Isolation and characterization of the *Schizosaccharomyces pombe* *rhp9* gene: a gene required for the DNA damage checkpoint but not the replication checkpoint

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ABSTRACT

Checkpoint controls exist in eukaryotic cells to ensure that cells do not enter mitosis in the presence of DNA damage or unreplicated chromosomes. In *Schizosaccharomyces pombe* many of the checkpoint genes analysed to date are required for both the DNA damage and the replication checkpoints, an exception being *chk1*. We report here on the characterization of nine new methylmethane sulphonate (MMS)-sensitive *S.pombe* mutants, one of which is defective in the DNA damage checkpoint but not the replication checkpoint. We have cloned and sequenced the corresponding gene. The predicted protein is most similar to the *Saccharomyces cerevisiae* Rad9 protein, having 46% similarity and 26% identity. The *S.pombe* protein, which we have named Rhp9 (Rad9 homologue in *S.pombe*) on the basis of structural and phenotypic similarity, also contains motifs present in BRCA1 and 53BP1. Deletion of the gene is not lethal and results in a DNA damage checkpoint defect. Epistasis analysis with other *S.pombe* checkpoint mutants indicates that *rhp9* acts in a process involving the checkpoint *rad* genes and that the *rhp9* mutant is phenotypically very similar to *chk1*.

INTRODUCTION

Multiple checkpoints exist in cells to prevent mitosis from occurring in the presence of DNA damage or incomplete DNA replication (1–6). DNA damage (radiation) checkpoints act both at G₁/S and G₂/M to prevent entry of cells into S phase and mitosis respectively (e.g. 2,7), while the S/M checkpoint prevents mitosis in the presence of unreplicated DNA (see for example 8). In *Saccharomyces cerevisiae*, G₂ arrest in the presence of DNA damage requires the *RAD9*, *RAD17*, *RAD24*, *MEC1/ESR1*, *RAD53* and *MEC3* genes (9,10). In addition, the *RAD9*, *RAD24* and *RAD53* genes are also required for S phase delay following irradiation of cells in G₁ (7,11,12). Two of the genes, *MEC1* and *RAD53*, are also involved in a separate checkpoint which is required for the prevention of entry into mitosis in the presence of incomplete DNA replication (10,11).

In *Schizosaccharomyces pombe* the DNA damage checkpoint requires the functions of at least seven genes, namely *rad1*, *rad3*,

rad9, *rad17*, *rad26*, *hus1* and *chk1* (3,5,6,13). All except *chk1* are also required for the S/M checkpoint, where entry into mitosis is dependent on completion of DNA replication (6,13).

A number of the *S.cerevisiae* and *S.pombe* checkpoint genes are related in sequence, e.g. *RAD17* and *rad1* (14), *RAD24* and *rad17* (15) and *MEC1* and *rad3* (16). The proteins encoded by these genes have been proposed to act as a guardian complex (15), however, the precise role of the gene products in the checkpoint process remains obscure. In addition to their checkpoint roles, the *S.cerevisiae* *RAD17* and *RAD24* gene products have been proposed to act in damage processing in *cdc13*-arrested cells, with the *RAD9* gene product having a regulatory role (14).

Here we report on the isolation of nine methylmethane sulphonate (MMS)-sensitive *S.pombe* mutants, six of which have an altered response to the DNA replication inhibitor hydroxyurea. In particular, we describe the further characterization of one new mutant, *rhp9-64*, which is defective in the DNA damage checkpoint. We have cloned and sequenced the *rhp9* gene and show that the gene product has 26% identity and 46% similarity to the *S.cerevisiae* Rad9 protein. The Rhp9 protein contains a domain present in the human and mouse BRCA1 proteins and 53BP1, a p53 binding protein. The *rhp9* null allele displays a slightly increased response to both UV and ionizing radiation compared with the original allele, but is less sensitive to both types of radiation than a checkpoint *rad* null allele. The *rhp9* gene has a DNA damage checkpoint role but is not required for the S/M checkpoint. Epistasis analysis indicates that *rhp9* functions in a process involving the checkpoint *rad* genes and, specifically, the *chk1* gene. Double mutant analysis also indicates that *rhp9* is likely to be required for an additional process independent of the arrest phenotype, possibly suggesting a damage processing role related to that reported for *S.cerevisiae* *RAD9*.

