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Components of the Ku-dependent non-homologous end-joining pathway are involved in telomeric length maintenance and telomeric silencing

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In the budding yeast, *Saccharomyces cerevisiae*, genes in close proximity to telomeres are subject to transcriptional silencing through the process of telomere position effect (TPE). Here, we show that the protein Ku, previously implicated in DNA double-strand break (DSB) repair and in telomeric length maintenance, is also essential for telomeric silencing. Furthermore, using an *in vivo* plasmid rejoining assay, we demonstrate that *SIR2*, *SIR3* and *SIR4*, three genes shown previously to function in TPE, are essential for Ku-dependent DSB repair. As is the case for Ku-deficient strains, residual repair operating in the absence of the *SIR* gene products ensues through an error-prone DNA repair pathway that results in terminal deletions. To identify novel components of the Ku-associated DSB repair pathway, we have tested several other candidate genes for their involvement in DNA DSB repair, telomeric maintenance and TPE. We show that *TEL1*, a gene required for telomeric length maintenance, is not required for either DNA DSB repair or TPE. However, *RAD50*, *MRE11* and *XRS2* function both in Ku-dependent DNA DSB repair and in telomeric length maintenance, although they have no major effects on TPE. These data provide important insights into DNA DSB repair and the linkage of this process to telomere length homeostasis and transcriptional silencing.
Keywords: DSB repair/Ku/Rad50/silencing/telomeres

Introduction

Ionizing radiation (IR) and radio-mimetic chemicals induce a variety of DNA lesions, the most lethal of which is the DNA double-strand break (DSB). The severity of this lesion is highlighted by the fact that a single DSB left unrepaired can be sufficient to induce cell death. Consequently, eukaryotic cells have developed mechanisms to sense DNA DSBs and mediate their effective repair. For example, work in mammalian systems has revealed that the enzyme DNA-dependent protein kinase (DNA-PK) functions in the DNA non-homologous end-joining (NHEJ) pathway of DSB repair (for reviews, see Jackson and Jeggo, 1995; Jackson, 1996; Lieber *et al*., 1997). DNA-PK is a multi-protein complex consisting of an ~465 kDa catalytic subunit (DNA-PK$_{cs}$; Hartley *et al*., 1995) and a DNA targeting component, termed Ku, which itself consists of two polypeptides of ~70 and 80 kDa (Ku70 and Ku80, respectively; Dvir *et al*., 1993; Gottlieb and Jackson, 1993). Notably, cells deficient in DNA-PK$_{cs}$ or Ku are hypersensitive to IR and display defects in DNA DSB rejoining. Furthermore, cells lacking functional DNA-PK are defective in V(D)J recombination, the genomic rearrangement process used in mammals to derive much of the antigen binding diversity of immunoglobulin and T-cell receptor molecules.

In contrast to mammalian systems, where NHEJ is the predominant DNA DSB repair pathway, in the yeast *Saccharomyces cerevisiae* this process is used less frequently. Recent work, however, has revealed that *S.cerevisiae* does possess homologues of both Ku70 and Ku80, and that these play crucial roles in DNA NHEJ (Boulton and Jackson, 1996a,b; Feldmann *et al*., 1996; Mages *et al*., 1996; Milne *et al*., 1996; Siede *et al*., 1996). This therefore suggests that the NHEJ system has been conserved highly throughout eukaryotic evolution. Consistent with this, recent studies have provided linkages between both mammalian DNA ligase IV and its yeast homologue, Lig4p (Dnl4p), in DNA NHEJ (Critchlow *et al*., 1997; Grawunder *et al*., 1997; Schar *et al*., 1997; Teo and Jackson, 1997; Wilson *et al*., 1997). *Saccharomyces cerevisiae* does not, however, possess a clear homologue of DNA-PK$_{cs}$ (Goffeau *et al*., 1996). Nevertheless, the existence of the DNA-PK$_{cs}$ relatives Tel1p and Mec1p in *S.cerevisiae*, and the involvement of such factors in DNA damage signalling (Greenwall *et al*., 1995; Carr, 1996; Jackson, 1996; Sun *et al*., 1996), suggests that one or other of these might function together with yeast Ku in DNA NHEJ.

Another mechanism of DNA DSB repair employs the process of homologous recombination and involves the exchange of genetic information between the damaged DNA molecule and an undamaged partner (for reviews, see Friedberg *et al*., 1995; Shinohara and Ogawa, 1995). Although this process can take place in mammalian cells, it is used less frequently than DNA NHEJ. By contrast, homologous recombination is the dominant DSB repair pathway in *S.cerevisiae*. Fundamental to homologous recombination in *S.cerevisiae* are the genes encoded by the *RAD52* epistasis group (*RAD50–57*, *MRE11* and *XRS2*). Strains carrying mutations in these genes display similar degrees of hypersensitivity to IR and show epistatic interactions when placed in pair-wise combinations. Nevertheless, the *RAD52* epistasis group can be divided into two sub-groups on the basis of the involvement of different genes in distinct types of recombinational repair. Thus, *RAD50*, *MRE11* and *XRS2* constitute a distinct sub-group since, unlike other members of the *RAD52* epistasis group, they have been linked to non-homologous chromosomal integration, and to illegitimate DSB joining of chromosomal and episomal DNA (Schiestl and Petes, 1991; Schiestl *et al*., 1993, 1994; Tsukamoto *et al*., 1997a). Significantly, recent work has revealed the existence of

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mammalian homologues of *RAD50* and *MRE11* (Petrini *et al*., 1995; Dolganov *et al*., 1996), suggesting that their functions are conserved throughout the eukaryotic kingdom.

DNA strand breaks existing within telomeres at the ends of linear eukaryotic chromosomes are unusual in that they are not normally recognized as DNA damage. One way that this may be achieved is via telomeric DNA being sequestered into a unique type of chromatin organization. In this regard, it is noteworthy that the telomeres of most eukaryotes contain repeats of simple repetitive sequences that are thought to function, in part, as recognition sites for telomere-associated proteins. For example, in *S.cerevisiae*, telomeres consist of tandem arrays of the consensus sequence $C_{1-3}A$ that include binding sites for the protein Rap1p, which plays an important role in telomere length maintenance (Blackburn, 1991; Marcand *et al*., 1997). As in many other eukaryotic systems, yeast appears to have evolved a number of mechanisms to maintain the $C_{1-3}A$ repeats to combat the gradual loss of terminal chromosomal sequences during DNA replication. The principal mechanism is believed to utilize the enzyme telomerase, a ribonucleoprotein complex that extends the $C_{1-3}A$ repeats by pairing its RNA component with existing telomeric $C_{1-3}A$ sequences then extending these through a reverse transcriptase-based elongation–translocation cycle (Cohn and Blackburn, 1995; Zakian, 1995).

