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The *rhp6*$^+$ gene of *Schizosaccharomyces pombe*: a structural and functional homolog of the *RAD6* gene from the distantly related yeast *Saccharomyces cerevisiae*

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The *RAD6* gene of *Saccharomyces cerevisiae* encodes a ubiquitin conjugating enyzme and is required for DNA repair, DNA-damage-induced mutagenesis and sporulation. Here, we show that *RAD6* and the *rhp6*$^+$ gene from the distantly related yeast *Schizosaccharomyces pombe* share a high degree of structural and functional homology. The predominantly acidic carboxyl-terminal 21 amino acids present in the RAD6 protein are absent in the *rhp6*$^+$-encoded protein; otherwise, the two proteins are very similar, with 77% identical residues. Like *rad6*, null mutations of the *rhp6*$^+$ gene confer a defect in DNA repair, UV mutagenesis and sporulation, and the *RAD6* and *rhp6*$^+$ genes can functionally substitute for one another. These observations suggest that functional interactions between RAD6 (rhp6$^+$) protein and other components of the DNA repair complex have been conserved among eukaryotes.

Key words: DNA repair/E2 enzyme/RAD6 gene/*rhp6*$^+$ gene/*Schizosaccharomyces pombe*

Introduction

The *RAD6* gene of *Saccharomyces cerevisiae* is involved in a variety of cellular processes. *rad6* mutants are highly sensitive to numerous DNA damaging agents, including UV, γ-rays and alkylating agents (Cox and Parry, 1968; Game and Mortimer, 1974; Prakash, 1974) and are defective in mutation induction by these agents (Prakash, 1974; Lawrence and Christensen, 1976; McKee and Lawrence, 1979). *rad6* mutants are defective in post-replication repair of UV damage: DNA strand discontinuities left during DNA replication in the newly synthesized DNA strand across from the non-coding UV lesion remain unrepaired in *rad6* mutants (Prakash, 1981). *rad6* mutants are also defective in sporulation (Game *et al.*, 1980; Montelone *et al.*, 1981), and they grow poorly and have poor plating efficiency.

The *RAD6*-encoded protein (M_r 19.7 kd) possesses a highly acidic carboxyl terminus in which 20 of the 23 residues are acidic (Reynolds *et al.*, 1985). The polyacidic sequence of RAD6 protein forms a disordered linear structure that is appended to the globular domain constituted by the first 149 residues (Morrison *et al.*, 1988). RAD6 protein is a ubiquitin-conjugating enzyme (E2) (Jentsch *et al.*, 1987) that mediates the attachment of multiple molecules of ubiquitin to histones H2A and H2B *in vitro* (Sung *et al.*, 1988). Multiple ubiquitination of histones may effect an open chromatin configuration, or it may mark histones for degradation by the ATP-dependent proteolytic system (Hershko *et al.*, 1984a,b; Hershko and Ciechanover, 1986). The acidic domain of RAD6 is required for the multiple ubiquitination of histones (Sung *et al.*, 1988). *rad6* mutants bearing a deletion of the acidic sequence fail to sporulate, but the DNA repair and UV mutagenesis functions are not affected (Morrison *et al.*, 1988). Mutation of the sole cysteine residue (Cys-88) in RAD6 to alanine or valine abrogates its E2 activity, and these mutants resemble *rad6* null mutants in being defective in DNA repair, UV mutagenesis and sporulation (Sung *et al.*, 1990), suggesting that RAD6 mediates all of its cellular functions via its role as an E2 enzyme.

Because of the central role of *RAD6* in DNA repair and in DNA-damage-induced mutagenesis, we have become interested in determining whether RAD6 is conserved among eukaryotes. A high degree of conservation of RAD6 would also suggest a parallel evolutionary conservation of proteins with which RAD6 might interact in its various cellular roles. In this paper, we report our studies with the RAD6 homolog from the evolutionarily divergent fission yeast *Schizosaccharomyces pombe*. Phylogenetic studies with 5S ribosomal RNAs indicate that *S.pombe* is evolutionarily closer to *Homo sapiens* than to *S.cerevisiae* (Huysmans *et al.*, 1983). *S.pombe* also resembles the higher eukaryotes in the control of the mitotic cell cycle (Russell and Nurse, 1986; Russell *et al.*, 1989), in the presence of introns in many of its genes and in the sequence requirements for the splicing of introns (Käufer *et al.*, 1985; Russell and Nurse, 1986). Therefore, a comparison of the structure and function of RAD6 from these two divergent yeast species should provide a good measure of evolutionary conservation of RAD6 among eukaryotes.

Our studies indicate a remarkable similarity in the amino acid sequences of the proteins encoded by the *S.cerevisiae RAD6* gene and by its homolog in *S.pombe, rhp6*$^+$ (rad homolog in *S.pombe*-6). The major difference between the two proteins is that the rhp6$^+$ protein lacks 21 carboxyl-terminal acidic residues present in RAD6. Like *rad6* mutations, null mutations of *rhp6*$^+$ confer a defect in DNA repair, mutagenesis and sporulation. We also show that the *RAD6* and *rhp6*$^+$ genes can functionally substitute for one another.

Results

Cloning of rhp6$^+$*, the S.pombe homolog of RAD6*

Southern blots of *S.pombe* genomic DNA were probed with the *S.cerevisiae* 0.5 kb *Eco*RI DNA fragment containing the *rad6-149* allele (Morrison *et al.*, 1988), in which the last 23 codons of the *RAD6* gene from nucleotide positions +448 to +516 (Reynolds *et al.*, 1985) are deleted. A single