MATERIALS AND METHODS

Plasmids, strains, growth conditions and general molecular biology techniques

The *S.pombe* plasmid pUR19 and the genomic library used in this study have been described elsewhere (17). The *S.pombe* *rad3*-containing plasmid (pSUB41) was a gift from S.Subramani (18). *Schizosaccharomyces pombe* strains used are shown in Table 1. Procedures and media used for routine growth and

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maintenance of *S.pombe* strains and general *S.pombe* genetic techniques were as described in our previous work (19). General molecular biology techniques were as described by Sambrook *et al.* (20). MMS was used at a concentration of 50 μ l/l (21).

Table 1. *Schizosaccharomyces pombe* strains used

Strain	Genotype
sp.011	<i>ade6-704, leu1-32, ura4-D18, h⁻</i>
sp.012	<i>ade6-704, leu1-32, ura4-D18, h⁺</i>
sp.096	<i>rad9::ura4, ade6-704, leu1-32, h⁻</i>
sp.218	<i>rad2::ura4, ade6-704, leu1-32, h⁻</i>
sp.222	<i>rad13::ura4, ade6-704, leu1-32, h⁻</i>
sp.310	<i>rad17::ura4, ade6-704, leu1-32, h⁻</i>
sp.304	<i>cdc10-V50, ura4-D18, h⁺</i>
sp.374	<i>chk1::ura4, h⁺</i>
sp.378	<i>rad1::ura4, leu1-32, ade6-704, h⁻</i>
sp.379	<i>rad3::ura4, leu1-32, ade6-704, h⁻</i>
sp.380	<i>hus1::leu1, h⁺</i>
sp.381	<i>rad26::ura4, ade6-704, leu1-32, h⁺</i>
sp.382	<i>cdc22-M45, ade6-704, ura4-D18, leu1-32, h⁺</i>
sp.383	<i>rad26-T12, h⁺</i>
sp.417	<i>hus1::leu1, h⁻</i>
Strains created during this study	
sp.346	<i>mms1, ade6-704, leu1-32, ura4-D18, h⁺</i>
sp.347	<i>mms8, ade6-704, leu1-32, ura4-D18, h⁺</i>
sp.348	<i>mms35, ade6-704, leu1-32, ura4-D18, h⁻</i>
sp.349	<i>mms38, ade6-704, leu1-32, ura4-D18, h⁻</i>
sp.350	<i>mms52, ade6-704, leu1-32, ura4-D18, h⁺</i>
sp.351	<i>mms57, ade6-704, leu1-32, ura4-D18, h⁻</i>
sp.352	<i>mms60, ade6-704, leu1-32, ura4-D18, h⁻</i>
sp.353	<i>rhp9-64, ade6-704, leu1-32, ura4-D18, h⁻</i>
sp.354	<i>mms70, ade6-704, leu1-32, ura4-D18, h⁻</i>
sp.391	<i>rhp9::ura4, ade6-704, leu1-32, h⁺</i>
sp.392	<i>rhp9::ura4, rad13::ura4, ade6-704, leu1-32, h⁺</i>
sp.394	<i>rhp9::ura4, hus1::leu1, h⁻</i>
sp.395	<i>rhp9::ura4, chk1::ura4, h⁻</i>
sp.397	<i>rhp9::ura4, rad2::ura4, ade6-704, leu1-32, h⁺</i>
sp.398	<i>rhp9::ura4, rad26-T12, h⁺</i>
sp.408	<i>rhp9::ura4, rad26::ura4, ade6-704, leu1-32, h⁺</i>
sp.409	<i>rhp9::ura4, rad3::ura4, ade6-704, leu1-32, h⁺</i>
sp.411	<i>rhp9::ura4, cdc10-V50, h⁻</i>
sp.412	<i>rhp9::ura4, cdc17, h⁺</i>
sp.413	<i>rhp9::ura4, cdc22-M45, h⁻</i>
sp.419	<i>chk1::ura4, hus1::leu1, h⁺</i>

Mutagenesis

10^7 cells (sp.011) were washed once in water and once in 0.1 M sodium citrate, pH 5.5, and then resuspended in 1 ml 0.1 M sodium citrate. An aliquot of 110 μ l 2 mg/ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanine (MNNG) was added and the cells were incubated at 30°C for 30 min. The cells were then diluted and washed twice in 0.1 M sodium citrate and twice in YES medium. Mutagenized cells were resuspended in 5 ml YES plus 1 ml glycerol and incubated at 30°C for 20 h.

