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Hus1p, a conserved fission yeast checkpoint protein, interacts with Rad1p and is phosphorylated in response to DNA damage

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The *hus1*$^+$ gene is one of six fission yeast genes, termed the *checkpoint rad* genes, which are essential for both the S-M and DNA damage checkpoints. Classical genetics suggests that these genes are required for activation of the PI-3 kinase-related (PIK-R) protein, Rad3p. Using a dominant negative allele of *hus1*$^+$, we have demonstrated a genetic interaction between *hus1*$^+$ and another *checkpoint rad* gene, *rad1*$^+$. Hus1p and Rad1p form a stable complex in wild-type fission yeast, and the formation of this complex is dependent on a third *checkpoint rad* gene, *rad9*$^+$, suggesting that these three proteins may exist in a discrete complex in the absence of checkpoint activation. Hus1p is phosphorylated in response to DNA damage, and this requires *rad3*$^+$ and each of the other *checkpoint rad* genes. Although there is no gene related to *hus1*$^+$ in the *Saccharomyces cerevisiae* genome, we have identified closely related mouse and human genes, suggesting that aspects of the checkpoint control mechanism are conserved between fission yeast and higher eukaryotes.
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Introduction

To complete a cell division cycle successfully the various events of the cell cycle must occur in a controlled progression. Cell-cycle regulatory mechanisms that ensure the proper order of cell-cycle events have been termed 'checkpoints' (Hartwell and Weinert, 1989). In fission and budding yeasts, 'relief of dependence' mutations have been identified which disrupt various cell-cycle checkpoints (Murray, 1992). Such mutations allow cell-cycle progression under conditions that would normally cause cell-cycle arrest. Two important checkpoints, collectively termed the G_2-M checkpoint, postpone mitosis when the DNA is incompletely replicated (the S-M checkpoint) or damaged (the DNA damage checkpoint). To understand the mechanism of these checkpoints, we have studied *Schizosaccharomyces pombe* mutants that enter mitosis in the presence of unreplicated or damaged DNA.

Many genes in fission yeast are required for the G_2-M checkpoint. One group, which includes *cdc18*$^+$, *cut5*$^+$, *cdt1*$^+$ and *pol1*$^+$ (Kelly *et al.*, 1993; Saka and Yanagida, 1993; Hofmann and Beach, 1994; Saka *et al.*, 1994; D'Urso *et al.*, 1995; McFarlane *et al.*, 1997), contains genes which are essential for DNA synthesis and also are required to couple mitosis to the completion of DNA synthesis, suggesting that elements of the replication complex are the origin of the G_2-M checkpoint signal. Another class of G_2-M checkpoint mutants was discovered by testing the checkpoint function of the *wee* mutants, known to be altered in regulation of the fission yeast cyclin-dependent kinase (cdk) Cdc2p (Enoch and Nurse, 1990). These studies suggest that tyrosine phosphorylation of Cdc2p is required for the S-M checkpoint in fission yeast and that Cdc2 is the downstream target of the checkpoint pathway. However, the role of Cdc2 activity in the S-M checkpoint remains controversial because Cdc2 kinase activity is not reduced upon activation of the S-M checkpoint (Knudsen *et al.*, 1996). Although the *wee* mutants are not defective in the DNA damage checkpoint (Sheldrick and Carr, 1993), recent studies suggest that inhibitory phosphorylation of Cdc2 is also required for the DNA damage response (O'Connell *et al.*, 1997; Rhind *et al.*, 1997).

At least nine other genes are involved in G_2-M checkpoint control in fission yeast: *chk1*$^+$, *cds1*$^+$, *crb2/rhp9*$^+$, *rad1*$^+$, *rad3*$^+$, *rad9*$^+$, *rad17*$^+$, *rad26*$^+$ and *hus1*$^+$ (Al-Khodairy and Carr, 1992; Enoch *et al.*, 1992; Rowley *et al.*, 1992; Walworth *et al.*, 1993; Al-Khodairy *et al.*, 1994; Murakami and Okayama, 1995; Willson *et al.*, 1997). In contrast to the previously mentioned *wee* mutants and genes involved in DNA synthesis, mutations in these genes disrupt G_2-M checkpoint control but do not affect cell cycle progression under normal growth conditions. This suggests that they are the signal transducers that couple the source of the checkpoint signal to the mitotic control genes. Mutations in *chk1*$^+$ or *crb2/rhp9*$^+$ disrupt the DNA damage checkpoint, and mutations in *cds1*$^+$ partially disrupt the S-M checkpoint. However, mutations in the *checkpoint rad* genes *rad1*$^+$, *rad3*$^+$, *rad9*$^+$, *rad17*$^+$, *rad26*$^+$ and *hus1*$^+$ abolish both the DNA damage and S-M checkpoints. These results suggest that although the *checkpoint rad* genes are required for both responses, the two checkpoints may function through distinct mechanisms. This idea is further supported by the fact that some *cdc2* alleles, an allele of *rad1*$^+$ (Kanter-Smoler *et al.*, 1995) and an allele of *rad26*$^+$ (Uchiyama *et al.*, 1997) are only defective for the DNA replication checkpoint.

A picture of the biochemical events underlying the DNA damage checkpoint response is beginning to emerge. An early step in this process may involve activation of the Rad3 checkpoint kinase (Seaton *et al.*, 1992; Bentley *et al.*, 1996). The checkpoint kinases Chk1p and Cds1p are themselves phosphorylated in a Rad3p-dependent

manner in response to checkpoint activation (Walworth and Bernards, 1996, Lindsay *et al.*, 1998), placing *chk1*$^+$ and *cds1*$^+$ downstream of *rad3*$^+$ in this pathway. Phosphorylation of Chk1p and Cds1p also requires the other five *checkpoint rad* genes, suggesting that they are required for the activation of Rad3p. Further, the Chk1p kinase has been shown to phosphorylate Cdc25p (Furnari *et al.*, 1997) and possibly Wee1p (O'Connell *et al.*, 1997), providing a potential link between the *checkpoint rad* proteins and the mitotic control apparatus (Weinert, 1997).

The Rad3p kinase is evolutionarily conserved. It is a member of the PI kinase-related (PIK-R) group of kinases (reviewed by Hoekstra, 1997) which are involved in checkpoint control and DNA damage repair in *Saccharomyces cerevisiae*, *Drosophila melanogaster* and humans (Lavin *et al.*, 1995). In humans, mutation of the PIK-R gene *ATM* causes the severe congenital disease ataxia-telangiectasia (A-T; Savitsky *et al.*, 1995). Children with this disease have a high incidence of cancer, particularly leukemias and lymphomas. Like *checkpoint rad* mutants, A-T cells have both DNA repair and cell-cycle defects, suggesting that the ATM and Rad3 kinases are functionally, as well as structurally, related. A second mammalian gene, *ATR* (ATM- and Rad3-related), is structurally similar to both *ATM* and *rad3*$^+$. Together with ATM, the ATR protein has been implicated in meiotic chromosome segregation (Keegan *et al.*, 1996). Another mammalian PIK-R kinase is the DNA-dependent protein kinase (DNA-PK; Hartley *et al.*, 1995). Although DNA-PK is not directly involved in cell-cycle controls, it plays an important role in the management of double-strand DNA breaks (Gottlieb and Jackson, 1993). Since many of the PIK-R kinases are involved in DNA metabolism, they may represent a new family of kinases which respond to specific DNA structures (Hoekstra, 1997).