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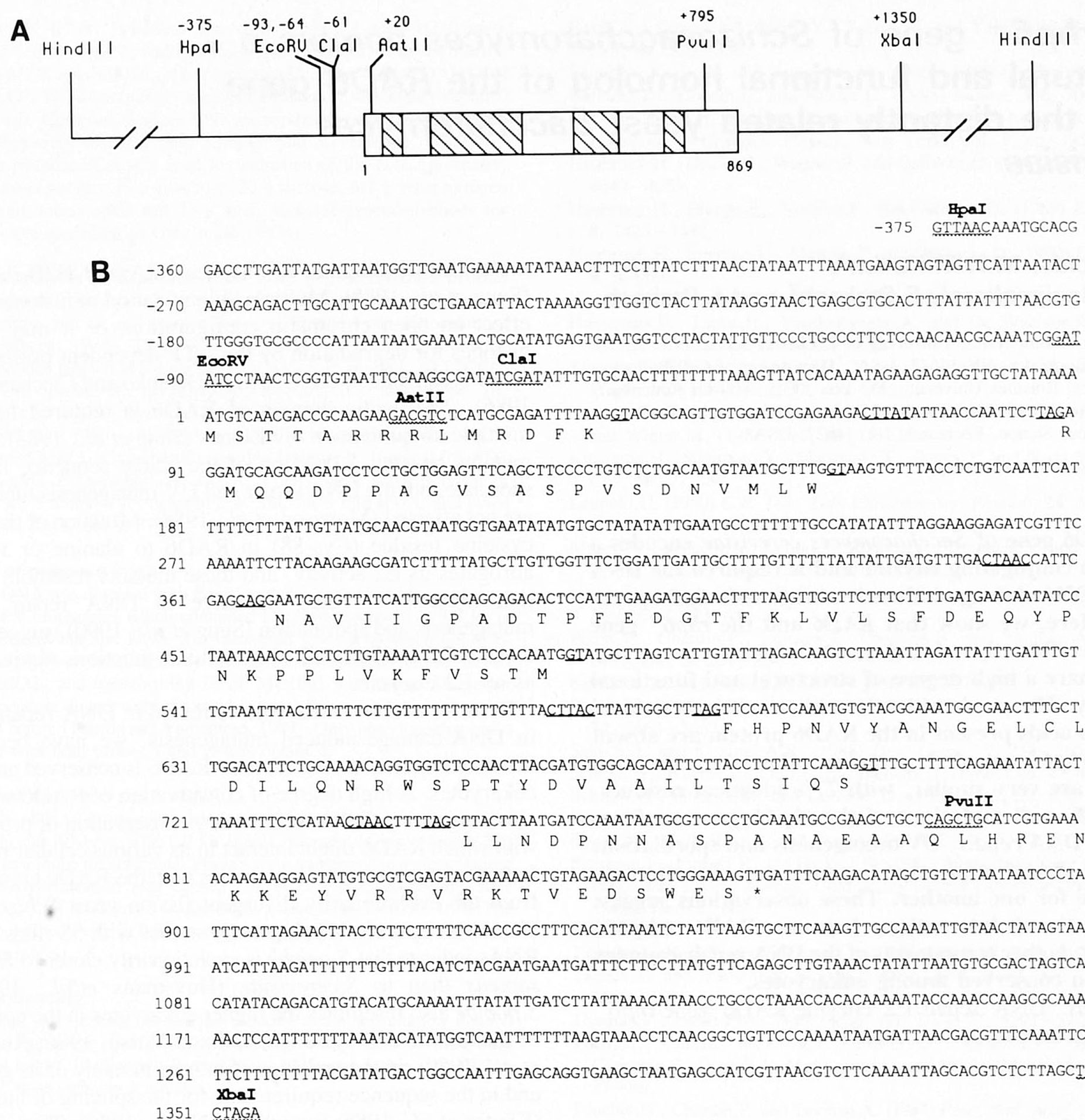

Fig. 1. Restriction map and nucleotide sequence of the *rhp6*$^+$ gene of *S.pombe*. (**A**) Partial restriction map of the 3.2 kb *Hin*dIII DNA segment originally isolated from the bacteriophage λ vector EMBL-3 harboring the *S.pombe* genomic sequence hybridizing to the *S.cerevisiae rad6-149* probe. Sequence analysis of the genomic region and of the cDNA synthesized by PCR predicted the exons (open boxes) and introns (hatched boxes). Numbers above the restriction sites refer to nucleotide position relative to the first ATG codon within the rhp6$^+$ ORF. (**B**) Nucleotide sequence of the *rhp6*$^+$ gene and amino acid sequence of its encoded protein. The first nucleotide of the first ATG codon in the rhp6$^+$ ORF is indicated at position 1. An asterisk marks the position of the termination TGA codon. Splice sequences in the introns are underlined. Restriction sites are marked by a wavy underline.

hybridizing band was detected in *Pvu*II, *Hin*dIII, *Pst*I or *Eco*RI digests of *S.pombe* genomic DNA when hybridization was carried out at 55°C in 1 M NaCl followed by two 5 min washes in 3 × SSC at 55°C and two 5 min washes in 1 × SSC at 55°C (results not shown). To isolate the *S.pombe RAD6* homolog, an *S.pombe* partial *Mbo*I genomic library was constructed in the λ vector EMBL-3 (Frischauf *et al.*, 1983) and screened with the *rad6-149* probe, using the hybridization conditions described above. Characterization of the DNA inserts in all 15 cross-hybridizing plaques obtained indicated that they originated from the same region of the *S.pombe* genome. The restriction map of the 3.2 kb *Hin*dIII fragment containing the *RAD6* homolog from *S.pombe rhp6*$^+$, is given in Figure 1(A).

Nucleotide sequence of the rhp6$^+$ *gene*

The *rhp6*$^+$ gene encodes a polyadenylated transcript of ~0.8 kb. The nucleotide sequence of *rhp6*$^+$ and its flanking regions is shown in Figure 1(B). The *rhp6*$^+$ open reading frame (ORF) is interrupted by the presence of four introns, all of which contain the consensus splicing signal sequences (Mertins and Gallwitz, 1987; Gatermann *et al.*, 1989). The 5′ splice site 5′-GTANGN-3′ is present in all the introns except for intron 4, which contains a T instead of an A at the third position. The branch sites have the conserved sequence 5′-CTPuAPy-3′, and this sequence is present 3–13 nt from the 3′ splice site PyAG. Nucleotide sequence analysis of *rhp6*$^+$ cDNA obtained by the polymerase chain reaction (PCR) (see Materials and

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MSTPARRRLMRDFKRMKEDAPPGVSASPLPDNVMVWNAMIIGPADTPYED  50   RAD6
||| |||||||||||| *|*|*||||||**||||*|||*||||||||*||
MSTTARRRLMRDFKRMQQDPPAGVSASPVSDNVMLWNAVIIGPADTPFED  50   rhp6+

GTFRLLLEFDEEYPNKPPHVKFLSEMFHPNVYANGEICLDILQNRWTPTY  100  RAD6
|||*|*| |||*|||||| |||*| |||||||||||*|||||||||*|||
GTFKLVLSFDEQYPNKPPLVKFVSTMFHPNVYANGELCLDILQNRWSPTY  100  rhp6+

DVASILTSIQSLFNDPNPASPANVEAATLFKDHKSQYVKRVKETVEKSWE  150  RAD6
|||*|||||||| |||| ||||| ||| | ** | *||*||* ||| |||
DVAAILTSIQSLLNDPNNASPANAEAAQLHRENKKEYVRRVRKTVEDSWE  150  rhp6+

DDMDDMDDDDDDDDDDDDDEAD                              172  RAD6

S                                                   151  rhp6+
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Fig. 2. Homology between the *S.cerevisiae* RAD6 and the *S.pombe* rhp6$^+$ proteins. The 172 amino acid long RAD6 protein is aligned with the 151 amino acid long rhp6$^+$ protein. The alignment of the two sequences is continuous throughout with the exception of the absence of the acidic carboxyl terminus in the rhp6$^+$ protein. Vertical lines between amino acids indicate identical residues and asterisks between amino acids indicate similar residues based on Dayhoff (1978).

methods) confirmed that all the introns are excised at these splice sites. The *rhp6*$^+$ ORF encodes a protein of 151 amino acids with an M_r of 17 097, containing 11.9% acidic and 12.6% basic residues. The predicted mol. wt of rhp6$^+$ protein is in good agreement with the 17 kd size estimated by SDS–PAGE.