Analysis of checkpoint functions

Cultures (50 ml) of exponentially growing cells were grown overnight to a cell density of $\sim 5 \times 10^6$ /ml. The cultures were split into three aliquots of 10 ml and treated with either 400 Gy irradiation or 20 mM hydroxyurea (HU), with the third aliquot acting as control. Samples of 100 μ l were taken at 30 min intervals into 1 ml methanol. Cells were then scored for per cent septa and 'cut' phenotype following staining with DAPI and calcofluor (22). Synchronous cultures were prepared on 7.5–30% lactose gradients as described by Carr and Murray (23). Cells were treated with a range of doses of ionizing radiation using a Gammacell 1000 ^{137}Cs source (12 Gy/min) as described by Al-Khodairy *et al.* (24).

RESULTS

Mutant isolation

In order to identify new recombination or checkpoint mutants we initiated a search for novel *S.pombe* mutants sensitive to the alkylating agent MMS. Sp.011 *S.pombe* cells (*rad⁺ mms⁺*) were mutagenized using MNNG to produce a killing rate of 98%. Approximately 10 000 independent colonies were either picked or replica plated to YES plates containing MMS (50 μ l/l). Following this selection, 120 colonies were rescreened on MMS-containing medium, resulting in nine MMS-sensitive mutants. These were back-crossed at least three times with the *rad⁺ mms⁺* strain sp.011 or sp.012.

Previous screens for *S.pombe* checkpoint mutants have resulted in the isolation of a disproportionately large number of alleles of *rad3* (see for example 6). To eliminate any *rad3* alleles from our screen, all the MMS-sensitive mutants were transformed with pSUB41, a *rad3*-containing plasmid (18). Of the nine mutants, the radiation sensitivity of one mutant (*mms70*) was complemented fully by pSUB41 (data not shown), indicating that this mutant is likely to be an allele of *rad3*.

Sensitivity of mutants to radiation and hydroxyurea

The *mms* mutants (excluding *mms70*) were screened for the presence of the radiation checkpoint by DAPI staining of mutant cells following exposure to 500 Gy ionizing radiation. Three mutants, *mms8*, *mms38* and *mms64*, displayed aberrant nuclear morphologies (Fig. 1). *mms64* cells appeared defective in the radiation checkpoint, i.e. they did not elongate following irradiation and displayed a 'cut' phenotype. *mms8* and *mms38* cells were more heterogeneous in appearance, with elongated cells containing fragmented nuclei as well as a small percentage of cells with a 'cut' phenotype.

To analyse the S/M checkpoint in the mutants, cells were plated on 6 mM HU. The mutants fell into three classes (summarized in Table 2). Wild-type cells, *mms52*, *mms57* and *mms60* are not sensitive to HU. *mms38* and *mms70* strains do not grow in the presence of 6 mM HU, while the remaining mutants grow in the presence of 6 mM HU but only form microcolonies. These results suggest that *mms38* and *mms70* are defective in the replication checkpoint and this is consistent with the complementation of *mms70* by pSUB1 described above, which suggests that *mms70* is defective in *rad3*. The formation of microcolonies by *mms1*, *mms8*, *mms35* and *mms64* implies that these mutants are not defective in the replication checkpoint but are altered in some other aspect of the response to S phase arrest.

Figure 1. Phenotype of exponentially growing cultures of selected MMS-sensitive mutants in response to ionizing radiation. Cells were exposed to 500 Gy ionizing radiation and incubated for a further 4 h at 30°C before fixing. *sp.011*, *rad*⁺ *mms*⁺; *mms64*, *rhp9-64*.

Figure 2. Radiation sensitivities of MMS-sensitive mutants and complementation data for *rhp9-64*. (a) Response of selected *mms* mutants to UV, (b) response of selected *mms* mutants to ionizing radiation and (c) complementation of the UV sensitivity of *rhp9-64* by pGa7. *sp.012*, *rad*⁺ *mms*⁺; pUR, pUR19 vector control.

The sensitivities of the *mms8*, *mms38* and *mms64* mutant strains to both UV and γ radiation were examined (Fig. 2a and b). All three mutants were sensitive to both types of radiation, with *mms38* being the most sensitive.

