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The RAD5 gene product is involved in the avoidance of non-homologous end-joining of DNA double strand breaks in the yeast *Saccharomyces cerevisiae*

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ABSTRACT

In wild-type yeast, the repair of a 169 bp double-strand gap induced by the restriction enzymes *Apa*I and *Nco*I in the *URA3* gene of the shuttle vector YpJA18 occurs with high fidelity according to the homologous chromosomal sequence. In contrast, only 25% of the cells of *rad5-7* and *rad5*Δ mutants perform correct gap repair. As has been proven by sequencing of the junction sites, the remaining cells recircularise the gapped plasmids by joining of the non-compatible, non-homologous ends. Thus, regarding the repair of DNA double-strand breaks, the *rad5* mutants behave like mammalian cells rather than budding yeast. The majority of the end joined plasmids miss either one or both of the 3′ and 5′ protruding single-strands of the restriction ends completely and have undergone blunt-end ligation accompanied by fill-in DNA synthesis. These results imply an important role for the Rad5 protein (Rad5p) in the protection of protruding single-strand ends and for the avoidance of non-homologous end joining during repair of double-strand gaps in budding yeast. Alternatively, the Rad5p may be an accessory factor increasing the efficiency of homologous recombination in yeast, however, the molecular mechanism of Rad5p function requires further investigation.

INTRODUCTION

DNA double-strand breaks (DSB) arise during cellular processes such as replication and recombination or due to external influences (1–4). It is generally assumed that increased radiation sensitivity in yeast and mammalian cell mutants is due to some deficiency in DSB repair (5–9). Several different cellular mechanisms are operative in the rejoining of DSB in diverse organisms.

In mammalian cells, the existing data suggest double-strand break rejoining to be the major pathway, rather than homologous recombination (10,11). The exact mechanism of DSB repair processes has not yet been elucidated. However, it is well proven that DNA-dependent protein kinase (DNA-PK) is involved in the rejoining of radiation-induced DSB as well as breaks arising during V(D)J recombination (12–16). DNA-PK consists of three subunits, the DNA end-binding Ku heterodimers Ku70 and Ku80 (polypeptides of ~70 and ~80 kDa respectively) and the catalytic subunit DNA-PK_{cs} of ~460 kDa (17). As has been deduced from restriction enzyme-cut plasmids transformed into mammalian cells, DNA ends of various configurations are predominantly repaired by end joining mechanisms without requiring extensive homology between the molecules to be joined (1,18–28). Such non-homologous end joining is often associated with sequence rearrangements and has been regarded as a mutagenic process (26,27). Commonly, the sequences of the DNA ends are preserved during repair of restriction enzyme-induced DSB and larger loss of information has been shown for a few junctional sequences (20,29). These results are in contrast to X-ray-induced DSB, where repair of damage is frequently associated with large deletions (30).

In contrast to mammalian cells, the yeast *Saccharomyces cerevisiae* repairs DSB primarily by homologous recombination controlled by the *RAD52* epistasis group of genes (7,31–35). Illegitimate non-homologous DNA end joining is detectable only in the absence of homology or in mutants incapable of homologous recombination. This process is invariably associated with deletion, insertion or duplication at the junction site (36–39). Recently, it has been shown that the yeast Ku70 homologue, the Hdf1 protein, controls an alternative process of DSB rejoining which is detectable only in the absence of homologous recombination. Obviously, this pathway is of minor importance for yeast, since hdf1 mutants do not exhibit sensitivity towards ionising radiation (40), however, they are sensitive to bleomycin (41). On the other hand, as indicated by the enhanced sensitivity of the corresponding mutants towards ionising radiation, some genes of the *RAD6* epistasis group (such as *RAD6*, *RAD5* and *RAD18*) may be of increased importance for the repair of X-ray-induced damage by a mechanism that can function, at least partially, in the absence of homologous chromosomes (42–44). Mutations in the *RAD5*, *RAD6* and *RAD18* genes cause a hyper-recombinagenic phenotype as opposed to mutants of the *RAD52* group of genes, which are mostly hypo-recombinagenic (45). This seems to be further support for the argument that in yeast these genes participate in the genetic control of an alternative pathway of DSB repair to *RAD52*-controlled homologous recombination. It is not known whether this presumed non-homologous mechanism is related to an end joining process and whether the *HDF1* gene plays a role

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in that putative pathway. So far, genetic analysis has disproved epistasis with the *RAD1* as well as the *RAD52* genes (40).