Homology between rhp6$^+$ and RAD6 encoded proteins

The alignment of the amino acid sequences of the *S.cerevisiae RAD6* and *S.pombe rhp6*$^+$ encoded proteins is shown in Figure 2. RAD6 protein is 172 amino acids long and 20 of its 23 carboxyl-terminal residues are acidic, whereas the *rhp6*$^+$ encoded protein contains 151 amino acids, and lacks the polyacidic carboxyl-terminal sequence. The first 151 residues of RAD6 share a high degree of homology with the rhp6$^+$ protein sequence. The alignment of the two sequences is continuous throughout, and 77% of the residues in these two sequences are identical. Although conservation of residues extends throughout the RAD6 and rhp6$^+$ proteins, the similarity between them is greater in the first 127 residues, which show 82% identity, than in the terminal 24 residues, with 50% identity. When conservative amino acid replacements are grouped according to Dayhoff (1978), the similarity between rhp6$^+$ and RAD6 proteins becomes 90%. Both proteins contain a single cysteine residue at position 88. Mutational studies with cysteine 88 in RAD6 indicate that this residue is essential for the E2 activity (Sung *et al.*, 1990).

rhp6$^+$ function is required in DNA repair, UV mutagenesis and sporulation in S.pombe

The high degree of homology between the *rhp6*$^+$ and *RAD6* encoded proteins strongly suggested that they are functionally similar. To determine this, we constructed a null mutation of *rhp6*$^+$ (*rph6*Δ) in which the genomic *rhp6*$^+$ sequence from the *Eco*RV site at position −93 to the *Pvu*II site at position +795 (Figure 1B) was deleted and replaced by the *ura4*$^+$ gene of *S.pombe*. We examined the effect of the *rhp6*Δ mutation on growth, sensitivity to DNA-damaging agents, UV mutagenesis and sporulation. We found that *rhp6*Δ mutants have a much slower growth rate than the *rhp6*$^+$ wild-type strains (Figure 3), and *rhp6*Δ strains accumulate longer cells. The *rhp6*Δ mutants are sensitive to UV light (Figure 4) and to γ-rays and to the alkylating agent methyl methanesulfonate (results not shown). The

Fig. 3. Deletion mutation of the *rhp6*$^+$ gene of *S.pombe* causes slow growth of cells. Cells were streaked onto minimal medium and plates were incubated at 30°C for 3 days. **Top**: *rhp6*$^+$ haploid; **left:** *rhp6*Δ haploid; **bottom:** *rhp6*$^+$/*rhp6*$^+$ diploid; **right:** *rhp6*Δ/*rhp6*Δ diploid.

Fig. 4. Survival after UV irradiation of the *S.pombe rhp6*Δ haploid strain PRZ61 carrying various plasmids. Strains were grown on media for maintaining selection for the plasmid. Strains PRZ55 and PRZ61 are isogenic (Table III). Symbols: ●, PRZ55 (*rhp6*$^+$); △, PRZ61 (*rhp6*Δ); ○, PRZ61 + plasmid pRR413 (*rhp6*$^+$); ■, PRZ61 + plasmid pRR415 (*RAD6*); □, PRZ61 + plasmid pRR417 (*rad6-149*).

*rph6*Δ mutant is not as UV sensitive (Figure 4) as the *S.cerevisiae rad6*Δ mutant (Figure 6); however, the UV sensitivity of *rph6*Δ mutants is equal to that of the most UV-sensitive mutants of *S.pombe* (Schüpbach, 1971; Nasim and Smith, 1975). The lower sensitivity of the *rhp6*Δ strain to UV light than that of *rad6*Δ may reflect the fact that *S.pombe* is more radioresistant than *S.cerevisiae* (Phipps *et al.*, 1985).

To determine the effect of *rhp6*Δ mutation on UV mutagenesis, we examined the frequency of forward