To determine whether any of the mutants showing altered response to HU are defective in the same gene, the *mms1*, *mms8*, *mms35*, *mms38* and *mms64* mutants were crossed with each other. The mutants fell into four complementation groups, with *mms1*

Figure 3. (A) Molecular analysis of the *rhp9* gene. (a) Restriction map of the *rhp9* gene region. B, *Bgl*II; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sal*I; V, *Eco*RV. (b) Complementation analysis of subclones of the *rhp9* gene region. (c) Position of the *rhp9* ORF. (d) The gene disruption construct. The *ura4* gene replaced the 1.4 kb *Hind*III-*Eco*RV fragment. (B) Alignment of the Rhp9 and Rad9 proteins. Upper sequence Rhp9, lower sequence Rad9. (C) Comparison of Rhp9 motifs with the BRCT domains. (D) Dendrogram showing relatedness of Rhp9 to other proteins. HS-0170, KIAA-0170; HS-BRCA1, human BRCA1; MM-BRCA1, mouse BRCA1 (32); SC-RAD9, *S. cerevisiae* Rad9; SP-07, SPAC19G10_7/SCHP0.

and *mms35* being allelic to one another. Having eliminated any *rad3* alleles by testing for complementation with pSUB1, *mms8*, *mms35* and *mms38* were checked for allelism with the other checkpoint *rad* mutants. The three mutants were crossed with *rad1*, *rad9*, *rad17*, *rad26* and *hus1* (5,6,15,25-27), with the result that none of the three mutants is defective in any of these checkpoint *rad* genes. Further characterization of *mms64* is described below.

A homologue of *S. cerevisiae* RAD9 complements the *mms64* mutation

An *S. pombe* genomic library (17) was used to transform the *mms64* strain, sp.353, to uracil prototrophy. A total of 30 000 *ura*⁺ transformants were pooled and subjected to three rounds of increasing doses of UV irradiation as previously described (19). Individual colonies were tested for co-instability of the *rad*⁺ and

Figure 4. Analysis of the radiation and replication checkpoint in *rhp9-d* cells. (a) Ionizing radiation checkpoint analysis and (b) S phase feedback control analysis. Synchronous cultures of wild-type (wt), *rhp9-64* and *rhp9-d* cells were exposed to (A) 0, 100 or 250 Gy ionizing radiation or (B) 10 mM hydroxyurea. Cells were assayed at intervals for per cent cells passing mitosis (A) or per cent septated cells (B).

ura⁺ phenotypes. Plasmid pGa7 (Fig. 3A) was isolated from a radiation-resistant colony and on retransformation into sp.353 was shown to complement the radiation-sensitive phenotype of *mms64* (Fig. 2c). Analysis of a stable integrant of pGa7 in *mms64* indicated that the plasmid integrated at the *mms64* locus.

Table 2. Summary of phenotypes of MMS-sensitive mutants

Strain	UV sensitivity	γ sensitivity	HU sensitivity
<i>mms1</i>	+	+	m
<i>mms8</i>	+++	+++	m
<i>mms35</i>	++	++	m
<i>mms38</i>	++	+++	+
<i>mms52</i>	+++	+	–
<i>mms57</i>	++	++	–
<i>mms60</i>	+	–	–
<i>mms64 (rhp9-64)</i>	++	+	m
<i>mms70</i>	++++	+++	+
sp.011 (wt)	–	–	–

HU sensitivity was assessed by plating 10³ cells on YES plates in the presence and absence of 6 mM HU. –, equal growth on +HU and –HU; +, no growth on HU; m, equal number of colonies on +HU and –HU but strains formed micro-colonies on HU.

The sequence of the 4.5 kb *Pst*I–*Bgl*III fragment of pGa7 was determined in both directions using the methods of Henikoff (28) and Morgan *et al.* (29). The sequence has DDBJ/EMBL/GenBank accession no. Y09431. Sequence analysis revealed an open reading frame (ORF) of 778 amino acids. Computer analyses indicate substantial homology between the ORF and the *S.cerevisiae* Rad9 gene product (30), with 26% identity and 46% similarity (Fig. 3B and Table 3). We have therefore renamed the gene which complements the *mms64* mutation *rhp9* (for *rad9* homologue in *S.pombe*) and the original *mms64* mutant strain *rhp9-64*. The regions of Rhp9 with the greatest homology to Rad9 are located in the C-terminal 350 and the N-terminal 260 amino acids.

The predicted Rhp9 protein also has homology to another open reading frame in *S.pombe* (SPAC19G10_7/SCHPO, DDBJ/EMBL/GenBank accession no. Z69909) and contains sequences which are very similar to the recently identified BRCT domains, present in human and mouse BRCA1 proteins, 53BP1 (a p53 binding protein) and another human ORF (KIAA0170/HUMAN, DDBJ/EMBL/GenBank accession no. D79992) (31) as shown in Figure 3C. Comparison of the overall levels of homology between these five proteins indicates that Rhp9 is most similar to *S.cerevisiae* Rad9 (Table 3 and Fig. 3D). Interestingly, Rhp9 is more similar to Rad9 than it is to the *S.pombe* ORF.