The *RAD5* gene of *S.cerevisiae*, a member of the *RAD6* epistasis group, has been cloned and analysed (46,47); the predicted Rad5 protein (Rad5p) possesses seven putative helicase domains, two zinc finger motifs and a leucine zipper motif. So far, analysis of the purified protein has demonstrated a functional nucleotide binding site, but no helicase activity has been detected (48). Strains carrying mutations in the *RAD5* gene are sensitive to UV, ionising radiation and certain chemicals and they show locus- and allele-dependent effects on spontaneous and UV-induced mutagenesis (49–53).

In order to investigate what role the *RAD5* gene plays in DSB repair in yeast, we have previously measured the repair of ^{60}Co γ-ray-induced DSB under non-growth conditions (buffer) applying pulsed-field gel electrophoresis for DSB quantitation (54–56). We could show that a diploid *rad5-7* mutant is capable of restoring chromosomal length DNA within 24 h to a level similar to that of the corresponding repair-competent wild-type diploid strain (~25% residual DSB). However, the initial rejoining was considerably slower in *rad5-7* mutants as compared with the wild-type. After 10 h, 80% residual DSB in the mutant as compared with 40% in the wild-type was found (Friedl, unpublished results). In addition, we observed that the *rad5-7* mutant exhibits a high percentage of incorrect repair of DSB and small double-stranded gaps (DSG) induced by restriction enzymes into a double-marker plasmid (57). Therefore, we assume that the radiosensitivity of *rad5* mutants is not due to a general defect in DSB rejoining, but due to a high percentage of misrepaired radiation-induced DSB. In this respect *rad5* mutants of yeast may be comparable with human ataxia telangiectasia cell lines and radiation-sensitive Chinese hamster cell line V79. In these cell lines the radiosensitivity is not due to a deficiency in rejoining DSB but to reduced fidelity of rejoining (58–60).

Here we provide more information on the role of Rad5p in DSG repair by use of our previously established plasmid system which allows homologous recombination to be distinguished from non-homologous processes at the DNA sequence level. We employed a screening system by use of the shuttle plasmid YpJA18 (57), which contains two selectable yeast genes (*TRP1* and *URA3*), a sequence for autonomous replication (*ARS*) in yeast and a centromeric region (*CEN4*). Plasmids of this kind show defined nucleosome structure and have been considered as circular minichromosomes (61,62). YpJA18 can be transformed into haploid yeast strains (*trp1-289*, *ura3-52*) of various repair capacities, either circular (as a control) or linearised by restriction enzymes yielding a DSB or a DSG (deletion of 169 bp by a *Nco*I–*Apa*I double-digest) in the *URA3* gene. Correct repair of the DSB and DSG restores uracil proficiency. Correct repair of a DSG can be achieved only by homologous recombination with the corresponding sequence retained in the chromosomal sequence (which is sufficiently distant from the *ura3-52* mutation; 63,64). Uracil auxotrophy reflecting misrepair can be due either to mutations caused by error-prone homologous recombination or to non-homologous processes such as end joining. Therefore, we have analysed the sequence of the gap junctions in misrepaired plasmids in order to discriminate between these processes. Our results indicate that the *RAD5* gene is required for correct DSB repair by homologous recombination in yeast, possibly to suppress non-homologous end joining of the plasmid DNA.

MATERIALS AND METHODS

Strains

The following haploid strains of the yeast *S.cerevisiae* were used:

MKP-0:	MATα, *can1-100, ade2-1, lys2-1, ura3-52,leu2-3-112,his3-Δ200,trp1-Δ901, RAD* (kindly provided by Dr B.A.Kunz, Geelong, Australia)
MKP-0 *rad5Δ*:	MATα, *can1-100, ade2-1, lys2-1, ura3-52, leu2-3-112, his3-Δ200, trp1-Δ901, rad5Δ::HIS3*
WS 8100–3A:	MATα, *ade2-1, trp1-289, ura3-52, arg4-17, his5-2, lys2-1, rad5-7.*

The bacterial strain *HB101* was used as the recipient for amplification of the plasmids in *Escherichia coli.*

Plasmids

The shuttle vector YpJA18 (57) was used for transformation of the yeast strains in order to perform the analysis of repair fidelity.

