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Identification of *Saccharomyces cerevisiae* DNA ligase IV: involvement in DNA double-strand break repair

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DNA ligases catalyse the joining of single and double-strand DNA breaks, which is an essential final step in DNA replication, recombination and repair. Mammalian cells have four DNA ligases, termed ligases I–IV. In contrast, other than a DNA ligase I homologue (encoded by *CDC9*), no other DNA ligases have hitherto been identified in *Saccharomyces cerevisiae*. Here, we report the identification and characterization of a novel gene, *LIG4*, which encodes a protein with strong homology to mammalian DNA ligase IV. Unlike *CDC9*, *LIG4* is not essential for DNA replication, *RAD52*-dependent homologous recombination nor the repair of UV light-induced DNA damage. Instead, it encodes a crucial component of the non-homologous end-joining (NHEJ) apparatus, which repairs DNA double-strand breaks that are generated by ionizing radiation or restriction enzyme digestion: a function which cannot be complemented by *CDC9*. Lig4p acts in the same DNA repair pathway as the DNA end-binding protein Ku. However, unlike Ku, it does not function in telomere length homeostasis. These findings indicate diversification of function between different eukaryotic DNA ligases. Furthermore, they provide insights into mechanisms of DNA repair and suggest that the NHEJ pathway is highly conserved throughout the eukaryotic kingdom.

Keywords: DNA ligase/DNA repair/double-strand break repair/Ku/radiosensitivity

Introduction

DNA joining is an essential final step in many cellular processes. DNA ligases, which catalyse this reaction, join together Okazaki fragments during lagging strand DNA synthesis, complete exchange events between homologous duplex DNA molecules and seal single or double-strand breaks in the DNA that are produced either by the direct action of DNA-damaging agents or by DNA repair enzymes removing DNA lesions (for review, see Lindahl and Barnes, 1992). In contrast to prokaryotic and yeast systems, where only a single species of DNA ligase has been described (Johnston and Nasmyth, 1978), four biochemically distinct DNA ligases have been identified in mammalian cells (Tomkinson *et al.*, 1991; Wei *et al.*, 1995; Robins and Lindahl, 1996). *In vitro* assays and studies of yeast and human cells containing mutated alleles of DNA ligase I suggest that this enzyme joins Okazaki fragments during DNA replication (Henderson *et al.*, 1985; Malkas *et al.*, 1990; Tomkinson *et al.*, 1991; Barnes *et al.*, 1992; Li *et al.*, 1994; Prigent *et al.*, 1994; Waga *et al.*, 1994). Furthermore, the sensitivity of DNA ligase I mutant cells to UV irradiation and some DNA-damaging agents suggests that DNA ligase I is involved in nucleotide excision repair and base excision repair (Henderson *et al.*, 1985; Lehmann *et al.*, 1988; Malkas *et al.*, 1990; Tomkinson *et al.*, 1991; Barnes *et al.*, 1992; Li *et al.*, 1994; Prigent *et al.*, 1994; Waga *et al.*, 1994).

Much less, however, is known about the function of the other three mammalian DNA ligases. It is currently unclear whether DNA ligases II and III arise from separate genes or by alternative splicing of the same gene (Roberts *et al.*, 1994; Wang *et al.*, 1994; Husain *et al.*, 1995). However, ligase II is induced in response to alkylation damage (Creissen and Shall, 1982), suggesting a role in DNA repair. Similarly, the elevation in levels of a splice variant of ligase III (ligase III-β) in spermatocytes undergoing meiotic recombination (Chen *et al.*, 1995; Husain *et al.*, 1995; Mackey *et al.*, 1997) and the association of another splice variant (ligase III-α) with the DNA repair protein XRCC1 (Thompson *et al.*, 1990; Caldecott *et al.*, 1994) are consistent with this enzyme joining DNA strand breaks to complete DNA recombination and repair (Jessberger *et al.*, 1993). Indeed, DNA ligase III, when present in a complex with XRCC-1, can reconstitute the ligation event necessary to complete base excision repair *in vitro* (Kubota *et al.*, 1996). A fourth enzyme, DNA ligase IV, has been purified recently from human cells and has biochemical properties distinct from other ligases (Robins and Lindahl, 1996). The physiological function of mammalian ligase IV is, however, unknown.

In prokaryotes, there is only one DNA ligase, and this enzyme catalyses all the DNA-joining events during replication, recombination and repair (Lindahl and Barnes, 1992). Similarly, genetic and biochemical data have suggested that there is only one DNA ligase in *Saccharomyces cerevisiae* (Lindahl and Barnes, 1992). However, the fractionation of yeast cell extracts has identified a second DNA ligase activity (Tomkinson *et al.*, 1992). In light of this, we searched for DNA ligase II/III or IV homologues in the *S.cerevisiae* genome, which was completely sequenced recently (Goffeau *et al.*, 1996; Oliver, 1996). Given that DNA ligase III appears to function in essential processes such as meiotic recombination and base excision repair (see above), it is perhaps surprising that we have been unable to detect an obvious candidate gene for this enzyme in *S.cerevisiae*. Instead, these searches identified a hitherto uncharacterized open reading frame (ORF) with sequence similarity along its entire length to mammalian DNA ligase IV. Here, we describe the effects of disrupting this gene, which we have termed *LIG4*, on DNA replication, homologous recombination and DNA repair in response

to a variety of DNA-damaging agents. These studies show that *LIG4* plays a crucial role in DNA double-strand break (DSB) repair via the non-homologous end-joining (NHEJ) pathway but does not have an essential role in other DNA repair pathways studied. Furthermore, we show that *LIG4* functions in the same DNA repair pathway that utilizes the DNA end-binding protein Ku. However, the phenotype of *lig4* mutant yeasts is not identical to those of yeasts disrupted for Ku function, revealing that Ku has additional roles in genome maintenance.