Gene deletion

A 2 kb region of the *rhp9* coding sequence (from the 5' *Eco*RV site to the 3' *Hind*III site within the *rhp9* coding sequence) was replaced by the *S.pombe ura4* gene (33). A disruption fragment

of 3.6 kb was excised by digestion with *Eco*RI (see Fig. 3A) and transformed into the haploid strain sp.012. *ura*⁺ transformants were checked by Southern blotting (data not shown) for homologous replacement of the wild-type *rhp9* gene by the disruption construct. Comparison of Figures 2a and 5a indicates that the *rhp9-d* null allele displays a somewhat increased level of sensitivity to UV radiation over that of the original *rhp9-64* allele. The *Pst*I–*Bgl*III fragment of pGa7 when cloned into pAL19 was able to complement the radiation sensitivity of the *rhp9-d* null allele to approaching wild-type levels (data not shown).

Table 3. Gene similarity and identity

	rhp9	SP-07	Rad9	BRCA1	53BP1	KIAA-0170
rhp9	100	40/20	46/26	39/22	45/22	39/21
SP-07		100	46/21	41/20	39/17	39/20
Rad9			100	44/22	43/20	40/17
BRCA1				100	42/21	36/18
53BP1					100	38/20
KIAA-0170						100

Figures indicate per cent similarity and identity respectively. SP-07, SPAC19G10_7/SCHPO, DDBJ/EMBL/GenBank accession no. Z69909; Rad9, *S.cerevisiae* Rad9; BRCA1, human BRCA1; KIAA-0170, human ORF, DDBJ/EMBL/GenBank accession no. D79992.

Checkpoint analysis

The 'cut' phenotype of the *rhp9-64* allele observed after exposure to ionizing radiation (Fig. 1) suggests that the *rhp9* gene is required for the DNA damage checkpoint. To investigate this and whether *rhp9* is also required for the replication checkpoint, synchronous cultures of *rhp9-64* and *rhp9-d* cells were exposed to a range of doses of ionizing radiation or 10 mM HU and cells analysed for G₂ arrest. Figure 4A indicates that the null allele lacks the damage checkpoint and that the *rhp9-64* allele is partially defective. In contrast, both the *rhp9-64* and *rhp9-d* alleles arrest in the presence of the DNA synthesis inhibitor HU (Fig. 4B). These results are reminiscent of *chk1*, which is proficient in the replication checkpoint, but defective in the damage checkpoint (6,13). Interestingly, the mitotic arrest in HU in the *rhp9* null mutant lasted until ~5 h after S phase arrest, at which time a relatively synchronous mitosis was observed. This is different from *chk1* and wild-type cells, where 'leakage' into mitosis is later and more asynchronous.

Epistasis analysis

To determine whether *rhp9* acts in a process involving other radiation checkpoint gene products or in any of the previously characterized repair pathways, epistasis analysis was carried out. Analysis of double mutants with the *rad2-d* and *rad13-d* null alleles indicates that *rhp9* functions in a process other than that involving *rad2* (the *S.pombe* equivalent of human FEN1; 34,35) and other than an excision repair pathway (36; Fig. 5a and b).

Figure 5. Epistasis analysis. Analysis of UV sensitivities of double mutants of *rhp9-d* with (a) *rad2-d* (sp.218), (b) *rad13-d* (sp.222), (c) *rad3-d* (sp.379), (d) *rad26-d* (sp.381), (e) *hus1-d* (sp.380), (f) *chk1-d* (sp.374) and (g) *hus1-d* (sp.417) with *chk1-d* (sp.374). *rhp9* is defective in a process requiring the checkpoint *rad* genes *rad3* and *rad26*. The *rhp9* gene functions in a process involving *chk1* and is required for a process which is not defective in *hus1-d* but is defective in *rad3-d* and *rad26-d*.

Figure 6. Phenotypes of *rhp9-d cdc* double mutants after 4 h at 36°C. At the non-permissive temperature *rhp9-d cdc22* double mutants display a 'cdc' phenotype, while *rhp9-d cdc10* and *rhp9-d cdc17* double mutants display a 'cut' phenotype.

