Allelism of PSO4 and PRP19 links pre-mRNA processing with recombination and error-prone DNA repair in Saccharomyces cerevisiae

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ABSTRACT
The radiation-sensitive mutant pso4-1 of Saccharomyces cerevisiae shows a pleiotropic phenotype, including sensitivity to DNA cross-linking agents, nearly blocked sporulation and reduced mutability. We have cloned the putative yeast DNA repair gene PSO4 from a genomic library by complementation of the blocked UV-induced mutagenesis and of sporulation in diploids homozygous for pso4-1. Sequence analysis revealed that gene PSO4 consists of 1512 bp located upstream of UBI4 on chromosome XII and encodes a putative protein of 56.7 kDa. PSO4 is allelic to PRP19, a gene encoding a spliceosome-associated protein, but shares no significant homology with other yeast genes. Gene disruption with a destroyed reading frame of our PSO4 clone resulted in death of haploid cells, confirming the finding that PSO4/PRP19 is an essential gene. Thus, PSO4 is the third essential DNA repair gene found in the yeast S.cerevisiae.

INTRODUCTION
At least 50 genes encoding proteins involved in DNA repair are known in the yeast Saccharomyces cerevisiae (1,2) and, since molecular studies on many of them have revealed a close relationship to DNA repair enzymes found in humans and mammals, this simple eukaryote has advanced to a model in which the complex mechanisms underlying DNA repair might be unraveled in the near future. The many DNA damage-sensitive yeast mutants were initially grouped into three phenotypic groups (3,4) which, by double mutant analysis that measured synergistic or epistatic interactions, were allocated to three epistasis groups (5–7). This classification more or less holds until today, with group I comprising ~20 genes that are RAD3-like and encode proteins for nucleotide excision repair, group II comprising ~12 genes that are RAD52-like and which seem to encode proteins involved in repair of DNA double-strand breaks via recombinational mechanisms and the largest, and by far the most heterogeneous, group III, defined by RAD6, comprising ~20 genes, many of which are responsible for error-prone DNA repair (1,2,8). Some DNA damage-sensitive mutants, e.g. several of the yeast mutants sensitive to photoactivated psoralens (pso mutants; 9) have been allocated to more than one epistasis group, depending on the mutagen applied and the biological end point scored in the respective double mutants (8). A mutant allele of gene PSO4, which is involved in error-prone repair and some types of recombination, i.e. gene conversion, crossing-over and intrachromosomal recombination (10,11,12), was originally isolated as X-ray-sensitive mutant xs9 (13); it was found to be particularly sensitive to photoactivated bifunctional 8-methoxy-psoralen and was thus renamed pso-4-1 (10). This yeast mutant is phenotypically similar to the recA mutant of Escherichia coli in that it combines mutation and radiation sensitivity with a block in recombination and induced mutagenesis. The pleiotropic repair phenotype caused by the singly existing pso-4-1 mutant led to allocation of PSO4 to more than one epistasis group (8,14).

More recent studies showed that heterologous expression of recA increased resistance to UV and ionizing radiation in S.cerevisiae wild-type but not in recombination-deficient rad52-1 mutant cells (15,16). However, recA-like yeast mutant pso-4-1 showed restored induced mutability after transformation with a multicopy vector containing the E.coli recA gene (17). Biochemical analysis proved normal incision of 8-MOP + UVA-induced interstrand cross-links (ICLS), but failure of DNA strand rejoining in pso-4-1 (16), which seems to depend on a recombinational step. The apparent role of gene PSO4 in DNA repair processes attributed to genes belonging to epistasis group II (recombination) and III (error-prone) makes it a very interesting candidate for our future understanding of interconnections between these repair processes in yeast. Progress in elucidating the function of Pso4 protein necessitated the molecular cloning of PSO4, which we wish to report in this communication.