Results

Saccharomyces cerevisiae possesses a second, hitherto uncharacterized, DNA ligase

We searched for DNA ligase II/III or IV homologues in the recently fully sequenced *S.cerevisiae* genome (Goffeau *et al.*, 1996) using the consensus sequence within the core catalytic domain of all published DNA ligases (see Materials and methods). In addition to detecting *CDC9*, which encodes DNA ligase I, these searches identified an ORF (YOR005c) present on chromosome XV as a highly significant hit. This ORF encodes a 944 amino acid residue polypeptide of predicted M_r 109 kDa that exhibits extensive similarity (24% identity; 43% similarity) in its central region to the 'core' ligase conserved domain of DNA ligase I (Figure 1A). Phylogenetic analyses of protein alignments over this region reveal that YOR005c is considerably more related to DNA ligases of eukaryotes and eukaryotic viruses than to those of prokaryotes and, in particular, is most closely related to human ligase IV (Figure 1B). Consistent with this, YOR005c shares an N-terminal extension with the mammalian enzymes that is lacking in prokaryotic DNA ligases (Figure 1C). Furthermore, it possesses an additional C-terminal extension that is homologous throughout its length to that of mammalian ligase IV (Figure 1A and C). We thus conclude that YOR005c encodes a homologue of mammalian DNA ligase IV, and designate this locus *LIG4*.

Disruption of LIG4 does not lead to marked hypersensitivity to a variety of DNA-damaging agents

To study *LIG4* function, we inactivated this gene in the haploid yeast strain W303α by a one-step gene disruption. Notably, resulting *lig4* mutants do not have readily observable growth defects when propagated at temperatures ranging from 18 to 37°C (Figure 2A and B, and data not shown). This contrasts markedly with *CDC9*, the gene encoding yeast ligase I, whose disruption results in lethality due to an inability to progress through S-phase (Johnston and Nasmyth, 1978). It is thus concluded that *LIG4* does not play an essential role in DNA replication, and that yeast ligase I is the only DNA ligase required for this process. Next, we tested whether *lig4* mutant yeast are defective in any of the predominant DNA repair pathways by assessing their sensitivity to killing by various DNA-damaging agents. Notably, *lig4* mutant strains are not hypersensitive to DNA damage induced by exposure to UV radiation (Figure 2C), showing that it is not essential in nucleotide excision repair. In addition, strains disrupted for *LIG4* are not hypersensitive to a range of concentrations of the radiomimetic drug, methyl methanesulfonate (MMS) in the growth medium (Figure 2D and data not shown). Finally, *lig4* mutant yeasts also do not display significantly elevated sensitivity to killing by ionizing radiation at a range of doses (0–45 kRad; Figure 3A and data not shown). Since radiation-induced DNA DSBs are repaired primarily by homologous recombination in *S.cerevisiae*, these data suggest that *LIG4* is not essential for the latter process. Consistent with this, we have been unable to detect significant differences in the efficiency of homologous recombination-mediated targeted integration into various loci in the yeast genome between wild-type and *lig4* mutant strains (data not shown).

LIG4 functions in the Ku-dependent NHEJ pathway of DNA double-strand break repair

In *S.cerevisiae*, radiation-induced DNA DSBs are repaired primarily by homologous recombination, which is mediated by genes in the *RAD52* epistasis group (Friedberg *et al.*, 1995). Thus, disruption of *RAD52* sensitizes yeast cells to ionizing radiation (Figure 3A). However, eukaryotic cells can also repair DNA DSBs by a second pathway, termed NHEJ, that utilizes gene products distinct from those employed in homologous recombination. In both yeast and mammals, one of these components is the DNA-binding protein Ku, comprising subunits of ~70 and ~80 kDa [Ku70 and Ku80 in mammals (Jackson and Jeggo, 1995); Yku70p/Hdf1p and Yku80p/Hdf2p in yeast (Feldmann and Winnacker, 1993; Boulton and Jackson, 1996a,b; Feldmann *et al.*, 1996; Mages *et al.*, 1996; Milne *et al.*, 1996; Siede *et al.*, 1996; Tsukamoto *et al.*, 1996)]. NHEJ appears to be the predominant pathway for DSB repair in mammals, but represents a minor pathway in yeast; consequently, disruption of *S.cerevisiae YKU70* or *YKU80* only results in significantly increased sensitivity to ionizing radiation or MMS when homologous recombination is inoperative (Boulton and Jackson, 1996a,b; Milne *et al.*, 1996; Siede *et al.*, 1996). We find that *lig4/rad52* double mutants are considerably more radiosensitive than are strains disrupted for *RAD52* alone (Figure 3B). This suggests that *LIG4* is involved in the repair of ionizing radiation-induced DNA damage and that it functions in a *RAD52*-independent pathway. Since the effects of disrupting *LIG4* are similar to those obtained by disrupting *YKU70* or *YKU80*, we assessed the radiosensitivity of *lig4/yku70/rad52* triple mutants. Such mutant strains are no more sensitive to ionizing radiation than are the *lig4/rad52* or *yku70/rad52* mutant strains (Figure 3B). Taken together, these data imply that Ku and *LIG4* function in the same DNA repair pathway.