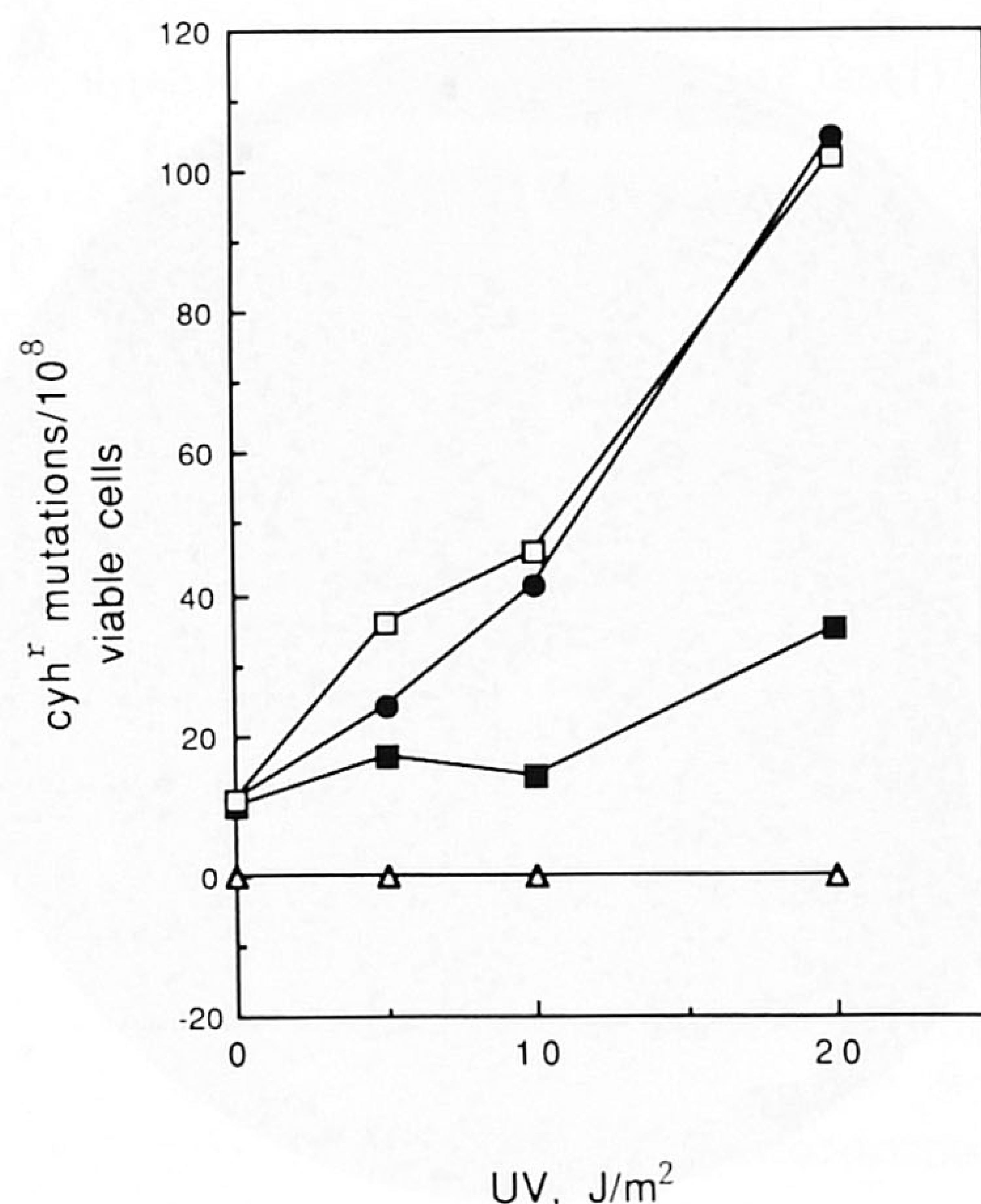

Fig. 5. UV-induced forward mutation to *cyh*^{r}. *rhp6Δ* strains carrying various plasmids were irradiated with UV light and the frequency of *cyh*^{r} mutants determined. Symbols: ●, PRZ55 (*rhp6*$^+$); △, PRZ61 (*rhp6Δ*); ■, PRZ61 + plasmid pRR415 (*RAD6*); □, PRZ61 + plasmid pRR417 (*rad6-149*).

Table I. Sporulation of the *S.pombe rhp6Δ/rhpΔ* strain in the presence of the *RAD6* or *rad6-149* gene

Strain	Genotype	% sporulation[a]
ZD6	*rhp6*$^+$/*rhp6*$^+$	57
ZD14	*rhp6*$^+$/*rhp6Δ*	54
ZD16	*rhp6Δ/rhp6+*	70
ZD18	*rhp6Δ/rhp6Δ*	0
ZD18 (pRR413)	*rhp6Δ/rhp6Δ* + *rhp6*$^+$	47
ZD18 (pRR415)	*rhp6Δ/rhp6Δ* + *RAD6*	29
ZD18 (pRR417)	*rhp6Δ/rhp6Δ* + *rad6-149*	43

[a]Based on a count of >500 cells for each strain.

mutations to cycloheximide resistance (*cyh*^{r}) in *rhp6*$^+$ and *rhp6Δ* strains. *cyh*^{r} mutations were induced by UV light in the *rhp6*$^+$ strain: at 10 J/m^2, the frequency of *cyh*^{r} mutants was 40/10^8 viable cells; and at 20 J/m^2, this frequency increased to >100/10^8 viable cells. In contrast, no UV-induced *cyh*^{r} mutants were observed in the *rhp6Δ* mutant strain (Figure 5).

To determine the role of *rhp6*$^+$ in sporulation, we examined sporulation in isogenic diploid strains *rhp6*$^+$/*rhp6*$^+$, *rhp6Δ/rhp6*$^+$ and *rhp6Δ/rhp6Δ* (Table I). Sporulation occurred at a frequency of 50–70% in *rhp6*$^+$ homozygous and heterozygous strains, whereas we observed no sporulation in *rhp6Δ/rhp6Δ* diploids. Thus, like the *rhp6Δ* mutation of *S.cerevisiae, the rhp6Δ* mutation of *S.pombe* results in defective DNA repair, UV mutagenesis and sporulation.

rad6-149 complements the rhp6Δ mutation of S.pombe more efficiently than the complete RAD6 gene

Next, we examined whether the *RAD6* gene of *S.cerevisiae* can functionally substitute for the *rhp6*$^+$ gene in *S.pombe*. Since the *rhp6*$^+$-encoded protein is devoid of the acidic carboxyl terminus, we also examined whether the rad6-149 protein lacking the carboxyl-terminal 23 predominantly acidic residues differs from the complete RAD6 protein in its capacity to complement the *rhp6Δ* mutation. To ensure adequate expression of the *RAD6* and *rad6-149* genes in *S.pombe*, these genes were placed downstream of the *rhp6*$^+$ promoter in the *S.pombe* vector pRR399 (see Materials and methods). As a control, the *rhp6*$^+$ gene was also cloned into this *S.pombe* vector. Western blots of total cellular protein from an *S.pombe rhp6Δ* strain transformed with these three plasmids—pRR413, pRR415 and pRR417—were probed with anti-RAD6 antibody. The RAD6, rad6-149 and rhp6$^+$ proteins were all present at about equal levels, and the amount of these proteins was somewhat higher than the amount of rhp6$^+$ protein present in the wild-type *S.pombe* strain (results not shown).

We examined the response to UV irradiation of an *rhp6Δ* strain carrying the *RAD6* or *rad6-149* gene (Figure 4). As expected, the *rhp6*$^+$ gene in plasmid pRR413 fully complements the UV sensitivity of the *rhp6Δ* mutation. The *rhp6Δ* mutant carrying the complete *S.cerevisiae RAD6* gene on plasmid pRR415 shows UV sensitivity that is intermediate between *rhp6Δ* and *rhp6*$^+$ strains, whereas a much higher level of UV resistance occurred with the *rad6-149* gene on plasmid pRR417.

Complementation for the UV mutagenesis defect of *rhp6Δ* by *RAD6* and *rad6-149* was tested by measuring the forward mutation frequency to *cyh*^{r} (Figure 5). The *rad6-149* gene restored wild-type levels of UV mutagenesis to the *rhp6Δ* strain, whereas the level of UV mutagenesis with the complete *RAD6* gene was intermediate between that of the wild-type *rhp6*$^+$ and *rhp6Δ* mutant strains.

The *rad6-149* gene restored nearly wild-type levels of sporulation in the *rhp6Δ/rhp6Δ* diploid, whereas sporulation was somewhat less efficient with the complete *RAD6* gene (Table I). The growth and morphology defects associated with the *rhp6Δ* mutation were also complemented to near wild-type levels by the *rad6-149* gene, and to a lesser extent, by the complete *RAD6* gene (results not shown). Thus, our observations clearly show that the *rad6-149* gene can carry out all of the functions of *rhp6*$^+$ in *S.pombe*. The lower efficiency with which the complete *RAD6* gene functionally substitutes for the *rhp6*$^+$ gene suggests that the polyacidic carboxyl-terminal region present in RAD6 interferes with its functioning properly in *S.pombe*.

