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Cloning and characterisation of the *rad9* DNA repair gene from *Schizosaccharomyces pombe*

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ABSTRACT

The *rad9.192* DNA repair mutant from the fission yeast, *Schizosaccharomyces pombe*, is sensitive to both UV and ionising radiation. The *rad9* gene has been cloned by complementation of the gamma-ray sensitivity of the mutant cell line. A 4.3kb *Hin*dIII fragment was found to confer resistance to both types of radiation. The region of complementation was further localised to a 2.6kb *Hin*dIII-*Eco*RV fragment, which, by DNA sequence analysis, was found to contain sequences capable of coding for a 427 amino acid protein, if three introns were postulated to remove stop codons. The introns were confirmed by sequence analysis of cDNA clones and PCR products derived from cDNA. The product of transcription is a 1.6kb mRNA of low abundance. The putative *rad9* protein shows no homology to any published sequence. A truncated protein is capable of complementing the radiation sensitivity of the *rad9.192* mutant. Deletion of the gene is not lethal and the null allele has a similar phenotype to the *rad9.192* mutant.

INTRODUCTION

In the fission yeast, *Schizosaccharomyces pombe*, mutants that are associated with defects in DNA repair have been assigned to 23 different complementation groups (1, 2). These *rad* mutants, unlike those identified in *Saccharomyces cerevisiae*, have not been extensively characterised. Most mutants are sensitive to both UV and gamma radiation. In this respect, they differ from the majority of *S. cerevisiae RAD* mutants, which are mostly sensitive to either UV- (excision repair) or gamma-irradiation (recombination repair) but not to both.

DNA repair is of fundamental importance in all living organisms. This is illustrated by the existence in man of a number of genetic disorders due to defects in DNA repair. The cloning and characterisation of *S. pombe* DNA repair genes should provide information relevant to DNA repair in other eukaryotes. This may be particularly relevant to the study of DNA repair in humans as the conservation of basic cellular mechanisms between *S. pombe* and higher eukaryotes has been well documented: as demonstrated, for example, in the cloning of a human cell cycle gene by functional complementation of an *S. pombe* mutant (3). *S. pombe* is also more like higher eukaryotes than *S. cerevisiae* in its prevalence of introns (4) and its ability to splice a mammalian intron correctly (5).

We report here the cloning of the *S. pombe rad9* gene. The *rad9.192* mutant is highly sensitive to both UV and gamma radiation. It also has a greater percentage of cell death during mitotic growth than wild type strains. It has been reported that, unlike rad$^+$ cells, the survival of *rad9.192* cells after irradiation is not decreased in the presence of caffeine, suggesting that *rad9* acts in a caffeine-sensitive repair pathway (1). It has been suggested that caffeine inhibits intergenic recombination in *S. pombe* (6) and that this implies that *rad9.192* may be defective in a recombination repair pathway. However, spontaneous intragenic recombination in *rad9.192* cells is approximately double that in wild type cells (1), indicating that cells containing the *rad9.192* mutation are still capable of undergoing some types of recombination. The UV-induced forward mutation rate has been reported to be slightly reduced (1).

MATERIALS AND METHODS

Strains and Plasmids

S. pombe strains and plasmids used are shown in tables 1 and 2 respectively. The *S. pombe* gene bank, constructed from partially *Hin*dIII digested genomic DNA, in the yeast shuttle vector pDB262, containing *S. cerevisiae LEU2* and 2μ sequences has been described previously (7). The *S. pombe* cDNA library was a gift from L. Guarente (8). Rescued plasmids were subcloned into the yeast vectors pSTA12 (7), pUR19 (9) and M13mp19.

Plasmids were grown in the *E. coli* strain DH5α, genotype: F- *endA1*, *hsdR17*, (*rk-*, *mk-*), *supE44*, *thi-1*, *l-*, *recA1*, *gyrA96*, *relA1*, *lacIqZ-M15(lacproAB)* (10) and rescued from *S. pombe* into the *recBC* strain JA226 (11). M13 derivatives were grown in DH5αF′ (genotype as for DH5α but containing an integrated F′).

Growth Conditions

S. pombe strains were grown and routinely maintained on complete medium (12). Selection for plasmid-containing cells utilised minimal medium or agar plates, as appropriate. Minimal

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medium agar plus sorbitol was used to plate cells after transformation (11). Minimal medium containing 0.05g/l NH_4Cl (low nitrogen) was used for mating and sporulation. Bacteria were grown in LB or 2YT (13) containing appropriate antibiotics.

Transformation

Yeast transformation was performed according to the spheroplast method of Beach *et al.*, (11). Bacterial transformation was by the calcium chloride method described by Maniatis *et al.* (13).

DNA Extraction and Purification

Plasmid and chromosomal DNA from yeast were purified according to the method of Aves *et al.*, (14). Plasmids from *E. coli* were prepared according to the alkali lysis method of Burke and Ish- Horowitz (13). Large scale preparations of plasmids were purified by caesium chloride-ethidium bromide centrifugation (13). DNA fragments were purified by extraction from low gelling temperature agarose using 'Geneclean' (Stratatech Scientific Ltd.) according to the manufacturer's recommended methods.