In addition to functioning in the maintenance of chromosome integrity, yeast telomeres exert profound influences on the transcription of adjacent genes. Thus, genes placed within or near to telomeres are subject to transcriptional repression as a result of the phenomenon termed the telomere position effect (TPE) (Gottschling *et al*., 1990; Aparicio *et al*., 1991; Shore, 1995). Several genes have been implicated in TPE, some of the best characterized of which are *SIR2*, *SIR3* and *SIR4 (*Aparicio *et al*., 1991), which were first identified in genetic screens looking for mutations that relieved transcriptional repression of the silent mating type loci (Klar *et al*., 1979; Rine *et al*., 1979). Notably, none of the Sir proteins appears to contain DNA binding motifs, and it is thought that they are targeted to telomeres and to the silent mating type cassettes via interactions with Rap1p (Shore *et al*., 1984; Shore and Nasmyth, 1987; Moretti *et al*., 1994). Once positioned at such loci, Sir2p, Sir3p and Sir4p are believed to bring about transcriptional silencing, at least in part, via their packaging DNA into a heterochromatin-like state. Despite the importance of the $C_{1-3}A$ repeats in TPE, the available evidence suggests that maximal telomeric silencing also requires a terminus-specific DNA end-binding factor that operates synergistically with $C_{1-3}A$-based silencing. For example, Stavenhagen and Zakian (1994) demonstrated that high-copy number plasmids possessing $C_{1-3}A$ repeat sequences de-repress $C_{1-3}A$-based silencing but have only a minor effect on TPE. In contrast, $C_{1-3}A$ repeat sequences containing extra-chromosomal linear plasmids relieve both TPE- and $C_{1-3}A$-based silencing (Wiley and Zakian, 1995). Together, these data suggest that a factor that can only be competed effectively by DNA termini plays a crucial role in telomeric silencing. Although candidate factors have been detected in several systems, the exact nature of the protein(s) functioning in this regard remains to be established.

In light of the above, it is noteworthy that we and others have demonstrated a role for yeast Ku in telomere length maintenance (Boulton and Jackson 1996b; Porter *et al.*, 1996). Thus, yeast cells defective in either of the genes for Ku70 or Ku80 (*YKU70* and *YKU80*, respectively; also termed *HDF1* and *HDF2*, respectively) lose the majority but not all of their terminal $C_{1-3}A$ repeats. Interestingly, a similar telomere-shortening phenotype is observed in strains lacking Tel1p, which is related to DNA-PK_{cs} in sequence. Together, these observations raise the possibility that Ku might interact with Tel1p, that Tel1p could be a component of the yeast NHEJ system and that Tel1p and/or Ku could function in chromosome terminus-mediated transcriptional repression. Furthermore, they suggest that certain other factors involved in telomere length control in yeast, such as Sir2p, Sir3p or Sir4p, could play important roles in DNA NHEJ. Here, we show that Ku is indeed required for telomere-mediated transcriptional silencing, whereas Tel1p is not required for NHEJ and only has a minor effect on TPE. Furthermore, and consistent with the very recent report by Tsukamoto *et al*. (1997b), we find that *SIR2*, *SIR3* and *SIR4* but not *SIR1* are required for NHEJ. Finally, we reveal that Rad50p, Mre11p and Xrs2p are essential components of the Ku-associated DNA NHEJ system and that they function in telomere length maintenance. However, unlike Ku and the Sir proteins, Rad50p, Mre11p and Xrs2p have only a minor effect on TPE. The significance of these findings in regard to DNA DSB repair, telomere length control and TPE are discussed.

Results

Yeast Ku is required for telomere-directed transcriptional silencing

Since Ku binds to DNA DSBs and functions in telomere length control in yeast, we were interested to learn whether it is involved in telomere-directed transcriptional silencing. To address this question, we utilized derivatives of the yeast strain UCC5 that contain the *URA3* gene integrated into the telomeric region of chromosome VII (Figure 1A). In wild-type strains, this *URA3* gene is subject to TPE and is consequently transcriptionally silent. However, *URA3* becomes de-repressed in yeasts carrying mutations in genes required for TPE, such as *SIR2*, *SIR3* and *SIR4* (Aparicio *et al.*, 1991). Using 5-FOA, a uracil analogue that is lethal to cells able to metabolize uracil, it is therefore possible to determine the transcriptional status of the telomere-associated *URA3* marker. Thus, wild-type strains exhibiting TPE do not express *URA3* and consequently, are able to grow either in the presence or absence of 5-FOA (Figure 1B; *SIR*). This is also the case for cells disrupted for *SIR1* function, as loss of this gene debilitates silencing at the mating-type loci but does not affect TPE (Aparicio *et al*., 1991). By contrast, disruption of *SIR2*, *SIR3* or *SIR4* leads to loss of TPE, the induction of *URA3* expression, and a consequential inability to grow on 5-FOA (Figure 1B). Strikingly, disruption of either *YKU70* or *YKU80* results in an inability to grow in the presence of 5-FOA, indicating that the telomere-associated *URA3* gene is transcriptionally active (Figure 1B). These data therefore reveal that Yku70p and Yku80p play crucial roles in TPE.