Previous work has shown that Ku functions in DNA NHEJ and that this process can be measured through employing an *in vivo* plasmid repair assay (Boulton and Jackson, 1996a,b; Milne *et al.*, 1996). In this assay, a yeast–*Escherichia coli* shuttle plasmid pBTM116 (Figure 4A; Boulton and Jackson, 1996a,b) is linearized by restriction enzyme digestion, then is introduced into *S.cerevisiae* by transformation. Since the plasmid must be recircularized to be propagated, the number of yeast transformant colonies obtained quantifies the ability of the strain to repair the plasmid. Furthermore, since the DNA DSB generated in these studies resides in a region that is not homologous to the yeast genome, homologous recombination is suppressed and repair operates predominantly via

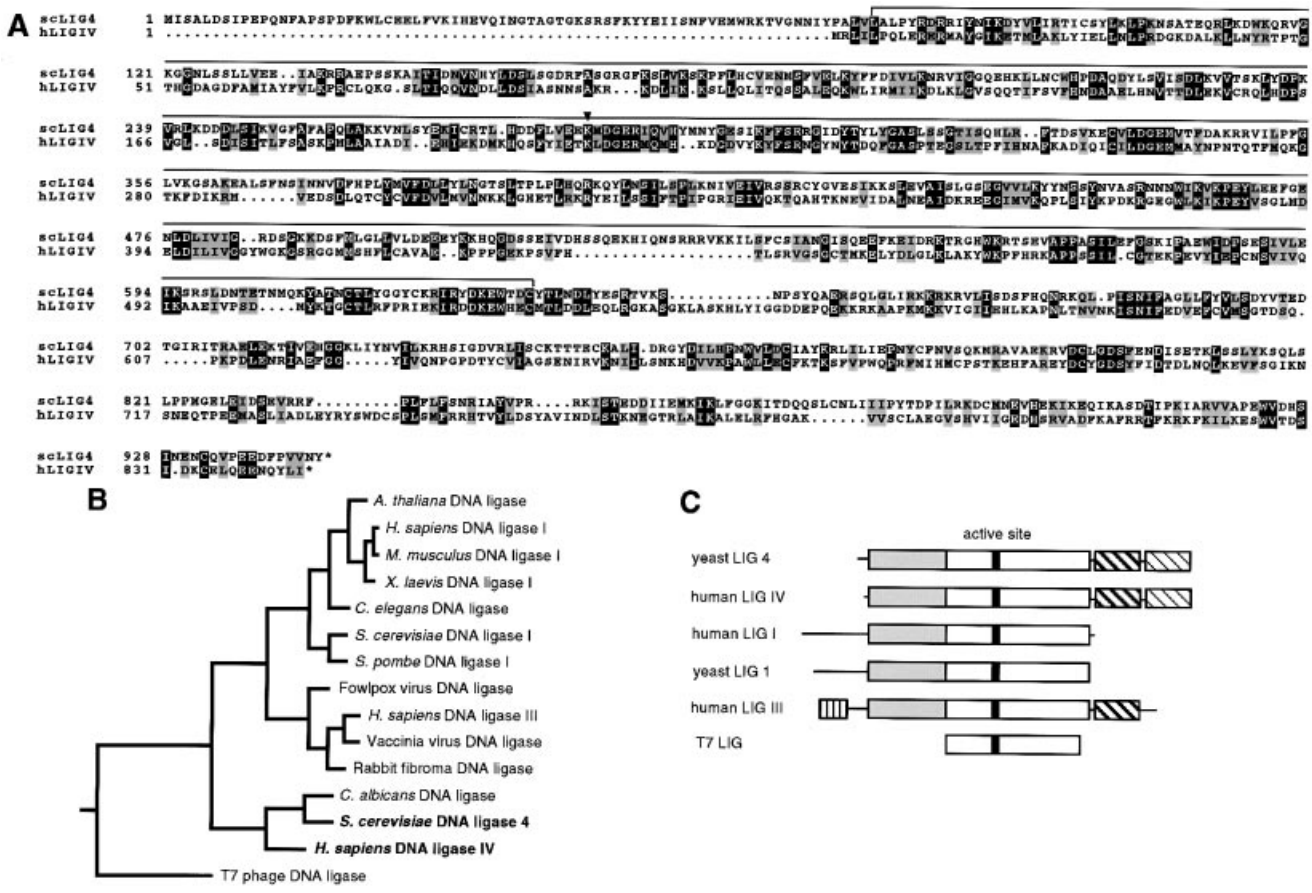

Fig. 1. YOR005c encodes a homologue of mammalian DNA ligase IV. (**A**) Amino acid sequence similarities between *S.cerevisiae* Lig4p (scLIG4; the product of the YOR005c ORF) and human DNA ligase IV (hLIGIV). The alignment was generated using the PILEUP program on the GCG (Genetics Computer Group, Wisconsin) package, and identical and similar amino acid residues are indicated by reverse shading and grey shading, respectively, using the BOXSHADE program. Amino acid residues are numbered from the amino-termini of the full-length polypeptides. Gaps were introduced for maximum alignment. The active site lysine residue is indicated with an arrowhead. The 'core' conserved region of DNA ligases of eukaryotes and eukaryotic viruses is delineated with a bar. (**B**) Phylogenetic tree showing the evolutionary relationship of DNA ligases from eukaryotes, eukaryotic viruses and T7 bacteriophage. The phenogram was generated using the PHYLIP package with the aligned 'core' conserved sequence of each protein as designated in (A) using the UPGMA method. DDBJ/EMBL/GenBank accession numbers are as follows: *Arabidopsis thaliana* I (X97924); *Candida albicans* (X95001); *Caenorhabditis elegans* I (Z73970); fowlpox virus (U00761); *Homo sapiens* I (M36067), III (X84740) and IV (X83441); *Mus musculus* I (U04674); rabbit fibroma virus (Z29716); *S.cerevisiae* I (X03246), IV (Z74913); *Schizosaccharomyces pombe* I (X05107); T7 bacteriophage (P00969); vaccinia virus (X16512); *Xenopus laevis* I (L43496). (**C**) Schematic representation of the putative domain structure (based on amino acid sequence homologies) of various DNA ligases. White box, 'core' conserved ligase domain; black box, active site residue; shaded box, N-terminal conserved domain of eukaryotic and eukaryotic viral DNA ligases; striped box, zinc finger-like DNA-binding domain; diagonally hatched box, putative BRCT domain (Koonin *et al.*, 1996; Callebaut and Mornon, 1997).