The function of the other *checkpoint rad* genes may also be conserved since in many cases there are related genes in other eukaryotes, including mammals. The *S.pombe rad1*$^+$ gene is structurally related to *RAD17* of *S.cerevisiae* (Siede *et al.*, 1996) and *REC1* of *Ustilago maydis* (Holden *et al.*, 1991; Long *et al.*, 1994), which are both involved in checkpoint control. The *S.pombe rad17*$^+$ gene, which shares some sequence similarity with Replication Factor-C (RF-C), has a budding yeast homolog, *RAD24* (Griffiths *et al.*, 1995). A potential homolog of *S.pombe rad9*$^+$ has also recently been identified in budding yeast, termed *DDC1* (Longhese *et al.*, 1997). The remaining two *S.pombe checkpoint rad* genes, *hus1*$^+$ and *rad26*$^+$, have no obvious structural homologs in budding yeast. As a result of the human and mouse EST sequencing projects, potential homologs of the *checkpoint rad* genes have also been discovered in mammals. A human gene related to the fission yeast *rad9*$^+$ gene has been characterized (Lieberman *et al.*, 1996) and sequences similar to *rad1*$^+$ and *rad17*$^+$ are also present in the database. As yet, no *rad26*$^+$-like mammalian sequences have been found. Components of the checkpoint pathway downstream of *rad3*$^+$ and the other *checkpoint rad* genes are probably also conserved, because homologs of the *chk1*$^+$ gene have been identified in higher eukaryotes (Fogarty *et al.*, 1997; Sanchez *et al.*, 1997).

To determine the function of the *checkpoint rad* genes in the checkpoint response, we carried out a genetic and biochemical analysis of *hus1*$^+$. The *hus1*$^+$ gene encodes a 33 kDa protein with no informative sequence motifs (Kostrub *et al.*, 1997). Using a dominant negative allele of *hus1*$^+$, we have demonstrated a genetic interaction between *hus1*$^+$ and another *checkpoint rad* gene, *rad1*$^+$. We have also discovered that Hus1p and Rad1p form a stable complex in both the presence and absence of checkpoint signals, and that the formation of this complex requires a third *checkpoint rad* gene, *rad9*$^+$. Further, Hus1p is phosphorylated in response to DNA damage, and this phosphorylation requires *rad3*$^+$ and all of the other *checkpoint rad* genes, but not the *chk1*$^+$ or the *cds1*$^+$ kinase. Aspects of this response may be conserved in mammals, as we have identified mouse and human genes with significant similarity to *hus1*$^+$.

Results

A dominant negative allele of hus1$^+$

Tagging Hus1p with a c-*myc* epitope at its N-terminus generates a dominant negative protein (see Materials and methods). The *myc*-tagged construct not only fails to rescue the checkpoint defect of *hus1*Δ yeast but also disrupts checkpoint control in wild-type yeast when overexpressed. As shown in Figure 1, expression of Myc–Hus1p does not affect cell growth on normal media but causes wild-type yeast to become hydroxyurea (HU) sensitive (Figure 1A). Myc–Hus1p also causes sensitivity to UV irradiation (Figure 1B) and the cells form 'cuts' when grown in HU (Figure 1C). These phenotypes caused by Myc–Hus1p are qualitatively similar to the phenotypes of the *hus1*Δ strain (Kostrub *et al.*, 1997), suggesting that Myc–Hus1p specifically interferes with the function of wild-type Hus1p. Overexpression of wild-type Hus1p has no effect on either cell growth or checkpoint control (data not shown); therefore, the dominant negative effect caused by Myc–Hus1p is a unique characteristic of the *myc–hus1* allele.

Myc–Hus1p may function as a dominant negative by titrating away components of the checkpoint complex. If this is the case, it should be possible to rescue the dominant negative phenotype by overexpressing other checkpoint proteins. To test this possibility, the *myc–hus1* construct was stably integrated into the genome and this strain, called *OPmyc–hus1* (TE866), was transformed with plasmids overexpressing the *checkpoint rad* proteins Hus1p, Rad1p, Rad3p, Rad9p or Rad17p. As illustrated in Figure 2, overexpression of wild-type Hus1p or Rad1p rescues the HU-sensitive phenotype of *OPmyc–hus1*, while only overexpression of Hus1p rescues the *hus1*Δ strain (data not shown). For the data presented in Figure 2, the *myc–hus1* construct was integrated at the *leu1* chromosomal locus, and the *checkpoint rad* proteins were overexpressed using *ura4*$^+$ plasmids. The experiment was also performed by integrating the *myc–hus1* construct at the *ura4* locus and overexpressing the *checkpoint rad* proteins on *LEU2* plasmids, with identical results (data not shown). Although overexpression of the remaining *checkpoint rad* protein, Rad26p, does not rescue *OPmyc–hus1*, this result is not interpretable because overexpression of Rad26p is toxic and disrupts growth of the cells even on normal media (data not shown). The failure of overexpression of Rad1p to rescue the *hus1*Δ strain suggests that it does not rescue

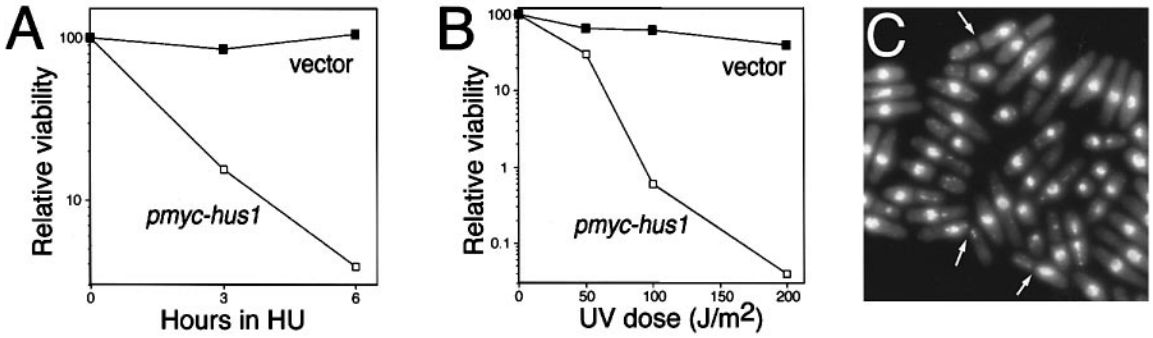

Fig. 1. Myc–Hus1p is a dominant negative protein. HU and UV sensitivity caused by the *myc–hus1* plasmid in wild-type (TE366) cells. See Materials and methods for detailed description of techniques. Graphs show (**A**) viability of strains in liquid media + 10 mM HU, and (**B**) viability of strains after various doses of UV irradiation. (**C**) Fluorescence microscopy of DAPI-stained wild-type yeast transformed with p*myc–hus1* and grown in liquid media with 10 mM HU for 6 h. White arrows indicate examples of septa in 'cut' cells.

Fig. 2. Overexpression of *hus1*$^+$ or *rad1*$^+$ rescues *OPmyc–hus1*. *OPmyc–hus1* (TE866) cells transformed with pREP2 vector, pREP2–*hus1*$^+$, pREP2–*rad1*$^+$, pREP2–*rad3*$^+$, pREP2–*rad9*$^+$ or pREP2–*rad17*$^+$, were grown on EMM plates then replica-plated to EMM plates (–HU) or EMM plates containing 10 mM HU (+HU). The small colonies seen in the vector field are probably revertants which arise after many generations of growth.

Myc–Hus1p by bypassing the requirement for Hus1p. Instead, it is likely to reflect a direct interaction between Myc–Hus1p and Rad1p. Indeed, Rad1p can be detected in *myc*-epitope immunoprecipitates from a strain over-expressing Rad1p and Myc–Hus1p (Figure 3A, lane 1).

Hus1p and Rad1p co-immunoprecipitate

To determine whether the genetic and physical interactions between Rad1p and Myc–Hus1p reflect a physiologically significant interaction, we investigated the physical interaction between Hus1p and Rad1p in wild-type cells using polyclonal antibodies to the proteins. As shown in Figure 3A (lanes 2–7), a specific interaction between Hus1p and Rad1p can be detected at normal expression levels. Rad1p is readily detected in Hus1p immunoprecipitates (Figure 3A, lane 2). Likewise, Hus1p is detected in Rad1p immunoprecipitates (Figure 3A, lane 5). The co-immunoprecipitation observed in wild-type cells appears to be specific, since Rad1p is not found in Hus1p immunoprecipitates from the *hus1*Δ strain (Figure 3A, lane 3), and Hus1p is not found in Rad1p immunoprecipitates from the *rad1*Δ strain (Figure 3A, lane 7). We note that soluble Hus1p levels are substantially decreased in the *rad1*Δ background (Figure 3A, lane 4). Total Hus1p levels are not affected by mutations in the other *checkpoint rad* genes when the proteins are extracted with 1% SDS under denaturing conditions (Kostrub *et al.*, 1997). Therefore, it is likely that the decrease in Hus1p levels observed under non-denaturing conditions is due to a change in the ability of Hus1p to be extracted in the absence of Rad1p, and not to degradation of Hus1p *in vivo*.