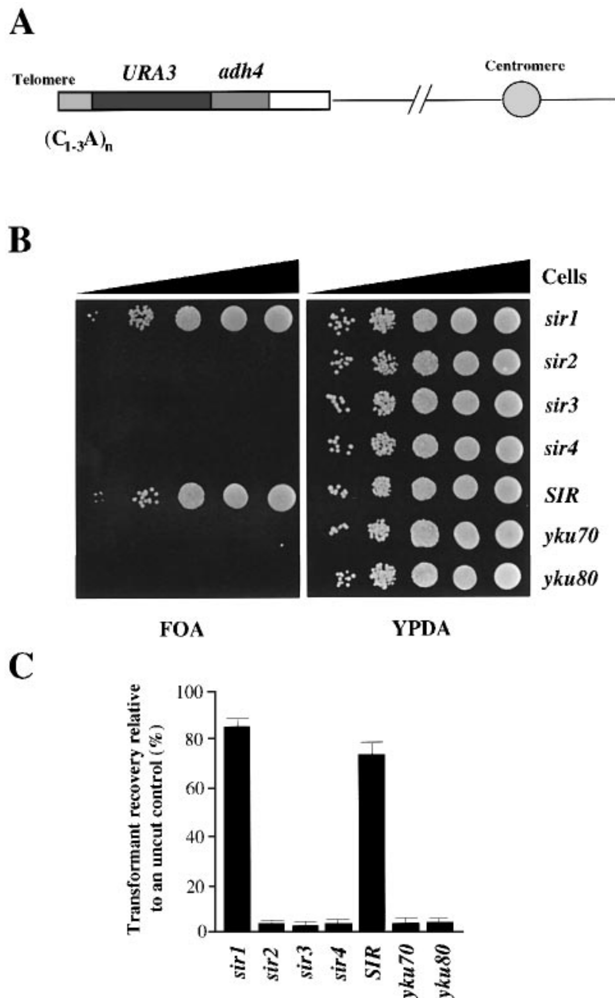

Fig. 1. Factors required for Ku-dependent DNA NHEJ and telomeric silencing. (**A**) Schematic representation of the organization of the *URA3*-marked telomere of strain UCC5. A *URA3* marker with homology to the *ADH4* gene and the telomere repeat sequences was used to insert *URA3* into the telomere region of chromosome VII. (**B**) TPE assays. *YKU70, YKU80* and *SIR1–4* disruptants were generated in the wild-type (*SIR*) strain UCC5 carrying the *URA3*-marked telomere. The telomeric *URA3* is transcriptionally active in yeast carrying mutations in genes required for telomere silencing, *SIR2–4* (UCC2, 3 and 4, respectively; Table II), resulting in lethality on 5-FOA. *SIR1* is not required for TPE, consequently UCC1 (Table II) is able to grow on 5-FOA. *YKU70* and *YKU80*, like *SIR2–4*, are required for TPE, as disruption of either abolishes *URA3* silencing at the telomere, resulting in lethality on 5-FOA. For each strain, a 10-fold serial dilution was made five times and 7.5 μl aliquots were plated onto 5-FOA or YPDA plates and incubated at 30°C for 3–4 days. (**C**) Disruption of *SIR2, SIR3* or *SIR4* impairs plasmid NHEJ. *sir1*, *sir2*, *sir3, sir4*, *SIR* (wild-type), *yku70* or *yku80* strains (UCC1–5, UCC70 and UCC80, respectively) were transformed, in parallel, with supercoiled or linear *Eco*RI-cleaved pBTM116 (or pRS414). Cells were plated in duplicate and incubated at 30°C for 3–4 days. The value plotted is the number of transformants obtained with *Eco*RI-linearized vector expressed as a percentage of the number obtained with the supercoiled vector.

SIR2, SIR3 and SIR4 but not SIR1 are required for Ku-dependent DNA NHEJ

The observation that yeast Ku, like Sir2p, Sir3p and Sir4p, is involved both in telomere length regulation and telomeric silencing suggested that further parallels might exist between the functions of these proteins. Specifically, we were interested to determine whether *SIR2*, *SIR3* and *SIR4* influence the Ku-dependent pathway of DNA NHEJ. To address this possibility, we utilized a transformation-based *in vivo* plasmid repair assay that we have developed to study Ku-dependent DNA NHEJ (Boulton and Jackson, 1996a,b; Teo and Jackson, 1997). In this assay, the strain under investigation is transformed with a yeast–*Escherichia coli* shuttle vector that has been linearized by treatment with a restriction enzyme. To correct for possible differences in transformation efficiencies between experiments and between strains, a supercoiled plasmid control is transformed into the yeast strain, in parallel. Since the linearized plasmid must be recircularized to be propagated, the number of transformants obtained with the linear plasmid normalized to that obtained with the supercoiled control reflects the yeast strain's ability to repair the DSB. Importantly, the plasmid used in these studies is devoid of yeast-derived sequences around the restriction enzyme cleavage site, so it is not a good substrate for homologous recombination and is repaired predominantly by DNA NHEJ. Strikingly, as is the case for loss of Ku function, the disruption of *SIR2*, *SIR3* or *SIR4* results in a dramatic (~50-fold) drop in plasmid NHEJ (Figure 1C). By contrast, *sir1* mutant strains have essentially wild-type levels of plasmid rejoining. These data are therefore consistent with those reported very recently by Tsukamoto *et al.* (1997b) and demonstrate that Sir2p, Sir3p and Sir4p are important components of the DNA NHEJ system.

In addition to measuring the efficiency of NHEJ, the plasmid repair assay also allows the accuracy of DSB rejoining to be ascertained by shuttling the repaired plasmids into *E.coli* and analysing these by restriction enzyme digestion and by DNA sequencing. Previous work has indicated that, in wild-type strains, the rejoining of DNA DSBs induced by restriction enzymes such as *Eco*RI or *Xba*I appears to take place almost exclusively by direct ligation, and no loss or addition of DNA sequences ensues. By contrast, in the absence of functional Ku, the low amount of residual DNA NHEJ that occurs results in deletion of terminal sequences and in DNA joining via short (usually 2–7 bp) direct repeat elements (Table I; some repair products are also generated via homologous recombination-mediated 'gap repair' with chromosomal sequences; Boulton and Jackson, 1996a,b; Schar *et al*., 1997; Teo and Jackson, 1997; Wilson *et al*., 1997). The available evidence suggests that this residual 'error-prone' DNA NHEJ in the absence of Ku takes place via the single-stranded annealing pathway (Kramer *et al*., 1994; Mezard and Nicolas, 1994; Moore and Haber, 1996). To determine the nature of the residual repair events taking place in *SIR2*, *SIR3* or *SIR4* disrupted strains, we retrieved a series of plasmid repair products arising in each case and subjected these to DNA sequence analyses. Significantly, as is the case for strains deficient in Ku, virtually all of the residual DNA NHEJ products had suffered deletions and were joined via short direct repeats (Table I; as with Ku-defective strains, some homologous recombination-mediated gap repair products were also generated). Furthermore, in each case, the spectrum of deletion products obtained was similar or identical to those derived from Ku-deficient strains. These data therefore suggest that Sir2p, Sir3p and Sir4p function at the same stage of the DNA NHEJ process as Ku.

Table I. Plasmid repair products generated in various genetic backgrounds (for strains, see Table II)

	Strain	Repair	Fidelity of repair	
		% to uncut control		
	Wt	75±10	accurate	a
	yKu70	2±1	inaccurate	b, c, d
	lig4	2±1	accurate/inaccurate	a, b, c, d
	rad52	40±5	accurate	a
	tel1	75±10	accurate	a
	rad50	2±1	predominately accurate	a, b, c
	mre11	2±1	predominately accurate	a, b, c
	xrs2	2±1	predominately accurate	a, b, c
yku70	*rad50*	2±1	inaccurate	b, c, d
yku70	*mre11*	2±1	inaccurate	b, c, d
yku70	*xrs2*	2±1	inaccurate	b, c, d
	sir1	75±10	accurate	a
	sir2, *3* or *4*	2±1	inaccurate	b, c, d

At least 30 repaired plasmids for each strain were shuttled to *E.coli* and were analysed by restriction enzyme digestion and DNA sequencing. As reported previously (Boulton and Jackson, 1996a,b), wt strains repair plasmids bearing 5′ overhanging cohesive ends with high efficiency and fidelity. Repair in *tel1* and *rad52* mutant strains is similar to that in wild-type, in that it is both efficient and accurate. *sir1* mutant strains also repair both efficiently and accurately. However, the residual inefficient repair observed in *yku70*, *yku80*, *sir2*, *sir3* and *sir4* mutant strains is inaccurate, resulting from repair between short repeats of homology and deletion of the intervening sequences. The inefficient repair observed in *lig4* mutant strains contains a mixture of accurate and inaccurate products (Teo and Jackson, 1997). The inefficient repair observed in *rad50*, *mre11* or *xrs2* mutant strains is virtually exclusively accurate—only one inaccurate product was observed in the *rad50* strain and consisted of a small deletion of 24 bp. In *rad50* and *mre11*, but not in the *xrs2* strains, a number of products had undergone gap-repair with the endogenous *ADH1* gene. Repair products observed when *RAD50*, *MRE11*, or *XRS2* were disrupted in a *yku70* mutant background were predominately inaccurate, similar to that seen for strains deficient in *YKU70* alone. Key: precise end-to-end joining (a), small deletions (b), gap repair with the endogenous *ADH1* gene (c), large deletions generated through repair between short direct homology repeats (d).