RNA Extraction and Purification

Total RNA was extracted from *S. pombe* by vortexing the cells in the presence of glass beads and phenol (15). Poly A^+ RNA was made by passing total RNA over oligo dT cellulose (13). For Northern analysis, polyA$^+$ RNA was run on 1% agarose gels after glyoxal treatment and transferred to Genescreen Plus (New England Nuclear Research Products) and probed by the manufacturer's recommended method.

DNA Sequence Analysis

Nucleotide sequencing was performed using Sequenase (US Biochemical Corp.) on DNA fragments subcloned into M13mp19. For the sequence of both strands of the complementing 4.3kb genomic fragment, nested series of deletions, in both directions, were made using exonuclease III according to the protocol of Henikoff (16). The sequence was analysed using the DNASTAR computer programs.

The sequence of the cDNA clones across the introns was determined in M13mp19 using the universal primer and oligonucleotides made to specific regions of the genomic sequence as alternative primers. These were:

O.3 (5'-ATGACTCCTACAATACGTATTCGTAC-3'),
O.11 (5'-ATGTTAATCTTCGGGACCTCGCAAG-3'),
O.13 (5'-AGTCTTCCTGAGAGAAAATGCCATGA-3'),
see figure 3.

Polymerase Chain Reaction

DNA sequences were amplified from cDNA made from *S. pombe* polyA$^+$ RNA, and from clones isolated from the cDNA library, using the polymerase chain reaction. Oligonucleotide sequences used as primers were as follows:

O.11 5'-ATGTTAATCTTCGGGACCTCGCAAG-3' upstream of intron I,
O.15 5'-GATGAGTCACTTTGATCAGTTTGCCA-3' spanning intron III,
O.17 5'-ATTTTATATGTTTTAATCACTCCGTGC-3' spanning intron II,

cDNA was synthesised with murine Moloney leukemia virus reverse transcriptase (Pharmacia) using a modification of the procedure of Yang *et al.* (17). To prime the reaction 1.5μg polyA+ RNA were annealed with 50ng oligo dT by heating to 80°C for 3 min and incubating at 37°C for 5 min. After cDNA synthesis for 1 hr at 37°C, 10% of the cDNA was used directly for PCR analysis.

PCR (18) was carried out for 30 cycles with 1 min at 94°C, 2 min at 58°C and 2.5 min at 70°C. 100ng plasmid DNA was used to amplify sequences from the cDNA clones, 1ng and 100ng genomic DNA used as control.

Pulse Field Gel Electrophoresis and General Molecular Methods

Molecular biology methods were as described in Maniatis *et al.* (13). Preparation of high molecular weight DNA, *Not*I digestion and transagonal pulse field gel electrophoresis of *S. pombe* chromosomal DNA was performed according to the Beckman protocol using the Beckman TAFE system. *Not*I digested *S. pombe* chromosomal DNA was separated in two phases. The first stage consisted of 4 sec pulses at 170mA for 30 min and the second a 60 sec pulse at 150mA for 18 h. The gel was stained with ethidium bromide to visualise the DNA bands. DNA was denatured and transferred to a Hybond-N filter (Amersham, UK).

Irradiation of *S.pombe* Cells

Gamma irradiation was routinely carried out using a ^{60}Co gamma source, with a dose rate of 5 Gy/min, on 1×10^7 cells/ml in minimal medium with appropriate supplements. The cells were then plated on minimal medium plates and colonies counted after 3 days at 30°C. Patch tests were carried out on

Table 1. *S. pombe* strains

sp.037	*rad9.192*, h^-: from the Canadian National Cell Repository.
sp.011	*leu1.32*, *ade6.704*, *ura4.D18*, h^-: from Paul Nurse, Oxford
sp.012	*leu1.32*, *ade6.704*, *ura4.D18*, h^+: from Paul Nurse, Oxford.
sp.014	*lys2.97*, h^-: from the National Yeast collection, Norwich.
	Strains created for this study:
sp.039	*rad9.192*, *leu1.32*, *ade6.704*, *ura4.D18*, h^-.
sp.016	*lys2.97*, *ade6.704*, *leu1.32*, *ura4.D18*, h^-.
sp.078	*leu1.32*, *ade6.704*, *ura4.D18*, *rad9*$^+$/*rad9*$^+$ + *sup3.5*, h^+.
sp.101	*leu1.32/leu1.32*, *ade6.704/ade6.704*, *ura4.D18/ura4.D18*, h^+/h^+.
	Deletion strains created:
sp.086	*leu1.32/leu1.32*, *ade6.704/ade6.704*, *ura4.D18/ura4.D18*, *rad9/rad9::ura4*, h^+/h^+.
sp.102	*leu1.32/leu1.32*, *ade6.704/ade6.704*, *ura4.D18/ura4.D18*, *rad9/rad9::ura4*, h^{90}/h^+.
sp.069	*leu1.32*, *ade6.704*, *ura4.D18*, *rad9*$^+$/*rad9::ura4*, h^-.
sp.070	*leu1.32*, *ade6.704*, *ura4.D18*, *rad9*$^+$/*rad9::ura4*, h^+.
sp.071	*leu1.32*, *ade6.704*, *ura4.D18*, h^-.
sp.072	*leu1.32*, *ade6.704*, *ura4.D18*, h^+.

sp.069-sp.072 are the products of a single tetrad of sp.102.