Media

YEPD, consisting of 2% dextrose, 2% Bacto peptone, 1% yeast extract solidified with 2% agar if required, was used as complete growth medium for yeast. Yeast transformants were plated on SC medium containing 4% dextrose, 0.33% yeast nitrogen base without amino acids and ammonium sulphate and 2% agar, supplemented with 9.8 g/l ammonium sulphate, 5 mg/l adenine, 20 mg/l arginine, 20 mg/l histidine, 40 mg/l lycine, 20 mg/l tryptophan and 20 mg/l uracil. For selection of *TRP*$^+$ and *URA*$^+$ prototrophs tryptophan (SC-Tryp) and uracil (SC-Ura) were omitted.

Escherichia coli was propagated in LB medium, containing 1% Bacto trypton, 1% sodium chloride, 0.5% yeast extract, pH 7.5, supplemented with 100 mg/l ampicillin. All chemicals for media were purchased from Difco.

Transformation and selection of transformants

Transformation of yeast was carried out according to the modified lithium acetate method (65); transformation of *E.coli* was performed according to the standard calcium chloride procedure.

DSG repair was assessed by transforming yeast cells with plasmid YpJA18 DNA either circular (as control) or linearised by a double digest with *Apa*I and *Nco*I, thus causing a 169 bp deletion (DSG). In order to avoid contamination with uncut, circular plasmids, only linear DNA purified by agarose electrophoresis was transformed into yeast. After transformation, the cells were first selected for *TRP*$^+$ expression, then the transformed colonies were replica plated on selective medium without uracil to score for *URA3* expression. Estimates of repair fidelity are based on the ratio of *Trp*$^+$*Ura*$^+$ to *Trp*$^+$ colonies.

General procedures were performed according to standard methods (66). Isolation of plasmid DNA from *E.coli*, restriction analysis, Southern blot analysis and elution of plasmid DNA from gels were performed as described previously (46). For the preparation of plasmids from transformed yeast cells a modified method of Robzyk and Kassier (67) was used.

Disruption of the *RAD5* gene

Plasmid pBR322 carrying a 1.1 kb *Cla*I fragment of the 3.5 kb *RAD5* reading frame was used to construct a *rad5*Δ disruption mutant. A 1.8 kb *Bam*HI fragment carrying the *HIS3* gene of

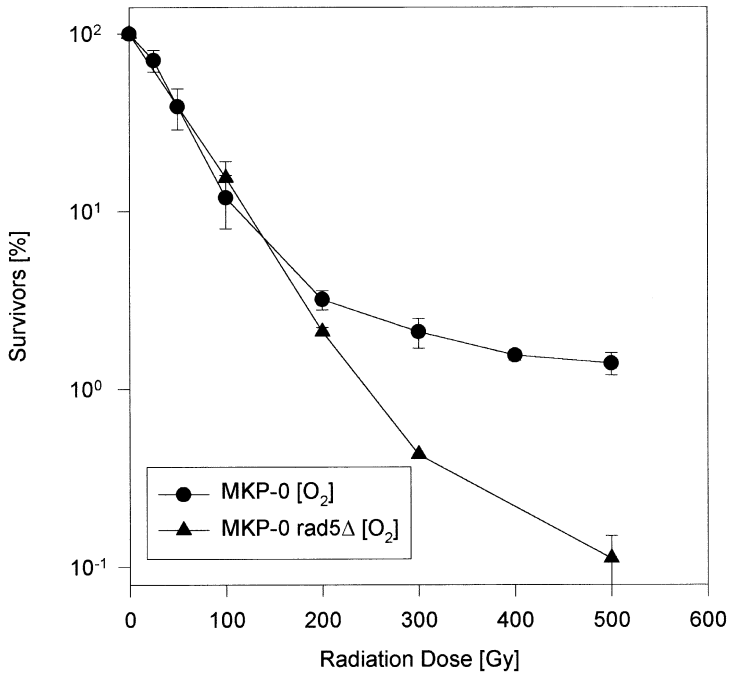

Figure 1. Survival of haploid strains following γ-ray treatment of stationary phase cells. Cells were irradiated with a ^{60}Co γ source at 20 Gy/min in suspension under O_2 aeration.