Table II. Yeast strains used in these studies

Strain	Genotype	Constructed by:
W303-1A	*MATα ade2 his3 leu2 trp1 ura3 can1-100*	Boulton and Jackson (1996a)
W303-1B	*MATa ade2 his3 leu2 trp1 ura3 can1-100*	Boulton and Jackson (1996a)
yku70α	*W303-1A yKu70::LEU2*	Boulton and Jackson (1996a)
yku70a	W303-1B yku70::URA3	Boulton and Jackson (1996a)
ligIV	*W303-1A ligIV::LEU2*	Teo and Jackson (1997)
tel1	*W303-1A tel1::HIS3*	this study
rad50	*W303-1A rad50::LEU2*	J.Downs
mre11	*W303-1A mre11::HIS3*	this study
xrs2	*W303-1A xrs2::HIS3*	this study
rad10	*W303-1A rad10::URA3*	this study
sgs1	*W303–1B sgs1::LEU2*	I.Hickson
UCC1	*JRY1705 TELadh4::URA3 sir1::HIS3*	D.Gottschling
UCC2	*JRY1706 TELadh4::URA3 sir2::HIS3*	D.Gottschling
UCC3	*JRY1264 TELadh4::URA3 sir3::LYS3*	D.Gottschling
UCC4	*JRY1263 TELadh4::URA3 sir4::HIS3*	D.Gottschling
UCC5	*DBY703 TELadh4::URA3*	D.Gottschling
UCC6	*DBY703 ura3–52::URA3*	D.Gottschling
UCCyku80	*UCC5 yku80::TRP*	this study
UCCtel1	*UCC5 tel1::HIS3*	this study
UCCrad50	*UCC5 rad50::HIS3*	this study
UCCmre11	*UCC5 mre11::HIS3*	this study
UCCxrs2	*UCC5 xrs2::HIS3*	this study

TEL1 is not required for Ku-dependent NHEJ and only has a minor effect on telomeric silencing

Given the homology between Tel1p and DNA-PK$_{cs}$, and since disruption of *TEL1* results in telomeric shortening, we tested for the potential involvement of Tel1p in TPE and DNA NHEJ. Thus, we analysed *tel1* mutant strains using the transformation-based plasmid repair assay described above. Notably, these studies revealed that *tel1* strains have approximately wild-type levels of plasmid rejoining (Figure 2A). In addition, analysis of the DNA NHEJ products arising in *tel1* mutant yeasts revealed that virtually all had been repaired by direct DNA end-ligation and had not suffered nucleotide loss or addition (Table I). These results therefore show that Tel1p is not an essential component of the yeast Ku-associated DNA NHEJ pathway. To establish whether *TEL1* plays a role in TPE, *TEL1* was disrupted in strain UCC5 that possesses the *URA3* gene integrated into the telomeric region of chromosome VII (Figure 1A). The resulting strain was then tested along with control strains for an ability to grow in the presence or absence of 5-FOA. Significantly, disruption of *TEL1* only results in a minor impairment of growth on 5-FOA, indicating that the *URA3* gene is repressed almost completely in this genetic background (Figure 2B). This contrasts markedly with the situation for strains deficient in *YKU70*, where growth on 5-FOA is almost fully

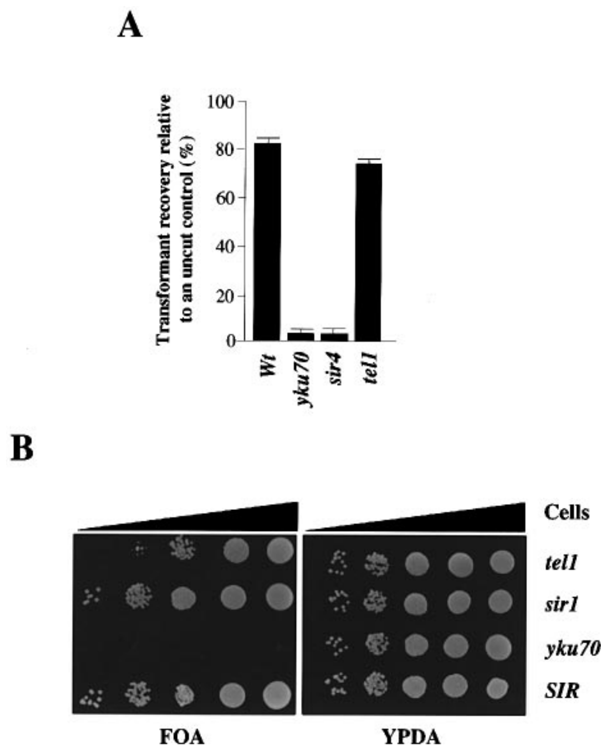

Fig. 2. Ku and Tel1p function in distinct pathways. (**A**) *TEL1* is not required for Ku-dependent plasmid NHEJ. Plasmid repair assays were conducted as outlined in the legend to Figure 1(C), using *Eco*RI-cut and supercoiled pBTM116. (**B**) Tel1p, unlike Ku, has only a minor effect in TPE. *TEL1* was disrupted in strain UCC5 (generating UCCTel; Table II) that has *URA3* inserted into the telomere region of chromosome VII. Assays were performed as outlined in the legend to Figure 1C.