We also tested whether activation of the DNA replication checkpoint affects the Hus1p–Rad1p interaction by incubating wild-type cells in 10 mM HU for 3 h prior to performing the co-immunoprecipitation (Figure 3A, lanes 8 and 9). The amount of the two proteins precipitated from HU-treated versus untreated cells is indistinguishable, suggesting that the Hus1p–Rad1p interaction does not change in response to activation of the checkpoint. However, the mobility of Hus1p changes in response to HU (Figure 3A, lane 9), suggesting that Hus1p is post-translationally modified in response to checkpoint activation. This subject is discussed in greater detail below.

To investigate what other proteins may be required for the Hus1p–Rad1p physical interaction, the co-immunoprecipitations were repeated in various cell-cycle mutant backgrounds. We tested deletion strains of all of the *checkpoint rad* genes, as well as deletions of the checkpoint kinases *cds1*$^+$ and *chk1*$^+$ (Figure 3B). Rad3p, Rad17p, Rad26p, Chk1p and Cds1p are not required for the Hus1p–Rad1p interaction (Figure 3B, lanes 2, 4–7, 9 and 11–14). However, in the *rad9*Δ background (Figure 3B, lane 3) Rad1p does not precipitate with Hus1p. In a manner analogous to the *rad1*Δ data (see Figure 3A, lane 4), Hus1p levels are decreased in the *rad9*Δ background (Figure 3B, lane 3). Although Rad1p levels are not significantly affected by the lack of Rad9p (Figure 3B, lane 10), Hus1p does not co-precipitate with Rad1p from

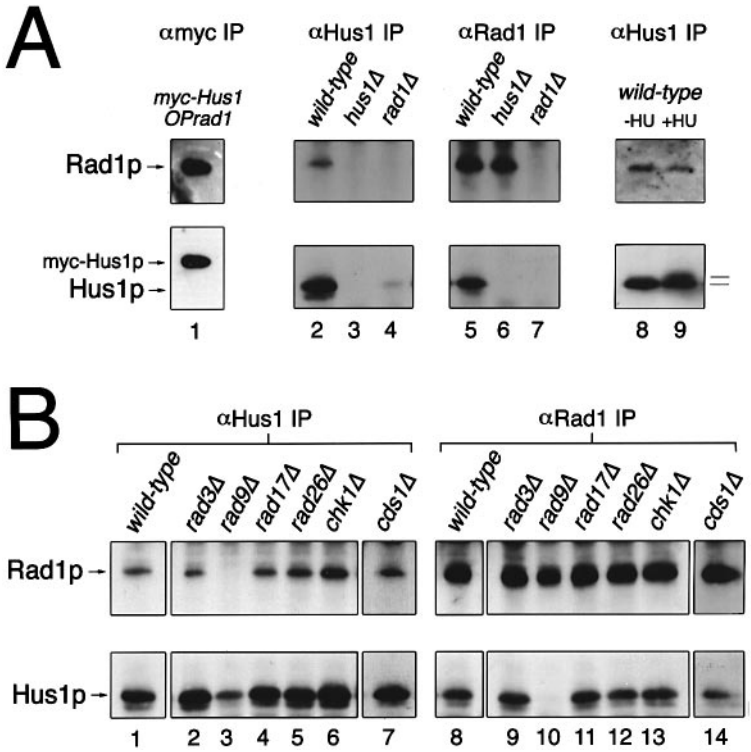

Fig. 3. Hus1p and Rad1p physically interact. Western blots of immunoprecipitations from fission yeast protein extracts probed with anti-Rad1p or anti-Hus1p antibody. (**A**) Lane 1 shows an anti-Myc immunoprecipitation from *OPmyc–hus1* (TE866) transformed with pREP2–*rad1*$^+$ probed with anti-Rad1p (top) or anti-Hus1p (bottom) antibody. Lanes 2–4 show anti-Hus1p immunoprecipitations from wild-type (TE366) yeast and the mutants *hus1::LEU2* (TE484) and *rad1::ura4*$^+$ (TE459) probed with anti-Rad1p (top) or anti-Hus1p (bottom) antibody. Lanes 5–7 show anti-Rad1p immunoprecipitations from the same strains probed in the same manner. Lanes 8 and 9 show anti-Hus1p immunoprecipitations from wild-type (TE366) yeast grown either in liquid media (–HU, lane 8) or liquid media with 10 mM HU for 3 h (+HU, lane 9) probed in the same manner. (**B**) Lanes 1–7 show anti-Hus1p immunoprecipitations from wild-type (TE366) yeast, and the mutants *rad3::ura4*$^+$ (TE570), *rad9::ura4*$^+$ (TE794), *rad17::ura4*$^+$ (TE864), *rad26::ura4*$^+$ (TE257), *chk1::ura4*$^+$ (TE548) and *cds1::ura4*$^+$ (TE700) probed with anti-Rad1p (top) or anti-Hus1p (bottom) antibodies. Lanes 8–14 show anti-Rad1p immunoprecipitations from the same strains probed in the same manner.

*rad9*Δ yeast. These results suggest that Rad9p is required for the stability of the Hus1p–Rad1p complex and raise the possibility that Hus1p, Rad1p and Rad9p form a trimeric complex. It is possible that Rad9p bridges the interaction between Hus1p and Rad1p.

hus1Δ yeast are supersensitive to DNA damage caused by bleomycin

To investigate the potential post-translational modification of Hus1p in response to checkpoint signals, we wished to simultaneously activate the G_2-M checkpoint in cells growing in liquid culture. Activating the checkpoint using HU is not ideal because HU only activates the checkpoint in S-phase cells. Checkpoint activation using UV irradiation poses technical difficulties: the cells must be plated out and dried, causing changes in temperature and nutritional environment, and it is difficult to obtain a large quantity of irradiated cells for biochemical experiments. As an alternative to gamma irradiation, we activated the checkpoint with bleomycin, a radiomimetic drug that causes double-stranded DNA breaks (Suzuki *et al.*, 1970). The effects of phleomycin, a relative of bleomycin, have been investigated in fission yeast (Belenguer *et al.*, 1995); we have extended this analysis to bleomycin.

Various doses of bleomycin were added to early log-phase cultures of both wild-type and *hus1*Δ fission yeast, and the survival of the yeast was tested after various incubation times. At low doses of bleomycin (<0.01 mU/ml) the viability of wild-type and *hus1*Δ yeast is not significantly affected (data not shown), presumably because at these levels the drug does not cause significant DNA damage. High doses of bleomycin (>10 mU/ml) are lethal to both *hus1*Δ and wild-type yeast (data not shown), probably because irreparable levels of DNA damage accumulate. As shown in Figure 4A, at a dose of 5 mU/ml bleomycin the relative viability of wild-type yeast remains unchanged for 3 h and then starts to decrease at later time points as high levels of DNA damage accumulate. In comparison, *hus1*Δ yeast at the same dose lose viability very quickly. The relative optical density of both cultures continues to increase after addition of 5 mU/ml bleomycin (Figure 4A, dashed lines), indicating that cell growth is not inhibited.

