVIIGPADT
               10^       20^            30^       40^
              60v       70v       80v       90v       100v
HUS5     SWEGGLYKLTMAFPEEYPTRPPKCRFTPPLFHPNVYPSGTVCLSILNEEEG
         : E:G :KL ::F E:YP :PP  :F : :FHPNVY::G :CL IL  ::
RHP6     PFEDGTFKLVLSFDEQYPNKPPLVKFVSTMFHPNVYANGELCLDIL--QNR
          50^       60^       70^       80^       90^
             110v      120v      130v      140v      150v
HUS5     WKPAITIKQILLGIQDLLDDPNIASPAQTEAYTMFKKDKVEYEKRVRAQAR
         W P:  :  IL :IQ LL:DPN ASPA::EA  : : :K EY :RVR
RHP6     WSPTYDVAAILTSIQSLLNDPNNASPANAEAAQLHRENKKEYVRRVRKTVE
          100^      110^      120^      130^      140^

HUS5     ENAP
         ::
RHP6     DSWES
         150^
```

Fig. 6. Amino acid identity to *rhp6*. Alignments of the *hus5* gene products with the *rhp6* product. These two proteins are 38% identical. *rhp6* is the *S. pombe* homolog to the *S. cerevisiae RAD6* gene and these two functional homologues share 77% identity. Standard single letter amino acid codes are used; : indicates conservative substitutions. The alignment was performed using the DNAstar package.

were backcrossed to *rad*$^+$ *ura4*$^-$ cells and 500 spores from each were plated onto non-selective media. When colonies had grown, the plates were replica plated separately to *ura*$^-$ media plates and to complete media phloxin B plates, which were then irradiated at 300 Jm^{-2}. Analysis of these plates 24 hours later showed that all colonies were radiation resistant and approximately 50% were uracil prototrophs and 50% uracil auxotrophs. This result demonstrates that in both cases the *hus5* complementing clone had integrated close to the *hus5.62* locus, indicating that the cloned region contained the *hus5*$^+$ gene.

Sequence analysis

The complementing clone contained 5.2 kb of genomic DNA (Fig. 5A). In order to localize the complementing activity a 3.3 kb *Hin*dIII fragment was subcloned into the pUR19N vector, transformed into *hus5.62* and tested for complementation of the radiation sensitivity. The complementing activity was contained within the 3.3 kb *Hin*dIII fragment. This DNA was subcloned into pGEM3$^+$ and subjected to sequence analysis (Fig. 5B). The sequenced region contained several short ORFs with conserved residues typical of ubiquitin conjugating enzymes. If five introns were postulated, a single ORF could be generated which potentially encodes a 157 amino acid protein with 38% homology to the *S. pombe rhp6* gene (Fig. 6).

A full length cDNA was isolated and sequenced to confirm the intron positions and to check that no errors had been introduced during PCR. This cDNA was then subcloned into the pREP41 thiamine regulatable expression vector. Under both induced and uninduced (basal) conditions this cDNA construct could complement the radiation sensitivity of both the *hus5.62* and the *hus5.17* alleles to wild-type levels.

Gene deletion

In order to ascertain if the phenotype of the *hus5.17* and *hus5.62* were the result of a partially or completely inactivated protein, a null allele of the *hus5* gene was generated. A deletion construct of *hus5* was prepared in which the *hus5*$^+$ gene had been replaced by the *ura4*$^+$ gene (Fig. 5A). This DNA was integrated at the *hus5* chromosomal locus in a diploid *h*$^+$/*h*$^+$ strain. Two independently isolated integrants were used to isolate h$^+$/h^{90} diploids, these were sporulated and the spores plated on complete media. Examination of these plates following colony formation revealed approximately equal numbers of healthy colonies and micro-colonies which contained elongated cells of variable length. Replica plating to *ura*$^-$ media plates and to complete media phloxin plates, which were then irradiated at 300 Jm^{-2} revealed that all the healthy colonies were *ura*$^-$ *rad*$^+$ while the micro-colonies were *ura*$^+$ and *rad*$^-$. This suggests that deletion of the *hus5* ubiquitin conjugating enzyme is detrimental to normal cell growth. The deleted (*ura4*$^+$) and wild-type *hus5* loci *(hus5.d* and *hus5*$^+$, respectively) segregated in the normal 2:2 manner when the spores from 10 tetrads were dissected. If micro-colonies were propagated for any length of time, suppressers and possible changes in ploidy occur which make the cultures unsuitable for most genetic, biochemical or cell biological studies.