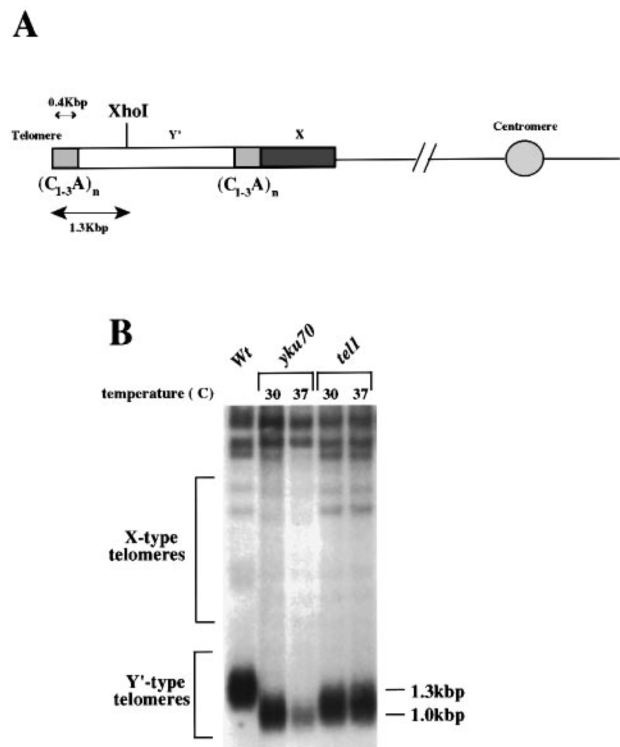

Fig. 3. Ku-deficient strains lose further telomeric repeats at 37°C. (**A**) Schematic representation of a yeast chromosomal arm, showing the locations of the centromeric and telomeric regions, and the relative positions of the $(C_{1-3}A)$ repeats, and the X′ and Y′ elements. *Xho*I cleaves in the conserved Y′ region present in most telomeres, generating chromosomal terminal fragments of ~1.3 kbp in wt yeast strains. This fragment includes ~400 bp of repeat $(C_{1-3}A)$ sequences. (**B**) Ku-deficient and *Tel1p*-deficient strains both display defects in telomere length maintenance, but unlike *tel1* strains, Ku-deficient strains lose further telomere repeat sequences at 37°C. In each case, 1 µg of *Xho*I-digested genomic DNA was electrophoresed on a 0.8% agarose gel, Southern transferred to a nylon membrane and hybridized with an end-labelled poly$(GT)_{20}$ probe that anneals to the telomere repeats.

abrogated. Thus, whereas Ku appears to be essential for TPE, *TEL1* has only a minor effect on this process. These results reinforce the notion that Tel1p and Ku function independently of one another. Furthermore, since loss of Ku or Tel1p leads to similar reductions in telomere length, this suggests that the abrogation of TPE in *yku70* or *yku80* mutant strains is not a simple consequence of the partial loss of telomeric repeats and, instead, is likely to reflect a distinct function of Ku in telomeric silencing.

Further loss of telomeric repeats is associated with the temperature-sensitive lethality of strains lacking Ku

We and others have shown previously that Ku-deficient yeast strains are temperature sensitive (ts), and are unable to grow at 37°C (Feldmann and Winnacker, 1993; Boulton and Jackson, 1996a,b; Barnes and Rio, 1997). In contrast, *tel1* mutant yeasts grow normally at 37°C (data not shown). These data therefore support models in which Ku and Tel1p operate independently from one another. Consistent with this idea, *tel1–yku70* or *tel1–yku80* strains are even more ts than are strains deficient in Ku alone, with growth becoming inhibited almost totally at 33.5°C (data not shown). Interestingly, previous studies have revealed that strains lacking Ku do not die immediately upon transfer to the restrictive temperature and, instead, continue dividing for several generations before arresting terminally in the G_2 phase of the cell cycle (Barnes and Rio, 1997). Given that this arrest phenotype is reminiscent of that displayed by strains lacking components of telomerase, we tested whether the death of Ku-deficient strains at 37°C is reflected in changes in telomeric composition. To this end, cultures of wild-type, *tel1*, *yku70* or *yku80* mutant strains were propagated at 30°C, then were split into two, and one half was propagated further at 30°C, whilst the other was grown at 37°C. Next, DNA was isolated, digested with the restriction enzyme *Xho*I and subjected to Southern blot hybridization analysis using the radiolabelled oligonucleotide, poly$(GT)_{20}$ that hybridizes to the telomeric repeat elements $(C_{1-3}A)$. As depicted in Figure 3A, *Xho*I cleaves within the sub-telomeric Y′ region that is found in the majority of yeast telomeres, thus generating a terminal restriction fragment of ~1.3 kbp that hybridizes with the poly$(GT)_{20}$ probe. In addition, a number of higher molecular weight bands are evident, which correspond to telomeric ends from the subset of telomeres that lack Y′ regions. As shown previously, at permissive growth temperatures, *yku70* or *yku80* mutant strains have greatly shortened telomeres, corresponding to a loss of ~65% of the $(C_{1-3}A)$ terminal telomeric repeat sequences. Strikingly, whereas telomere length is not affected when wild-type strains or *tel1* mutant yeasts are incubated at 37°C, the transfer of *yku70* or *yku80* mutant

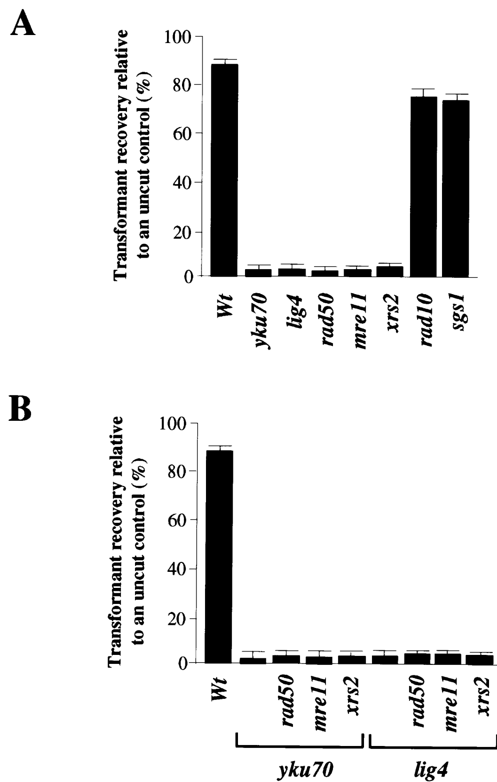

Fig. 4. *RAD50, MRE11* and *XRS2* are required for Ku-dependent NHEJ. (**A**) Strains disrupted for *RAD50, MRE11* or *XRS2,* but not *RAD10* or *SGS1* (Table II), display defects in plasmid rejoining. (**B**) The *rad50, mre11* and *xrs2* plasmid rejoining defect is epistatic with defects associated with *yku70* or *lig4* mutations. Plasmid repair assays were conducted as described in the legend to Figure 1(C), using *Eco*RI-cut and supercoiled pBTM116.

strains to 37°C leads to a further dramatic loss of telomeric repeats [Figure 3B; the further shortened telomeres are not detected by hybridization to the poly(GT)$_{20}$ probe presumably because they have now lost virtually all of their terminal telomeric repeat sequences]. Indeed, quantitation of the hybridization signal reveals that the number of repeats is reduced to ~5% of that found in wild-type strains. Since previous studies with telomerase-deficient yeasts have shown that growth arrest ensues when telomeres are shortened to this extent, these data suggest that the death of Ku-deficient yeast strains at 37°C is a consequence of the loss of telomeric repeats.