S.cerevisiae was inserted into the unique *Bgl*II restriction site on the 1.1 kb *Cla*I fragment generating a 2.8 kb *Cla*I–*Eco*RV fragment. This fragment was isolated and transformed into haploid *S.cerevisiae* wild-type cells (*RAD5*, strain MKP-0). His$^+$ transformants were selected; disruption of the *RAD5* gene was verified by Southern blot analysis (data not shown).

DNA sequencing

DNA was subjected to sequence analysis according to Sanger employing a T7 DNA polymerase sequencing kit (Pharmacia). The reactions were performed according to the supplier's protocol using 2 µmol sequencing primer for each reaction. Primers P1 (5′-TGGAGTTAGTTGAAGCATTA) and P2 (5′-CTGCTAACATCAAAAGGCCT) were used for sequencing ~250 bp (both strands) at the site of the *Nco*I–*Apa*I-derived DSG.

Survival experiments

For determination of the γ-ray sensitivity of the *rad5*Δ disruption mutant standard procedures were employed as described previously (40).

RESULTS

Construction and characterisation of a mutant disrupted in the *RAD5* gene

As previously reported, we observed that ~20% of *rad5-7* clones transformed with linearised gapped plasmid YpJA18 were Ura$^+$, indicating repair by homologous recombination (57). In order to eliminate the possibility that this remaining level of correct repair of DSB and DSG was due to some residual Rad5p activity in the point mutant, we constructed a *RAD5*::*HIS3* disruption (see Materials and Methods). Disruption in the *RAD5* gene was confirmed by Southern blot analysis and absence of the transcript by RT-PCR (data not shown). The *rad5*Δ mutant is UV sensitive as compared with the *RAD5* wild-type strain; the survival curves are similar to those of the non-isogenic *rad5-7* point mutant used in a previous work (57; data not shown). For ^{60}Co γ-irradiation, the disruption mutant shows moderate sensitivity in comparison with the wild-type strain (Fig. 1). This indicates some role for Rad5p in DSB repair supplementary to homologous recombination as the main DSB repair pathway, which is in contrast to the role of the Hdf1 protein, which seems to be important for DSB rejoining only when homologous recombination is impaired (40,68).

Selection of correctly and incorrectly repairing transformants

Using the previously constructed vector YpJA18 our studies on the repair of DSG are comparable with similar studies in mammalian cells (30,69). Uncut and 'gapped' plasmids linearised by digestion with *Nco*I (recessed 3′-end) and *Apa*I (recessed 5′-end) and thus lacking a 169 bp fragment in the *URA3* gene were transformed into haploid yeast strains (*trp1*, *ura3-52*) to complement the auxotrophies for tryptophan and uracil. Initial selection for *Trp*$^+$ prototrophs allowed identification of transformant clones irrespective of the fidelity of DSG repair in the *URA3* gene. The transformation frequencies were ~3×10^3 transformants/µg DNA in the wild-type and both *rad5* mutant strains. In the next step, the transformed colonies were selected for *URA3* expression. Uracil proficiency was taken as an indication of correct repair of the restriction enzyme-generated DSG by homologous recombination with the chromosomal gene. The percentage of transformants repairing correctly was calculated as described in Materials and Methods.

Table 1 shows the transformation frequencies as well as the rate of correct repair for circular and gapped plasmid DNA transformed