Table 2. Plasmids

pUR19	pUC19 based *ura4 ars6* plasmid (9).
pSTA12	pUC12 based *sup3.5* plasmid (7).
pDB262	*LEU2* 2μ plasmid (11).
p9/4	4.3kb *Hin*dIII *rad9.192* complementing insert in DB262.
pRAD9	2.6kb *Hin*dIII-*Eco*RV fragment from p9/4 in pUR19.
pS9	4.3kb *Hin*dIII fragment from p9/4 in STA12.
pS9U4	pS9 with *Cla*I-*Mlu*I replaced with 1.7kb *ura4* fragment.
pA4.5	A4.5 *Exo*III deletion in pUR19.
pS13	S13 *Exo*III deletion in pUR19.
pES13	*Eco*RI-end S13 deletion in pUR19.

6 cm plates that were lowered directly into the chamber of the source for a time calibrated to deliver a 500 Gy dose.

UV irradiation was carried out in a Stratagene 'Stratalinker' directly on freshly plated cells. For the survival curves, four plates were counted for each point and the analysis repeated at least twice.

RESULTS

Complementation of the *rad9* Mutation

An *S. pombe* genomic library containing partially *Hin*dIII digested DNA in the shuttle vector pDB262 was used to transform the *rad9.192* strain sp.039. 40 000 transformants were selected for their ability to grow in the absence of leucine and the cells were then irradiated with a dose of 500 Gy in a ^{60}Co source, to select for plasmids complementing the radiation sensitivity. At this dose the difference in survival between wild type and *rad9.192* cells was maximal. After a 500 Gy dose, approximately 80% of wild type cells, but less than 1% of *rad9.192* cells, can form colonies (see fig 2a). After three rounds of selection, individual colonies were patched out on 6 cm minimal plates and tested for sensitivity to gamma-radiation. Resistant clones were then tested for co-instability of the leu$^+$ and rad$^+$ phenotypes. This indicated that the complementation was due to plasmid-borne sequences rather than to mutations in the genome.

DNA was prepared from the resistant colonies for plasmid rescue into the *E. coli* strain JA226. Plasmids were rescued from two independent radiation resistant colonies. These were retransformed into the *rad9.192* strain sp.039 and found to complement the *rad9.192* phenotype. The complementing plasmids were identical, and contained four *Hin*dIII fragments of 4.3kb, 1.7kb, 1.5kb and 0.4kb. The *rad9* complementing region was found, on routine subcloning, to be contained within the 4.3kb fragment, contained in plasmid p9/4. Further analysis localised the *rad9* gene to a 2.6kb *Hin*dIII-*Eco*RV fragment, in plasmid prad9 (see fig. 1A).

Mapping of the Chromosomal Locus of p9/4

The 4.3kb *Hin*dIII insert from p9/4 was subcloned into the non-*ars* plasmid pSTA12 (7) which contains the *sup3−5* suppressor