The rhp6$^+$ gene complements the rad6Δ mutation of S.cerevisiae

We also examined whether the *rhp6*$^+$ gene complements the DNA repair, UV mutagenesis and sporulation defects of the *rad6Δ* strain of *S.cerevisiae*. Since *S.pombe* introns are spliced inefficiently in *S.cerevisiae* (Beach *et al.*, 1982; Booher and Beach, 1986), we cloned the *rhp6*$^+$ cDNA into *S.cerevisiae* low copy *CEN* and multicopy 2μ plasmid vectors (see Materials and methods). We also cloned the *rhp6*$^+$ cDNA downstream of the highly expressed *S.cerevisiae* alcohol dehydrogenase I (*ADC1*) promoter. These plasmids were introduced into the *S.cerevisiae rhp6Δ* strain, and the level of the rhp6$^+$ protein examined by Western analysis using the anti-RAD6 antibodies. The level of rhp6$^+$ protein in the *rad6Δ S.cerevisiae* strain carrying the *rhp6*$^+$ gene on the *CEN* plasmid pRR425 was about the same as the level of RAD6 protein present in the wild-type *S.cerevisiae* strain. The rhp6$^+$ protein level increased ~10-fold in *rad6Δ* cells

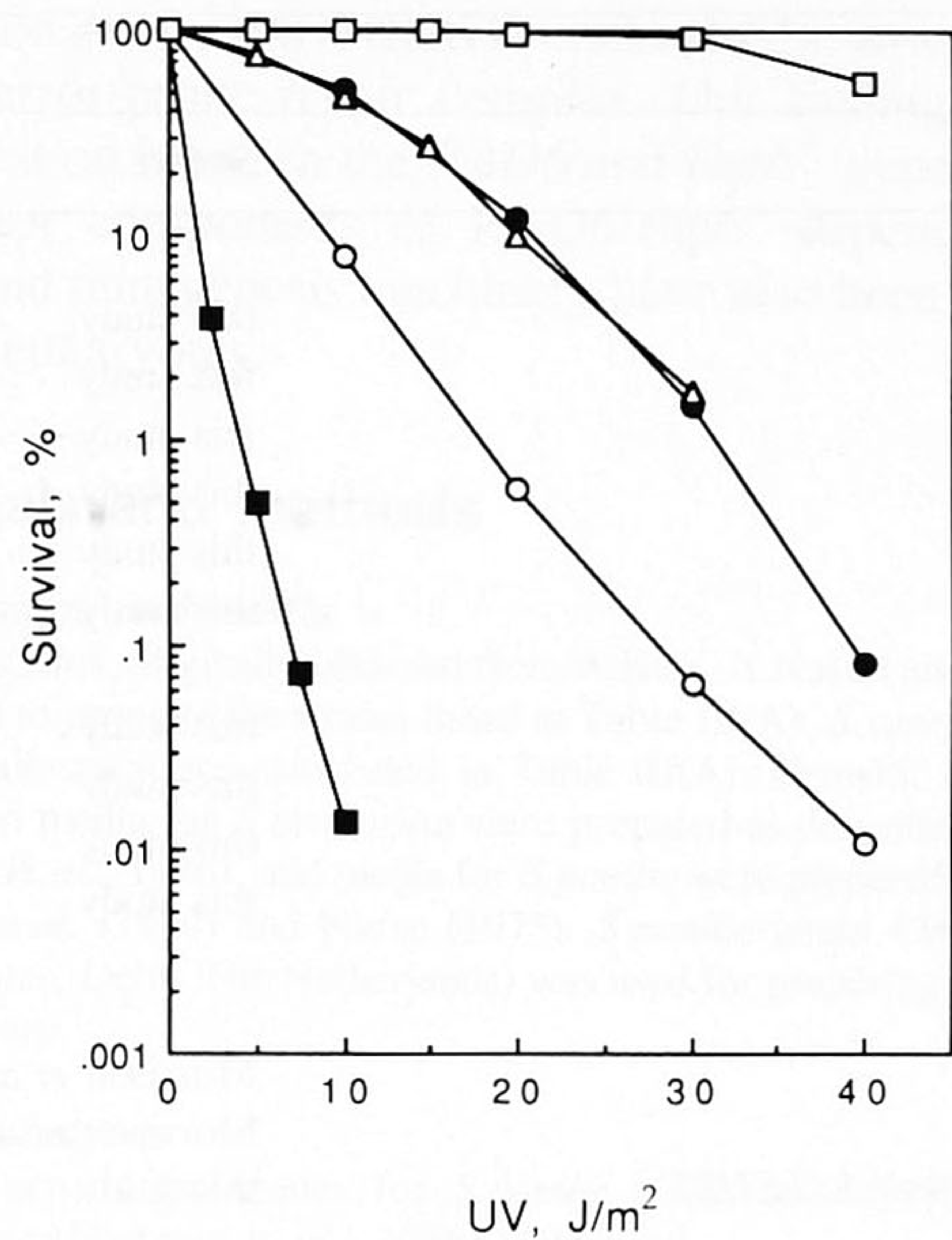

Fig. 6. Survival after UV irradiation of the *S.cerevisiae rhp6Δ* haploid strain EMY7 carrying various plasmids. Strains were grown on media for maintaining selection for the plasmid. Symbols: ■, EMY7 + plasmid pTB236 (2μ vector); ○, EMY7 + plasmid pRR425 (*CEN rhp6*$^+$); ●, EMY7 + plasmid pRR428 (2μ *rhp6*$^+$); △, EMY7 + plasmid pRR429 (*ADC1::rhp6*$^+$); □, EMY7 + plasmid pR67 (*CEN RAD6*).