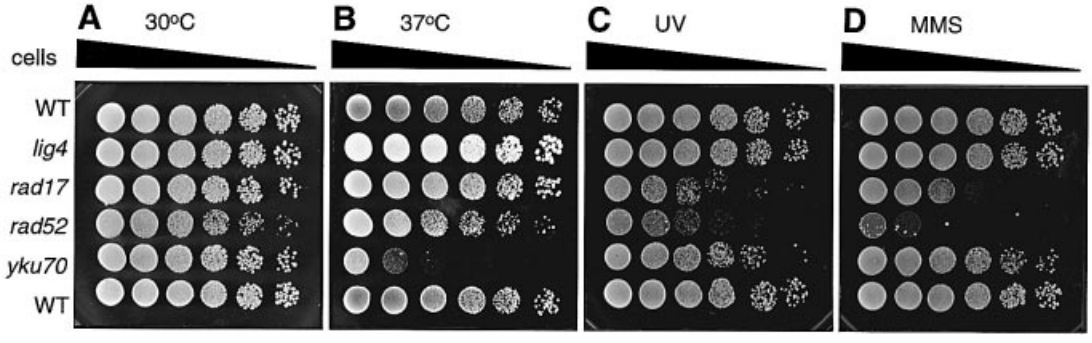

Fig. 2. *lig4* mutant strains are not temperature-sensitive and are not hypersensitive to various DNA-damaging agents. Aliquots (15 µl) of serial 5-fold dilutions of mid-log phase yeast cultures were spotted onto YPDA plates and were grown for 36 h at 30°C (**A**, **C** and **D**) or 37°C (**B**). Strains on plate C were exposed to 50 J/m^2 UV-C radiation (Stratalinker; Stratagene). On plate D, YPDA medium contained 0.0025% MMS. In other studies, *lig4* mutant strains did not display hypersensitivity to MMS (0.0005 and 0.005% in the growth medium) nor to UV-C (20–150 J/m^2).

NHEJ. We therefore analysed the ability of *lig4* mutant yeasts to repair pBTM116 after cleavage with various restriction endonucleases. As with strains disrupted for *YKU70* or *YKU80*, *lig4* mutant strains are severely impaired in plasmid NHEJ, and this is observed both with 5′ and 3′ overhanging DNA ends (Figure 4B).

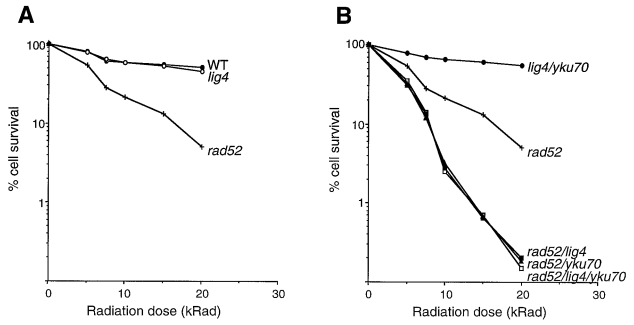

Fig. 3. *LIG4* functions in the Ku-dependent pathway for repairing ionizing radiation-induced DNA damage. The sensitivity of various yeast strains to killing by ionizing radiation was judged by exposure to various radiation doses. Error bars are not shown for simplicity; standard deviation is <5% of each value point. (**A**) Unlike *rad52* mutants, *lig4* mutant yeast are as resistant to ionizing radiation as parental strains: *lig4* mutants were not significantly more sensitive even at high doses (up to 45 kRad). (**B**) As in the case of *YKU70*, disruption of *LIG4* hypersensitizes yeast to ionizing radiation in *rad52* mutant backgrounds. Furthermore, *lig4/yku70/rad52* triple mutants are not appreciably more radiosensitive than are *yku70/rad52* double mutant or *lig4/rad52* double mutant strains, indicating that Lig4p and Yku70p function on the same *RAD52*-independent repair pathway.