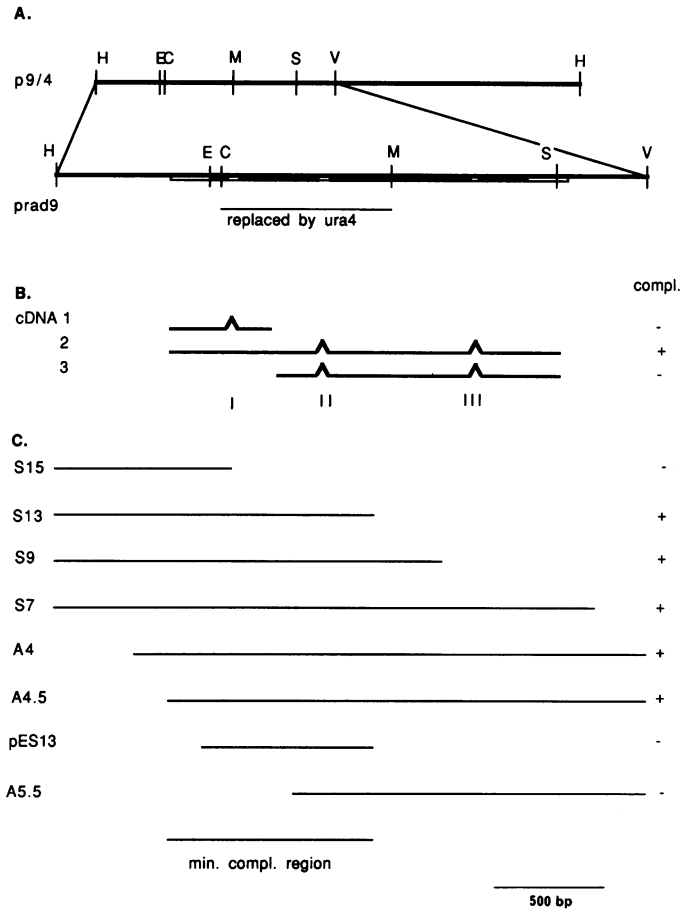

Figure 1. Physical maps of the rad9 region and complementation data. **a.** Restriction map of the 4.3kb *Hin*dIII insert of p9/4 and below this the restriction map of the 2.6kb *Hin*dIII-*Eco*RV subclone, prad9. H = *Hin*dIII, E = *Eco*RI, C = *Cla*I, M = *Mlu*I, S = *Ssp*I, V = *Eco*RV. The boxed line represents the transcribed region, the filled box the exons and the open box the untranslated leader, introns and 3′ sequence. The region (*Cla*I−*Mlu*I) replaced by a 1.7kb fragment containing the *ura4* gene in the disruption is indicated below the coding region. **b.** Diagram to show the position of three cDNA clones with respect to the genomic sequence. Introns spliced out of these sequences are indicated and the complementation analysis is given in the right hand column. **c.** The functional complementation data are drawn with respect to the prad9 map above. The regions present in the *Exo*III deletions S7, S9, S13, S15, A4, A4.5, A5.5 are represented by bars and the complementation analysis is given in the right hand column. The minimum complementing region (end of A4.5−end of S13) is shown.

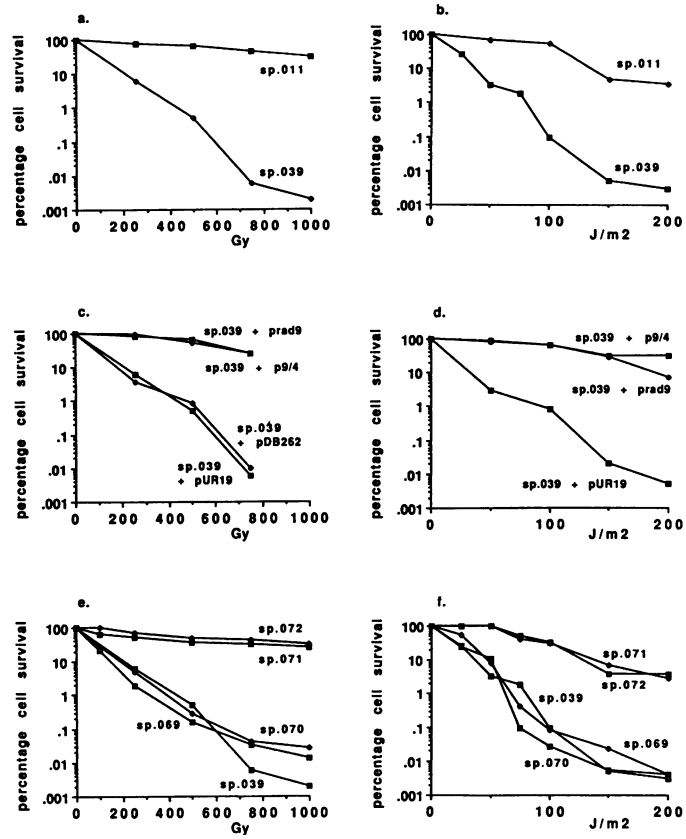

Figure 2. Survival curves of S. pombe strains following gamma or UV irradiation. **a.** and **b.** Survival of the rad$^+$ strain sp.011 and the *rad9.192* strain sp.039 following gamma- and UV-irradiation. **c.** Survival of strain the *rad9.192* strain sp.039 containing the complementing plasmids p9/4 and prad9 or the control vectors pUR19 and DB262, following gamma-irradiation. **d.** Survival of strain the *rad9.192* strain sp.039 containing the complementing plasmids p9/4 and prad9 or the control vector pUR19, following UV-irradiation. **e.** and **f.** Gamma- and UV-irradiation survival of the four products of a tetrad of the diploid deletion strain sp.102 and a *rad9.192* control, sp.039. The two null mutants are sp.069 and sp.070 and the two with wild type sensitivity to gamma-irradiation are sp.071 and sp.072.

tRNA that can suppress the *ade6.704* phenotype. This construct (pS9) was used to transform the rad$^+$ strain sp.011. Stable integrants were selected as white colonies on medium lacking adenine. One of these integrants, sp.078, was then crossed to the *ade6.704 lys2* strain sp.016. Spores were germinated on complete medium and the phenotypes of resulting colonies were scored with respect to adenine and lysine prototrophy. Table 3 shows that the site of integration is linked to the *lys2* locus at a distance of 3.9 cM. This is consistent with the published genetic map, which places *rad9* 4.3 cM from lys2 (19).

The chromosomal localisation was confirmed by pulse field gel electrophoresis using the Beckman TAFE system. The 4.3kb *Hin*dIII fragment containing the *rad9* gene was found to hybridise to a 1.2Mb fragment (data not shown). This is consistent with the position of the *rad9* locus on the *Not*I genomic map of *S. pombe* (20).

