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Cloning and characterisation of the *Schizosaccharomyces pombe* rad8 gene, a member of the SNF2 helicase family

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ABSTRACT

The *Schizosaccharomyces pombe* rad8 mutant is sensitive to both UV and gamma irradiation. We have cloned the rad8 gene by complementation of the UV sensitivity of a rad8.190 mutant strain. The gene comprises an open reading frame of 3.4 kb which does not contain any introns and is capable of encoding a 1133 amino acid protein of 129 kDa. Deletion of the gene indicates that it is not essential for cell viability. Recognisable motifs are present for a nuclear localisation signal, a RING finger and helicase domains. The predicted protein is a member of the SNF2 subfamily of proteins and shows particular homology to the Saccharomyces cerevisiae RAD5 protein. Double mutant analysis demonstrated that the rad8 mutant is not epistatic to mutants in the excision repair pathway (rad13) or checkpoint pathway (rad9). Analysis of radiation sensitivity though the cell cycle indicates that, unlike most other rad mutants, rad8 is most sensitive to irradiation during the G1/S period.

INTRODUCTION

DNA repair mechanisms have important roles in the maintenance of the integrity of DNA. The fundamental requirement for these repair mechanisms is illustrated by a number of human genetic disorders which have been associated with defects in the repair of DNA, such as Xeroderma pigmentosum and Cockayne’s syndrome. Many of the genes involved in these processes have been cloned from humans and from model systems, such as the budding and fission yeasts. A number of striking homologies have been identified between genes from different species (1). The degree of conservation appears to be particularly good for genes which contain putative helicase domains, e.g. the human genes ERCC2 and ERCC3 have high levels of sequence similarity to their yeast homologues, and this homology is most apparent in the conserved helicase domains (2).

The fission yeast, *Schizosaccharomyces pombe*, has proved to be an important model system for the study of basic processes in higher eukaryotes, most notably cell cycle control (3). Eukaryotic DNA repair mechanisms have been most extensively studied in the budding yeast *Saccharomyces cerevisiae* (4,5), but the recent developments in the study of DNA damage responses in *S. pombe* now provides an important complementary model system. Radiation sensitive mutants in *S. pombe* were originally assigned to approximately 23 complementation groups (6,7), but more recent evidence suggests that not all of these are distinct (8). The functions of the corresponding genes is unknown in most cases, but they have been shown to be required for a number of different mechanisms including excision repair, e.g. *rad13* (9, rad15 (10,11), rad16 (8), error-prone repair, e.g. *rhp6* (12), recombination repair, e.g. *rhp51* (13), and mitotic arrest following DNA damage, e.g. *rad1*, *rad3*, *rad9*, *rad17* (14,15). Analysis of the cloned genes indicates that many of them possess significant sequence identity to DNA repair genes in budding yeast and man, e.g. *rad15*, which is homologous to *S. cerevisiae RAD3* and human *ERCC2* (10).

The rad8 mutant was initially reported to be sensitive to UV and gamma irradiation, but not to be further sensitised by caffeine treatment after irradiation and to have a particularly low UV-induced mutation rate (6). This phenotype suggests that the rad8 gene may be involved in recombination and/or error-prone repair processes. In this paper we report the cloning and characterisation of the rad8 gene, which is capable of encoding a putative protein of 129 kDa with homology, predominantly within the helicase domains, to the SNF2 family of proteins. This is the first reported *S. pombe* gene which is a member of the SNF2 helicase family (16).

MATERIALS AND METHODS

Plasmids, strains and growth conditions  
The *S. pombe* plasmids, pUR19 and pWH5, and the genomic library used in this study have been described elsewhere (17,18). The *S. cerevisiae* RAD5-containing plasmid pBM118 (19), and RAD54-containing plasmid pYPE13-RAD54 (20) were generous gifts from R.Johnson and D.Schild, respectively. Plasmids were grown in *E. coli* strain DH5a, (endA1, hsdR17, (rk-,mk-), supE44, thi-1, recA1, lacIqZ-M15 (lacproAB)). M13 derivatives were grown in DH5aF' (as DH5a except that it contains an integrated F'). *S. pombe* strains used in this study are shown in

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Irradiation of S. pombe cells

UV irradiation for survival curves of log phase asynchronous cultures was carried out directly on freshly plated cells using a Stratagene 'Stratalinker'. Survival curves represent the data from a minimum of four plates obtained from at least two separate experiments.