MATERIALS AND METHODS
Yeast strains and plasmids
The yeast strains used are listed in Table 1. Single copy vector pRS316 (18) and multicopy vector pRS426 (19), both containing URA3 as selectable marker, were used for subcloning, complementation tests and for sequencing. The original complementing isolate pMG470 is based on the multicopy plasmid YES24 (20). Hence, subcloning was performed using multicopy vector pRS426

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Figure 1. (A) Subcloning of the psso4-complementing gene from the passenger DNA of pMG470 into multicopy plasmids. The restriction maps of the passenger DNAs of the pMG470-derived plasmids are shown. pMG470: the original clone isolated from the gene bank (20) harboring a 16 kb passenger. Only the 6.5 kb area that overlaps with the second positive clone, pMG490 (not shown), is depicted in detail. pMG471, pMG473 and pMG475; non-complementing subfragments of pMG470. pMG480: fragment containing just one gene, PRP19, that is still able to complement all phenotypes of a psso4-1 mutant. Abbreviations for cleavage sites: B/S, ligation site of BamHI and Sau3A; E, EcoRI; L, SalI; X, XbaI; Y, BstYI. +/–: Plasmid complements/does not complement a psso4-1/pso4-1 and a psso4-1/prp19-0 mutant. (B) Non-complementation of the psso4-1 and prp19-0 alleles. Diploids containing different allele combinations were constructed and tested for survival at 30 and 36°C by streaking cell suspensions on twoYEPD plates which were subsequently incubated at 30 and 36°C respectively. (C) Survival and mutagenesis after irradiation with UV254 light of heteroallelic psso4-1/prp19-0 diploid MG5101 transformed with either empty plasmid pRS426 (triangles) or pMG480 containing just PRP19 (squares) and the heterozygous psso4-1/PRP19 diploid MG5103 transformed with empty plasmid (circles) as positive control.

to also allow for screening of a possible multicopy suppressor of PSO4 (Fig. 1A).

Cloning of PSO4

Gene PSO4 was cloned by transforming diploid strain MG5128 homozygous for psso4-1 with a yeast genomic library (20) according to the lithium acetate-based protocol described by Gietz et al. (21). Due to the previously introduced heterozygous CAN1/canl marker it was possible to select for forward mutants homozygous for canl after replica-plating onto synthetic medium lacking both arginine and uracil and supplemented with 20 mg/l canavanine (SynCo–Arg–Ura+Can). After irradiation with UV254 light (20 J/m²) the plates were incubated at 30°C in the dark for 3–5 days. Clones with more than three papillae on SynCo–Arg–Ura+Can were isolated from the corresponding master plate and tested for the ability to sporulate.

Table 1. Saccharomyces cerevisiae strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
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<td>xs9</td>
<td>MATαlys2 psso4-1</td>
<td>A.C.Schenberg</td>
</tr>
<tr>
<td>W303</td>
<td>MATαMATαura1-1ura3-1 ade2-1ade2-1 rpl1-1rpl1-1 leu2-3,112his3-1,15his3-1,15 can1-100can1-100 PSO/PSO</td>
<td>R.Rothstein</td>
</tr>
<tr>
<td>MG5100</td>
<td>MATαMATαura1-1ura3-1 ade2-1ade2-1 rpl1-1rpl1-1 leu2-3,112his3-1,15his3-1,15 can1-100can1-100 PSO/psso4-1</td>
<td>This study</td>
</tr>
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<td>MG5101</td>
<td>MATαMATαura1-1ura3-1 ade2-1ade2-1 rpl1-1rpl1-1 leu2-3,112his3-1,15his3-1,15 can1-100can1-100 PSO/psso4-1</td>
<td>This study</td>
</tr>
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<td>MG5103</td>
<td>MATαMATαura1-1ura3-1 ade2-1ade2-1 rpl1-1rpl1-1 leu2-3,112his3-1,15his3-1,15 can1-100can1-100 PSO4/pso4-1</td>
<td>This study</td>
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<tr>
<td>MG5128</td>
<td>MATαMATαura1-1ura3-1 ade2-1ade2-1 rpl1-1rpl1-1 leu2-3,112his3-1,15his3-1,15 can1-100can1-100 PSO4/pso4-1</td>
<td>This study</td>
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</table>
Mutagenicity assay and survival