To determine whether *hus1*Δ yeast are supersensitive to bleomycin because they lack the DNA damage cell-cycle checkpoint, the bleomycin-treated cells were examined microscopically. As shown in Figure 4B, wild-type cells elongate in bleomycin, suggesting that they undergo cell-cycle arrest. In contrast, *hus1*Δ yeast do not become elongated (Figure 4C) and the culture contains many recently divided cells. Often the DNA in these cells appears to be unevenly distributed between the daughter cells; clear examples of this are indicated by the white arrows in Figure 4C. It is possible that double-strand DNA breaks create pieces of chromosomes that lack centromeres and cannot segregate properly. We conclude that *hus1*Δ yeast are super-

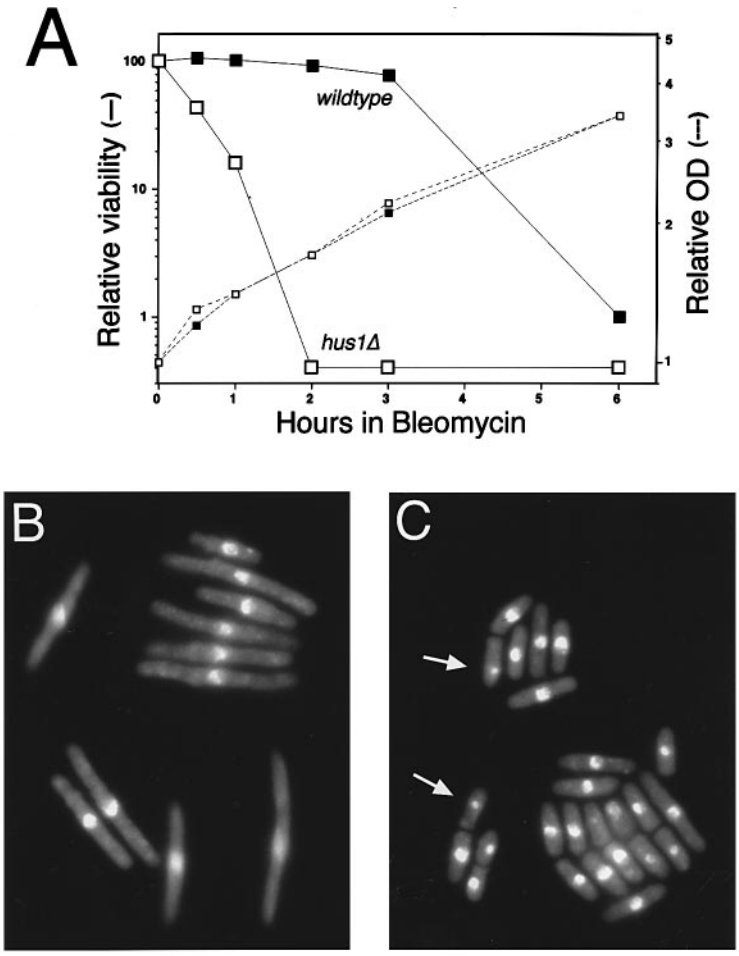

Fig. 4. *hus1*Δ yeast are supersensitive to bleomycin. Sensitivity of wild-type (TE366) and *hus1::LEU2* (TE484) yeast to bleomycin. The graph (**A**) shows viability (solid lines) and relative optical density (dashed lines) of strains in liquid media with 5 mU/ml bleomycin. The bottom panels show fluorescence microscopy of DAPI-stained (**B**) wild-type and (**C**) *hus1::LEU2* yeast grown in liquid media with 5 mU/ml bleomycin for 3 h. White arrows indicate clear examples of uneven distribution of DNA to the daughter cells.

sensitive to bleomycin because they fail to arrest the cell cycle in response to the DNA damage caused by the drug.

Treatment of the other *checkpoint rad* mutants, *rad1*Δ, *rad3*Δ, *rad9*Δ, *rad17*Δ and *rad26*Δ, with bleomycin results in a phenotype similar to *hus1*Δ (data not shown). This phenotype is similar to the cut phenotype seen when checkpoint mutants are exposed to HU, but has significant differences. Exposing *checkpoint rad* mutants to HU results in cut cells with only one nucleus, or one nucleus bisected by the septum. In contrast, exposing the *checkpoint rad* mutants to bleomycin results in septated cells with two nuclei. The different terminal phenotypes probably result because HU-treated cells are arrested in S-phase with unreplicated chromosomes, while log-phase cultures treated with bleomycin contain mostly G_2 cells with replicated chromosomes that can attempt chromosome segregation and nuclear division.

Hus1p is phosphorylated in response to DNA damage

As mentioned in the previous section, the physical interaction between Hus1p and Rad1p is not changed by activation of the DNA replication checkpoint using HU. However, the Hus1p from cells incubated in HU does not run as a sharp band on SDS–PAGE gels (Figure 3A, lane 9). We suspected that Hus1p may be post-translationally modified in response to S-phase inhibition.

Using bleomycin, we investigated whether DNA damage affects either Hus1p, Rad1p or the interaction between them. The amount of Hus1p and Rad1p co-precipitated from bleomycin-treated wild-type cells did not change relative to untreated cells (data not shown), suggesting that the Hus1p–Rad1p complex exists prior to checkpoint activation and is not regulated by induction of the checkpoint signal. However, as displayed in Figure 5A, Hus1p shifts mobility in response to bleomycin (compare lanes 3–7 to lane 1). The mobility shift is observed as early as 0.5 h after addition of bleomycin (lane 3), is maximal between 1 and 2 h (lanes 4 and 5) and remains at this level for at least 6 h (lanes 6 and 7). This mobility shift is due to phosphorylation of Hus1p, because treatment of the shifted form with phosphatase converts Hus1p to the unshifted form (Figure 5A, lane 9). Further, the phosphorylation of Hus1p in response to DNA damage requires an intact Rad3p kinase; the phosphorylation is not observed in *rad3*Δ yeast treated with bleomycin (Figure 5A, lane 10). This suggests that the Rad3p kinase directly or indirectly mediates the phosphorylation of Hus1p in response to DNA damage.