Gamma-irradiation was carried out in liquid YES medium at a cell density of 10^7/ml in a Gammacell 1000 using a 137Cs source with a dose rate of 12 Gy/min.

Radiation survival through the cell cycle

S. pombe cultures were synchronised using lactose gradients as described by Mitchison and Carter (23). Cells from the top of the gradient, which are in G2, were inoculated into fresh YES medium. At 15 minute intervals samples were taken for the following treatments. UV irradiation: 10^3 cells were plated and irradiated under a UVC lamp with a dose rate of 6 J/m^-2, Gamma irradiation: 0.1 ml of cells at a density of 10^4 cells/ml in YES were irradiated in a Gammacell 1000 137Cs source (dose rate 12 Gy/min) prior to plating. Control: 5 x 10^3 cells were plated as a negative control for each time point; mitotic and septation indices: a sample of cells were fixed in 80% methanol, air dried onto a microscope slide, stained with DAPI (0.1 µg/ml) and Calcofluor (0.5 µg/ml) and observed by fluorescence microscopy.

Genetics and molecular biology methods

S. pombe genetic and molecular biology methods have been described in our previous work (21). General molecular biology protocols were as detailed in Sambrook et al. (24).

RESULTS

Isolation of the rad8 gene

An S. pombe genomic library (17) was used to transform the rad8 mutant strain, sp.559, to uracil prototrophy. Approximately 50,000 ura+ colonies were pooled, plated at a density of 1x10^6 cells per plate on selective medium and subjected to UV irradiation at a dose of 100 J/m^-2. After two further rounds of selection, individual colonies were tested for co-instability of the ura+ phenotype. The correcting plasmid, pRAE2, was extracted from UV-resistant transformants and used to transform the E. coli strain DH5a. Retransformation of the transformant diploid strain sp.101 to uracil prototrophy. Stable integrants of the rad8 gene (27). A linear 5.4 kb fragment containing this disrupted region was then used to transform the diploid strain sp.559 with pRAE2 restored the UV sensitivity to approximately wild-type levels.

Chromosomal mapping of pRAE2 to the rad8 locus

The rad8 locus has been reported to be on the most telomere proximal NotI fragment on the left arm of chromosome 1 (25). To map the rad8 locus more precisely, crosses were carried out between a rad8 mutant strain, sp.559, and a cdc25 mutant strain, sp.628, or a cdc25 mutant strain, sp.574. Analysis of the UV- and temperature-sensitive phenotypes of the resulting progeny indicated that the rad8 gene maps 8cM on the telomere proximal side of the cdc12 gene. To confirm that the cloned gene is the rad8 gene, rather than a suppressor of the rad8 mutant phenotype, its chromosomal location was mapped. A stable integrant of the pRAE2 plasmid in the rad8 mutant strain sp.559 was obtained and crossed to the rad+ strain, sp.011. Analysis of the resultant spores showed that no UV sensitive isolates were obtained. This indicates that pRAE2 had integrated at the rad8 locus and thus contains the rad8 gene. The rad8 gene has also been hybridised to the grided S. pombe cosmid array of Lehrach and colleagues and its position on the physical map has been reported (26), further defining the rad8 locus.

Gene deletion

In order to test whether the rad8 protein is required for cell viability, a null allele was constructed. A plasmid construct was made in which the 0.8 kb BclI-BglII fragment of the rad8 ORF (Figure 1A) was replaced with a 1.7 kb marker fragment containing the ura4+ gene (27). A linear 5.4 kb HindIII fragment containing this disrupted region was then used to transform the diploid strain sp.101 to uracil prototrophy. Stable ura+ colonies were isolated and Southern analysis was used to confirm that a single copy of the ura4+ gene had integrated at the rad8 locus (data not shown). An h9 derivative (a revertant of h+ which produces 90% of cells able to sporulate on low.

Table 1. Procedures and media used for the routine growth and maintenance of S. pombe strains were as reported in our previous work (21).