DNA Sequence Analysis and Expression of the *rad9* gene

The sequence of the entire 4.3kb *Hin*dIII fragment that complemented the *rad9* defect was determined in both directions by sequencing sets of overlapping clones, created by exonuclease III deletion, in M13mp19. The sequence was analysed using the DNASTAR computer program. Analysis of all six possible reading frames revealed two major open reading frames in a tail to tail configuration. One of these, consisting of about 200 amino acids, was located within the 2.6kb *Hin*dIII - *Eco*RV fragment that complemented the rad9 mutation (figure 3). (The second open reading frame, on the non-complementing portion of the 4.3kb insert, was found to be a homologue to the rat L7 ribosomal protein (21)).

The putative *rad9* ORF could be further extended if three introns were postulated on the basis of *S. pombe* intron consensus sequences (22). In order to further investigate this possibility an internal 1.5kb *Eco*RI - *Ssp*I fragment (figure 1a) was used as a probe to identify cDNA clones of *rad9* from an *S.pombe* cDNA library (8). A number of clones were identified and three of these were subcloned into M13mp19 for further analysis. Two were found to be truncated, one at the 3′ end and the other at the 5′ (see figure 1b). The third, presumed to be full length, was the only one to complement the *rad9.192* mutant strain. This contained a 1.6kb insert.

Further sequence analysis of the two large cDNA clones (cDNAs 2 and 3, figure 1b) using oligonucleotides made to specific parts of the sequence as primers, O.3 and O.13 (see figure 3), confirmed the absence in the cDNAs of sequences corresponding to the two introns (II nt 1147−1204 and III nt 1818−1874). Both introns have the consensus splice junctions but intron III has an unusual branch site to 3′ acceptor distance, resulting in a smaller intron than predicted from consensus splice data.

Sequence analysis of the small cDNA corresponding to the 5′ end of the gene (cDNA 1, figure 1b) indicated that splicing had occurred to remove bases 760−817, suggesting that these nt comprise intron I. However this sequence was not spliced in the complementing full length cDNA clone (cDNA 2, figure 1b) and it had a 5′ donor sequence that was divergent from the consensus (22).

Amplification of sequences by PCR from the full length cDNA clone and *S. pombe* cDNA, using oligonucleotides made to coding sequences flanking the splice junctions of introns II or III, as primers, confirmed the use of these splice sites in the *S. pombe* RNA. The fact that PCR products could be obtained from cDNA but not from genomic DNA using these spanning primers indicates that the introns are spliced out in the RNA and also

Table 3. Random spore analysis of a cross between an integrated *rad9 + sup3.5* strain (sp.078) and an *ade6.704 lys2.97* strain (sp.016)

no. of colonies analysed					
ade$^+$lys$^+$	ade$^+$lys$^-$	ade$^-$lys$^+$	ade$^-$lys$^-$	total	% recombinants
1015	41	38	980	2074	3.9%

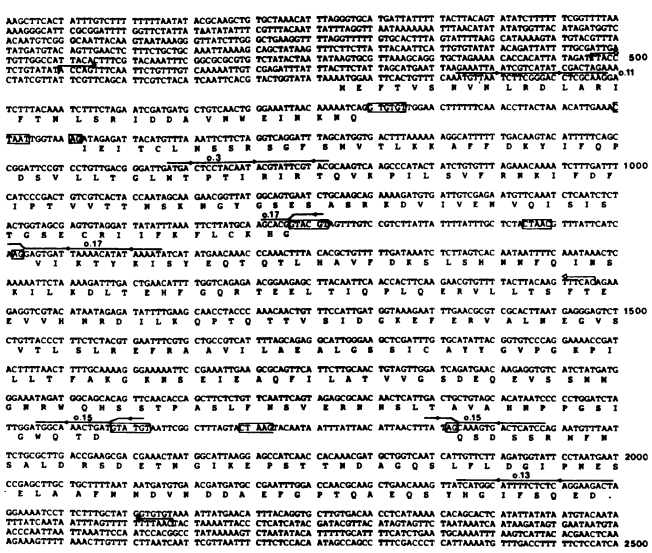

Figure 3. Nucleotide and putative amino acid sequence of the rad9 coding region. Amino acids are shown in single letter code from the putative start of translation. Consensus splice signals, including the putative branch sites are boxed. The sites of initiation and termination of the full length cDNA are indicated by solid headed arrows and the minimum region necessary for functional complementation defined by open headed arrows. The positions of the oligonucleotides are overlined with the 5′-3′ direction given by internal arrows. The EMBL accession number is X58231.