Exponentially growing cells cultivated in liquid synthetic medium supplemented with the appropriate amino acids and bases were washed and resuspended in phosphate buffer (67 mM, pH 7) to a final density of 10⁵ cells/ml and irradiated with different UV doses. For the determination of survival the cells were plated on complete medium (SynCo) and for the determination of forward mutations (can1) on SynCo without arginine and plus canavanine (20 mg/l) respectively.

DNA sequencing

DNA sequencing was performed as part of the framework of the European Union BIOTECH program to sequence the entire chromosome XII. A set of random subclones in plasmid cloning vector pUC18 was generated from the insert region of cosmid 1F17. These subclones served as the starting points for a primer walking strategy. Double-stranded template DNA of the cosmid and DNA of the subclones was purified using the QIAwell Purification System (Qiagen). Cycle sequencing reactions were carried out using Taq DNA polymerase and fluorescent terminators. Gel electrophoresis and automated base calling were done on an ABI 373A Stretch DNA Sequencer (Applied Biosystems). Contig assembly and DNA sequence editing were done using the DNASTAR software package (Lasergene).

RESULTS

Cloning of DNA repair gene PSO4

Diploids homozygous for mutant allele psd4-1 show higher than wild-type UV sensitivity, lower than wild-type UV-induced mutation and mitotic recombination and sporulate very poorly (<1% asc) on appropriate medium (8). PSO4 was molecularly cloned by screening for transformants in diploid MG5128 homozygous for psd4-1 and heterozygous for CAN1 (Table 1), in which inducibility of mutagenesis by UV₂₅₄ had been restored (CAN1→can1¹). Amongst 6000 transformants two were found to show wild-type-like UV-induced mutagenesis and furthermore restored sporulation (40% in the transformants and the respective wild-type versus a maximal number of <1% asci in the mutant) and restored resistance to DNA cross-linking mutagens. Restriction analysis of the two complementing plasmids, named pMG470 and pMG490, showed that the both passengers contained an identical 6.5 kb overlapping fragment (depicted in Fig. 1A). Hybridization of a 5'-2P-labeled 2 kb EcoRI fragment from this area (Fig. 1A, pMG475) revealed that the passengers were part of chromosome XII (not shown). Two subfragments obtained by restriction of plasmid pMG470 at the SalI site yielded the two plasmids pMG471 and pMG473 (Fig. 1A), both unable to restore the PSO phenotype (Table 2A), indicating that SalI is located within the psd4-1 complementing open reading frame (ORF) or its promoter. We determined the DNA sequence in this area and found a match with gene PRP19 (22), containing the SalI site. Further sequencing revealed that PSO4/PRP19 is located ~1.5 kb upstream of UBI4 and that it shares a promoter region of ~320 bp with another ORF (L0916, Fig. 1A) upstream of PRP19 encoding a hitherto uncharacterized protein containing a putative ATP/GTP binding site. Subcloning of a 2.4 kb fragment containing only gene PRP19 (pMG480, Fig. 1A) revealed that this gene alone is sufficient to restore the wild-type-like phenotype when transformed into a psd4 mutant (Fig. 1C and Table 2A).

The putative Pso4 protein

We determined the size of the PSO4/PRP19 gene to be 1512 bp, which corresponds to a 56.7 kDa protein consisting of 503 amino acids, instead of 502, as originally described for PRP19 by Cheng et al. (22), owing to one additional proline at position 239. Sequence analysis of the putative protein revealed one myb-like DNA binding domain [W(5ST)²X₄[DE]₅X₄[LIV]] at the C-terminus of the Psd4/Prp19 protein (positions 457–465, WTKDEESAL). The retroviral oncogene v-myc and its cellular counterpart c-myc encode nuclear DNA binding proteins. In S.cerevisiae, the myb-related genes include the DNA-binding proteins RBEI (23) and BASSI (24). However, the Psd4/Prp19 protein does not share any considerable homology with the putative proteins encoded by these two other yeast genes. The nucleotide sequence of PSO4 will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession no. X99770.