<table>
<thead>
<tr>
<th>Strains Created For This Study:</th>
</tr>
</thead>
<tbody>
<tr>
<td>sp.152 rad8.190, ade6.704, leu1.32, ura4D18, h-</td>
</tr>
<tr>
<td>sp.193 rad8::ura4, ade6.704/ade6.704, leu1.32/leu1.32, ura4D18/ura4D18, h+/h-</td>
</tr>
<tr>
<td>sp.197 rad8::ura4, ade6.704, leu1.32, ura4D18, h-</td>
</tr>
<tr>
<td>sp.199 rad8::ura4, rad21, h-</td>
</tr>
<tr>
<td>sp.201 rad8::ura4, rad13::ura4, ade6.704, leu1.32, ura4D18, h-</td>
</tr>
<tr>
<td>sp.203 rad8::ura4, rad27, ade6.704, leu1.32, ura4D18, h+</td>
</tr>
<tr>
<td>sp.204 rad8::ura4, rad18, ade6.704, leu1.32, h+</td>
</tr>
<tr>
<td>sp.206 rad8::ura4, rad18, ade6.704, leu1.32, ura4D18, h-</td>
</tr>
</tbody>
</table>

Table 1. S. pombe strains.

| sp.611 ade6.704, leu1.32, ura4D18, h- |
| sp.612 ade6.704, leu1.32, ura4D18, h+ |
| sp.689 rad21, h- |
| sp.101 ade6.704/ade6.704, leu1.32/leu1.32, ura4D18/ura4D18, h+/h- |
| sp.118 rad9::ura4, ade6.704, leu1.32, ura4D18, h- |
| sp.138 rad11::ura4, ade6.704, leu1.32, ura4D18, h- |
| sp.145 rad4, ade6.704, leu1.32, h- |
| sp.151 rad18, ade6.704, leu1.32, ura4D18, h- |
| sp.559 rad8::ura4, ade6.704, leu1.32, ura4D18, h+ |
| sp.574 cdc25.22, ura4D18, h- |
| sp.628 cdc12, h- |
The predicted rad8 protein sequence contains several interesting features. There is a putative nuclear localisation signal at amino acid position 246 with the sequence RKKSKK with two potential casein kinase II phosphorylation sites (29) in close proximity. One is at amino acid position 191 with the sequence TPLD, and the other is at amino acid position 286 with the sequence TLME. The protein sequence also contains the RING finger motif between residues 874 and 925 (Figure 3a). This sequence has been found in a number of other proteins, including the S. cerevisiae DNA repair proteins RAD18 and RAD5 (30,19). This type of zinc finger has been shown to bind both DNA and zinc in vitro (30).

**Rad8 homologies**

Database searches reveal that the rad8 ORF has homology to the SNF2 helicase subfamily (16,31). This homology is largely confined to the helicase domains (Figure 3b). There are four DNA repair proteins reported to date as belonging to this group, namely the S. cerevisiae RAD54 (32), RAD16 (33,34) and RAD5 (REV2) (19,35) and human ERCC5 proteins (36). Comparison of the rad8 putative protein sequence with these proteins indicates that S. cerevisiae RAD5 more closely resembles rad8 than do the other proteins (Figure 3b and c). The predicted sizes of the rad8 and RAD5 ORFs are very close, 1133 and 1169 amino acids respectively. The two proteins have similar spacings of the helicase domains and both possess a RING finger motif (30) at approximately the same position (Figure 3c). Additionally, the phenotypes of both mutant strains are similar: rad8 and rad5 mutants are sensitive to both UV and gamma irradiation and are implicated in error-prone repair processes.

In order to test whether RAD5 can functionally substitute for rad8, a 4.4 kb fragment from the plasmid pBM1118 (19) containing the RAD5 gene, was subcloned into the S. pombe vector pH5 (18) and used to transform the rad8 deletion strain sp187. The RAD5-containing plasmid pYEP13-RAD54 (20) was also tested for the ability to complement the rad8 deletion mutation. Transformants were selected for leucine prototrophy, replica plates were subjected to a UV dose of 300 J/m² and colonies grown up overnight. Examination of the irradiated plates showed that in both cases the transformants were more UV sensitive than wild-type cells. The survival of the transformants was tested at various doses of UV irradiation (Figure 2c) and confirmed that the RAD54-containing plasmid did not complement the rad8 mutation. Transformants containing the RAD5 plasmid showed an approximate four-fold increase in UV resistance at high doses when compared to the rad8 deletion strain. This low level of complementation may be due to the fact that the RAD5 protein cannot functionally substitute for rad8 (a possibility which would seem likely since the level of homology between RAD5 and rad8 outside the helicase domains is very low) or that insufficient RAD5 protein is produced in the transformants.