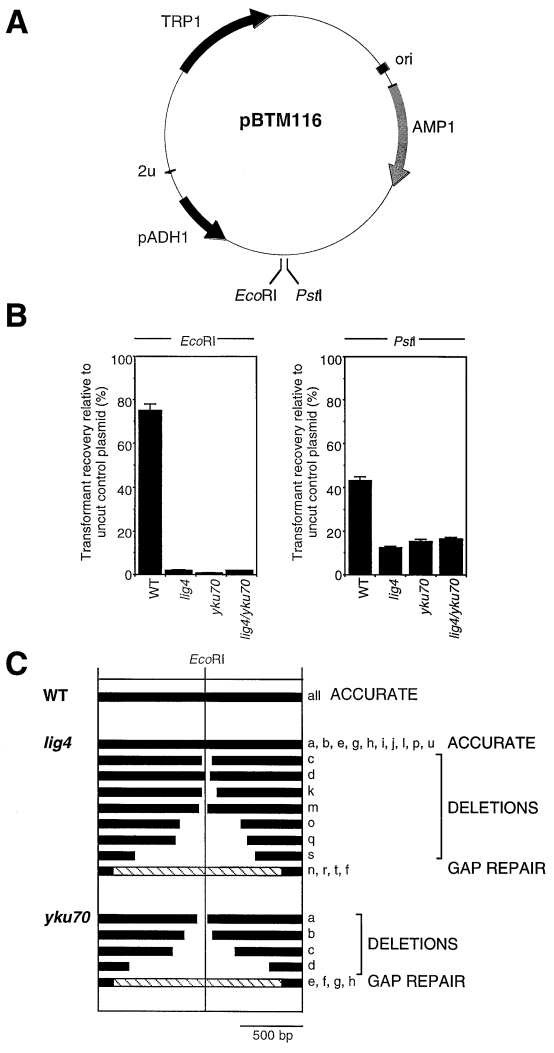

Fig. 4. (**A**) Plasmid map of the shuttle pBTM116 showing the locations of the yeast selectable marker *TRP1*, the β-lactamase gene, the *ADH1* promoter and the restriction enzyme cleavage sites. (**B**) Disruption of *LIG4* results in a dramatic reduction in the ability to repair restriction enzyme-generated cohesive DNA DSBs in plasmid (pBTM116) DNA. Cells for each strain were transformed in parallel with supercoiled pBTM116 or with an equivalent amount of pBTM116 that had been digested to completion with *Eco*RI or *Pst*I, as indicated. For each experiment, the value plotted is the number of transformants obtained with the linear plasmid expressed as a percentage of the number obtained for supercoiled DNA. Each experiment was repeated at least three times and, in each case, cells were plated and counted in duplicate. (**C**) In the absence of functional *LIG4*, cohesive DNA termini are repaired by an inefficient error-prone DNA repair pathway. The plasmid pBTM116 contains the *ADH1* promoter, and some repair products have been generated by a gap repair process involving the chromosomal *ADH1* gene; the striped region represents DNA derived from the genomic locus. Gaps indicate deletions. A single letter in lower case represents a single plasmid analysed in this study (representative transformants are represented).

Interestingly, these studies reveal that the effect of *lig4* mutations is less pronounced with 3′ overhanging DNA ends than it is with 5′ overhanging ends. Although other alternatives exist, it is possible that this reflects differences in the mechanisms by which the two types of DNA ends can be repaired or is due to differential sensitivities of the different end structures to nuclease attack. Notably, DNA repair is not impaired further in *yku70*/*lig4* double mutant strains (Figure 4B). In addition, although the precise reason for this effect is not known, as is the case for *yku70* or *yku80* mutants, *lig4* mutant yeasts have a slightly elevated ability to rejoin pBTM116 bearing blunt ends (data not shown). Taken together, these results reveal that Lig4p plays a crucial role in the repair of plasmid molecules bearing cohesive DNA DSBs *in vivo*. Secondly, they show that, although purified DNA ligase I (*CDC9*) has been shown to be capable of catalysing DSB joining *in vitro* (Tomkinson *et al.*, 1992), this enzyme does not play a major role in this pathway as assayed by *in vivo* plasmid DSB rejoining, and is unable to substitute efficiently for Lig4p in this process. Finally these results also show that Lig4p plays an important role in the Ku-dependent NHEJ pathway.

Although plasmid repair is reduced dramatically in *lig4* mutant strains, it is not abolished. To determine precisely the types of DNA repair events that are dependent or independent of *LIG4*, repaired plasmids were recovered and then analysed by restriction enzyme digestion and DNA sequencing (Figure 4C). Of the large number of plasmids recovered from parental strains, all had been repaired by direct ligation of the cohesive DNA termini, thus regenerating the restriction enzyme cleavage site (Boulton and Jackson, 1996a,b; Figure 4C). Plasmid repair products recovered from *yku70* or *lig4* mutant strains, however, were found to fall into several categories. Some of these corresponded to 'gap repair' products which we have shown are generated via *RAD52*-dependent homologous recombination with yeast genomic DNA (sequence analyses reveal that homologous recombination is employed in the generation of these products and their production is abolished by disruption of *RAD52*; Boulton and Jackson, 1996a,b, and data not shown). This therefore provides further evidence that *LIG4*, like *YKU70* and *YKU80*, does not play a crucial role in homologous recombination processes. In *yku70* or *yku80* mutant strains, virtually all of the residual repair products were found to have suffered deletions (Boulton and Jackson, 1996a,b; Figure 4C). In contrast, although many of the residual