Construction and phenotype of a psd4 null allele

After in vitro disruption of the Psd4/Prp19-encoding ORF at the SalI site by inserting HIS3 (cf. Fig. 1), we performed one-step disruption experiments in haploid and diploid wild-type cells. While no haploid transformants with correct insertion could be found, diploid disruptants (MG5100, Table 2) showed only two surviving His auxotrophic ascospores upon sporulation and tetrad analysis. The absence of any His prototrophic spores confirmed the previously reported essentiality of the PSO4/PRP19 gene (22). However, it has never been demonstrated whether this essentiality is restricted to spore germination. Therefore, the heterozygous diploid MG5100 was transformed with pMG480 (URA3; PSO4), resulting in four viable spores after sporulation. Plasmid loss experiments with spores containing the HIS3 disruption marker did not yield any viable Ura auxotrophs, while haploid His auxotrophic non-disruptants showed plasmid loss of ~20% within 36 h. This indicates that the essentiality of PSO4 is not restricted to spore germination, since loss of the plasmid results in lethality of haploid disruptants. As essentiality could be verified with another construct using URA3 as reporter gene (not shown), the disrupted alleles will be generally named psd4-0 for the purpose of simplification during discussion. PSO4 is allelic to PRP19

Besides the fact that gene PRP19 alone was sufficient to complement the psd4-1 mutation, final evidence for the allelism of the cloned ORF with PSO4 was obtained by showing that null allele prp19::HIS3 cannot complement any phenotype of the psd4-1 mutant in a heteroallelic diploid which was constructed by crossing the original psd4-1 mutant x9 with the haploid prp19::HIS3 disruptant MG5100-1C of opposite mating-type, the latter containing PSO4-harboring plasmid pMG480 to ensure viability of the haploid disruptant. After plasmid loss, the resulting Ura auxotrophic diploid MG5101, heteroallelic for psd4-1/prp19::HIS3, was viable and showed the pleiotropic phenotype typical of a homozygous psd4-1 diploid, including a strong reduction in sporulation and in induced mutagenesis together with sensitivity to several DNA cross-linking mutagens (Table 2 and Fig. 1C). Since the prp19-0 allele is unable to complement a psd4-1 mutant phenotype, we have final proof that both genes are allelic. Thus the terms psd4::HIS3 (psd4-0) and prp19::HIS3 (prp19-0) can be considered as synonymous.
Table 2. (A) Complementation analysis of different subclones of pMG470 (see also Fig. 1A); (B) complementation analysis of the same set of plasmids transformed into the heteroallelic diploid MG5101; (C) non-complementation of the prp19 and pso4 mutant alleles (MG5101) as evidence for their allelism (see also Fig. 1B and C).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Plasmid</th>
<th>Sporulation</th>
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<th>30°C</th>
<th>36°C</th>
<th>Mutability</th>
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<tbody>
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<td>(A)</td>
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<td></td>
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<tr>
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<td>S</td>
<td>+</td>
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<td>None</td>
<td>35%</td>
<td>R</td>
<td>-</td>
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R, wild-type-like resistance; S, pso4 mutant-like sensitivity to HN2.
+/- corresponds to viability/non-viability at 30 or 36°C and mutability/reduced mutability respectively.