**Expression of the rad8 gene**

Northern analysis was carried out on total RNA (Figure 4) from wild-type (sp.01) cells. Using a 1.4 kb Psrl–BglII fragment from the 5’ end of the gene as a probe, four transcripts were detected in both preparations of RNA, with the sizes 4 kb, 3 kb, 2 kb and 1.1 kb (Figure 4, lane B). Similar results were observed with several RNA preparations probed with three other regions.
of the rad8 gene (Figure 1C); namely an EcoRI–KpnI 0.6 kb fragment from the 3' end of the rad8 gene, a rad8 internal 2 kb BglII–BamHI fragment and a rad8 internal 1.6 kb EcoRI–BamHI fragment, except that the smallest 1.1 kb RNA is not seen with either of the rad8 internal fragment probes. Analysis of transcripts in the rad8 deletion mutation strain (sp.187) indicates the absence of the 4 kb band (Figure 4, lane A), implying that this 4 kb RNA is transcribed from the rad8 gene. This size is consistent with the size of the rad8 predicted ORF.

**Radiation sensitivities of double rad mutant strains**

Initial characterisation of the S.pombe DNA repair mutants classified them according to the UV and gamma sensitivities of the single mutants, since double mutant data required for epistasis analysis were limited (6). In order to determine the functional relationship of the rad8 protein with other rad proteins, a number of double mutant strains were isolated following the crossing of rad8 mutants with the appropriate rad mutant strains and tetrad dissection. The UV survival of the double mutant strains was then assessed relative to each of the single mutants. A synergistic increase in UV sensitivity was observed when the rad8 deletion mutation was combined with the rad13 (sp.138) excision repair deletion mutation (9) (Figure 2d) or with the rad9 (sp.118) checkpoint deletion mutation (14,21) (Figure 2e), as expected. Similarly, UV sensitivity was increased when the rad8 deletion mutation was in combination with the rad21 mutation (sp.089) (Figure 2f) which is defective in double-strand break repair (35), and also with the rad18 mutation (sp.151) (Figure 2g) and the rad4 (sp.145) (38) mutation (Figure 2h) which are involved in as yet undetermined processes. These observations suggest that the rad8 protein does not function in excision repair, and direct measurements on removal of cyclobutane and 6-4 pyrimidine photoproducts are in agreement with this (39). Similarly, the double mutant data are consistent with previous reports that rad8 does not seem to be involved with the G2 checkpoint pathway, although it has been noted that in a cdc25 background, the survival of a rad8 mutant after UV irradiation is increased if mitotic entry is delayed by incubation at the restrictive temperature for cdc25 (14). Additionally, the rad8 protein does not function in combination with either the rad21, rad18 or rad4 proteins. Thus, on the basis of these epistatic data, we have not been able to define the pathway in which the rad8 protein acts, but the conserved excision repair and the checkpoint pathways have been excluded. We cannot rule out the possibility that the rad8 protein affects the efficiency of a process that still occurs in rad8 mutants. However any such process must still operate in all the mutants tested.

**S phase requirement for functional rad8 protein**

To determine whether rad8 enhances DNA damage survival at a specific point in the cell cycle, experiments were carried out
Figure 2. Survival curves of rad8 strains following UV or gamma irradiation. (a and b) Survival of rad8 strain sp.011 (wt), the rad8 deletion strain sp.559 (rad8), the rad8 deletion strain sp.187 (rad8D) and rad8 strain sp.559 containing the complementing plasmid pRAE2 (RAE2) after UV and gamma irradiation respectively.