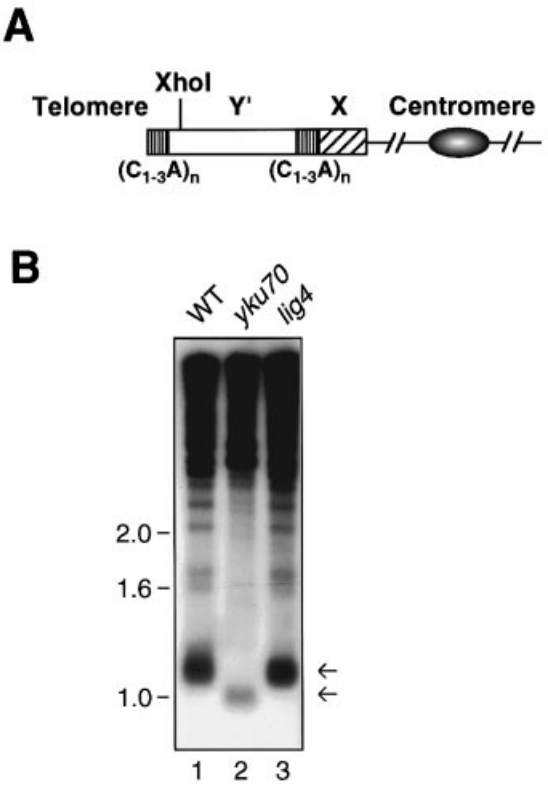

Fig. 5. Unlike disruption of *YKU70*, disruption of *LIG4* does not have any appreciable effect on telomere length. (**A**) Depiction of a typical yeast telomere, showing that cleavage with the restriction enzyme *Xho*I yields an ~1.3 kb fragment from the conserved Y′ region present in many yeast telomeres. This fragment includes ~400 bp of repeating ($C_{1–3}A$) sequence and is detected by Southern hybridization using a radiolabelled poly$(GT)_{20}$ oligonucleotide. (**B**) Southern blots of *Xho*I-digested genomic DNA from wild-type (WT), *yku70* mutant or *lig4* mutant strains was probed with the radiolabelled poly$(GT)_{20}$ oligonucleotide to visualize telomeric sequences. The top arrow indicates the location of full-length telomeric fragments present in wild-type and *lig4* mutant strains; the bottom arrow indicates the shortened telomeric fragments present in the *yku70* mutant strain.

repair products generated in *lig4* mutants had also suffered deletion of terminal sequences, some were rejoined accurately (Figure 4C). Collectively, these results provide insights into the distinct roles performed by Lig4p and Ku in DNA NHEJ (see Discussion).

Lig4p, unlike Ku, does not appear to function in telomere length maintenance

Telomeres occur at the ends of eukaryotic chromosomes, are structurally distinct and have unusual replication intermediates for which it is unclear whether a distinct DNA ligase is necessary (Blackburn, 1991; Zakian, 1995; Lundblad and Wright, 1996). Recent work has demonstrated that Ku functions in telomere homeostasis, since disruption of either *YKU70* or *YKU80* results in a dramatic reduction in telomeric length (Boulton and Jackson, 1996b; Porter *et al.*, 1996). Given that Lig4p and Ku function together in DNA NHEJ, we tested whether *LIG4* is involved in telomere length control. To do this, yeast genomic DNA was digested with the restriction enzyme *Xho*I, which in wild-type strains produces a predominant telomeric fragment of ~1.3 kb that is detected by Southern hybridization to an oligonucleotide probe that binds to the repetitive telomeric sequences (Figure 5). Notably, whereas disruption of *YKU70* results in telomeric shortening, loss of *LIG4* function has no detectable effect. These data therefore reveal that, although Ku and Lig4p function together in DNA DSB repair, Ku but not Lig4p has an additional essential function in telomere length homeostasis.

Discussion

In this report, we have described the identification of an ORF in the *S.cerevisiae* genome that displays extensive sequence similarity along its length with mammalian DNA ligase IV. This gene, which we have designated *LIG4*, is not essential for DNA replication, *RAD52*-dependent homologous recombination nor the pathways of nucleotide excision repair and base excision repair. Instead, we have shown that *LIG4* is specifically involved in the rejoining of DNA DSBs by the process of DNA NHEJ, which does not demand homology between the two recombining DNA molecules and does not require *RAD52*. Notably, genetic epistasis experiments reveal that *LIG4* acts in the same DNA repair pathway as Ku, a nuclear protein that specifically recognizes DNA strand breaks. We have thus identified a novel *S.cerevisiae* DNA ligase and have shown that it is involved specifically in the Ku-dependent NHEJ pathway of DNA DSB repair.

In light of the above, and given that mutations in *YKU70* or *YKU80* result in dramatic telomeric shortening in yeast (Boulton *et al.*, 1996b; Porter *et al.*, 1996), we have also assessed the potential involvement of *LIG4* in telomere length homeostasis. Telomeres are the protein–DNA structures at the ends of eukaryotic chromosomes that ensure the complete replication of chromosome ends, protect these ends from degradation and prevent chromosomal termini from activating DNA damage signalling pathways or engaging in fusion and recombination reactions with other loci (for reviews, see Blackburn, 1991; Zakian, 1995; Lundblad and Wright, 1996). In most organisms, telomeres are composed of variable numbers of simple repeat sequences and, at least in *S.cerevisiae*, the length of these sequence arrays is maintained by a combination of telomerase activity and *RAD52*-dependent and -independent recombination. In yeast, deficiencies in Ku result in an ~70% reduction in the number of telomeric repeat sequences (Boulton *et al.*, 1996b; Porter *et al.*, 1996). Given that Ku binds to the ends of double-stranded DNA (Mimori and Hardin, 1986; Paillard and Strauss, 1991), one possibility is that Ku may interact directly with telomeric DNA ends and potentiate telomere lengthening by protecting telomeric DNA termini from nucleases or by augmenting telomerase recruitment. An alternative explanation is that the effect of Ku inactivation on telomere length is indirect—perhaps the DNA repair defects that are associated with Ku-deficient yeasts result in changes in cell physiology that impinge indirectly on telomere length control. Although it is not possible at present for us to identify precisely how Ku affects telomere length, the fact that mutations in *LIG4* have essentially the same DNA repair defect as Ku but do not alter telomere length argues for a specific role for Ku in telomere homeostasis that is distinct from its activities in DNA DSB repair. In this regard, it will be of interest to see whether mutated derivatives of Ku can be generated

that have no effect on DNA repair but do result in defective telomeric maintenance.