As shown in Figure 5B, analysis of Hus1p phosphoryl-

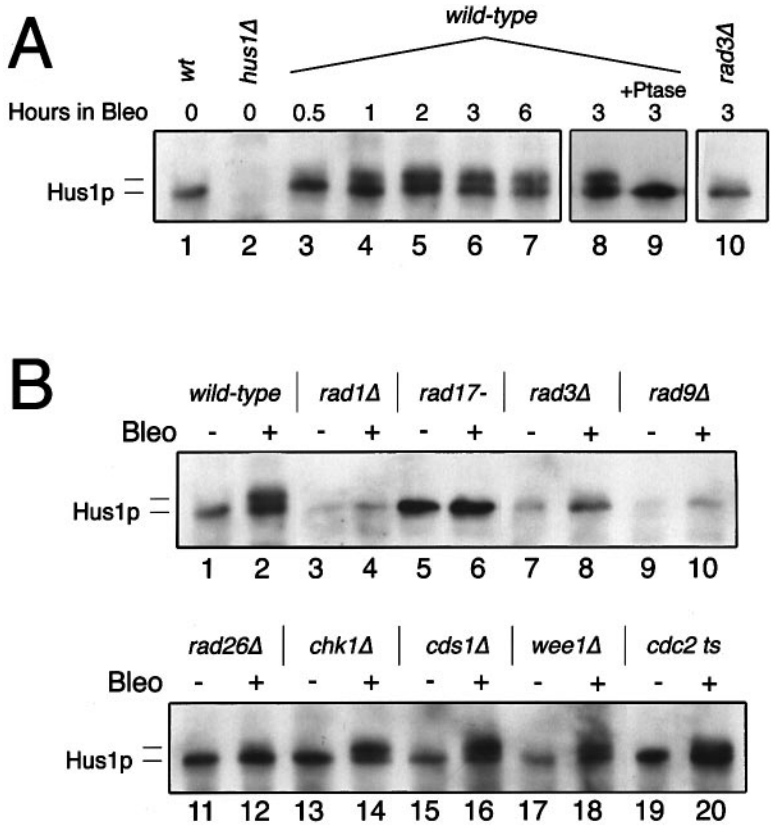

Fig. 5. Hus1p is phosphorylated in response to DNA damage. Western blots of anti-Hus1p immunoprecipitations from fission yeast protein extracts, probed with anti-Hus1p antibody. (**A**) Lanes 1 and 2 are immunoprecipitations from wild-type (TE366) and *hus1::LEU2* (TE484) yeast grown in liquid media. Lanes 3–9 show Hus1p from wild-type yeast grown in liquid media with 5 mU/ml bleomycin for the times indicated. In lane 9, the immunoprecipitated protein was treated with λ-phosphatase prior to Western blotting. Lane 10 shows Hus1p from *rad3::ura4*$^+$ (TE570) yeast grown in liquid media with 5 mU/ml bleomycin for 3 h. (**B**) shows Hus1p from wild-type (TE366) yeast and the mutants *rad1::ura4*$^+$ (TE459), *rad17-h11* (TE18), *rad3::ura4*$^+$ (TE570), *rad9::ura4*$^+$ (TE794), *rad26::ura4*$^+$ (TE257), *chk1::ura4*$^+$ (TE548), *cds1::ura4*$^+$ (TE700), *wee1::ura4*$^+$ (TE391) and *cdc2–33* (TE275) grown either in liquid media (–) or liquid media with 5 mU/ml bleomycin for 3 h (+). In lanes 19 and 20, the yeast were shifted to 36°C starting 3 h prior to addition of bleomycin to inactivate the Cdc2^{ts} protein. In some lanes, the Hus1p levels appear to increase in response to bleomycin treatment, but this is not consistently observed. Also, the decrease in Hus1p levels in the *rad3*Δ is not consistently observed (see Figure 3B).

ation in mutant backgrounds establishes that all of the *checkpoint rad* genes are required for the phosphorylation of Hus1p in response to bleomycin (Figure 5B, lanes 3–12). As discussed above, Hus1p levels are significantly decreased in the *rad1*Δ and *rad9*Δ backgrounds, but the remaining Hus1p appears as a single unshifted band, not as a shifted doublet (Figure 5B, compare lane 2 with lanes 4 and 10). Four other cell-cycle kinases are not required for the phosphorylation of Hus1p; it is still phosphorylated after bleomycin treatment in the *chk1*Δ, *cds1*Δ and *wee1*Δ deletion strains, and in a *cdc2*^{ts} strain shifted to restrictive temperature for 3 h prior to bleomycin addition (Figure 5B, lanes 13–20). These results confirm the previously proposed model that the cell-cycle kinases Cds1p, Chk1p, Wee1p and Cdc2p function downstream of the Rad3p kinase and the other *checkpoint rad* proteins (Walworth and Bernards, 1996). The phosphorylation of Hus1p in the *chk1*Δ strain is particularly significant because these cells fail to arrest the cell cycle in response to bleomycin treatment (data not shown). This establishes that the phosphorylation of Hus1p is not an indirect effect of cell-cycle arrest, but rather is the result of activation of the checkpoint pathway.

Potential human and mouse homologs of the yeast hus1$^+$ *gene*

We have identified genes in the human and mouse genomes with sequence similiarity to the yeast *hus1*$^+$ gene by searching the human and mouse EST databases. The amino acid sequences of the three proteins are aligned in Figure 6. The complete sequences of the human and mouse proteins were assembled from two incomplete but overlapping EST clones corresponding to each gene. The three proteins are almost exactly the same size, and they are similar throughout, suggesting that their general structure has been conserved. The human protein is 30% identical and 57% similar and the mouse protein is 31% identical and 56% similar to Hus1p. Based on a BLAST search, the sequence matches are significant, with $P=1.0\times10^{-21}$ for the human protein and $P=1.4\times10^{-31}$ for the mouse protein. The human and mouse proteins are 86% identical and 93% similar to each other, and they are not similar to any other proteins in the database besides yeast Hus1p, suggesting that they are homologs of each other, and not members of a larger family of human and mouse proteins. To test whether these mammalian proteins can functionally substitute for yeast Hus1p we expressed

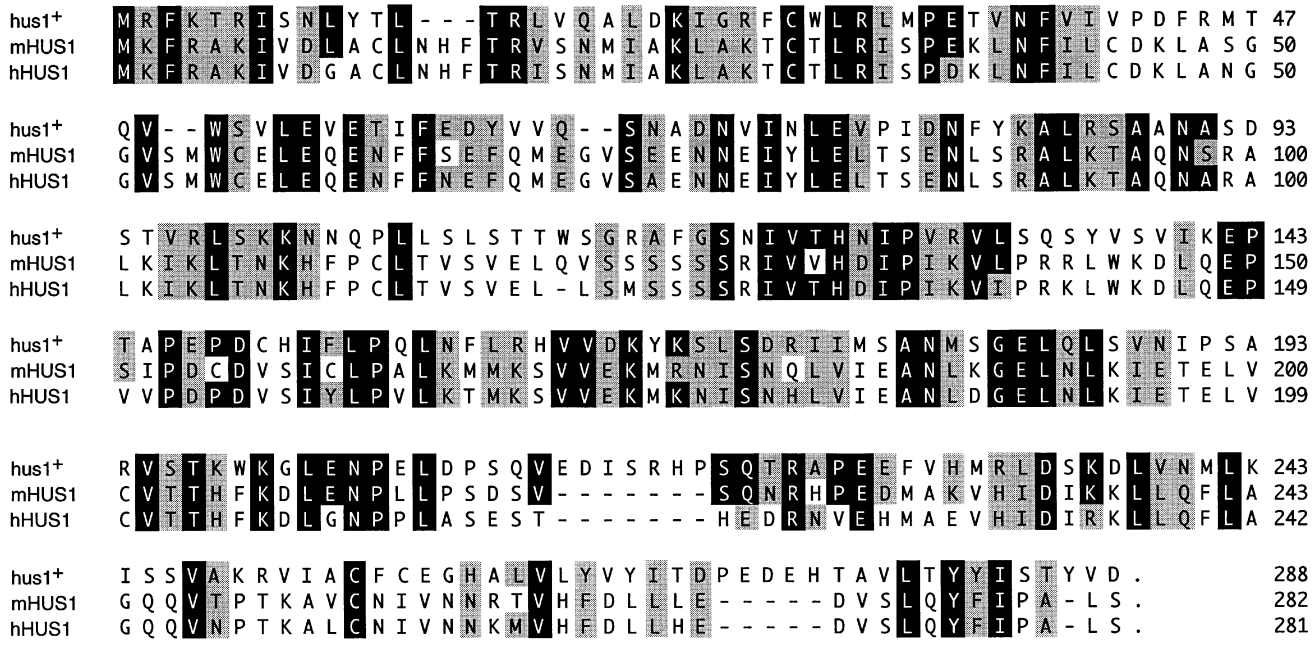

Fig. 6. Amino acid comparison of hus1$^+$ with its human and mouse homologs. Residues identical to yeast *hus1*$^+$ are boxed in black, and conservative differences are shaded in gray. Based on a BLAST database search, the similarities to Hus1p are significant, with $P=1.0\times10^{-21}$ for hHUS1 and $P=1.4\times10^{-31}$ for mHUS1.

the human and mouse proteins in *hus1*Δ yeast, but they were unable to rescue the HU-sensitivity of the cells (data not shown). This result does not necessarily imply that the mammalian genes are not homologs of *hus1*$^+$, because we have previously found that *rad1*$^-$, *rad3*$^-$ and *rad17*$^-$ mutants cannot be rescued by their *S.cerevisiae* homologs, *RAD17*, *MEC1* and *RAD24* (Griffiths *et al.*, 1995; H.Ghazizadeh and T.Enoch, unpublished). It is probable that the related proteins cannot interact properly with all of the other components of the fission yeast checkpoint mechanism.