(c) Survival of rad8 strain sp.011 (wt), the rad8 deletion strain sp.187 (rad8), and the rad8 deletion strain, sp.187 (rad8D), transformed with either pH5 S.pombe cloning vector (pWH5), the S.cerevisiae RAD54 containing plasmid pYEP-RAD54 (RAD54) or the S.cerevisiae RAD5 gene cloned into pWH5 (RAD5), plated on selective minimal agar plates. (d–h) Survival of double mutants of the rad8 deletion in combination with various other rod mutations. The rad8 deletion (rad8D), rad13 deletion mutant (excision repair) (rad13D), the rad8/rad13 double deletion (rad8D,13D), the rad9 deletion mutant (G2 arrest deficient) (rad9D), the rad8/rad9 double deletion (rad8D,rad9D), the rad21 mutant (double-strand break repair deficient) (rad21), the rad8 deletion/rad21 double mutant (rad8D,rad21), the rad18 mutant (rad18), the rad8 deletion/rad18 double mutant (rad8D,rad18), the rad4 mutant (rad4), the rad8 deletion/rad4 double mutant (rad8D,rad4).

to determine the UV sensitivity and gamma sensitivity of the rad8 mutant through the cell cycle. Synchronous cultures of rad8 deletion (sp.187), rad13 deletion (sp.138) and wild-type (sp.011) strains were prepared using lactose gradients. At 15 min intervals samples of cells were removed, diluted, plated and UV irradiated. Simultaneously samples were taken to determine the septation index and mitotic index. The results are shown in Figure 5. Several conclusions can be drawn from the results. Wild-type cells are most sensitive to irradiation during the G1/S period of the cell cycle and showed maximal resistance during G2. In contrast, the rad13 deletion strain which has a defect in excision repair (9,39) shows maximal sensitivity to radiation at a time comensurate with mitosis. rad8 deletion cells show a profile of sensitivity similar to that seen with wild type cells, being most sensitive to irradiation during the G1/S period of the cycle. rad8 was the only mutant of ten tested (rad2, rad4, rad8, rad9, rad13, rad17, rad18, rad21, rad26 and rad27 (40)) which showed maximal sensitivity during G1/S phase, and this suggests that the rad8 protein may act to either repair or tolerate damage induced at this time. It should be noted, however, that the doses used in these experiments are relatively low (in order to limit the duration of their consequences to less than one cell cycle) and may not reflect the consequences of higher levels of damage administered at equivalent times.

DISCUSSION

We have cloned the rad8 gene by complementation of the UV-sensitivity of a rad8 mutant strain. The isolated plasmid, pRAE2, can restore both UV and gamma sensitivities to near wild-type levels and has been shown to map to the rad8 locus, which is approximately 8cM proximal to the cdc12 gene on chromosome 1. The transcript of the rad8 gene is approximately 4 kb in size. Multiple transcripts are detected using rad8 probes, as has been observed for several members of the SNF2 helicase gene family, for example, human ERCC5 (41), Drosophila Ldr (42) and mouse...
**Figure 4.** Northern analysis of *S. pombe* total RNA hybridised with a rad8 specific, *Prtl*-BagII 1.4 kb, probe. Lane A: total RNA isolated from a rad8 deletion strain, sp.187; lane B: total RNA from a wild-type strain, sp.011. Four transcripts of 4 kb, 3 kb, 2 kb and 1 kb are seen in the wild-type strain. The 4 kb transcript is absent from the deletion strain; this is presumed to be due to premature termination of transcription in this strain.

**Figure 5.** Cell cycle survival profiles. Cells were synchronised in G2 on lactose gradients and at 15 min intervals survival after UV and gamma irradiation was assayed. At 30 min intervals a sample was fixed and stained with DAPI and Calcofluor to determine the percentage of septated cells and the mitotic index. These provide an estimate of the position of cells in the cell cycle at the time of irradiation. The wild type and rad8 deletion strain were irradiated with either 30 J/m² UV or 75 Gy ionising radiation. The rad13 deletion strain was irradiated at 15 J/m² UV (to give equivalent percentage survival) or 75 Gy ionising radiation. Mitotic idox, mitotic index.