Strains disrupted for RAD50, MRE11 or XRS2 are deficient in NHEJ but the precise nature of their repair defects differs from that of Ku-deficient strains

Previous studies have revealed that *RAD50*, *MRE11* and *XRS2* are involved in illegitimate recombination processes (Schiestl *et al.*, 1994; Milne *et al.*, 1996; Tsukamoto *et al.*, 1997a). To define the roles of these genes in DNA DSB repair, we tested *rad50*, *mre11* or *xrs2* mutant strains in the transformation-based plasmid NHEJ assay discussed above. Notably, as is the case for strains debilitated in *YKU70*, *YKU80* or *LIG4*, *rad50*, *mre11* and *xrs2* mutant yeasts display an ~40 to 50-fold reduction in plasmid rejoining (Figure 4A). By contrast, disruption of the DNA repair-associated genes *RAD10* or *SGS1* has little or no effect on DNA NHEJ. Importantly, *rad50–yku70*, *mre11–yku70* or *xrs2–yku70* double mutant yeasts are not significantly more debilitated in plasmid repair than are the single mutant strains (Figure 4B). Similarly, *rad50–lig4*, *mre11–lig4* or *xrs2–lig4* double mutants are not appreciably more impaired than are the single mutants (Figure 4B). Taken together, these data indicate that Rad50p, Mre11p and Xrs2p function epistatically with Ku and Lig4p in DNA NHEJ.

To determine the nature of the residual plasmid repair events taking place in *rad50*, *mre11* and *xrs2* mutant strains, we retrieved a series of the plasmid repair products arising in each case and subjected these to DNA sequence analyses. As is the case with strains deficient in Ku, a subset of the repair products arising in *rad50* or *mre11* mutant strains had been generated by homologous recombination-mediated gap repair with chromosomal sequences (Table I). In contrast, none of the products arising in *xrs2* mutant strains was of this type, which is in line with the demonstrated role of *XRS2* in *RAD52*-dependent homologous recombination. More significantly, as shown in Table I, virtually all of the DNA NHEJ products derived from *rad50*, *mre11* or *xrs2* mutant yeasts had been ligated accurately, without nucleotide loss or addition (the only exception to this was a single NHEJ product arising in the *rad50* mutant, which had lost a small amount of terminal sequences before ligation had ensued; see Table I legend). This therefore contrasts markedly with the situation for Ku-deficient strains, where nearly all residual NHEJ products suffer deletions. Taken together, these results show that, although Rad50p, Mre11p and Xrs2p are required for efficient DNA NHEJ, these proteins have different roles from Ku in this process (see Discussion). To gain insights into the epistatic relationships between the two distinct phenotypes for residual NHEJ, we analysed the nature of the residual repair products arising in *yku70–rad50*, *yku70–mre11* or *yku70–xrs2* double mutant yeasts. Significantly, virtually all the residual plasmid rejoining in such strains is inaccurate, with plasmids containing deletions of various sizes (Table I). These products are hence similar or identical to those observed in strains lacking Ku alone. This indicates that, with regard to DNA NHEJ, the repair phenotype associated with lack of Ku is dominant over that associated with disruption of *RAD50*, *MRE11* or *XRS2* (see Discussion).

Disruption of RAD50, MRE11 or XRS2 results in telomeric shortening but has only a minor effect on telomeric silencing

The above results establish a role for *RAD50*, *MRE11* and *XRS2* in DNA NHEJ. Given that some (*YKU70*, *YKU80*, *SIR2*, *SIR3* and *SIR4*) but not all (*LIG4*) of the other genes involved in NHEJ function in telomere length maintenance and TPE, we tested whether *RAD50*, *MRE11* and *XRS2* are involved in these processes. Thus, genomic DNA was recovered from cultures of the appropriate strains, was digested with *Xho*I and analysed by Southern hybridization analysis using a radiolabelled poly(GT)$_{20}$ probe that anneals to the $C_{1–3}A$ telomeric repeat elements. Notably, these studies revealed that *rad50*, *mre11* and *xrs2* mutant strains suffer dramatic telomeric attrition, losing ~65% of their terminal repeat sequences (Figure 5A). Furthermore, no further telomere shortening is evident in *yku70–rad50*, *yku70–mre11* or *yku70–xrs2* double mutant strains

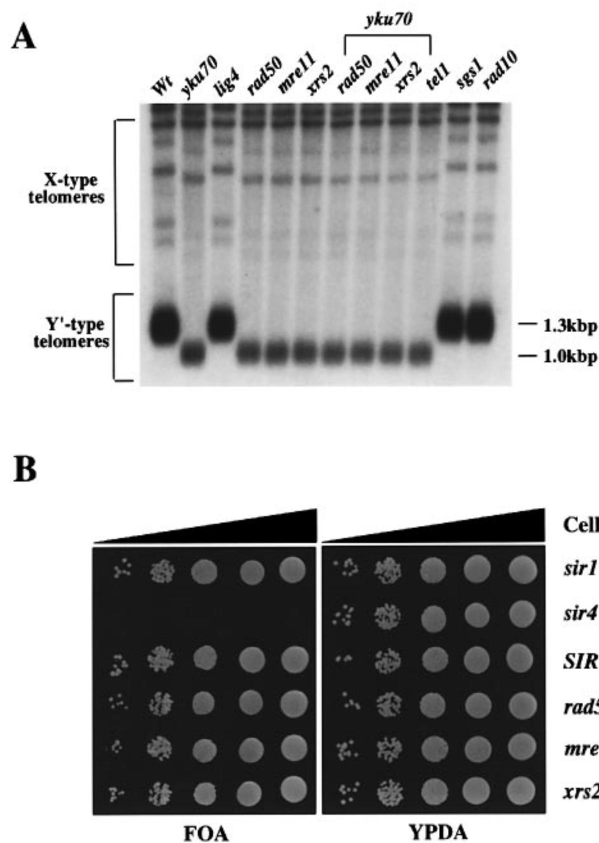

Fig. 5. Rad50p, Mre11p and Xrs2p are required for telomeric length maintenance but not for TPE. (**A**) *rad50*, *mre11* and *xrs2* strains display dramatic telomeric attrition, a defect that is epistatic with the telomeric length defect of Ku-deficient strains. Telomere length was determined as outlined in the legend to Figure 3B. (**B**) To determine the involvement of *RAD50*, *MRE11* and *XRS2* in TPE, disruptions of each gene were made in the strain UCC5 (*URA3*-marked telomere), generating UCCrad50, UCCmre11 and UCCxrs2, respectively. TPE assays were determined as outlined in the legend to Figure 1B.