Having established a role for Rhp9 in the damage checkpoint, double mutants were created with several of the checkpoint *rad* mutants known to be defective in both the S phase and damage checkpoints. *rhp9-d* double mutants with either *rad3-d* (3) or *rad26-d* (6) showed no increase in radiation sensitivity over that of the single *rad3-d* or *rad26-d* mutants (Fig. 5c and d), indicating that *rhp9* functions in a process involving the checkpoint *rad* genes. Double mutants with other checkpoint *rad* mutants, namely *rad9-d* and *rad17-d*, (15,26) also showed no increase in sensitivity over that of the most sensitive single mutant (data not shown). Another mutant generally classified with the checkpoint *rad* mutants is *hus1* (27). Interestingly, an *rhp9-d hus1-d* double mutant was more sensitive than the most sensitive single mutant, *hus1-d* (5,27; Fig. 5e), suggesting that *hus1-d* retains a function which is defective in the *rad3-d*, *rad9-d*, *rad17-d* and *rad26-d* mutants.

To further define the process involving *rhp9*, two additional *rhp9-d* double mutants were created. A double mutant with *chk1-d* (which is defective in the damage checkpoint but not the S phase checkpoint; 6) demonstrated no increase in radiation sensitivity over that of *rhp9-d* (Fig. 5f). The *rad26-T12* mutant has previously been characterized as having no obvious defect in the mitotic checkpoint but displays radiation and HU sensitivity (6). This has been interpreted as a defect in a DNA damage tolerance process (6,37). A *rad26-T12 rhp9-d* double mutant is more sensitive to radiation than the most sensitive single mutant, *rhp9-d* (data not shown), suggesting that *rhp9* is not required for the damage tolerance process.

These results suggest that in addition to their requirement in the S phase and damage checkpoints, the checkpoint *rad* genes *rad3*, *rad9*, *rad17* and *rad26* may also be involved in an additional

Figure 7. Model for the possible roles of *rhp9* in the DNA damage response in *S. pombe*. *rhp9* is required for the DNA damage checkpoint and for a process which does not include *hus1*. We were unable to produce a *rhp9 rad1* double mutant, possibly because the two genes are linked, and therefore have not determined whether *rad1* is required for this second process.

process which is independent of *hus1* but which requires *rhp9*. To determine whether *chk1* is required for this process, a further double mutant was created, *chk1-d hus1-d*. The double mutant displayed no increase in radiation sensitivity over that of the *hus1-d* mutant, unlike the *rhp9-d hus1-d* double mutant, indicating that the additional process is independent of *chk1*.

Genetic interactions

The *chk1* gene has been shown to be required to prevent mitosis following cell cycle arrest in *cdc10* and *cdc17* mutants as well as following exposure to DNA damaging agents (38). To investigate whether *rhp9* is required in a similar manner, double mutants were created with *cdc10-V50* (V.Simanis, personal communication), *cdc17-K42* and *cdc22-M45* (39). An *rhp9 cdc22* double mutant displayed a 'cdc' phenotype after 4 h incubation at 36°C (Fig. 6), consistent with the HU phenotype shown in Figure 4. In contrast, *rhp9 cdc10* and *rhp9 cdc17* double mutants displayed a 'cut' phenotype. These results are consistent with those obtained with *chk1* (38).

DISCUSSION

We have isolated a number of new MMS-sensitive mutants, some of which are also altered in response to the DNA replication inhibitor HU. Of these mutants, two, which show no growth on HU, are likely to be defective in the DNA replication checkpoint. Three further mutants, which form microcolonies on HU, are likely to be defective in some other aspect of S phase arrest. We have cloned two of the corresponding wild-type genes by complementation of the radiation-sensitive phenotypes. Sequence analysis indicates that the gene which fully complements the radiation sensitivity of *mms8* (data not shown) is the *S. pombe rad4/cut5* gene (40,41).

The *mms64* mutant phenotype is complemented fully by a novel gene which we have called *rhp9*. The *rhp9* gene product, which is identical (apart from a couple of amino acid differences) to a recently identified sequence (Crb2) in the GenBank database (accession no. D86478) is most homologous to the *S. cerevisiae* Rad9 protein and contains motifs resembling the BRCT domains in human and mouse BRCA1 proteins. Human BRCA1 was identified as a breast cancer susceptibility gene, with mutations in BRCA1 accounting for 45% of families with high incidence of breast cancer and for 80–90% of families with both breast and

ovarian cancer (42). The biological role of the BRCT domains is unknown, however, two missense mutations in the C-terminal 160 amino acids of BRCA1 (which include the BRCT domains) are associated with breast cancer (43,44). Additionally, the C-terminal 270 amino acids of 53BP1 are sufficient for binding p53 (45).