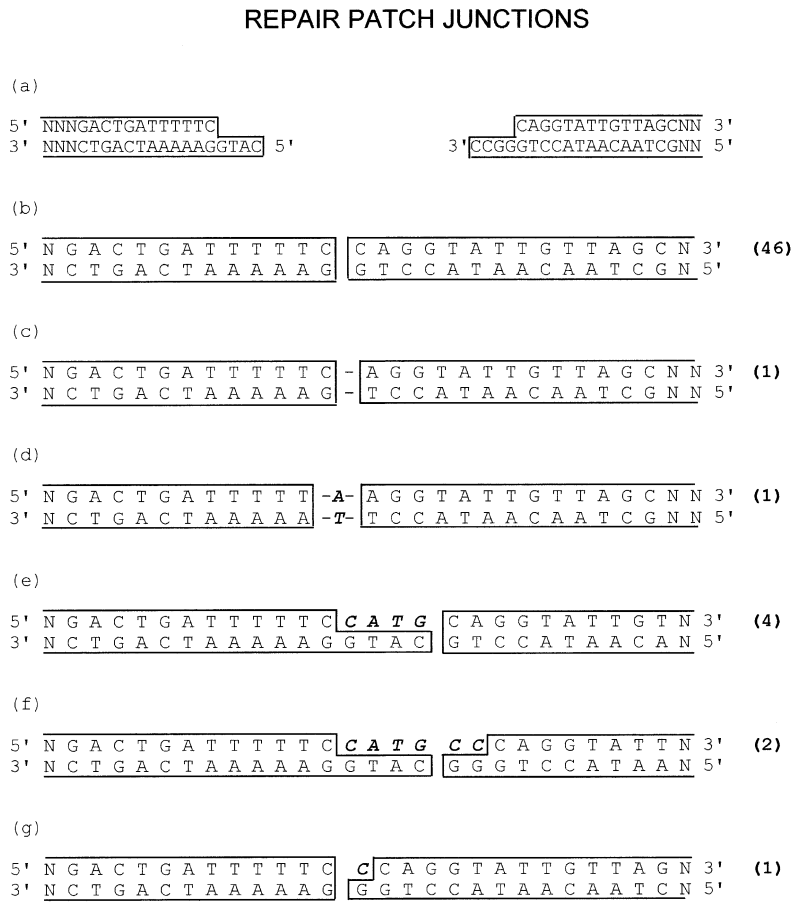

Figure 2. Sequences of the repair patch junctions. (**a**) 5′ and 3′ protruding ends were created by the restriction enzymes *Nco*I and *Apa*I. (**b**) Blunt–blunt ligation after degradation of the single-stranded protruding ends. (**c**) Blunt–blunt ligation after deletion of one base pair (CG). (**d**) Blunt–blunt ligation (deletion of two CG and insertion of one AT base pair). (**e**) Blunt ligation after fill-in of the single stranded 5′-region by polymerase activity and degradation of the 3′ protruding ends by exonuclease. (**f**) Intermediate of filled-in 5′-ends and partially degraded 3′-end. (**g**) Degraded 5′-PSS and partially degraded and filled-in 3′-ends. Bold letters indicate newly synthesised base(s). The total numbers of events are given in brackets on the right.

into haploid recipient strains. For the uncut plasmid, the *URA3* gene was expressed in ~90–100% of all transformants irrespective of the DNA repair capacity of the yeast strain used. In the repair-competent *RAD* wild-type strain, 97% of the transformants with the gapped plasmid were Ura$^+$. Of special interest are the findings for the *rad5*-7 point mutant and the *rad5*Δ disruption mutant. For the cut plasmid, both *rad5* mutant strains showed transformation efficiencies similar to the wild-type strain, but uracil proficiency was detected in 18 and 26% of the *Trp*$^+$ transformants only. Thus, both the point and the disruption mutations of the *RAD5* gene result in erroneous DSG repair in 74–82% of cells of the population and only 18–26% of the cells correctly restored the gap in the plasmid DNA by recombination with the homologous chromosome. It seems unlikely that this effect is due to residual Rad5p activity in the point mutant, since the disruption mutant shows the identical phenotype.

Table 1. Recovery of clones indicating correct repair of the restriction enzyme-induced (*Apa*I–*Nco*I) double-strand gap in the two-marker YpJA18 plasmid transfected into haploid repair-competent wild-type and repair-deficient *rad5* mutant yeast strains

Yeast strain	Uncut plasmid Transformation frequency (10^3/μg DNA)	Trp$^+$Ura$^+$/Trp$^+$ [a]	Ura$^+$ (%)[b]	Gapped plasmid Transformation frequency (10^3/μg DNA)	Trp$^+$Ura$^+$/Trp$^+$ [a]	Ura$^+$ (%)[b]
Wild-type (*RAD*)	2.98	370/372	100	3.15	584/600	97
rad5-7	3.00	173/188	92	3.20	146/796	18
*rad5*Δ	2.99	297/300	99	3.00	78/300	26

[a]The number of uracil-proficient colonies (Trp$^+$Ura$^+$) was identified among the total number of Trp$^+$ colonies scored in five independent experiments.
[b]The percent numbers indicate the proportion of Trp$^+$ clones correctly repairing the *URA3* gene.