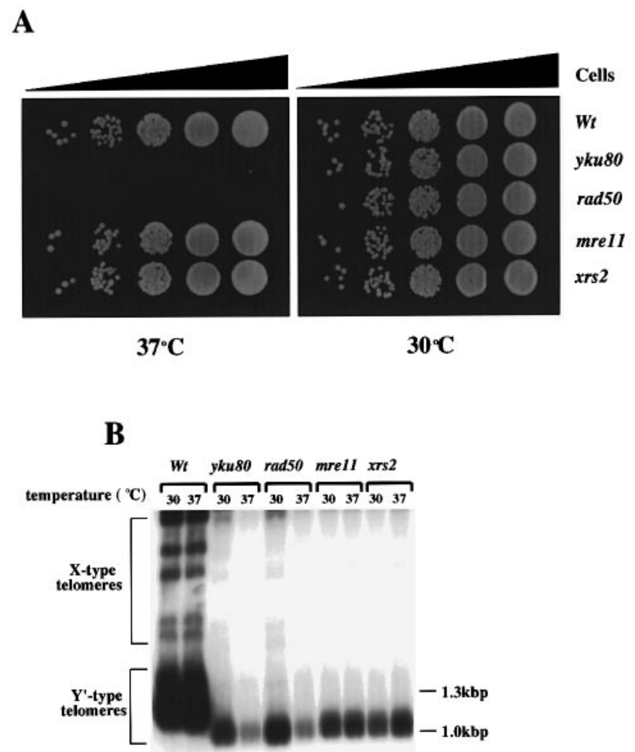

Fig. 6. The *rad50* mutation, unlike the *mre11* and *xrs2* mutations, leads to ts growth defect and a concomitant loss of additional telomeric repeats at 37°C, phenotypes similar to that observed for Ku-deficient strains. (**A**) The temperature sensitivity of wt, *yku80*, *rad50*, *mre11* and *xrs2* mutant strains was determined by plating 15 µl aliquots of a 5× 10-fold serial dilution in duplicate onto YPDA plates followed by growth at either 30 or 37°C for 3–4 days. (**B**) *rad50* strains but not *mre11* or *xrs2* mutant strains, lose additional telomere sequences at 37°C. Growth conditions and assays were as outlined in the legend to Figure 2C.

(Figure 5A). These results therefore indicate that *RAD50*, *MRE11* and *XRS2* function epistatically with Ku in telomere length maintenance.

In light of the above, we assessed the potential involvement of *RAD50*, *MRE11* and *XRS2* in TPE. To do this, *RAD50*, *MRE11* or *XRS2* disruptions were constructed in strain UCC5 containing a *URA3*-tagged telomere, and telomeric silencing of *URA3* was assessed by testing the resulting strains for growth in the presence or absence of 5-FOA (Figure 5B). Significantly, these studies revealed that *URA3* remains fully repressed in *rad50*, *mre11* and *xrs2* mutant strains. Therefore, despite functioning in a manner similar to *YKU70* and *YKU80* in telomere length control and DNA NHEJ, *RAD50*, *MRE11* and *XRS2* differ from the Ku components in that they are not essential for TPE.

rad50 but not mre11 or xrs2 mutant strains are ts and lose additional terminal telomeric sequences at the non-permissive temperature

Since Ku-deficient yeasts are ts, we tested whether this is also the case for yeasts disrupted for *RAD50*, *MRE11* or *XRS2*. Thus, overnight cultures of wild-type, *rad50*, *mre11*, *xrs2* or *yku70* mutant strains were diluted serially and each dilution was plated, in duplicate, onto YPDA media plates. One plate was then incubated further at 30°C, whilst the other was incubated at 37°C. Perhaps surprisingly, whereas *mre11* and *xrs2* mutant strains are able to grow effectively at 37°C, the growth of *rad50* strains is impaired dramatically at this temperature (Figure 6A). Importantly, this ts growth defect was observed with several independently derived *rad50* strains, indicating that it is indeed a consequence of the *rad50* mutation. As demonstrated above, Ku-deficient strains are ts and lose further telomeric sequences at the restrictive temperature, whereas mutants such as *tel1*, which are not ts, do not. To see whether this correlation extends further, we employed Southern blot-hybridization to analyse the effects on telomere length of culturing *rad50*, *mre11* and *xrs2* mutant strains at 30 or 37°C (Figure 6B). Notably, as is the case for *tel1* mutants, the shortened telomeres of *mre11* or *xrs2* mutant yeasts are not shortened further upon transfer to 37°C. In marked contrast, the shortened telomeres of *rad50* mutant yeasts growing at 30°C undergo a dramatic further reduction in length upon transfer to 37°C (Figure 6C). Thus, as with strains bearing mutations in *YKU70* or *YKU80*, the temperature sensitivity of *rad50* mutant strains correlates with further loss of telomeric sequences, suggesting that telomeric attrition causes the growth arrest of these strains at 37°C.

Discussion

Previous studies have revealed that Yku70p and Yku80p function in DNA NHEJ and also play a role in telomere length maintenance. Here, we show that cells disrupted for Ku function are also debilitated in telomere-associated transcriptional silencing. Since the telomeric shortening and loss of TPE phenotypes of Ku-deficient strains are very similar to those reported for strains disrupted for *SIR2*, *SIR3* or *SIR4*, we investigated the potential involvement of these three latter genes in DNA NHEJ. Notably, and consistent with the recent report by Tsukamoto *et al.* (1997b), by using a transformation-based *in vivo* plasmid DNA DSB rejoining assay, we find that *sir2*, *sir3* or *sir4* mutant strains are highly deficient in DNA NHEJ. Moreover, by analysing the precise nature of the residual repair products that arise in *sir2*, *sir3* or *sir4* mutant strains, we discovered that their repair defects are indistinguishable from one another and from those of strains deficient in Yku70p or Yku80p. Thus, such strains are incapable of ligating two cohesive restriction enzyme-induced DNA termini effectively, and the residual repair that does occur results in the deletion of terminal sequences and the joining of the resected DNA ends via short direct sequence repeats. Hence, Sir2p, Sir3p and Sir4p behave as components of the Ku-associated DNA NHEJ apparatus.

The association of Ku with telomeric silencing is in accord with studies indicating that DNA ends contribute to transcriptional silencing at yeast telomeres and with work in vertebrate systems, which has revealed that Ku, either alone or in association with DNA-PK_{cs}, is a potent inhibitor of transcription (Kuhn *et al*., 1995; Labhart, 1995). The association of Ku with TPE is also in line with the fact that Yku70p interacts with Sir4p in the yeast two-hybrid system (Tsukamoto *et al*., 1997b). An attractive model, therefore, is that yeast Ku recognizes telomeric DNA ends directly and, through its interactions with Sir4p, helps to recruit the Sir2p–Sir3p–Sir4p (Sir) complex to the telomere. Since such a mechanism would be distinct from the recruitment of the Sir complex via interactions with the telomere repeat binding protein Rap1p, this could help explain how $C_{1-3}A$ repeats and DNA termini function synergistically in establishing telomeric silencing. A requirement of DNA termini for Ku to influence silencing would also be consistent with the fact that Ku has no apparent role in silencing at the yeast silent mating type loci, which are located within the body of the chromosome and are not apposed closely to DNA ends.