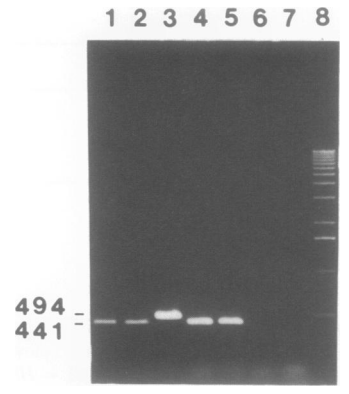

Figure 4. Analysis of intron usage. The products of PCR amplification using oligonucleotides O.11 and O.17, electrophoresed on a 0.8% agarose gel. The PCR reactions were as follows: 1. and 2. 150ng two different batches of *S. pombe* cDNA. 3. 100ng unspliced, *rad9.192* complementing cDNA clone (cDNA 2: figure 1B). 4. 100ng spliced cDNA clone (cDNA 4). 5. 100ng cDNA library DNA. 6. and 7. 1ng and 100ng *S. pombe* genomic DNA. 15% of the reaction mixture was loaded per track. The size markers in track 8. are a 1kb ladder (BRL). Oligonucleotide O.11 is homologous to sequences upstream of intron I, O.17 spans intron II. The sizes of the PCR bands are given in base pairs.

eliminates the possibility of contaminating DNA obscuring the result (see figure 4). The major PCR product, seen in cDNA and in DNA from the cDNA library, using oligonucleotides O.11 (upstream of intron I) and O.17 (spanning intron II) is about 440bp. This is compatible with the predicted product of 441bp, expected if intron I is spliced out. A band of slightly higher molecular weight (494bp) is seen in the unspliced cDNA clone (cDNA 2, figure 1B) and also as a minor band in cDNA and the cDNA library. This is consistent with intron I being unspliced in a minority of products, as shown by our isolation of a full length cDNA clone containing an unspliced intron I.

In the truncated cDNA clone with a splice at intron I, the AG at 815 is used as splice acceptor (this was determined by sequence analysis of both strands), this splicing does not bring an upstream exon into frame with the main ORF. However, sequence analysis of PCR products derived from two further cDNA clones showed that the splice acceptor was the AG at 811, giving rise to a 445bp PCR product. When this is the case an ORF of 35 amino acids is spliced in frame onto the main ORF. Thus intron I extends from nt 760−813.

It is likely that the start of the complementing cDNA clone (cDNA 2, figure 1b) at 510 is the 5′ start of transcription of the *rad9* gene, as the truncated cDNA clone (cDNA 1, figure 1b) started at the same base. No obvious TATA motif is present upstream of this putative start site in the genomic sequence. However, this is not unusual in *S. pombe* genes, especially those that are expressed at low levels (22).

From these studies we conclude that the mRNA starts at base 510 and that initiation of protein synthesis is probably at 655, giving a maximum sized predicted protein of 427 amino acids if all three introns are spliced out. This leaves an untranslated leader region of 145bp upstream of the putative initiation codon. The putative *rad9* polypeptide does not exhibit a codon usage bias as calculated according to the method of Russell and Hall (23). This suggests that the *rad9* gene is not expressed at a high level. Low levels of expression have also been found for most *S. cerevisiae* DNA repair genes (24).

Northern blot analysis indicates that the *rad9* gene is indeed expressed at a low level, as a transcript was only visualised with 5μg polyA$^+$ RNA. A single band at 1.6kb was seen with polyA$^+$ RNA when probed with the internal 1.5kb *Eco*RI−*Ssp*I fragment (figure 5). This is consistent with the size of the complementing clone isolated from the cDNA library.

The predicted *rad9* ORF would encode a protein with a molecular weight of 47512D and an isoelectric point at pH 5.15.

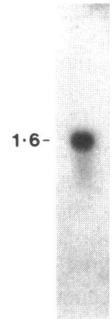

Figure 5. Northern blot analysis of the rad9 transcript. 5μg polyA$^+$ RNA was probed with a 1.5kb *Eco*RI-*Ssp*I fragment from prad9. *Eco*RI *Hin*dIII digested single stranded lambda was used as size markers (not shown). From this the estimated size of the transcript is 1.6kb.

Computer searches, using the DNASTAR programmes and TFASTA at the Daresbury Laboratory, of the EMBL and PIR data bases for homology to the predicted *rad9* protein revealed no significant homology to any known protein or class of protein.

Mapping the Functional Limits of the *rad9* Gene

In order to map the functional limits of the *rad9* gene, the ExoIII deletions of the *rad9* sequence in mp19 were cotransformed with pUR19, into the *rad9.192* mutant strain (sp.039). Transformants were selected for their ability to grow in the absence of uracil and then replica plates compared for the ability of colonies to grow after UV irradiation at a Stratalinker setting of 300 Jm^{-2}. The smallest region that complemented the UV sensitivity was found to extend from nucleotide 496 to nucleotide 1397, in exon III. (see figure 1c for details of the deletions used). This was confirmed by repeating the functional analysis with subclones of deletions A4.5 and S13 (see figure 1c) in pUR19. These plasmids complemented the UV sensitivity when introduced into the *rad9.192* strain. This implies that only the 900bp region containing the first two exons are required to complement the mutant phenotype.

The region from the *Eco*RI site, at nucleotide 658, to the end of the S13 deletion, at nucleotide 1397 (pES13, figure 1c) was found not to complement the UV sensitivity of the *rad9.192* strain. *Eco*RI cuts one base inside the proposed initiating ATG in exon I, supporting the view that this exon is necessary for a functional *rad9* protein.