Anti-tubulin and spindle pole body staining

In order to further understand the consequences of loss of *hus5*$^+$ function, several *hus5.d* micro colonies were 'pooled' and propagated for a brief period. The cells were then fixed and examined by DAPI staining and by in situ immunofluorescence with anti-tubulin (Woods et al., 1989) and anti-*sad1* antibodies to localize the microtubules and spindle pole bodies, respectively. Fig. 7 shows a typical field of such cells: (A) stained with DAPI to show the chromatin; (B) stained with anti-tubulin antibody; (C) stained with anti-*sad1* antibodies. A variety of defects can be seen. In a number of cells (16%) the chromatin is highly condensed, indicative of a strong mitotic delay. In many mitoses (for example see inset), portions of chromatin that are not being correctly segregated by the mitotic spindle are evident, indicating an abortive mitosis. Finally, there are many cells whose morphology (an abnormal sized lump of chromatin lacking a nucleolus positioned at one end of the cell) bear witness to previous lethal 'cut' like attempts at division.

The long S shape of the spindle seen in the cell in the inset is unusual and indicates that, in addition to the problems in chromatin separation, spindle elongation has proceeded for longer than normal and septation has occurred without prior activation of the central microtubule organizing center of the PAA, which is always the case in wild-type cells (Hagan and Hyams, 1988). As errors in chromosome segregation would be expected to lead to gene loss and karyotypic instability (reminiscent of the karyotypic instability seen in the oncogenic state) it is not possible to conclude any more from these data than that mitosis is abnormal in *hus5* null mutants.

Similar staining was undertaken with the *hus5.62* and *hus5.17* mutants during exponential growth at 25°C in yeast extract media. The *hus5.17* mutant has previously been reported to have an associated growth defect at 36°C (Enoch et al., 1992) and thus this analysis was also performed one, two and three hours after a temperature shift to 36°C. Consistent with previous observations, the phenotype presented by the mutants at 25°C was very heterogeneous and resembled the collection of abnormalities seen in *hus5.d* (Table 2). Whilst

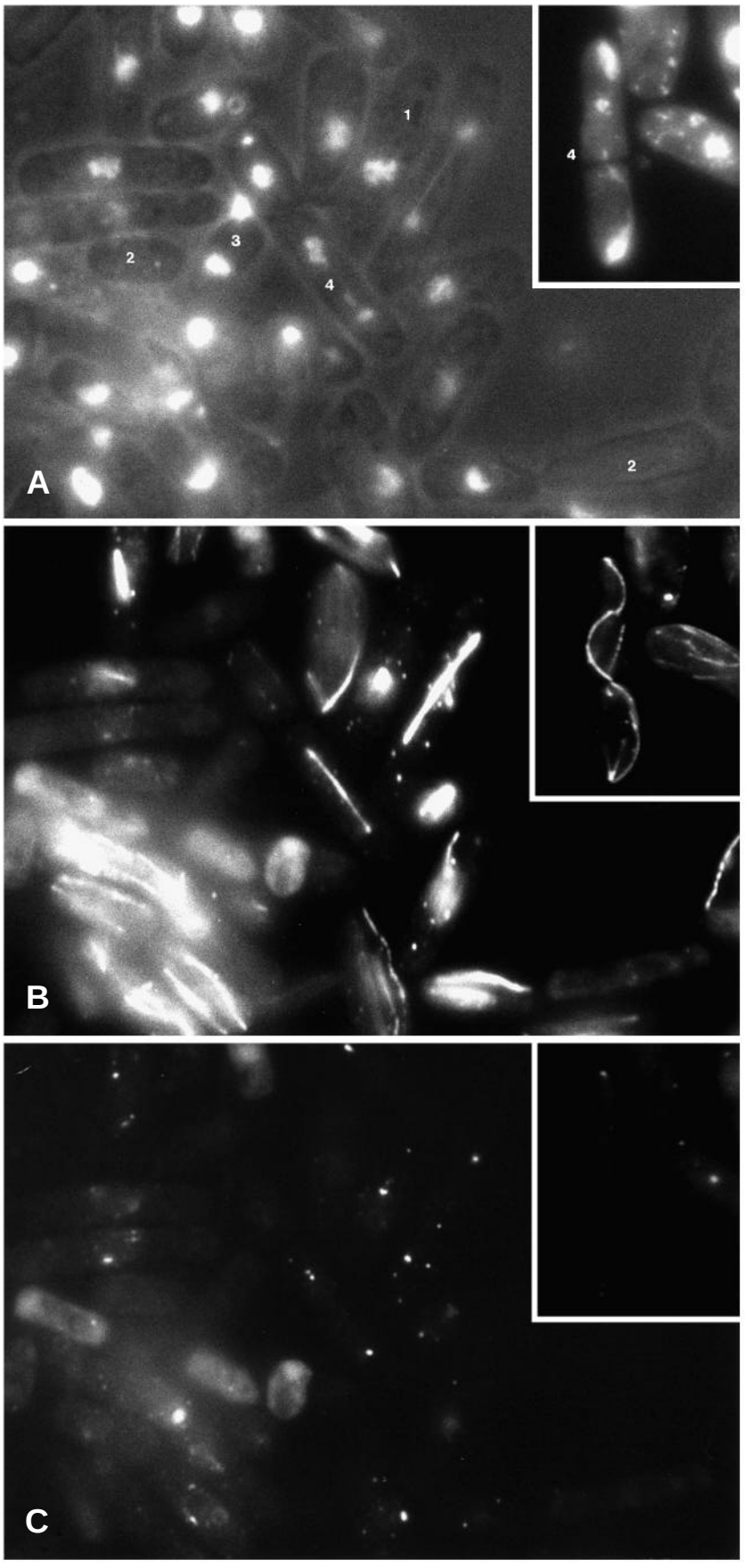

Fig. 7. Immunofluorescent staining of *hus5* deletion cells. *hus5* deletion cells were stained with anti tubulin and anti *sad1* (spindle pole body protein) antibodies: (A) shows the DNA by DAPI staining and (B) and (C) show the anti tubulin fluorescence and anti *sad1* fluorescence, respectively. In addition to the size heterogeneity, several phenotypes can be seen: 1, condensed chromosomes; 2, enucleate cells; 3, cells that are the product of a lethal 'cut' abortive mitosis; 4, cells in which the chromatin is not segregating properly along the spindle and is either stretched along the spindle, or is located at some point along it.