Discussion

Genetic evidence for an interaction between the checkpoint rad genes hus1$^+$ *and rad1*$^+$

Checkpoint control in fission yeast is likely to require activation of the PIK-R kinase, Rad3p. Five other *checkpoint rad* fission yeast genes, *rad1*$^+$, *rad9*$^+$, *rad17*$^+$, *rad26*$^+$ and *hus1*$^+$, are apparently required for activation of Rad3p (Al-Khodairy and Carr, 1992; Enoch *et al.*, 1992; Rowley *et al.*, 1992). The exact role of these genes is not known, although they probably function with *rad3*$^+$, as either activators or substrates of the Rad3p kinase. It is also unclear whether all the genes function together as a complex or whether subsets have distinct functions.

To explore this problem, we have undertaken a genetic and biochemical analysis of one of these proteins, Hus1p. Hus1p is a 33 kDa protein with no informative sequence motifs (Kostrub *et al.*, 1997). Previous genetic studies have failed to detect specific interactions between *hus1*$^+$ and the other *checkpoint rad* genes. Double mutants display the same phenotypes as single mutants (Murray *et al.*, 1997; C.F.Kostrub, unpublished), overexpression of *hus1*$^+$ does not suppress any of the other *checkpoint rad* mutants and overexpression of the other *checkpoint rad* genes does not suppress *hus1*$^-$ mutants (C.F.Kostrub, unpublished). Here we have been able to detect a specific genetic interaction between *hus1*$^+$ and *rad1*$^+$ using a dominant negative allele of *hus1*$^+$, *myc–hus1*. Overexpression of *myc–hus1* disrupts checkpoint control in wild-type cells and this phenotype can be suppressed by overexpression of either *hus1*$^+$ or *rad1*$^+$ (Figures 1 and 2). To explain these observations, we propose that Hus1p interacts with Rad1p and at least one other protein. The addition of the *myc* tag to Hus1p may disrupt some of these interactions, sequestering Rad1p into non-functional complexes. The defect can be rescued by overexpressing Rad1p, presumably because binding of Rad1p to *myc*–Hus1p becomes saturated and enough Rad1p remains to permit assembly of a normal checkpoint complex. The alternative explanation, that overexpression of Rad1p bypasses the requirement for Hus1p function, is unlikely because overexpression of Rad1p does not suppress *hus1* deletions.

None of the other *checkpoint rad* genes can rescue overexpression of *myc–hus1*, suggesting that they interact with Hus1p with less affinity than Rad1p, although we cannot expect to detect any interaction that is disrupted by the *myc* tag. It may be possible to identify other specific interactions between the *checkpoint rad* genes by extending this analysis to other dominant negative alleles of *hus1*$^+$ and by generating dominant negative alleles of other *checkpoint rad* proteins. As discussed below, the interaction we identified using this approach appears to be biologically relevant since we also detect an interaction between Hus1p and Rad1p using biochemical methods.

Biochemical evidence for a complex consisting of Hus1p, Rad1p and Rad9p

As shown in Figure 3, we have biochemically detected a physical interaction between wild-type Hus1p and Rad1p

at endogenous protein levels. This demonstrates that Hus1p and Rad1p form a complex under normal physiological conditions in wild-type cells. The Hus1p–Rad1p complex is present in log-phase growing cells and the interaction does not appear to change in response to activation of the checkpoint by HU or bleomycin, indicating that Hus1p and Rad1p form a stable complex in the absence of checkpoint stimuli. The Hus1p–Rad1p interaction requires Rad9p, which could mean that Rad9p, Hus1p and Rad1p form a trimeric complex. We also note that we did not detect any genetic evidence for an interaction between Myc–Hus1p and Rad9p. One explanation is that the *myc* tag blocks the Hus1p–Rad9p interaction. Indeed, the inability of Myc–Hus1p to interact with Rad9p may be the cause of its dominant negative effect. We will be able to investigate these possibilities once we obtain antibodies to Rad9p, which are currently being developed.

Because formation of the Hus1p–Rad1p complex does not require the other *checkpoint rad* proteins Rad3p, Rad17p and Rad26p, these proteins may be part of a separate complex or the Hus1p–Rad1p (and possibly Rad9p) complex may interact less stably with them. For example, the Hus1p–Rad1p–(Rad9p) complex may unite with Rad3p, Rad17p and Rad26p upon stimulation of the checkpoint. This is consistent with our finding that Hus1p is phosphorylated in response to checkpoint signals, and that phosphorylation requires all of the other *checkpoint rad* proteins, not only Rad1p and Rad9p (Figure 5B). It is also possible that all the *checkpoint rad* proteins form a large, stable complex, but that Hus1p only contacts Rad1p and Rad9p and therefore the absence of the other proteins does not affect Hus1p–Rad1p interaction. Elucidation of the details of these interactions will be possible as new antibodies become available.

Phosphorylation of Hus1p in response to checkpoint signals

Although the interaction between Hus1p and Rad1p is not affected by checkpoint activation, Hus1p is phosphorylated in response to checkpoint signals (see Figure 5). Phosphorylation requires the Rad3p kinase but no other kinases known to be involved in checkpoint control, including Cds1p, Chk1p, Wee1p and Cdc2p. It therefore seems probable that Hus1p is a substrate of Rad3p, but demonstrating this conclusively will require analysis of purified proteins *in vitro*. Two other potential Rad3p substrates have been identified in fission yeast, the kinases Cds1p and Chk1p (Walworth and Bernards, 1996; Lindsay *et al.*, 1998). Putative substrates of Mec1p and Tel1p, the *S.cerevisiae* PIK-R kinases, have also been identified (Cohen-Fix *et al.*, 1996; Sanchez *et al.*, 1996; Sun *et al.*, 1996; Longhese *et al.*, 1997). Genetic analysis suggests that all of these proteins are downstream of the Rad3p and Mec1p kinases in the checkpoint pathway, as mutation of any of these substrates leads to only a subset of the phenotypes observed when Rad3p or Mec1p is mutated. In contrast, *hus1*$^{-}$ and *rad3*$^{-}$ mutants have indistinguishable phenotypes, which may indicate that they act at the same point in the checkpoint pathway. It is possible that Hus1p is both an activator and a substrate of Rad3p. We do not know the function of Hus1p phosphorylation in response to checkpoint signals at present. It may be required for activation of the checkpoint, regulation of the checkpoint response, or it may simply reflect the proximity of Hus1p to the activated Rad3p kinase. Once the phosphorylation site has been identified this question can be investigated by making appropriate mutants.

We note that the phosphorylation of Hus1p in response to HU versus bleomycin is quantitatively different. This may be because bleomycin activates the checkpoint in all cells simultaneously while HU activates the checkpoint only as the cells become arrested in S-phase. Since we used an asynchronous population of cells for this study, HU did not induce S-phase arrest simultaneously in all the cells in the culture. Alternatively, different complexes could be involved in the S-phase and DNA damage checkpoints. All of the *checkpoint rad* proteins are clearly required for both processes, but they may form two physically distinct complexes, one monitoring S-phase progression and the other monitoring DNA damage. We note that only ~50% of the Hus1p is phosphorylated in response to DNA damage (Figure 5). Unphosphorylated Hus1p may not be in a complex or may be in a complex that does not respond to DNA damage.

Possible significance of checkpoint rad protein interactions

One, although certainly not the only, interpretation of these studies is that Hus1p, Rad1p and Rad9p form a separate complex with a distinct function in checkpoint control. Rad1p is related to the *REC1* gene of *U.maydis* and the *RAD17* gene of *S.cerevisiae*. Purified Rec1p has been shown to be active as an exonuclease *in vitro* (Thelen *et al.*, 1994), and in *S.cerevisiae* loss of *RAD17* can decrease the rate at which single-stranded DNA is generated *in vivo* (Lydall and Weinert, 1995). To explain these observations, it has been suggested that activation of the checkpoint response involves conversion of primary lesions to single-stranded DNA by an exonuclease (Lydall and Weinert, 1995). Interestingly, C-terminal truncations of *REC1* abolish checkpoint control without affecting exonuclease activity (Onel *et al.*, 1995, 1996) indicating that Rec1p exonuclease activity is not sufficient for checkpoint function. It is possible that these truncations abolish the ability of Rec1p to interact with essential accessory proteins. This is consistent with our studies showing that in fission yeast, Rad1p forms a stable complex with Hus1p and possibly Rad9p. The truncation deletes a region that is conserved between Rec1p and Rad1p. It will be interesting to determine what effect equivalent truncations of Rad1p have on the Hus1p–Rad1p–(Rad9p) complex.