DNA sequence analysis of the repair patch junctions

Misrepair of a DSG can be due either to error-prone homologous recombination or, alternatively, to non-homologous end joining. Sequence analysis of the junction sites in the misrepaired plasmids allows discrimination between these alternative sources of error and the spectra of sequence alterations allow conclusions on the function of Rad5p in DSB repair.

Therefore, plasmids from eight $Trp^+Ura^-$and 10 Trp^+Ura^+ clones of the *RAD* wild-type strain and 56 Trp^+Ura^- (19 of *rad5-7*, 37 of *rad5*Δ) and 10 Trp^+Ura^+ clones of the *rad5* strains were isolated following propagation in SD-Trp. Starting from the two sequencing primer binding sites P1 (53 bp upstream of the *Nco*I restriction site) and P2 (55 bp downstream of the *Apa*I restriction site) up to 250 bp were sequenced on both strands. The sequences of the repair patch junctions are depicted schematically in Figure 2.

All sequences derived from plasmids restoring the Ura^+ phenotype showed exact restitution of the 169 bp gap without any alteration within the junction site if isolated from the *RAD* wild-type or the *rad5* mutant strains. In three of eight plasmids isolated from Trp^+Ura^- clones derived from the *RAD* wild-type, the gap was reconstituted and within 250 bp no mutation was found. Apparently, a mutational change possibly unrelated to the recombination event had occurred at a greater distance. Of the remaining five clones, two contained sequences of unknown origin and three shuttle vectors could not be sequenced by use of primers P1 and P2 and were excluded from further analysis. Not a single case of end joining was found in *RAD5* wild-type clones. In contrast, only one out of 56 Trp^+Ura^- clones of the *rad5* mutants showed erroneous recombination upon restoring the 169 bp deletion. In the remaining 55 Trp^+Ura^- clones the ends were joined without incorporation of the missing 169 bp sequence. End joining was accompanied by various modifications of the DNA ends. No differences at the junction sites between clones derived from the *rad5*-7 and the *rad5*Δ mutant were observed.

Analysis of junctional breakpoints in the *rad5*-derived plasmids

Sequence analysis of the junction sites in plasmids circularised by end joining revealed that the majority of the alterations were confined to the protruding single strands introduced by the restriction enzymes *Nco*I (5′-protruding single strand, 5′-PSS) and *Apa*I (3′-PSS). In most cases (46 out of 55), the terminal sequences were shortened by elimination of the protruding 4 nt from both the 5′-PSS and 3′-PSS, resulting in the fusion of two blunt ends (‘blunt–blunt’, Fig. 2b). In four cases (Fig. 2e), the 5′-PSS remained intact while the 3′-PSS was completely removed. Subsequently, the single-stranded region was presumably filled in by polymerase activity from the recessed 3′-end of the 5′-PSS. In another two cases (Fig. 2f), the 3′-PSS was shortened by 2–3 nt without any change in the 5′-PSS and the resulting 6–7 nt single-stranded region was filled in. Approximately 96% of the deletions affected the terminal single-stranded ends (2–4 nt), whereas only 3% extended 1–2 nt further into the adjacent duplex region (Fig. 2d). The maximum size of a deletion was 10 bp. This suggests that the end joining system operating in the *rad5* mutants but not in the wild-type strain eliminates single-stranded overhangs preferentially to produce blunt ends which are then ligated. Further deletions affecting such newly formed blunt ends were infrequent and restricted to 1 nt at one blunt end (Fig. 2c). In one case (Fig. 2d), a single extra nucleotide was added at the junction site of two blunt-ended sequences. In summary, the obtained junctions include those which result from joining of two double-stranded ends (DD junctions, 84%), joining of the 5′-PSS with a double-stranded end (DS junction, 11%) and joining of the 5′- with the 3′-PSS (SS junction, 5%). We did not observe any short patches of homologies at the break points and exclude therefore that a single-strand annealing mechanism was involved. Thus, analysis of the junction sites revealed a mechanism of DSG repair that preferentially joins abutting ends without any homologies (two blunt ends or one blunt end with a PSS tail) in the *rad5* mutant strains.