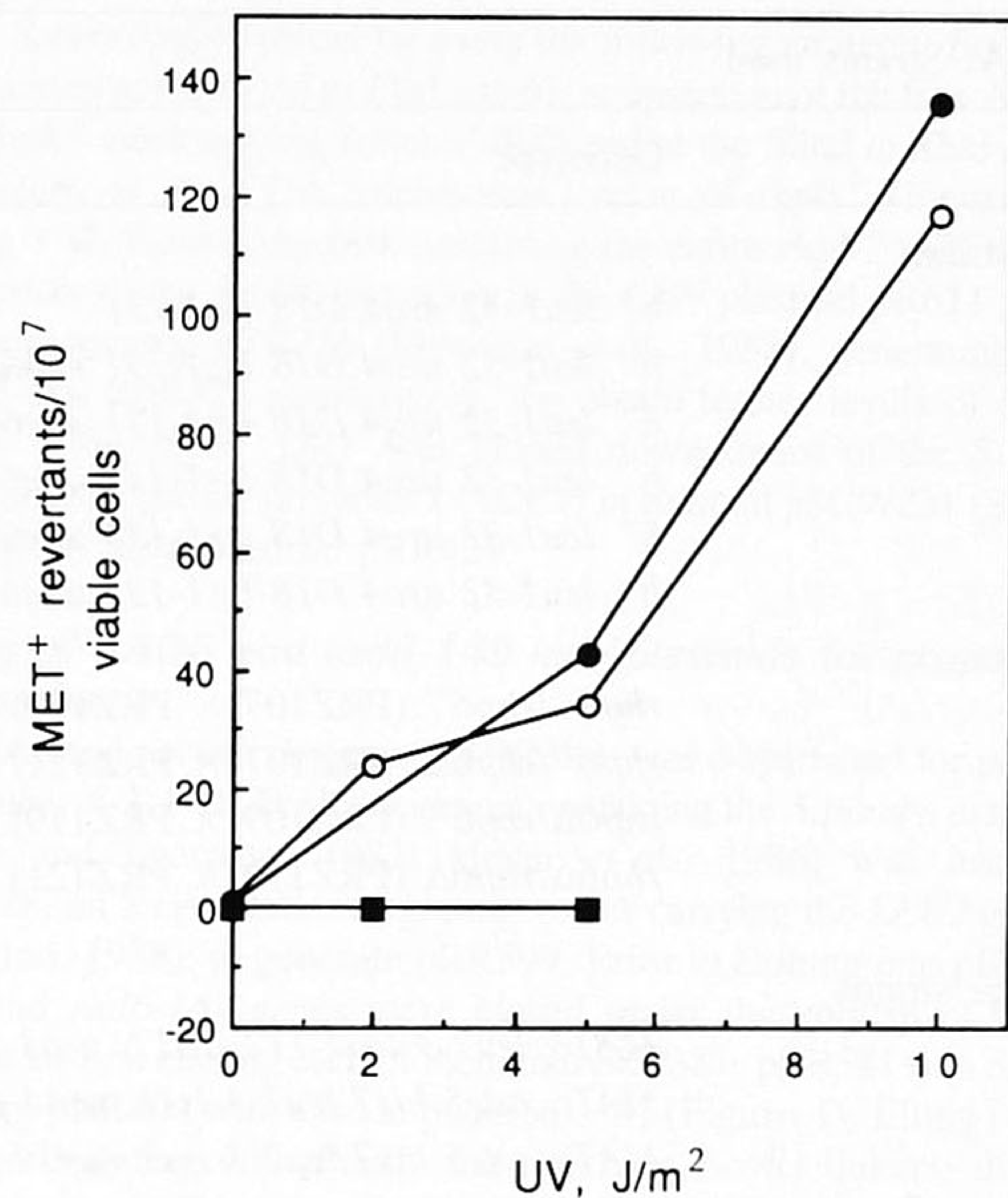

Fig. 7. UV-induced reversion of *met14* in the *rhp6Δ* strain EMY7 carrying various plasmids. Cells were irradiated with UV light and the frequency of *MET*$^+$ revertants determined. Symbols: ■, EMY7 (*rhp6*△); ●, EMY7 + plasmid pRR425 (*CEN rhp6*$^+$); ●, EMY7 + plasmid pR67 (*CEN RAD6*).

carrying the *rhp6*$^+$ gene on a 2μ multicopy plasmid pRR428, and a further ~10-fold increase occurred with plasmid pRR429 in which the *rhp6*$^+$ gene is fused on the *ADC1* promoter (results not shown).

The *rad6Δ* mutant is highly sensitive to UV light: at 10 J/m^2, survival is reduced 10^{-4}-fold (Figure 6). Transformation of the *rad6Δ* strain with the low copy *CEN rhp6*$^+$ plasmid pRR425 greatly enhanced the UV resistance of the *rad6Δ* strain, such that at 10 J/m^2, UV survival increased 10^3-fold to 10% (Figure 6). The *rad6Δ* strain carrying the multicopy 2μ *rhp6*$^+$ plasmid pRR428 showed a further increase in UV resistance; however, the UV resistance of these cells was still below the wild-type level. Additional overproduction of *rhp6*$^+$ protein by the *ADC1::rhp6*$^+$ plasmid pRR429 did not raise the UV resistance of *rad6Δ* cells further.

To examine whether the *rhp6*$^+$ gene can perform the UV mutagenesis function of *RAD6* in *S.cerevisiae*, we examined the reversion of a *met14* mutation in a *rad6Δ* strain carrying the *CEN rhp6*$^+$ plasmid pRR425. As shown in Figure 7, the *CEN rhp6*$^+$ plasmid restored UV mutability to the *rad6Δ* strain to the same extent as does the *CEN RAD6* plasmid pR67. Similar results were obtained with the 2μ *rhp6*$^+$ plasmid pRR428 and the *ADC1::rhp6*$^+$ plasmid pRR429 (results not shown).

Although the *rhp6*$^+$ gene of *S.pombe* restored the UV resistance and UV mutability to the *rad6Δ* strain, the sporulation defect of the *rad6Δ/rad6Δ* strain was not complemented by the *rhp6*$^+$ gene carried on the low copy *CEN* plasmid pRR425 (Table II). Little sporulation occurred with the *rhp6*$^+$ gene on the multicopy 2μ plasmid pRR428, but the introduction of the *ADC1::rhp6*$^+$ plasmid pRR429 in the *rad6Δ/rad6Δ* strain increased sporulation to 7% (Table II). Even though *rad6-149* mutants of *S.cerevisiae* are defective in sporulation (Morrison *et al.*, 1988), overproduction of the rad6-149 protein from the *ADC1* promoter

Table II. Sporulation of *S.cerevisiae rad6Δ/rad6Δ* diploids carrying the *rhp6*$^+$ gene on different plasmids

Plasmids		Vector	Gene	% sporulation[a]
S.cerevisiae	*S.pombe*			
pR611		CEN	*rhp6Δ*	0
pR67		CEN	*RAD6*	34
	pRR425	CEN	*rhp6*$^+$	0
	pRR428	2μ	*rhp6*$^+$	1
	pRR429	ADC	*rhp6*$^+$	7
pR619		ADC	*rad6-149*	12

All CEN and 2μ plasmids are in *S.cerevisiae* strain EMY26, whereas the ADC plasmids are in strain EMY28.
[a]Based on a count of >500 cells for each strain.

also conferred a low level of sporulation ability to the *rad6Δ/rad6Δ* strain (Table II).

Discussion

We have cloned the *rhp6*$^+$ gene of *S.pombe* and show that it bears strong structural and functional homology to the *RAD6* gene from the distantly related yeast *S.cerevisiae*. The rhp6$^+$ protein differs from RAD6 in not possessing the last 21 residues, of which 18 are acidic. The two proteins are highly homologous, sharing 77% identical residues and 90% similar residues when conservative replacements are grouped together. The conservation of RAD6 suggests that the other components of the ubiquitin conjugation pathway, such as the ubiquitin-activating enzyme E1, which transfers ubiquitin to a cysteine residue in the E2 enzymes, and the other E2 enzymes, are likely to be conserved among eukaryotes.