Studies of another PIK-R kinase, DNA-PK, provide a precedent for the existence of subcomplexes with distinct functions. The catalytic subunit of this kinase resembles Rad3p in that it is a large protein with a PI-3 kinase-related domain at the C-terminus (Hartley *et al.*, 1995). The catalytic subunit is guided to DNA ends by interactions with two smaller subunits, Ku70 and Ku80 (Gottlieb and Jackson, 1993). The stable association of DNA ends, the Ku proteins and DNA-PK leads to activation of kinase activity. A Ku70–Ku80 complex which is not associated with the catalytic subunit can also be detected (Mimori and Hardin, 1986). It is possible that the *S.pombe* Hus1p–Rad1p–(Rad9p) complex is acting similarly; in the absence of checkpoint signals these proteins may form a separate 'sentinel' complex that scans the genome for lesions.

When such lesions are detected, the complex may process them and then recruit the Rad3p kinase and other *checkpoint rad* proteins to these sites. Although this model is highly speculative, we believe it provides a useful framework for future studies.

Mammalian HUS1 genes

Using the EST database, we have identified mouse and human genes with significant sequence similarity to the fission yeast *hus1*$^+$ gene (Figure 6). The mouse and human genes do not complement the *hus1*Δ strain, possibly because the mammalian proteins cannot interact with other components of the fission yeast checkpoint complex. We have previously found that *rad1*$^-$, *rad3*$^-$ and *rad17*$^-$ mutants cannot be rescued by the related *S.cerevisiae* genes *RAD17*, *MEC1* and *RAD24* (H.Ghazizadeh and T.Enoch, unpublished), so lack of complementation by the mouse gene is not entirely unexpected.

The identification of mammalian *HUS1* genes is remarkable since there is no *S.cerevisiae* gene that is significantly related to *hus1*$^+$. Similarly, there is a human gene related to the fission yeast *rad9*$^+$ gene, and the human and fission yeast genes are much more closely related to each other than they are to the most similar *S.cerevisiae* gene, *DDC1* (Lieberman *et al.*, 1996; Longhese *et al.*, 1997). This is interesting given our evidence for interactions between Hus1p, Rad1p and Rad9p in fission yeast. *Saccharomyces cerevisiae* does have a gene that is closely related to *rad1*$^+$, *RAD17*, and both the *S.cerevisiae* and *S.pombe* genes are approximately equally related to genes in other organisms, including humans and mice. As discussed above, this gene is proposed to encode an exonuclease. Its evolutionary conservation suggests a universally important role for such an activity in checkpoint activation. However, the function of this activity could be modified in different organisms by association with different regulatory subunits. It is also interesting to note that the *S.cerevisiae RAD17* gene is only required for the DNA damage checkpoint (Weinert *et al.*, 1994), while *rad1*$^+$ and the *U.maydis REC1* gene are required for both the DNA damage and S-phase checkpoints (Rowley *et al.*, 1992; Onel *et al.*, 1996). Perhaps the function of the Hus1p and Rad9p proteins is to direct the Rad1p exonuclease to lesions at either replication forks or sites of DNA damage. In this regard, it would be interesting to know whether *U.maydis* has genes related to either *hus1*$^+$ or *rad9*$^+$. Whatever the function, the existence of Hus1p- and Rad9p-related proteins in mammalian cells suggests that certain aspects of the checkpoint response may be conserved between fission yeast and humans. Our results suggest that after checkpoint activation, Hus1p is phosphorylated by the Rad3p kinase. Mammalian cells have two kinases that are related to Rad3p, Atmp and Atrp (see Introduction; Savitsky *et al.*, 1995; Keegan *et al.*, 1996). It should soon be possible to determine whether the mammalian Hus1 proteins are substrates of either or both of these kinases.

Large scale sequencing projects are currently identifying novel mammalian genes at a remarkable rate. One popular approach to determining the function of these genes is to search the completely sequenced *S.cerevisiae* genome for a related gene of known function. Our results show that non-redundant information can be obtained by extending this analysis to other simple eukaryotes such as fission yeast, illustrating the value of studying eukaryotic cell biology using multiple model organisms.

Materials and methods

Schizosaccharomyces pombe physiological methods

The strains and plasmids used in this study are listed in Table I. Strains were grown under standard conditions (Moreno *et al.*, 1991) and yeast cells were transformed by the overnight lithium acetate procedure (Elble, 1992). To assess the HU-sensitive phenotype on plates, cells were replica-plated to minimal or yeast extract plates containing 10 mM HU and 5 mg/ml phloxine B (PB). Under these conditions, HU-sensitive cells fail to form colonies while wild-type (HU resistant) cells form colonies of elongated cells after 48 h.

To examine the HU- and bleomycin-sensitive phenotypes quantitatively, cells were grown in EMM (Edinburgh minimal medium; Bio101) liquid culture with appropriate supplements to early log phase (1×10^6 cells/ml). HU sensitivity was measured by adding HU to a concentration of 10 mM and testing the viability of the cells after indicated times. Bleomycin sensitivity was measured by adding bleomycin sulfate (Sigma) to 5 mU/ml, and testing the viability of the cells after indicated times. Bleomycin sulfate was prepared as a 3 U/ml stock solution in water. To determine viability, aliquots of cells were taken from the culture, diluted into fresh media, sonicated briefly to disrupt clumps and plated onto EMM solid media. Colonies were counted after a 3–5 day incubation. To observe the cut phenotype, cells from liquid cultures were fixed in methanol, stained with 4,6-diamidino-2-phenylindole (DAPI), and examined by fluorescence microscopy. To assess sensitivity to UV radiation, cells were grown to early log phase in liquid culture and aliquots were plated as described above. After the plates had dried, they were irradiated with the indicated doses of UV light using a Stratalinker 2400 (Stratagene).

Constructing plasmids and strains

To construct p*myc–hus1*$^+$ (pTE554), the *Nde*I–*Bam*HI fragment encoding the *hus1*$^+$ open reading frame (ORF) (Kostrub *et al.*, 1997) was cloned into the pREP42 vector, modified to contain two copies of the c-*myc* tag upstream of the initiator methionine (adapted from Griffiths *et al.*, 1995). This construct adds the sequence MGSSHHHHHHAEEQKLISEEDLSMAEEQKLISEEDLH to the N-terminus of Hus1p. To construct the strain *leu1::pJK148-nmt′-myc–hus1*$^+$ (TE866), the *Pst*I–*Sac*I fragment from p*myc–hus1*$^+$, which contains *myc–hus1*$^+$ plus the *nmt′* promoter and terminator, was cloned into the pJK148 vector for integration at *leu1* (Keeney and Boeke, 1994), creating the plasmid pTE560. To integrate this plasmid, it was linearized with *Nru*I, and transformed by electroporation (Prentice, 1992) into strain TE236. To create pREP2–*hus1*$^+$ (TE574), the *Nde*I–*Bam*HI fragment encoding the *hus1*$^+$ ORF (Kostrub *et al.*, 1997) was cloned into the pREP2 vector. The pREP2–*rad9*$^+$ (TE575) construct was created in two steps. First, the C-terminal *Nde*I–*Bam*HI fragment from pREP41–*rad9*$^+$ (gift from A.Carr) was cloned into the pREP2 vector. Then, the N-terminal *Nde*I–*Nde*I fragment of *rad9*$^+$ was cloned into the unique *Nde*I site. To create pREP2–*rad3*$^+$ (pTE569) and pREP2–*rad17*$^+$ (pTE570), the *Nde*I–*Sac*I fragments from pREP1–*rad3*$^+$ and pREP41–*rad17*$^+$ (gifts from A.Carr), respectively, were cloned into pREP2. pREP2–*rad1*$^+$ (TE172) was a gift from A.Carr.