Yeast cells mutated in *LIG4* have pronounced defects in DNA NHEJ, showing that Lig4p plays a crucial role in this process that cannot be complemented efficiently by yeast DNA ligase I. Conversely, yeast *CDC9* and human DNA ligase I mutants are defective in DNA replication and, at least *in vitro*, this function is not performed efficiently by other enzymes. This suggests that yeast DNA ligases I and IV have distinct and largely separate cellular functions and cannot substitute effectively for one another. Thus, DNA ligase I plays a crucial role in DNA replication and also appears to seal single strand DNA breaks that are the end-products of nucleotide and base excision repair and, moreover, is likely to complete recombination events between homologous duplex DNA molecules (see Introduction). There are also data suggesting that mammalian DNA ligase III is specialized towards particular functions. One splice variant (DNA ligase III-α), as a complex with XRCC-1, may operate in a separate pathway for base excision repair while another variant (DNA ligase III-β) has been implicated in meiotic recombination. Notably, there are no obvious homologues of mammalian DNA ligase II/III in *S.cerevisiae*. However, sequence analyses (Figure 1; Colinas *et al.*, 1990; Kerr *et al.*, 1991; Husain *et al.*, 1995) reveal that these ligases are related more closely to DNA ligases encoded by cytoplasmic poxviruses than they are to DNA ligase I, suggesting that ligases II and III may have arisen fairly recently in vertebrate evolution. Interestingly, and largely consistent with the proposed functions of mammalian ligase III, inactivation of poxvirus DNA ligase does not affect viral DNA replication or recombination but renders the mutant virus more sensitive to DNA damage induced by UV or bleomycin (Colinas *et al.*, 1990; Kerr *et al.*, 1991). Collectively, these data suggest that DNA ligase I and perhaps DNA ligase II/III are involved predominantly in the rejoining of single-stranded nicks whereas, at least in yeast, DNA ligase IV is the major enzyme catalysing the joining of DSBs. In light of these points, and given that *LIG4* functions in the highly evolutionarily conserved Ku-dependent NHEJ pathway, it seems likely that mammalian DNA ligase IV will also play a key role in Ku-dependent DNA DSB rejoining. It will therefore be of great interest to determine whether, as is the case for Ku (reviewed in Jackson and Jeggo, 1995), deficiency in mammalian ligase IV results in cellular radiosensitivity and an inability to rejoin site-specific V(D)J recombination intermediates.

Although the available data suggest diversification of function for the different eukaryotic DNA ligases, it is unclear whether this arises from intrinsic differences in catalytic activity or from differences conferred, for example, by the distinct C- and N-terminal extensions of the enzymes (Figure 1). At least *in vitro*, purified human DNA ligases I, III and IV show differing capacities to join single-stranded breaks in hybrid polynucleotide substrates (Arrand *et al.*, 1986; Tomkinson *et al.*, 1991; Robins and Lindahl, 1996). Furthermore, purified mammalian DNA ligases differ in their abilities to rejoin DNA DSBs. It is noteworthy, however, that in contrast to the available *in vivo* data, these studies show that purified ligase I but no other mammalian DNA ligase is able to catalyse the joining of blunt DNA ends effectively *in vitro* (Arrand *et al.*, 1986; Tomkinson *et al.*, 1991, 1992; Robins and Lindahl, 1996). One possible explanation for this discrepancy between the *in vitro* and *in vivo* data is that at least some of the eukaryotic DNA ligases may not have high intrinsic affinity for DNA and, within the cell, are targeted to appropriate DNA lesions by accessory factors. Consistent with this idea, the N-terminal regulatory domain of human ligase I has been shown to target this enzyme to discrete nuclear foci called 'replication factories' (Montecucco *et al.*, 1995), and DNA ligase III, via its C-terminal extension, interacts with the DNA repair factor XRCC1 (Caldecott *et al.*, 1994) which may in turn act as a 'scaffold protein' by interacting specifically with other proteins necessary for the repair process (Kubota *et al.*, 1996). Most notably with regard to the data described in this study, the chromatographic behaviour of human DNA ligase IV is consistent with it being associated with another factor (Robins and Lindahl, 1996). If this is so, it will be of interest to identify this factor and to determine whether it co-operates with ligase IV in DNA DSB repair.