The biological functions of the *rhp6*$^+$ gene product in *S.pombe* are identical to those of *RAD6* in *S.cerevisiae*. Strains carrying null mutations of both genes are defective in DNA repair, UV mutagenesis and in sporulation. In addition, both mutations affect growth rate and plating

Table IIIA. Strains used

Strain	Genotype	Source
S.pombe strains		
PRZ55	*h⁻ leu1-32 ura4.D18 lys1-131*	this study
PRZ61	*h⁻ leu1-32 ura4.D18 lys1-131 rhp6Δ::ura4⁺*	this study
PRZ107	*h⁺ leu1-32 ura4.D18 lys1-131 ade6-210*	this study
PRZ109	*h⁻ leu1-32 ura4.D18 lys1-131 ade6-216*	this study
PRZ119	*h⁺ leu1-32 ura4.D18 lys1-131 ade6-210 rhp6Δ::ura4⁺*	this study
PRZ121	*h⁻ leu1-32 ura4.D18 lys1-131 ade6-216 rhp6Δ::ura4⁺*	this study
ZD6	*rhp6⁺/rhp6⁺* (PRZ107 × PRZ109)	this study
ZD14	*rhp6⁺/rhp6Δ* (PRZ107 × PRZ121)	this study
ZD16	*rhp6Δ/rhp6⁺* (PRZ109 × PRZ119)	this study
ZD18	*rhp6Δ/rhp6Δ* (PRZ119 × PRZ121)	this study
S.cerevisiae strains		
EMY1	*MATα leu2-3 leu2-112 trp1Δ ura3-52 rad6Δ::LEU2⁺*	Morrison *et al.* (1988)
EMY7[a]	*MATα ade5 his7 leu2-3 lys1 metl4 pet5 ura3 rad6Δ::LEU2⁺*	Morrison *et al.* (1988)
EMY8	*MATα ade5 his7 leu2-3 lys1 met14 pet5 ura3 trp1Δ::URA3⁺ rad6Δ::LEU2⁺*	this study
EMY26	*rad6Δ/rad6Δ* (EMY1 × EMY7)	
EMY28	*rad6Δ/rad6Δ* (EMY1 × EMY8)	

[a]EMY7 is isogenic with EMY8. They differ only in that EMY8 was made *trp1Δ* by replacing the *TRP1* gene with the *URA3* gene, thus making EMY8 Ura⁺.

Table IIIB. Plasmids used in this study

Plasmids	Gene; vector
pRR399	*S.cerevisiae LEU2⁺: S.pombe ars1* vector
pRR413	*rhp6⁺* in *S.pombe ars1* vector
pRR415	*rhp6⁺ promoter::RAD6* in *S.pombe ars1* vector
pRR417	*rhp6⁺ promoter::rad6-149* in *S.pombe ars1* vector
pRR425	*RAD6 promoter::rhp6⁺* in *S.cerevisiae CEN* vector
pRR428	*RAD6 promoter::rhp6⁺* in *S.cerevisiae 2μ* vector
pRR429	*ADC1 promoter::rhp6⁺* in *S.cerevisiae 2μ* vector
pR67	*RAD6* gene in *S.cerevisiae* CEN vector
pR611	*rad6Δ* gene in *S.cerevisiae CEN* vector
pR619	*ADC1 promoter::rad6-149* gene in *S.cerevisiae 2μ* vector

efficiency adversely. We find that the *rhp6⁺* and *RAD6* genes can functionally substitute for one another. In the presence of the *rhp6⁺* gene on a low copy plasmid, the UV resistance of the *S.cerevisiae rad6Δ* strain is greatly enhanced and UV mutagenesis occurs at wild type rates. The *rhp6⁺* gene did not complement the sporulation defect of the *rad6Δ/rad6Δ* strain, unless the *rhp6⁺* gene product was overproduced in high amounts from the *ADC1* promoter. The *rad6-149* allele, which resembles *rhp6⁺* in the absence of the polyacidic carboxyl terminus, also does not support sporulation (Morrison *et al.*, 1988) except when rad6-149 protein is overproduced from the *ADC1* promoter (Table II). The *RAD6* and *rad6-149* genes of *S.cerevisiae* differ in their ability to function in *S.pombe*. Interestingly, the complete *RAD6* gene functions less efficiently in *S.pombe* than the *rad6-149* gene. In the *rhp6Δ S. pombe* strain carrying the *rad6-149* gene, UV resistance increases to near wild-type levels, and UV mutagensis and sporulation occur at normal frequencies, whereas the complete *RAD6* gene provides a lower level of complementation of all these defects, indicating that the polyacidic carboxyl terminus of RAD6 inhibits its proper functioning in *S.pombe*. Thus, it appears that the *S.cerevisiae* DNA repair proteins have evolved to adapt the RAD6 polyacidic sequence.

The acidic carboxyl terminus of RAD6 is required for sporulation in *S.cerevisiae* (Morrison *et al.*, 1988) and for efficient polyubiquitination of histones *in vitro* (Sung *et al.*, 1988). Our observation that the rhp6⁺ protein lacking the polyacidic carboxyl terminus is essential for sporulation in *S.pombe* raises the possibility that the rhp6⁺ and RAD6 proteins ubiquitinate non-histone protein substrates in sporulation. The absence of the polyacidic sequence from the rhp6⁺ protein may mean that either polyubiquitination of histones is not as necessary for sporulation in *S.pombe* as in *S.cerevisiae*, or there is an alternate E2 in *S.pombe* that mediates histone polyubiquitination during sporulation.

The high degree of structural and functional homology between the *RAD6* and *rhp6⁺* genes lends credence to the idea that the other proteins with which RAD6 and rhp6⁺ proteins interact in mediating their different cellular roles have also been conserved during evolution. The various proteins involved in DNA repair and mutagenesis in *S.cerevisiae* with which RAD6 may interact could include the proteins encoded by genes in the *RAD6* epistasis group, such as RAD18, REV1, REV2 and REV3. The *RAD18*-encoded protein contains three putative DNA binding zinc finger domains and a Walker type A sequence for the binding and hydrolysis of purine nucleotide(s) (Jones *et al.*, 1988). Both *rad6* and *rad18* mutants are highly defective in post-replication repair of UV-damaged DNA (Prakash, 1981). Since the RAD6 protein by itself does not bind DNA (P.Sung, unpublished observations), presumably RAD6 is brought to the site of DNA damage via its interaction with other proteins that bind the damage sites in DNA. The RAD18 protein could be the damage recognition factor and the interacton of RAD6 with RAD18 could target RAD6 to the sites of DNA lesions, where it may facilitate repair via ubiquitination of chromosomal proteins. The *REV* genes are required for UV mutagenesis (Lemontt, 1971) and *REV3* encodes a protein that shows homology to DNA polymerases

(Morrison *et al.*, 1989). RAD6 could also be an integral part of the error-prone repair complex. Our finding of strong conservation between the *RAD6* and *rhp6*$^+$ genes suggests that other components of RAD6/rhp6$^+$-dependent DNA repair and mutagenesis machinery have also been conserved among eukaryotes.