Gene Deletion

A null allele of the *rad9* gene was constructed in order to test the deletion phenotype of the gene. A plasmid construct was made in which the 0.76kb *Cla*I to *Mlu*I fragment (see figure 1a) was replaced with a 1.8kb DNA fragment containing the *ura4* gene (25). The resulting 3.5kb *Hin*dIII to *Eco*RV fragment was then used to transform the diploid strain sp.101 to uracil prototrophy. Colonies not requiring uracil were tested for stability and chromosomal DNA was prepared from stable clones. Southern blot analysis of one such stable transformant, sp.086 (figure 6) showed that the fragment had integrated into the chromosome by homologous recombination. Hybridisation of *Hin*dIII digested DNA from the rad$^+$ strain sp.011 with prad9 revealed a single 4.3kb band. A similar digest of the diploid strain heterozygous for the deletion, contained this band and an additional 5.3kb band.

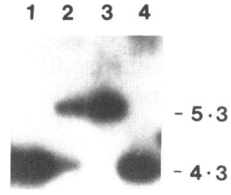

Figure 6. Southern blot analysis of the null allele of rad9. DNA from the rad$^+$ strain sp.011 (track 1), the diploid deletion strain sp.086 (track 2), and two products of meiosis of a h^{90} derivative of sp.086, one ura$^+$ (sp.069) (track 3) and the other ura$^-$ (sp.071) (track 4), was digested with *Hin*dIII, southern blotted and hybridised with the 2.6kb *Hin*dIII-*Eco*RV fragment from prad9.

Table 4. Random spore analysis of the deletion mutant sp.086

no. of colonies analysed				
rad^+ura^+	rad^+ura^-	rad^-ura^+	rad^-ura^-	total
0	857	903	0	1760

This is the result of the disruption of one of the two copies of the *rad9* gene by the *ura4* gene. An h^{90}/h^+ derivative (sp.102) was then selected and used to generate haploid spores.

The spores were then subjected to random spore analysis (Table 4) and tetrad analysis. Ten tetrads were dissected and all showed a 2:2 segregation of uracil prototropy. Ura^+ was always associated with radiation sensitivity. This showed that the the *rad9* gene is not essential for mitotic growth.

Southern blot analysis (figure 6) showed the 5.3kb *ura4*-containing band was inherited with the radiation-sensitive phenotype in one product of meiosis (sp.069) of the diploid deletion strain, while the ura- product (sp.071) inherited the 4.3kb band. This formally shows that the sp.069 deletion strain contains a disrupted copy of the *rad9* gene.

Complementation Data

The *rad9.192* strain is sensitive to both UV and gamma irradiation. At a fluence of 200 Jm^{-2} less than 0.01% of *rad9.192* cells survive, compared to about 10% of wild type cells. Similarly, with ionising radiation, at 1000 Gy less than 0.01% of *rad9.192* cells survive, compared to about 50% survival of wild type cells. The survival curves following UV or gamma radiation, of the *rad9.192* strain sp.039 and the equivalent rad^+ control strain, sp.011, are shown in figures 2a and b.

The gamma-radiation and UV irradiation survival curves of the *rad9.192* strain sp.039 containing the complementing plasmids p9/4 and prad9, are shown in figures 2c and 2d. p9/4 is the 4.3kb *Hin*dIII fragment in DB262 and prad9 is the 2.6kb *Hin*dIII-*Eco*RV fragment subcloned from p9/4 in the vector pUR19. The presence of the plasmids increases the UV and gamma survival of cells to almost wild type levels. The gamma-radiation survival curves of the *rad9.192* strain sp.039 containing non-complementing vectors are also shown in figure 2c, for comparison. The equivalent UV irradiation survival curves are shown in figure 2d. No increase in survival after either UV or gamma irradiation was seen with either vector.

The null allele has also been subjected to the same analysis. The four products of a single tetrad were tested for survival after gamma- and UV-irradiation. The two null mutant strains (sp.069 and sp.070) show phenotypes virtually identical to the *rad9.192* allele and the two ura- strains (sp.071 and sp.072) show wild type survival (figure 2e and 2f).

DISCUSSION

We have cloned the *rad9* gene of fission yeast by complementation of the *rad9.192* strain with an *S. pombe* gene bank. Selection was carried out over three rounds of gamma-irradiation and the resulting resistant colonies were shown to be co-unstable for radiation resistance and leucine prototrophy. Thus the complementation resulted from plasmid borne sequences. Plasmid rescued from the resistant colonies complemented the radiation sensitive phenotype on reintroduction into the *rad9.191* mutant and the complementing region was localised to a 2.6kb *Hin*dIII-*Eco*RV fragment. This sequence was found to integrate at a genomic locus 3.9cM from the *lys2* gene and to hybridise to the expected 1.2Mb *Not*I fragment. This is consistent with the localisation of *rad9* on the genetic and long range maps of *S. pombe* (19, 20).