**Figure 3.** Homology of the rad8 putative protein to other proteins. (a) Alignment of putative RING Zn²⁺ finger binding regions in rad8 (SpRad8) and *S. cerevisiae* RAD5 (SCRAD5), RAD16 (SCRAD16), and RAD18 (SCRAD18). Residues conserved between a protein and the rad8 predicted protein are represented by a dot. The conserved cysteines and histidines are indicated with open boxes. Numbers in parentheses represent residue positions. (b) Alignment of the putative RING Zn²⁺ finger binding regions in rad8 (SPrad8) and *S. cerevisiae* RAD5 (43), and *S. cerevisiae* RAD54 (32). In most of these cases the phenomenon has been attributed to alternative polyadenylation. However, analysis of the transcripts in the rad8 deletion strain implicates the 4 kb RNA as being transcribed from the *rad8* gene, since three of the transcripts are still observed in a rad8 deletion mutation strain. The identity of the other three transcripts is not clear, but they may represent transcripts from other genes in *S. pombe* with homology to the rad8 gene. We also find that the rad8 transcript is induced at low levels after UV irradiation, (Shayeghi et al., manuscript in preparation.) The related *S. cerevisiae* genes RAD5 (REV2), RAD16 and RAD54 have also been reported as being UV-inducible.
Analysis of the rad8 sequence predicts a protein of 1133 amino acids with a Mₐ of 129 kDa. This sequence contains several recognisable motifs. A putative nuclear localisation signal with associated casein kinase II phosphorylation sites suggests the rad8 protein acts in the nucleus, which is consistent with a DNA repair function. Phosphorylation at casein kinase II sites is thought to affect the rate of nuclear protein transport (29). A RING zinc finger motif has also been observed. This motif has been found in a number of proteins including some with roles in DNA repair and recombination (30). This suggests that the rad8 protein binds to DNA. The other major motifs identified in the sequence are seven helicase domains, including the ATPase motif, suggesting that the protein has DNA unwinding activity.

Homology searches reveal that rad8 is the first reported S.pombe member of a subfamily of proteins, found in a wide range of both prokaryotic and eukaryotic organisms, which show a particular type of conservation over the helicase regions (16,31). This subfamily is defined as proteins that have significant homology to the S.cerevisiae protein SNF2 (44), which was the first of the group to be identified and is the most well characterised. SNF2 is a transcriptional activator of numerous genes, possibly acting by altering chromatin structure, and is thought to form part of a large multi-subunit complex (45). ATPase, but not helicase, activity has been demonstrated for this protein (46). The family also contains other transcriptional activators, for example Drosophila Brm (47), and a negative transcription regulator, S.cerevisiae MOT1 (48).

Four DNA repair proteins have been reported with homology to SNF2, namely the S.cerevisiae proteins RAD54 (32), RAD16 (33,34), RAD5 (19), and human ERCC6 (36), which seem to represent four separate functions in repair of DNA. The human disease, Cockayne’s syndrome, has been shown to be associated with a defective ERCC6 protein (35). This excision repair protein is thought to be associated with the preferential repair of lesions from the transcribed strand of active genes. S.cerevisiae RAD16 is also involved with damage excision but, in contrast to ERCC6, is thought to be required for the removal of damage from the inactive DNA. RAD54 is required for recombination repair and RAD5 for a postreplication repair pathway. None of these proteins is thought to be essential for cell viability. It has been speculated that the existence of this type of helicase in a number of different repair pathways may indicate a function required in each of the processes which must also have some pathway specificity. The RAD5 protein is the most similar to S.pombe rad8 (see Figure 2) but does not appear to complement efficiently the rad8 deletion mutation. This may be because these two genes are not functional homologues. Alternatively, the two genes may encode proteins with similar functions, but which have diverged to an extent where they can no longer substitute for each other’s function, e.g. in specific protein—protein interactions. The fact that homology between the two proteins is mainly confined to the helicase domains would support this. The increased radiation sensitivity of the rad8 mutant during the G1/S period of the cell cycle is consistent with the previous report that the UV sensitivity of a rad8 mutant can be partially rescued by transiently preventing cell cycle progression using the cdc25.22 mutant following irradiation (14) and suggests that the mutant may be defective in a DNA damage response affecting DNA replication. However, since the null allele is viable, it is unlikely that the protein is required directly for replication. We are currently characterising the rad8 protein with a view to elucidating its precise role.

ACKNOWLEDGEMENTS

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