Materials and methods

Yeast strains and media

S.pombe strains, originally obtained from A.Klar, A.Nasim and V.Simanis, were used to generate the strains listed in Table III(A). *S.cerevisiae* strains used in this study are also listed in Table III(A). Growth, minimal and sporulation media for *S.cerevisiae* were prepared as described previously (Sherman *et al.*, 1986), and media for *S.pombe* were prepared as described by Gutz *et al.* (1974) and Nurse (1975). *S.pombe* strain CBS356 (Yeast Stock Center, Delft, The Netherlands) was used for preparing the genomic DNA library.

Genetic analyses

Standard genetic techniques for *S.pombe* (Gutz *et al.*, 1974) and for *S.cerevisiae* (Sherman *et al.*, 1986) were used.

Transformation and other procedures

Yeast transformations were performed according to the method of Ito *et al.* (1983). *E.coli* transformations and DNA treatment were carried out by previously published methods (Maniatis *et al.*, 1982; Frischauf *et al.*, 1983).

Survival after UV irradiation and induction of mutations by UV light were as described previously (Morrison *et al.*, 1988).

Isolation of total RNA and poly(A) RNA from *S.pombe* and Northern hybridizations were as described by Madura and Prakash (1986). Polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970). Preparation of anti-RAD6 antibody and Western blotting were as described by Morrison *et al.* (1988).

The nucleotide sequence of the *rhp6*$^+$ gene was determined by the deoxy chain termination method of Sanger *et al.* (1977) using ([α-^{35}S]-thio)triphosphate (Biggin *et al.*, 1983). DNA fragments obtained by a variety of restriction enzymes recognizing six-base and four-base sequences were cloned into M13 derivative phages.

Construction of S.pombe plasmids and generation of a genomic rhp6$^+$ *deletion mutation in S.pombe*

To facilitate genetic manipulations with the *rhp6*$^+$ gene, the 3.2 kb *Hin*dIII DNA fragment containing the *S.pombe rhp6*$^+$ gene (Figure 1A) was cloned into pUC18 in which the *Aat*II site had been deleted and the 322 bp *Pvu*II fragment spanning the polylinker had been replaced by a *Hin*dIII site for cloning the 3.2 kb *Hin*dIII fragment, generating the plasmid pRR404.

Plasmid pRR394 contains the *rhp6*$^+$ gene on the 3.2 kb *Hin*dIII DNA fragment (Figure 1A), in which the blunt-ended 1.8 kb *Hin*dIII *ura4*$^+$ fragment (Grimm *et al.*, 1988) has replaced the *rhp6*$^+$ gene from the *Eco*RV site at −93 to the *Pvu*II site at +795 (Figure 1B). The resulting 4.1 kb *Hin*dIII fragment from pRR394 was used to transform *ura4.D18 S.pombe* strains to Ura$^+$. The slow growing transformants were examined by Southern blotting of genomic DNA and shown to carry the *rhp6*Δ mutation (results not shown). The frequency of genomic *rhp6*Δ mutations among Ura$^+$ transformants was ~2%.

Isolation of rhp6$^+$ *cDNA and cloning into S.cerevisiae vectors*

Plasmid pRR404 was gapped at the unique *Aat*II and *Pvu*II sites in the first and last exons of *rhp6*$^+$ (Figure 1). The gap was filled by a 362 nt *Aat*II−*Pvu*II fragment containing *rhp6*$^+$ cDNA prepared by PCR (Saiki *et al.*, 1985), using the protocol described by Rotenberg *et al.* (1989). The two oligonucleotide primers employed for PCR were 89.023: 5′-TTTCACGATGC**AGCTG**AGCA-3′, which hybridizes to *rhp6*$^+$ mRNA and spans the *Pvu*II site in the last exon of the *rhp6*$^+$ gene; and 89.024: 5′-ACCGCAAGAA**GACGTC**TCAT-3′, which hybridizes to the DNA strand coding for *rhp6*$^+$ mRNA and spans the *Aat*II site. The *Pvu*II site and the *Aat*II sites are indicated in bold letters in 89.023 and 89.024 respectively. The 362 nt reaction product was purified from an agarose gel and subjected to a second round of PCR. This amplified fragment was digested with *Aat*II and *Pvu*II and cloned into gapped plasmid pRR404, generating plasmid pRR405. The cDNA sequence of *rhp6*$^+$ in plasmid pRR405 was confirmed by dideoxy sequencing using oligonucleotides 89.023 and 89.024 as primers.

The *rhp6*$^+$ cDNA was cloned downstream of the *RAD6* promoter in various *S.cerevisiae* vectors by using the following strategy. *Eco*RI linkers were inserted at the filled in *Cla*I site 61 nt upstream of the first ATG codon in the rhp6$^+$ open reading frame (ORF) and at the filled in *Xba*I site 481 nt downstream of the TGA termination codon of rhp6$^+$ (Figure 1B) The resulting 1 kb *Eco*RI fragment containing the entire *rhp6*$^+$ ORF was cloned downstream of the *RAD6* promoter in the *CEN* plasmid pR611 and the 2μ multicopy plasmid pTB236 (Morrison *et al.*, 1988), generating plasmids pRR425 and pRR428 respectively. To obtain higher levels of expression of *rhp6*$^+$, the *rhp6*$^+$ ORF was cloned downstream of the *S.cerevisiae* alcohol dehydrogenase promoter I (*ADC1*) in plasmid pSCW231 (Sung *et al.*, 1987), generating plasmid pRR429.

Cloning of RAD6 and rad6-149 into plasmids for propagation in S.pombe

A new plasmid vector, designated pRR399, was constructed for propagation in *S.pombe*. A 1.1 kb *Eco*RI fragment containing the *S.pombe arsl* sequence (Losson and Lacroute, 1983; Heyer *et al.*, 1986) was inserted into YIplac128, an *S.cerevisiae* integrating vector carrying the *LEU2* gene (Gietz and Sugino, 1988), to generate pRR399. Prior to cloning into pRR399, the *RAD6* and *rad6-149* genes were placed under the control of the *rhp6*$^+$ promoter by first cloning each of them into pRR381. pRR381 was constructed by cutting pRR404 with *Cla*I at position −61 (Figure 1), filling in the *Cla*I site, then digesting with *Pvu*II, and attaching *Eco*RI linkers; this creates a gap deleting 85% of the *rhp6*$^+$ ORF. The *RAD6* gene on the 0.61 kb *Eco*RI fragment from positions −48 to +565, which includes the entire RAD6 ORF along with 49 5′ flanking nucleotides and 66 3′ flanking nucleotides (Reynolds *et al.*, 1985), and the *rad6-149* gene on the 0.57 kb *Eco*RI fragment (Morrison *et al.*, 1988) were then each inserted into the *Eco*RI site of pRR381, generating plasmids pRR409 and pRR411 respectively. The *rhp6*$^+$ promoter*::RAD6* and *rhp6*$^+$ promoter*::rad6-149* genes from plasmids pRR409 and pRR411 were cloned into pRR399 as 3 kb *Hin*dIII fragments, generating plasmids pRR415 and pRR417 respectively.

A summary of plasmids used in this study is given in Table III(B).

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