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 RAD24 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 rad5-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 µl/l (21).

### Table 1. Schizosaccharomyces pombe strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>sp.011</td>
<td>ade6-704, leu1-32, ura4-D18, h-</td>
</tr>
<tr>
<td>sp.012</td>
<td>ade6-704, leu1-32, ura4-D18, h+</td>
</tr>
<tr>
<td>sp.096</td>
<td>rad9::ura4, ade6-704, leu1-32, h-</td>
</tr>
<tr>
<td>sp.218</td>
<td>rad2::ura4, ade6-704, leu1-32, h+</td>
</tr>
<tr>
<td>sp.222</td>
<td>rad13::ura4, ade6-704, leu1-32, h-</td>
</tr>
<tr>
<td>sp.310</td>
<td>rad17::ura4, ade6-704, leu1-32, h+</td>
</tr>
<tr>
<td>sp.304</td>
<td>cdc10-V50, ura4-D18, h+</td>
</tr>
<tr>
<td>sp.374</td>
<td>chk1::ura4, h-</td>
</tr>
<tr>
<td>sp.378</td>
<td>rad1::ura4, leu1-32, ade6-704, h-</td>
</tr>
<tr>
<td>sp.379</td>
<td>rad3::ura4, leu1-32, ade6-704, h+</td>
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<tr>
<td>sp.380</td>
<td>has1::leu1, h-</td>
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<td>sp.381</td>
<td>rad26::ura4, ade6-704, leu1-32, h+</td>
</tr>
<tr>
<td>sp.382</td>
<td>cdc22-M45, ade6-704, ura4-D18, leu1-32, h+</td>
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<tr>
<td>sp.383</td>
<td>rad26-T12, h+</td>
</tr>
<tr>
<td>sp.417</td>
<td>has1::leu1, h+</td>
</tr>
</tbody>
</table>

Strains created during this study

| sp.346 | mms1, ade6-704, leu1-32, ura4-D18, h+ |
| sp.347 | mms8, ade6-704, leu1-32, ura4-D18, h+ |
| sp.348 | mms35, ade6-704, leu1-32, ura4-D18, h+ |
| sp.349 | mms38, ade6-704, leu1-32, ura4-D18, h+ |
| sp.350 | mms52, ade6-704, leu1-32, ura4-D18, h- |
| sp.351 | mms57, ade6-704, leu1-32, ura4-D18, h+ |
| sp.352 | mms60, ade6-704, leu1-32, ura4-D18, h+ |
| sp.353 | rhp9-64, ade6-704, leu1-32, ura4-D18, h- |
| sp.354 | mms70, ade6-704, leu1-32, ura4-D18, h+ |
| sp.391 | rhp9::ura4, ade6-704, leu1-32, h+ |
| sp.392 | rhp9::ura4, rad13::ura4, ade6-704, leu1-32, h+ |
| sp.394 | rhp9::ura4, has1::leu1, h- |
| sp.395 | rhp9::ura4, chk1::ura4, h- |
| sp.397 | rhp9::ura4, rad2::ura4, ade6-704, leu1-32, h+ |
| sp.398 | rhp9::ura4, rad26-T12, h+ |
| sp.408 | rhp9::ura4, rad26::ura4, ade6-704, leu1-32, h+ |
| sp.409 | rhp9::ura4, rad3::ura4, ade6-704, leu1-32, h+ |
| sp.411 | rhp9::ura4, cdc10-V50, h- |
| sp.412 | rhp9::ura4, cdc17, h+ |
| sp.413 | rhp9::ura4, cdc22-M45, h+ |
| sp.419 | chk1::ura4, has1::leu1, h+ |

### Mutagenesis

10⁷ 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 µl 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 ∼5 × 10⁶/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 µl 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 (C)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 µl/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 mms38 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.

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

<table>
<thead>
<tr>
<th>Table 2. Summary of phenotypes of MMS-sensitive mutants</th>
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<tbody>
<tr>
<td>Strain</td>
</tr>
<tr>
<td>mms1</td>
</tr>
<tr>
<td>mms8</td>
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<tr>
<td>mms35</td>
</tr>
<tr>
<td>mms38</td>
</tr>
<tr>
<td>mms52</td>
</tr>
<tr>
<td>mms57</td>
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<tr>
<td>mms60</td>
</tr>
<tr>
<td>mms64 (rhp9-64)</td>
</tr>
<tr>
<td>mms70</td>
</tr>
<tr>
<td>sp.011 (wt)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 3. Gene similarity and identity</th>
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<tbody>
<tr>
<td>Gene</td>
</tr>
<tr>
<td>rhp9</td>
</tr>
<tr>
<td>SP-07</td>
</tr>
<tr>
<td>Rad9</td>
</tr>
<tr>
<td>BRCA1</td>
</tr>
<tr>
<td>53BP1</td>
</tr>
<tr>
<td>KIAA-0170</td>
</tr>
</tbody>
</table>

Records 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.

The sequence of the 4.5 kb *PstI*-*Bgl*II 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′ EcoRV site to the 3′ HindIII 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 EcoRI (see Fig. 3A) and transformed into the haploid strain sp.012. *ura4* 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 *PstI*-*Bgl*II 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).

**Checkpoint analysis**

The 'cwr' 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).
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-d 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 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 ‘cut’ 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 mms6 (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 (Cr2) 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 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 hsl1 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 hsl1 double mutant over that of hsl1 suggests that hsl1 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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