DNA sequence analysis of the 2.6kb *Hin*dIII-*Eco*RV fragment revealed the presence of an open reading frame which could be extended by the postulation of three introns. The existence of the introns was verified by the isolation of a full length, complementing, cDNA clone and by PCR and sequence analysis of this and other cDNA clones. These studies were necessary as the splicing of the *rad9* transcript proved to be contrary to the accepted consensus data. The results indicate that the *rad9* transcript starts at base 510, with initiation of protein synthesis at base 655. Three introns (I nt 760−813, II nt 1147−1204 and III nt 1818- 1874) are spliced out resulting in a predicted protein of 427 amino acids.

The third intron has an unusual branch site to 3′ acceptor distance, resulting in a smaller intron than predicted from consensus splice data (22). The consensus branch site, as found in intron II, is CTPuAPy, 11 to 23 bases upstream of the 3′ end of the intron (22). Intron III, however, contains the sequence CTAAG 35 bases upstream of the 3′ junction. This may be analogous to the situation found in the *cam1* intron which contains the sequence CTGAA starting 34 bases upstream of the 3′ junction (22).

The first intron also proved to be unusual. PCR analysis of cDNA using flanking sequences as primers, indicates that splicing occurs at this intron in the majority of cases. The first exon, with an ATG at nucleotide 655, is spliced into frame with the major ORF if the AG at 811 is used as the 3′ acceptor. However, sequence analysis of a truncated cDNA clone indicated that in this case the AG at 815 was used, suggesting that the first exon could not be functional. Further analysis of sequences amplified from two other cDNA clones indicates that the AG acceptor at 811 is utilised and we believe that the splicing seen in the truncated clone is aberrant.

Intron I contains a degenerate 5′ donor site (GTGTGT) as opposed to the consensus GTANGT (22) and this may explain the high level of message unspliced or mis-spliced at this intron. A transition in the third position has been shown to reduce the efficiency of splicing of a functional artificial intron in the *S. pombe ura4* gene (26). The full length cDNA clone, originally isolated from the cDNA library, is not spliced at this intron but is capable of complementing the *rad9.192* mutant. Therefore, either splicing of the transcript from this cDNA clone is completed *in vivo* or the first exon is not required for the DNA repair function. The non-complementation of a plasmid pES13, missing the ATG at 655 suggests that the first exon is required.

Alternatively, the non-splicing could be specific to a subpopulation of molecules, giving rise to an alternative protein. In this case the first exon could not be used and the *rad9* gene would contain an unusually long leader sequence, with translation starting at the ATG at base 855 in intron II. It is possible that exon I is not translated even when spliced and has a regulatory function as, for example, the untranslated first exon of the oncogene *c-myc* (27). However, this possibility awaits confirmation by N-terminal sequence analysis of the *rad9* protein.

The size of the full length cDNA clone (1.6kb) is consistent with the size of the transcript seen in northern analysis of $polyA^+$ RNA when probed with the *rad9* sequence. However, the functional complementation data show that a 900bp fragment spanning the first two exons was sufficient to confer radiation

resistance to the *rad9.192* mutant. This suggests that the mutation in the *rad9.192* strain is contained within the first two exons and that only this region is required for the protein's role in DNA repair. It is possible that the *rad9* protein has more than one role and that different functions are executed by different domains of the protein. However, none of these roles are essential for mitotic growth under normal conditions, as the *rad9* null mutant is viable. The null mutant, which has the same radiation sensitive phenotype as the *rad9.192* mutant, has most of the first exon, all the second exon, and most of the third replaced by the *ura4* gene and therefore the protein is unlikely to be active.

Analysis of the 427 amino acid sequence using the DNASTAR computer program revealed no sequence motifs common to known classes of proteins and no significant homology to any known sequences was found during searches of the EMBL and NBRF-PIR data bases using the TFASTA program at the Daresbury Laboratory. Thus, the *rad9* gene, presented here, like the *S. pombe rad1* gene (28), is a novel DNA repair gene.

The *S. pombe rad9.192* and *rad1.1* mutants are, like most *S. pombe* repair deficient mutants, highly sensitive to both UV and ionising radiation (1). This is in contrast to the situation in *S. cerevisiae* in which the majority of mutants are sensitive to either UV or ionising radiation (24). This may reflect a difference in response to DNA damage between the two organisms. *S. pombe*, unlike *S. cerevisiae*, spends the greater part of its cell cycle in G2, where a replicated genome is available for recombination repair, and thus, may preferentially repair its DNA damage by a recombination mechanism, while in *S. cerevisiae* the dominant form of repair may be excision repair. This may be reflected in the differences in types of DNA repair mutants isolated in the two organisms. However, there is evidence that repair pathways are conserved between the two highly divergent yeasts, as a homologue to the *S. cerevisiae* gene *RAD6*, which codes for a ubiquitin carrier protein, has been identified in *S. pombe* (29). We also have evidence that at least three further DNA repair genes, all in the excision repair pathway are conserved between the two yeasts (30, 31). It will be of interest to determine whether the *rad9* gene is conserved across species boundaries. The identification of such genes would provide evidence for the evolutionary conservation of pathways other than excision repair.

Further work will include the characterisation of the *rad9* protein with a view to elucidating the role of this gene in DNA repair.

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