The *S.cerevisiae* *RAD9* gene has been shown to be required for DNA damage-specific checkpoints in both G₁ and G₂ (1,7,12,30) and more recently roles for *RAD9* in DNA damage processing (14) and damage-dependent transcription (46) have also been demonstrated. Data presented here indicate that *rhp9* is also required for the damage checkpoints in both G₁ and G₂ (Figs 4 and 6). Epistasis analysis indicates that *rhp9* functions in one or more processes requiring the *rad3*, *rad9*, *rad17* and *rad26* genes, with one of these processes likely to be the damage checkpoint.

The *S.pombe* checkpoint *rad* mutants *rad1*, *rad3*, *rad9*, *rad7*, *rad26* and *hus1* have all been shown to be defective in both the replication and DNA damage checkpoints (3,5,6). Double mutants of *rhp9-d* with *rad3*, *rad9*, *rad17* and *rad26* null alleles display no increase in radiation sensitivity over that of the most sensitive single mutant, indicating that the genes are required for one or more common processes (Fig. 7). The increased sensitivity of an *rhp9 hus1* double mutant over that of *hus1* suggests that *hus1* is not required for all of the functions which are defective in other checkpoint *rad* mutants such as *rad3*, *rad17* and *rad26*. This additional function may be reflected in the response of the *rhp9* null allele to HU (Fig. 4). In response to HU, synchronous cultures of *rhp9-d* do not undergo septation for at least 5 h following exposure to HU, indicating an intact replication checkpoint. After 5 h, the expected length of replication delay induced by HU exposure (47), *rhp9-d* cells undergo septation, unlike *chk1* cells, which do not. This may reflect an inability of *rhp9-d* cells to properly regulate the processing of DNA structures which occur as a result of the HU block. This potential defect may then lead to loss of the arrest signal after a particular time of delay. Such an *rhp9*-dependent DNA processing function may be analogous to that carried out by *RAD9* in *S.cerevisiae* (14).

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REFERENCES

- Weinert, T.A. and Hartwell, L.H. (1988) *Science*, **241**, 317–322.
- Hartwell, L.H. and Weinert, T.A. (1989) *Science*, **246**, 629–634.
- Al-Khodairy, F. and Carr, A.M. (1992) *EMBO J.*, **11**, 1343–1350.
- Rowley, R., Subramani, S. and Young, P.G. (1992) *EMBO J.*, **11**, 1335–1342.
- Enoch, T., Carr, A.M. and Nurse, P. (1992) *Genes Dev.*, **6**, 2035–2046.
- Al-Khodairy, F., Fotou, E., Sheldrick, K.S., Griffiths, D.J.F., Lehmann, A.R. and Carr, A.M. (1994) *Mol. Biol. Cell*, **5**, 147–160.