Table 2. Percentage of aberrant morphologies in cultures of *hus5* mutant strains prepared for immunofluorescent microscopy

Strain	Time at 36	Enucleates	Chromatin stretched along the spindle	Condensed chromosomes	cdc	cut	Uncoupled
hus5.62	0	0	3	4	6	7	0
	1	1	2	5	4	6	1
	2	1	4	5	9	7	1
	3	2	2	5	9	9	1
hus5.17	0	1	3	3	4	5	1
	1	4	1	5	4	5	1
	2	3	1	3	6	6	1
	3	1	1	2	6	8	0
hus5.d	-	5	1	16	16	11	1

Cells were prepared as described in the text and scored for the relative frequency of a number of morphologies. Many of these morphologies are presented in the micrograph in Fig. 7. Enucleate strains are those lacking nuclei (Fig. 7, class 2). Cells with chromatin stretched along the spindle correspond to Fig 7, class 4. Cells with condensed chromosomes correspond to Fig. 7, class 1. cdc refers to all cells that on visual screening were longer than the standard cell size at division (14 μm). 'cut' refers to cells that display the unusual phenotype of an abnormal sized lump of chromatin at one end of the cell. This phenotype is indicative of a lethal mitotic cut division in the previous nuclear division (Fig. 7, class 3). The frequency of cells in which cell division is occurring without nuclear division and which will give rise to an enucleate cell and a cell with twice the amount of chromatin the parent cell had are presented in the last column.

there was no noticeable increase in the numbers of *hus5.17* cells exhibiting mitotic abnormalities upon temperature shift, the number of cells that have arisen from a 'cut'-like lethal mitosis (a very sensitive indicator of the relative severity of mitotic defects) did increase. Thus there is not a sharp transition in the phenotype of the *hus5.17* mutant upon temperature shift, rather the severity of the already pleiotropic defect is accentuated. This is not surprising considering the pleiotropic phenotype seen with a complete loss of hus5 function in *hus5.d* cells.

DISCUSSION

hus5$^+$ is not directly involved in checkpoint control

Mutations in the *hus5*$^+$ gene have been isolated in two independent screens for mitotic entry checkpoint mutants. However, we have found no evidence that *hus5* mutations disrupt the mitotic entry checkpoint. *hus5* mutants arrest relatively normally in HU, although they display some evidence of aberrant mitosis at the restrictive temperature when combined with a temperature sensitive *cdc22* mutation (Fig. 2; *cdc22*$^+$ encodes ribonucleotide reductase), and they arrest in an identical fashion to wild-type cells after treatment with ionizing radiation. A deletion of *hus5*$^+$ was virtually inviable making analysis of checkpoint control in this strain impossible. However, morphological analysis suggests that the deletion phenotype is most likely to represent a more extreme version of the phenotypes of the mutants initially isolated in the genetic screens.