Inactivation of either yeast Ku or Lig4p results in a similar dramatic reduction of NHEJ in the *in vivo* plasmid DNA DSB repair assay. Because of this and since the level of DNA repair does not fall further in yeast strains defective in both Ku and Lig4p, we conclude that these two factors function in the same illegitimate recombination pathway. However, it is apparent that Ku and Lig4p have distinct functions in DNA NHEJ, as evidenced by the different spectra of residual plasmid repair products that are generated in the respective mutant strains. Thus, whereas nearly all the residual plasmid repair products arising in *yku70* mutants suffer deletions, in *lig4* mutant strains these correspond to a mixture of deletion products and products generated by accurate DNA end-joining. Collectively, these results suggest that Ku may function in at least two ways to potentiate DNA repair. Firstly, it may protect exposed DNA ends from nuclease attack. Secondly, it might serve to recruit specifically Lig4p, directly or indirectly, to the sites of DNA damage, perhaps via the Lig4p C-terminal extension that is absent from DNA ligase I. Consequently, the phenotypes of strains defective in Ku or Lig4p can both be explained to result from an inability to target a ligase to DNA DSBs efficiently. In Ku-deficient strains, the ready access of nucleases to the DNA ends may lead to deletions in virtually all the residual NHEJ repair products, which presumably arise via inefficient DNA end-joining by untargeted ligase I or Lig4p. In contrast, when Lig4p is absent, Ku is still able to protect the DNA ends, and this can explain how some accurate repair can still occur, this presumably being mediated by DNA ligase I. However, the reduced repair kinetics in *lig4* mutant yeast may mean that, even in the presence of Ku, nucleases ultimately gain access to the DNA termini and lead to deletions in a large proportion of the residual repair products. Consistent with the above model, we find that virtually all of the residual NHEJ products generated in *yku70/lig4* double mutants have sustained terminal deletions (S.Teo and S.P.Jackson, unpublished data). Clearly, some of the major goals for future investigations will be to determine the precise roles performed by Ku and ligase IV in DNA NHEJ in yeast and mammalian systems, and to investigate the functions

of additional polypeptides such as Rad50p, Mre11p and Xrs2p, which have been implicated in the DNA NHEJ pathway (Dolganov *et al.*, 1996; Milne *et al.*, 1996).

Materials and methods

Protein sequence analyses

Protein sequence alignment was performed using the 'core' catalytic domain of all the DNA ligases listed in Figure 1 using the PILEUP program in GCG (Genetics Computing Group, Wisconsin) package. The resultant pileup was edited using LINEUP and used to generate a consensus matrix using the PROFILEMAKE program which was in turn used in BLAST database searches. The pileup was also used to generated a phylogenetic tree using the PHYLIP package and the UPGMA method.

Gene disruptions

Full-length *LIG4* was amplified by PCR with primers *LIG4*-1 and *LIG4*-2 (5′-TCAGTAGTTGACTACGGGAAAGTCT-3′ and 5′-ATGATATCA-GCACTAGATTCTATAC-3′, respectively) using the Expand High Fidelity DNA polymerase (Boehringer Mannheim). After cloning into pGEM-T (Promega), the resultant plasmid was digested with *Eco*RI, treated with *Pfu* DNA polymerase and then digested with *Xba*I. The *HIS3* marker was inserted to replace the *LIG4* ORF between residues 289 and 592. The disruption fragment was excised with *Sph*I and *Spe*I and was used to transform the appropriate yeast strains to His^+. Gene disruption was verified by using *LIG4* and *HIS3* primers in PCR. Two *RAD52* disruption constructs were provided by D.Weaver and have *TRP1* and *URA3* selection respectively.

Ionizing-irradiation survival assays

Three independent isolates of each strain were inoculated either into minimal media lacking the appropriate amino acid(s) or into YPDA and were grown overnight at 30°C. Cultures were diluted in sterile water to an $OD_{600\ nm}$ value equivalent to 1×10^7 cells/ml and 1 ml aliquots were irradiated using a ^{137}Cs source at a dose of 0.18 kRad/min. Irradiated samples and unirradiated controls were then diluted and plated in duplicate using an automated spiral plater (Whitley) on YPDA or minimal media. Colony numbers were ascertained following incubation at 30°C for 3–4 days.

Plasmid repair assay

Plasmid repair assays were performed as described previously (Boulton and Jackson, 1996a,b). Briefly, the yeast–*E.coli* shuttle plasmid pBTM116 (2–5 μg), which contains *TRP*1 for selection in yeast, was digested with the appropriate restriction enzyme to completion and the enzyme was inactivated by treatment at 65°C for 20 min. Linearized DNA was then used to transform yeast by the lithium acetate method (Ausubel *et al.*, 1987). Parallel transformations were performed with an equivalent amount of uncut plasmid to enable normalization for slight differences in transformation efficiency. Diluted samples were plated in duplicate on minimal media lacking the appropriate amino acids, and colonies were counted following incubation at 30°C for 3–4 days. To analyse plasmid repair products, DNA from single yeast transformants was isolated via the Yeast DNA Isolation kit (Stratagene) and this was used to transform *E.coli* XL1-Blue cells (Stratagene) to ampicillin resistance. Plasmid DNA was then isolated and was analysed by restriction enzyme digestion and by DNA sequencing.

Yeast DNA extraction and analyses of telomeric DNA

Genomic DNA from *S.cerevisiae* was isolated essentially as described (Ausubel *et al.*, 1987). For telomere analyses, 2 μg of genomic DNA was digested with 30 U of *Xho*I (Boehringer Mannheim) at 37°C overnight. The digested DNA was then separated on a 1.2% agarose 1× TAE gel and was transferred to Hybond Nfp+ membrane (Amersham) by capillary transfer in 20× SSC as suggested by the manufacturer. Membranes were pre-hybridized in 0.5 M sodium phosphate, pH 7.2, 1% SDS and then hybridized with 3 ng/ml of ^{32}P-end-labelled $poly(GT)_{20}$ oligonucleotide (sp. act. $>10^9$/μg) in a Church-based buffer (0.2 M sodium phosphate, pH 7.2, 1% bovine serum albumin, 6% polyethyleneglycol 6000, 1% SDS) overnight at 62°C. Finally, membranes were washed twice at room temperature for 30 min in 0.2 M sodium phosphate, 0.1% SDS, then exposed to pre-flashed X-ray film at –70°C.

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