DISCUSSION

Some of the genes of the *RAD6* epistasis group of repair genes in the yeast *S.cerevisiae* control a mechanism of DSB repair which presumably is an alternative to the dominant route of homologous recombination controlled by the *RAD52* epistasis group, as it is probably independent of homologous chromosomes (42–44). However, its molecular mechanism is unknown so far. We have shown that the efficiencies of DSB rejoining in the diploid *rad5-7* mutant are comparable with wild-type cells in chromosomal DNA as well as the plasmid system. However, the initial rate of DSB repair in chromosomes (Friedl, unpublished results) as well as the fidelity of DSB and gap (DSG) repair analysed in plasmid DNA is reduced (44,57).

In this study we made use of our previously established shuttle vector YpJA18, exhibiting nucleosomal structure in yeast (61,62), to further characterise the possible function of Rad5p in DSG repair. We introduced a gap of 169 bp in the *URA3* gene of YpJA18 which was then transformed into isogenic strains differing in their *RAD5*-controlled repair capacities. A DSG can be correctly repaired only by recombination with a homologous sequence. The original sequence of the gap cannot be restored by non-homologous end joining or illegitimate recombination, an alternative but rare pathway in yeast (36,38,70). End joining possibly re-establishes the information disrupted by a DSB but does not restore the sequence of a DSG; additionally, error-prone recombination can result in misrepair of DSB as well as DSG. These two possibilities can be discriminated by sequence analysis of the repair patch sites.

In *RAD* wild-type strains the transformation frequencies of gapped and circular plasmids are nearly identical and only 3% of the gapped plasmids are misrepaired. This indicates the high efficiency and high fidelity of DSG as well as DSB repair in yeast. In contrast, *rad54-1*, *rad50-1* and *rad51-1* mutants of the *RAD52* group of genes, which are deficient in homologous recombination, show 50- to 100-fold reduced transformation frequencies of linearised as compared with circular plasmids and ~96–98% of the gapped plasmids are misrepaired in these mutants (57). Non-circularised plasmids cannot be propagated in yeast. Therefore, the low transformation frequency in the recombination-deficient mutants indicates a low efficiency of gap repair and the high frequency of misrepaired gaps refers to an alternative, obviously error-prone pathway. These results are consistent with the current opinion (i) that in yeast DSG repair is preferentially performed by homologous recombination with high sequence fidelity and that (ii) at least one alternative pathway exists which is error-prone and might be non-homologous end joining or illegitimate recombination.

Based on extensive genetic analysis of DNA repair pathways (35) and the hyper-recombinogenic phenotype of *rad5* mutants (49; Kupiec personal communication), it can be assumed that the *RAD52*-dependent mechanism for DSB repair is active in *rad5* mutants. As expected, the transformation frequencies for the linearised plasmid in the *rad5*Δ disruption mutant are almost identical to those for uncut plasmids, cut plasmids in the isogenic *RAD* wild-type and of a *rad5*-7 point mutant of a different genetic background. However, in ~75–80% of the clones of both *rad5*-7 and *rad5*Δ mutant strains the gaps in the *URA3* gene on the plasmids are misrepaired despite a homologous sequence on the chromosome. Experiments with a different plasmid system indicate that this effect is not specific to the *URA3* gene (unpublished data).

Our results suggest that in addition to the gene products of the *RAD52* group required for gap repair by homologous recombination and the presence of homologous sequences, Rad5p is required for DSG repair by homologous recombination and for avoiding non-homologous end joining. The fact that 26% of the cells of the *rad5*Δ disruption mutant are capable of correctly performing DSG repair supports the genetic evidence that homologous recombination can function in the absence of Rad5p. Nevertheless, the remaining 76% of the *rad5*Δ cells restore the gap by end joining without restoring *URA3* function. This might indicate that Rad5p antagonises some factor(s) which initiates end joining and that in the absence of Rad5p this/these factor(s) might gain importance, increasing the fraction of end joining. Alternatively, some other factor(s) could partially replace Rad5p function or Rad5p may be an accessory factor increasing the efficiency of homologous recombination. Our data support the notion that Rad5p is neither involved in the processes of homologous recombination nor in non-homologous end joining. Rather, we suggest an important regulatory or 'channeling' role of Rad5p for the avoidance of non-homologous end joining in the yeast *S.cerevisiae*.