Like all of the known mitotic entry checkpoint mutants, *hus5* mutants are temperature sensitive for viability when combined with *wee1-50*, a temperature sensitive allele of *wee1*$^+$ (Fig. 2). This could reflect a checkpoint defect that is too subtle to detect in the *hus5* single mutants, or it could reflect a defect in a recovery process that requires *hus5*$^+$ function (see below). Such a recovery process may become essential in the absence of *wee1*$^+$. An alternative possibility is that the synthetic lethality results from the mitotic defect seen in *hus5* cells. Morphological analysis suggests that *hus5* mutants undergo significant levels of aberrant mitoses, even under normal conditions. This may be exaggerated in *wee1* mutants, where there is even less time for mitosis.

The design of both of the genetic screens which identified the two *hus5* mutants can account for their isolation, despite the lack of checkpoint defects. In one screen (Enoch et al., 1992), HU sensitive mutants were isolated and then examined cytologically for evidence of entry into mitosis in the presence of HU. Cells that enter mitosis without completing DNA replication display a characteristic 'cut' phenotype consisting of a single nucleus cleaved by a septum (in some cases, the septum misses the nucleus giving rise to one anucleate daughter) (Enoch and Nurse, 1990). However, the 'cut' phenotype is also observed in mutants with defects in chromosome segregation (Hirano et al., 1986). Thus *hus5-17*, which is both HU sensitive and has defects in chromosome segregation that gives rise to 'cuts', was isolated in this screen.

In the second screen, mutants that were sensitive both to irradiation and to HU were selected (Al-Khodairy et al., 1994). This strategy was based on the observations that, while most of the fission yeast repair mutants were not sensitive to HU (Al-Khodairy and Carr, 1992) and most replication mutants were not sensitive to irradiation, the checkpoint mutants, *rad1*, *rad3*, *rad9* and *rad17* were sensitive to treatment with both agents.

Using such approaches, a large number of checkpoint deficient mutants were identified. However, as the studies reported here illustrate, additional criteria are required to establish that mutants isolated by such strategies are truly defective in checkpoint controls.

A role for *hus5*$^+$ in a checkpoint-related 'recovery' process?

The largest category of fission yeast mitotic entry checkpoint mutants (the 'checkpoint *rad*' mutants) consists of mutants that cannot arrest in either S-phase following a block to DNA synthesis or arrest in G_2 following DNA damage. In addition to these checkpoint phenotypes, these mutants also manifest a further defect which results in poor recovery from treatments that either induce S-phase arrest or cause DNA damage (Enoch et al., 1992; Al-Khodairy et al., 1994). Mutations in *rad1*, *rad3*, *rad9*, *rad17*, *rad26* and *hus1* all share this phenotype. As a

result of the additional defect, these strains are significantly more sensitive to S-phase arrest or to DNA damage than other classes of checkpoint mutants which only affect the mitotic arrest checkpoints (*chk1* and *cdc2.3w*). We have previously proposed that this additional sensitivity represents a defect in a 'recovery' processes of an unknown nature (Enoch et al., 1992; Al-Khodairy et al., 1994), which may relate specifically to DNA synthesis (Al-Khodairy et al., 1994).

hus5 mutants appear to express a subset of phenotype common to the checkpoint mutants: they are sensitive both to S-phase arrest and to irradiation; they display a rapid death phenotype (linked with increased levels of catastrophic mitosis) when combined with S phase and late S/G_2 phase *cdc* mutants and they are also synthetically lethal with loss of *wee1* function. As *hus5* checkpoint controls appear to be normal, some or all of these phenotypes could be the result of a defect in the S-phase related 'recovery' processes. We have shown that *rad17 hus5* double mutants are no more sensitive to radiation than *rad17* single mutants and that, in contrast, the radiation sensitivities of *hus5* and *chk1* (which is defective only in the checkpoint control) are additive and have a radiation sensitivity similar to the *rad17* single mutant. Classically, non-additive radiation sensitivities (*hus5* and *rad17*) have been considered to imply that the two different mutations affect genes in the same 'epistasis group' and function in the same radiation response pathway. Additive or synergistic sensitivities (*hus5* and *chk1*) have been taken to imply that the mutations affect genes acting in different radiation responses (see Friedberg et al., 1991, for review). One interpretation of our results is that *hus5* mutants share a defect in a mitotic arrest independent recovery process with the 'checkpoint *rad*' class of mutants. However, in order to establish this conclusively it will be necessary to know more about this 'recovery' process.