*psd4-1* is a non-lethal allele of the essential gene PSO4/PRP19

Although a disruption of PRP19 is lethal, mutants containing the *psd4-1* allele are viable but show the above-mentioned phenotype. Often, viable mutants of essential genes are isolated as sensitive to temperatures >30°C. We therefore tested our diploids harboring different alleles of P304 for possible temperature sensitivity. In fact, both diploids either homoallelic for *psd4-1* or heteroallelic (pso4-1/prp19-0) were sensitive to 36°C. Again, the *prp19-0* allele failed to complement this sensitivity, thus once more confirming allelism of both genes (Fig. 1B). When comparing the growth yield of our diploid strains at 30°C we found that heteroallelic pso4-1/pso4-0 diploid MG5101 can grow at 30°C (cf. above) but has a severely retarded growth rate with a generation time >3 h in YEPD medium, while the isogenic P304/pso4-0 diploid MG5103 showed a generation time of ~1.5 h, comparable with other wild-types (not shown).

DISCUSSION

Repair of DNA double-strand breaks in *S.cerevisiae* involves genes of the RAD52 epistasis group. The corresponding proteins are supposed to act via a recombinational mechanism and some of them have been shown to constitute a protein complex which has been termed a recombinosome (25,26). A *psd4-1* mutant has previously been shown to exhibit a pleiotropic phenotype, i.e. sensitivity to many DNA damaging agents, nearly blocked induced mutagenesis, lowered spontaneous and induced recombination and nearly totally blocked pre-meiotic DNA synthesis and sporulation (8,12,27). We have now isolated gene P304, which is a member of the RAD52 epistasis group by complementation of the *psd4-1* mutant’s drastically reduced induced mutability and blocked sporulation. Characterization of DNA repair gene P304 showed its allelism to the yeast gene *PRP19*, which encodes a 502 amino acid spliceosome-associated protein (22,28). In contrast, our P304 sequence contains one additional proline-coding CCC triplet and thus encodes a 503 amino acid protein. However, this discrepancy may be due to a strain-specific difference and is presently most likely irrelevant for discussion of the putative protein.

The allelism of P304 and PRP19 implicates that this yeast gene encodes a protein that has functions in RNA splicing and in error-prone DNA repair, recombination and sporulation in this organism. It is thus the first DNA repair gene with a function linked to the processing of RNA and its essentiality underscores the vital function in the latter process. Next to RAD3 and SSL2/RAD25, P304/PRP19 is the third DNA repair gene that is essential for growth and viability of *S.cerevisiae*.

The Prp19 protein has been demonstrated to be essential for splicing of pre-mRNA (28). However, biochemical characterization revealed that it is not tightly associated with snRNAs, but is associated with the spliceosome during the splicing reaction (22). Interestingly, Cheng *et al.* discussed the fact that Prp19 is distinct from other Prp proteins or other spliceosomal components regarding the protein sequence and that it does not contain any of
the four motifs found in other Prp sequences. It could be further demonstrated that the Prp19 protein is associated with a protein complex different from the spliceosome, consisting of at least seven proteins in addition to Prp19, itself most likely present in an oligomeric form (29). However, the Prp19-associated complex, itself about as big as the spliceosome, is unlikely to bind to the latter complex (29). The authors suggest that the Prp protein may be released to become associated with the splicing complex. This event was found to be concomitant with or just after dissociation of the U4 snRNA and an ATP-dependent conformational change before formation of a functional spliceosome, suggesting that Prp19 may function in this step of spliceosome assembly (30).

What could be, on the other hand, the function of the PRP19/PSO4-encoded protein in recombinational and error-prone repair? All evidence gathered from genetic and biochemical experiments using allele prs4-1 point to a late function in repair of DNA lesions missing in the mutant strain. The excision of 8-MOP + UVA-induced ICLs from DNA is thought to proceed normally, with production of DNA double-strand breaks as repair intermediates which, however, are not rejoined in prs4-1 (14). It has been suggested that repair of DNA lesions of the ICL type would require two modes of repair (31,32), the first being removal of the damage by the repairosome postulated for nucleotide excision repair (33) and the second another enzyme complex for repair of strand breaks, in which the PSO4/PRP19 encoded protein would have a function. The existence in S. cerevisiae of such a DNA double-strand repair-specific recombinoasme, a complex containing at least proteins encoded by RAD51, RAD52, RAD55 and RAD57, has recently been suggested (26). We suggest that the Prp19/Prp4-associated protein complex found by Tarn and co-workers (29) might be the above-mentioned recombinosome.