The postulated role of Rad5p as a 'deterrent' against non-homologous end joining was further supported by sequence analysis of the repair junction sites obtained by end joining, which show similarity with those observed in other yeast systems (36,38) and in mammalian cells (21,39). In our system, the single-stranded overhangs (PSS) introduced by the *Apa*I and *Nco*I restriction digests are non-compatible and there is no sequence homology between the two ends. In the *rad5* mutants we found no single junction with both 5′- and 3′-PSS being completely intact. We found three different types of junctions: blunt end plus blunt end due to complete loss of both PSS, 5′-PSS plus blunt end and 5′-PSS plus partial 3′-PSS (see Fig. 2b–g). The complete deletion of the 5′- and 3′-PSS generating blunt ends in the majority of cases (84%) may indicate an increased sensitivity towards nucleolytic degradation of PSS. Blunt ends, however, do not seem to be suitable substrates for further degradation, as indicated by the very low frequency (3%) of deletions extending into the double-stranded end. One can only speculate on whether the Rad1–Rad10 protein complex, an endonuclease cleaving at the junction between duplex DNA and the 3′-PSS (71,72), and the Rad2 protein, cleaving at the junction between duplex DNA and the 5′-PSS (73), are responsible for modification of the PSS. Non-homologous DNA blunt end joining accompanied by loss of terminal bases has also been reported for *Schizosaccharomyces pombe* (36).

In ~10% of cases we found that the 5′-PSS remained intact while the 3′-PSS was deleted. The joining of a 5′-PSS with a blunt end requires fill-in DNA synthesis as envisaged in mammalian cells (21,26,29) and in yeast (36,38). In the least abundant category we observed joining of intact 5′-PSS with partially deleted 3′-PSS (Fig. 2c and d). One or two remaining nucleotides of the 3′-PSS may result either from partial degradation of the 3′-PSS or, possibly, from duplication of the terminal nucleotide, as has been described for mammalian cells (9,27,74). Both the Klenow fragment of DNA polymerase I from *E.coli* and the native *Taq* DNA polymerase are capable of synthesising across discontinuous templates *in vitro* (75,76). The joining sequences depicted in Figure 2f indicate that some yeast polymerase has a similar capacity *in vivo*. Finally, we found one junction sequence with a single nucleotide (A/T) added to the blunt terminal sequence (Fig. 2d). This is consistent with previous findings showing rare addition of mostly a single nucleotide for non-homologous end joining. (18,36,37,77). In our case, this insertion of a single nucleotide was accompanied by deletion of a nucleotide at both blunt ends. DNA polymerase is able to add single nucleotides to blunt-ended DNA *in vitro* (77) and possibly is responsible for the insertion of a single nucleotide or a simple duplication in yeast as well.

It should be emphasised that both specific loss of nucleotides at the protruding single-stranded end and a high percentage of end joining are normally not observed in yeast when the conditions for homologous recombination are met.The *rad5* mutants are unique and 'mammalian cell-like' in that they perform error-prone gap repair by non-homologous end joining three times more frequently than by homologous recombination. Mammalian cells show high frequencies of end joining as opposed to homologous recombination and frequently they also modify DNA ends by terminal deletions and insertion of extra bases at the cleavage site (27). In yeast end joining of DSB and modification of protruding ends may be related processes and both of these events may be increased in the absence of *RAD5* function.

In conclusion, in *S.cerevisiae* the *RAD5* gene product seems to be involved in maintaining a balance which acts in favour of error-free homologous recombination and in disfavour of error-prone non-homologous end joining during DSB and DSG repair in a minichromosome system. The question whether these results concerning the repair of restriction enzyme-mediated gaps are relevant to the repair of radiation-induced DSB in chromosomal DNA will require further investigation. Of special interest will be the identification of genes that control non-homologous end joining of broken chromosomal DNA in yeast and their relationship to *RAD5*. One candidate of interest is the *HDF1* gene, which mediates DSB repair by some mechanism of illegitimate recombination.

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