- Siede, W., Friedberg, A.S. and Friedberg, E.C. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 7985–7989.
- Navas, T.A., Zhou, Z. and Elledge, S.J. (1995) *Cell*, **80**, 29–39.
- Weinert, T.A. and Hartwell, L.H. (1993) *Genetics*, **134**, 63–80.
- Weinert, T.A., Kiser, G.L. and Hartwell, L.H. (1994) *Genes Dev.*, **8**, 652–655.
- Allen, J., Zhou, Z., Siede, W., Friedberg, E.C. and Elledge, S.J. (1994) *Genes Dev.*, **8**, 2416–2428.
- Siede, W., Friedberg, A.S., Dianova, I. and Friedberg, E.C. (1994) *Genetics*, **138**, 271–281.
- Walworth, N., Davey, S. and Beach, D. (1993) *Nature*, **363**, 368–371.
- Lyall, D. and Weinert, T.A. (1995) *Science*, **270**, 1488–1491.
- Griffiths, D.J.F., Barbet, N.C., McCready, S., Lehmann, A.R. and Carr, A.M. (1995) *EMBO J.*, **14**, 5812–5823.
- Bentley, N.J., Hawkins, G., Keegan, K., DeMaggio, A., Holtzman, D., Ford, J.C., Hoekstra, M. and Carr, A.M. (1996) *EMBO J.*, **15**, 6641–6651.
- Barbet, N., Muriel, W.J. and Carr, A.M. (1992) *Gene*, **114**, 59–66.
- Seaton, B.L., Yucel, J., Sunnerhagen, P. and Subramani, S. (1992) *Gene*, **119**, 83–89.
- Murray, J.M., Doe, C.L., Schenk, P., Carr, A.M., Lehmann, A.R. and Watts, F.Z. (1992) *Nucleic Acids Res.*, **20**, 2673–2678.
- Sambrook, J., Fritsch, E.F. and Maniatis, T.E. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- DeVaux, L.C., Hoagland, N.A. and Smith, G.R. (1992) *Genetics*, **130**, 251–262.
- Moreno, S., Klar, A. and Nurse, P. (1991) *Methods Enzymol.*, **194**, 795–823.
- Carr, A.M. and Murray, J.M. (1996) In Adolph, K.W. (ed.), *Microbial Genome Methods*. CRC Press, Boca Raton, FL, pp. 133–147.
- Al-Khodairy, F., Enoch, T., Hagan, I.M. and Carr, A.M. (1995) *J. Cell Sci.*, **108**, 475–486.
- Sunnerhagen, P., Seaton, B.L., Nasim, A. and Subramani, S. (1990) *Mol. Cell. Biol.*, **10**, 3750–3760.
- Murray, J.M., Carr, A.M., Lehmann, A.R. and Watts, F.Z. (1991) *Nucleic Acids Res.*, **19**, 3525–3531.
- Kostrub, C.F., Carr, A.M., Al-Khodairy, F., Ghazizadeh, H. and Enoch, T. (1997) *Mol. Gen. Genet.*, in press.
- Henikoff, S. (1984) *Gene*, **28**, 351–359.
- Morgan, B.A., Conlon, F.L., Manzanarez, M., Millar, J.B.A., Kanuga, N., Sharpe, J., Krumlauf, R., Smith, J.C. and Sedgwick, S.G. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 2801–2806.
- Schiestl, R.H., Reynolds, P., Prakash, S. and Prakash, L. (1989) *Mol. Cell. Biol.*, **9**, 1882–1896.
- Koonin, E.V., Altschul, S.F. and Bork, P. (1996) *Nature Genet.*, **13**, 266–268.
- Lane, T.F., Deng, C.X., Elson, A., Lyu, M.S., Kozak, C.A. and Leder, P. (1995) *Genes Dev.*, **9**, 2712–2722.
- Grimm, C., Kohli, J., Murray, J.M. and Maundrell, K. (1988) *Mol. Gen. Genet.*, **215**, 81–86.
- Murray, J.M., Tavassoli, M., Al-Harithy, R., Sheldrick, K.S., Lehmann, A.R., Carr, A.M. and Watts, F.Z. (1994) *Mol. Cell. Biol.*, **14**, 4878–4888.
- Harrington, J.J. and Lieber, M.R. (1994) *Genes Dev.*, **8**, 1344–1355.
- Carr, A.M., Sheldrick, K.S., Murray, J.M., Al-Harithy, R., Watts, F.Z. and Lehmann, A.R. (1993) *Nucleic Acids Res.*, **21**, 1345–1349.
- Shayeghi, M., Doe, C.L., Tavassoli, M. and Watts, F.Z. (1997) *Nucleic Acids Res.*, **25**, 1162–1169.
- Nasmyth, K. and Nurse, P. (1981) *Mol. Gen. Genet.*, **181**, 119–124.
- Carr, A.M., Moudjou, M., Bentley, N.J. and Hagan, I.M. (1995) *Curr. Biol.*, **5**, 1179–1190.
- Fenech, M., Carr, A.M., Murray, J.M., Watts, F.Z. and Lehmann, A.R. (1991) *Nucleic Acids Res.*, **19**, 6737–6741.
- Saka, Y. and Yanagida, M. (1993) *Cell*, **74**, 383–393.
- Easton, D.F., Bishop, D.T., Ford, D., Crockford, G.P. and Breast Cancer Consortium (1993) *Am. J. Hum. Genet.*, **52**, 678–701.
- Miki, Y. et al. (1994) *Science*, **266**, 66–71.
- Futreal, P.A. et al. (1994) *Science*, **266**, 120–122.
- Iwabuchi, K., Bartel, P.L., Li, B., Marraccino, R. and Fields, S. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 6098–6102.
- Aboussekh, A., Vialard, J.E., Morison, D.E., Angeles de la Torre-Ruiz, M., Cernakova, L., Fabre, F. and Lowndes, N.F. (1966) *EMBO J.*, **15**, 3912–3922.
- Sazer, S. and Nurse, P. (1994) *EMBO J.*, **13**, 606–615.