The fact that both existing viable alleles of the essential gene PSO4/PRP19, as represented by the previously described prp19 mutant (22,34) and by the prs4-1 mutant respectively, exhibit a temperature sensitivity implies that the 503 amino acid protein encoded by PSO4/PRP19 might have two or more functional domains, of which one or more would be still active in the protein expressed by haploid and diploid prs4-1 mutants: since there is no obvious growth retardation at 30°C in prs4-1 haploid and homoaelfic diploid mutants we must assume that RNA splicing proceeds with an efficiency close or identical to that of the wild-type. This hypothesis is underscored by the fact that the viable prp19 mutant accumulates unprocessed pre-mRNA at the non-permissive but not at the permissive temperature (34), suggesting that viability of a prs41/prp19 mutant is dependent on functional splicing. Survival of a prs4-1 mutant but not of prs4-0 therefore suggests that the former mutant allele still expresses a partially functional protein at 30°C; while its role in spliceosomal assembly and function would be about normal its function in repair of DNA single- and double-strand breaks via recombinational processes would be significantly perturbed. We suggest that these phenotypes could be due to disturbed binding of the other proteins to PSO4 in the multi-enzyme complex found by Tarn et al. (29), indicating that at least one of these hitherto unidentified proteins may belong to the RAD52 epistasis group.

An alternative, but in our opinion highly unlikely, explanation for the repair phenotype of the prs4-1 mutant would be the incorrect processing of transcripts of DNA repair genes. Amongst the many repair genes in yeast only one, namely RAD14, which is involved in incision of damaged DNA, is intron-containing (35) and hence would require splicing for proper function. Since prs4-1 mutants are able to incise DNA (14), the only known splicing-dependent repair gene is functional, once again pointing to normal splicing activity in prs4-1 mutants at 30°C. Also, if non-functional splicing always resulted in repair deficiency phenotypes, at least some of the ~50 DNA repair genes cloned ought to encode proteins needed for splicing, i.e. they should have turned out to be al lelic to one of the many other PRP genes.

The severe handicap in growth in complete medium at 30°C of heteroallelic ps4-1/prs4-0 but not of heterozygous ps4-1/PSO4 diploids indicates that the quantity of protein produced from one ps4-1 mutant allele is not sufficient for normal spliceosome activity, whereas the protein encoded in one PSO4 wild-type allele of a normal growing PSO4/prs4-0 diploid apparently satisfies cellular demand for the spliceosome-associated protein function. In this context the partial complementation of the yeast ps4-1 mutant’s sensitivity to photoactivated 8-MOP + UVA by overexpression of RecA protein (17) has new significance. RecA protein can bind to single- and double-stranded DNA, thereby facilitating initiation of recombination (36). Interstrand cross-link repair in E. coli as well as in yeast has been shown to proceed via double-strand breaks (cf. 2,31); these secondary lesions would be a substrate for RecA or RecA-like proteins which, after helical filament formation, would initiate recombinational repair. The RecA and Rad51 proteins share considerable sequence homology and there is strong evidence that Rad51 binds to the Rad52 protein (26). The sensitivity of the ps4-1 mutant to 8-MOP + UVA, its partial complementation by overexpressed RecA protein and the epistatic interactions of the ps4-1 mutant with rad51 and rad52 mutant alleles (17) has lead to the hypothesis that it functions in the repair of primary or secondary induced DNA strand breaks via a recombinational mechanism. The association with a functional protein complex, perhaps in forming a recombinoasme (26), could be one function of the PSO4/Prp19 protein; the other, more essential to the yeast cell, would be its role as a spliceosome-associated protein in pre-mRNA processing. Recently, a similar two-way association has been described for yeast transcription factor TFIIF: it participates in formation of a holocomplex active in RNA polymerase II transcription initiation and can also be found in the repairosome, which is specific for nucleotide excision repair (37,38).

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