Construction of human and mouse HUS1

Full length mouse *HUS1* (*mHUS1*) was constructed using the EST clones 604141 (pTE623) and 658922 (pTE625; IMAGE consortium). Nucleotides 1–780 of clone 604141 encode the 5′ portion of the *mHUS1* mRNA. The first 109 nucleotides of clone 658922 overlap with the last 109 nucleotides (nt 672–780) of 604141, and nucleotides 110–496 of 658922 encode the remaining 3′ portion of the *mHUS1* message. The amino acid sequence in Figure 6 is a compilation of these sequences. To express *mHUS1* in yeast, the *mHUS1* ORF was PCR-amplified as a *Nde*I–*Bam*HI fragment, using the primers HIM-5N (5′-CCATATGAAGTTTCGCGCCAAGATCGTG) and HIM-E (5′-TATGTCAATGTGCACCTTGG) for clone 604141 and the primers HIM-F (5′-CCAAGGTGCACATTGACATA) and HIM-3B (5′-AGGATCCTAGGACAAGGCTGGGATGAAATA) for clone 658922. The two resulting products were combined by crossover-PCR (the HIM-E and HIM-F primers overlap). The resulting PCR product was cloned using the TA Cloning Vector (Invitrogen) and this *Nde*I–*Bam*HI fragment encoding the *mHUS1* ORF was cloned into pREP42 for expression in yeast. The

Table I. *Schizosaccharomyces pombe* strains and plasmids

Strain	Genotype	Origin of strain
TE18	*rad17-h11 ade6–704 ura4-D18 leu1–32 h*$^-$	Enoch *et al.* (1992)
TE236	*leu1–32 ura4-D18 h*$^-$	this paper
TE257	*rad26::ura4*$^+$ *ade6–704 leu1–32 ura4-D18 h*$^-$	Al-Khodairy *et al.* (1994)
TE275	*cdc2–33 h*$^-$	Nurse *et al.* (1976)
TE366	*ura4-D18 h*$^-$	Grimm *et al.* (1988)
TE391	*wee1::ura4*$^+$ *leu1–32 ura4-D18 h*$^-$	Russell and Nurse (1987)
TE459	*rad1::ura4*$^+$ *leu1–32 his*$^-$	Sunnerhagen *et al.* (1990)
TE484	*hus1::LEU2 leu1–32 ura4-D18 h*$^-$	Kostrub *et al.* (1997)
TE548	*chk1::ura4*$^+$ *ura4-D18 h*$^-$	Al-Khodairy *et al.* (1994)
TE570	*rad3::ura4*$^+$ *ade6–704 leu1–32 ura4-D18 h*$^-$	Bentley *et al.* (1996)
TE700	*cds1::ura4*$^+$ *leu1–32 ura4-D18 h*$^-$	Murakami and Okayama (1995)
TE794	*rad9::ura4*$^+$ *ade6–704 leu1–32 ura4-D18 h*$^-$	Murray *et al.* (1991)
TE864	*rad17::ura4*$^+$ *ade6–704 leu1–32 ura4-D18 h*$^-$	Griffiths *et al.* (1995)
TE866	*leu1::pJK148-nmt'-myc–hus1*$^+$ *ura4-D18 h*$^-$	this paper
Plasmid	**Construct**	**Origin of plasmid**
pTE554	pREP42[a]–*myc–hus1*$^+$ this paper	
pTE560	pJK148[b]–*nmt'-myc–hus1*$^+$	this paper
pTE574	pREP2[c]–*hus1*$^+$	this paper
pTE172	pREP2–*rad1*$^+$	gift of A.Carr
pTE569	pREP2–*rad3*$^+$	this paper
pTE575	pREP2–*rad9*$^+$	this paper
pTE570	pREP2–*rad17*$^+$	this paper
pTE623	EST #604141	IMAGE consortium
pTE625	EST #658922	IMAGE consortium
pTE624	EST #711713	IMAGE consortium
pTE622	EST #F1-1279D	gift of S.S.Choi
pTE628	pREP42–*mHUS1*	this paper
pTE629	pREP42–*hHUS1*	this paper

[a]Basi *et al.* (1993); [b]Keeny and Boeke (1994); [c]Barbet *et al.* (1992).

resulting pREP42-*mHUS1* construct (pTE628) was sequenced to ensure that no mutations had been introduced during the PCR steps.

Full length human *HUS1* (*hHUS1*) was constructed using the EST clones F1-1279D (pTE622; gift from S.S.Choi) and 711713 (pTE624; IMAGE consortium). Nucleotides 1–575 of clone 711713 encode the 5′ portion of the *hHUS1* mRNA. The first 325 nucleotides of clone F1-1279D overlap with the last 325 nucleotides (nt 250–575) of 711713, and nucleotides 326–800 of 658922 encode the remaining 3′ portion of the *hHUS1* message. The amino acid sequence in Figure 6 is a compilation of these sequences. To express *hHUS1* in yeast, the *hHUS1* ORF was PCR-amplified using the primers HIH-5N (5′-GCATATGAA-GTTTCGGGCCAAGATCGTG) and HIH-E (5′-ACCGGTTCTTGTAA-GTCCTTCCAC) for clone F1-1279D and the primers HIH-3B (5′-AGG- ATCCTAGGACAGCGCAGGGATGAAATA) and HIH-F (5′-GGTGTCTCTGCAGAAAACAATGAG) for clone 711713. The two PCR products were cloned using the TA Cloning Vector (Invitrogen) and then subcloned into pREP42 as *Nde*I–*Pst*I and *Pst*I–*Bam*HI fragments using a three-way ligation. The resulting pREP42–*hHUS1* construct (pTE629) was sequenced to ensure that no mutations had been introduced during the PCR steps.

Protein extracts and immunoprecipitations

Antibodies were raised against Rad1p using a β-galactosidase–Rad1p fusion protein. The fusion protein was purified from bacteria and injected into rabbits. Production of the Hus1p antibody is described in Kostrub *et al.* (1997).

Yeast protein extracts for immunoprecipitations were prepared from early log phase cultures. The entire procedure was carried out at 4°C to minimize protease and phosphatase activity. 1×10^9 cells were collected by centrifugation, and washed in 1 ml IPB (25 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, 0.1% NP-40, 0.5 mM DTT, 15 mM $MgCl_2$, 15 mM EDTA, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 1 mM Na-orthovanadate, 0.1 mM NaF). 40 μl of IPB and 3 ml glass beads (Sigma) were added to the cells, the slurry was vortexed for 4 min and cell lysis was confirmed using phase contrast microscopy. After lysis, another 800 μl IPB were added and the samples were centrifuged to pellet cell debris. For co-immunoprecipitation experiments, this 800 μl sample was then separated into two 400 μl samples, one for anti-Hus1 immunoprecipitation and one for anti-Rad1 immunoprecipitation. For each immunoprecipitation, 20 μl Sepharose-A beads (Sigma) were pre-incubated with 10 μl polyclonal serum in 100 μl IPB for 30 min, then washed with 100 μl IPB. These beads were then incubated with the protein extract for 1 h, and washed 3 times with 500 μl IPB.

For phosphatase treatment, the immunoprecipitates were washed 3 times with 500 μl IPB minus phosphatase inhibitors (25 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, 0.1% NP-40, 0.5 mM DTT, 15 mM $MgCl_2$, 15 mM EDTA, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin), and treated with 800 U λ-phosphatase for 1 h at 37°C as recommended by the manufacturer (NEB).

For Western blot analysis, the proteins were separated by SDS–PAGE, and transferred to nitrocellulose using semi-dry electrotransfer. Buffer B (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Carnation Dry Milk, 1% BSA) was used for blocking the membrane and diluting antibodies. Immunodetection was accomplished using horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody and the ECL chemiluminescence method (Amersham).

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