hus5$^+$ encodes a novel member of the E2 family of ubiquitin conjugating enzymes (UBCs)

The protein predicted by the *hus5*$^+$ DNA sequence is very likely to encode a member of the E2 family of the ubiquitin conjugating enzymes (UBCs) with an evolutionarily conserved function in many aspects of cell cycle progression. The predicted protein shows 38% similarity to the protein encoded by the fission yeast *rhp6*$^+$ gene (Reynolds et al., 1990) which is the homologue of the budding yeast RAD6 gene (Reynolds et al., 1985). It shows 80% similarity to a novel budding yeast UBC, isolated by PCR using information derived from the *hus5*$^+$ gene sequence (J. Spence and D. Finley, personal communication). Thus it seems likely that budding yeast has a *hus5* equivalent and that its role is evolutionarily conserved.

UBCs mediate attachment of ubiquitin molecules to a variety of protein substrates. One function of this modification can be to target the protein for destruction by ubiquitin-dependent proteases. However, ubiquitination may alter protein function in other ways as well (see Finley and Chau, 1991, for review). Here we have shown that *hus5*$^+$ mediated ubiquitination is required for the response to radiation damage and for normal mitosis. Another UBC, *rhp6*$^+$, is also required for the response to radiation (Reynolds et al., 1990) and is highly conserved in both budding and fission yeasts. It will be interesting to determine whether these gene functions overlap.

It is becoming increasingly clear that ubiquitination of regulatory molecules plays a crucial role in cell cycle progression. Transitions from one cell cycle stage to the next in eukaryotic cells require 'cell division kinases' which consist of 34 kDa catalytic subunits and regulatory subunits called cyclins. Degradation of cyclins inactivates the kinase and may be required for transitions from one stage of the cell cycle to the next (Glotzer et al., 1991). In budding yeast, a UBC encoded by the CDC34 gene appears to be involved in degradation of mitotic cyclins during anaphase and G_1 (Tyers et al., 1992; Amon et al., 1994). While no fission yeast equivalent of the CDC34 gene has been identified, a human equivalent has been reported. Interestingly, this gene was detected as a high copy suppresser of an *S. cerevisiae* mitotic entry checkpoint mutant (Plon et al., 1993).

Protein ubiquitination has also been shown to be required for chromatid separation and the initiation of anaphase using a *Xenopus* cell-free system (Holloway et al., 1993). *S. pombe* mutants which delayed anaphase in a similar manner might be expected to show a 'cut' phenotype. Interestingly, mutations in the *S. pombe mts*$^+$ gene, which encodes a subunit of a ubiquitin dependent protease, also display a 'cut' phenotype (Gordon et al., 1993), although it appears to be more severe than the one we observe for *hus5* disruptions. Conceivably, these phenotypes could reflect stabilization of the same protein; in the case of *hus5* mutants the protein is not ubiquitinated and therefore not targeted for degradation, whilst in the *mts* mutant the ubiquitinated protein is not degraded due to the absence of the protease.

Clearly, by targeting key regulatory proteins for degradation, UBCs play an important role in many aspects of cell proliferation. The activity of these enzymes must be regulated temporally and coordinated with other cellular events in order to ensure precise integration of a diverse array of cellular processes. Identifying UBC targets and understanding how UBC activity is controlled is therefore of considerable importance. The molecular, physiological and genetic analysis of the *S. pombe hus5*$^+$ gene presented here is a step in this process.

This work was supported, in part, by CEC contract F13P-CT920007 and by NIH grant GM50015-02 to T.E. F.Al-K. was supported by the Saudi Arabian Government. T.E. is a Harcourt General New Investigator. I.H. is suported by the Cancer Research Campaign. A.C. is supported by the MRC. We thank Andrew Sutcliffe for assistance with immunofluorescence.

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(Received 26 August 1994 - Accepted 29 September 1994)

Note added in proof

Recent work suggests that *hus5* is homologous to *S. cerevisiae UBC91*, which is involved in the degradation of both S and M phase cyclins (Seufert et al. (1995) *Nature* (in press)).