Investigations into the mechanisms of TPE in yeast and the related phenomenon of position effect variegation in *Drosophila melanogaster* have indicated that transcriptionally repressed regions of the genome exist in a condensed heterochromatin-like state. Although the mechanism by which this state is established is unknown, the ability of Sir3p and Sir4p to interact with the N-terminal tails of histones H3 and H4, and the requirement of these tails for Sir-mediated silencing, suggest that it is brought about by the Sir protein complex interacting directly with nucleosomal DNA and causing its condensation (Hecht *et al.*, 1995; Grunstein, 1997; Jackson, 1997). The involvement of Sir2p, Sir3p and Sir4p in the Ku-dependent DNA NHEJ system and the interaction of Ku with Sir4p therefore suggest that, upon association with sites of DNA damage, Ku recruits the Sir protein complex and this then leads to the DNA in the vicinity of the DNA DSB becoming assembled into transcriptionally silenced heterochromatin. There are several ways in which this could potentiate DNA repair. First, because silenced chromatin is largely inaccessible to DNA-modifying enzymes, it could prevent nuclease-mediated degradation of damaged DNA ends. Consistent with this, the low level of DNA NHEJ operating in Ku-deficient or Sir protein-deficient yeasts leads to repair products that have suffered deletions. In addition, the Ku–Sir protein complex assembled at the site of DNA damage could, either alone or via the formation of heterochromatin, prevent processes such as transcription and possibly DNA replication from interfering with the assembly of the DNA repair apparatus. Alternatively, or in addition, the condensation of the damaged DNA could aid the juxtaposition of the two broken DNA ends and thus increase the efficiency of their ligation. Finally, the condensation of DNA in the vicinity of DNA DSBs could help prevent the DNA ends from engaging in undesirable recombination reactions with other loci.

Although Ku is involved in telomere length control and TPE, we have noted that inactivation of the gene for another component of the Ku-associated DNA NHEJ system, Lig4p, has no appreciable effect on either of these two processes (Teo and Jackson, 1997; S.J.Boulton, unpublished data). Hence, loss of TPE and normal telomere length control in *yku70* or *yku80* mutant strains is not apparently a simple consequence of a DSB repair defect. In light of the above, we investigated the potential involvement of several other factors in DNA NHEJ, telomere length control and TPE. Although *TEL1* was a good candidate for functioning in these processes, disruption of *TEL1* has no detectable effect on either the efficiency or accuracy of DNA NHEJ, and telomeric silencing is only debilitated slightly in *tel1* mutant strains. Other genes that we investigated are *RAD50*, *MRE11* and *XRS2*, as previous studies have indicated that cells disrupted for these are, amongst other things, defective in illegitimate recombination. Significantly, we found that *rad50*, *mre11* or *xrs2* mutant strains are essentially as debilitated in DNA NHEJ as are those disrupted for *YKU70*, *YKU80* or *LIG4*. Furthermore, disruption of *RAD50*, *MRE11* or *XRS2* in *yku70* or *lig4* mutant backgrounds does not lead to a greater defect in NHEJ than those exhibited by singly mutated strains. Together with previous studies revealing epistatic relationships between Ku and Lig4p in DNA DSB rejoining, this indicates that Rad50p, Mre11p, Xrs2p, Yku70p, Yku80p and Lig4p function together in the same DNA NHEJ repair pathway. Notably, although Rad50p, Mre11p and Xrs2p are essential for efficient plasmid rejoining, the residual repair observed in their absence is predominantly accurate, in contrast to the mainly error-prone repair observed in *yku70*, *yku80*, *lig4* or *sir* mutant strains. A possible explanation for this is that, in the absence of Rad50p, Mre11p or Xrs2p, the Ku–Sir protein complex is still (directly, or indirectly through establishing transcriptionally silenced heterochromatin) able to prevent the access of nucleases and the error-prone repair machinery. Consistent with this model, disruption of *YKU70* or *YKU80* in either *rad50*, *mre11* or *xrs2* mutant backgrounds leads to error-prone repair.

The role(s) played by Rad50p, Mre11p and Xrs2p in NHEJ is unknown. However, the fact that Rad50p and Mre11p share homology with *E.coli* SbcC and SbcD, which together constitute an ATP-dependent exonuclease (Connelly and Leach, 1996), raises the possibility that they process DNA ends prior to their ligation. Such a role would be essential for the repair of IR-induced DSB breaks, which normally possess damaged termini that preclude repair by simple ligation (Friedberg *et al.*, 1995). DNA end processing is, however, unlikely to be the only function of the Rad50p–Mre11p–Xrs2p complex in NHEJ, because there is no obvious requirement for such an activity in repairing restriction enzyme-generated DNA DSBs in the plasmid rejoining assay. Hence, an additional function of the Rad50p–Mre11p–Xrs2p complex may be to serve as a bridging factor between Ku and other components of the NHEJ pathway. Interestingly, human Mre11 was identified initially in a yeast two-hybrid screen for proteins that interact with DNA ligase I (Petrini *et al.*, 1995), raising the possibility that the Rad50p–Mre11p–Xrs2p complex recruits Lig4p to the DSB. Finally, it is noteworthy that, as for inactivation of *YKU70* or *YKU80*, disruption of either *RAD50*, *MRE11* or *XRS2* leads to telomeric attrition. Since the products of these genes appear to operate together in NHEJ, it is tempting to speculate that they also function together in telomere length control. At present, the role(s) of Yku70p, Yku80p, Rad50p, Mre11p and Xrs2p in telomere maintenance remains unclear but one possibility is that they constitute a telomeric end binding complex that facilitates the recruitment of other proteins to telomeric DNA. The recent finding that Ku associates with Sir4p (Tsukamoto *et al.*, 1997b) supports this idea. Notably, Sir4p also interacts with Rap1p, a key regulator of telomere length (Marcand *et al.*, 1997). Thus, it is possible that loss of Ku or the Rad50p–Mre11p–Xrs2p complex affects the localization of Sir4p and its associated Rap1p, and it is this that results in the defect in telomere length homeostasis.

Materials and methods

Media, growth conditions and plasmid repair assays

Non-selective (YPED), selective media, pre-sporulation and sporulation media were as described by Sherman *et al.* (1979). TPE was determined as described by Aparicio *et al.* (1991). Briefly, yeast colonies were picked into dH_2O and diluted five times by 10-fold serial dilution. Aliquots (7.5 μl) of each dilution were spotted in duplicate onto YPED plates with or without 5-FOA and then incubated at 30°C for 3–4 days. The ts phenotype was assayed by spotting 7.5 μl aliquots of serially diluted culture in duplicate onto non-selective media followed by incubation at 30 or 37°C for 3–4 days. Plasmid repair assays were performed as described by Boulton and Jackson (1996a,b).

Gene disruptions

The *RAD50* disruption construct was generated by PCR cloning a 1.46 kb fragment (Rad50-1/Rad50-2 primers) into pGEM-T (Promega), and the open reading frame (ORF) was disrupted by inserting either a *URA3* or *HIS3* marker into the unique *Xba*I site. The resulting disruption fragment was excised using *Spe*I/*Sph*I and used to transform the appropriate strains. The *mre11*::*HIS3* disruption was generated by PCR cloning a 2.459 kbp fragment (Mre11-A/Mre11-B primers) into pCRscript (Stratagene), and the ORF was disrupted by insertion of a *HIS3* marker into the *Stu*I site. The disruption fragment was liberated by *Pvu*II digestion. The *xrs2*::*HIS3* disruption construct was generated by PCR cloning a 2.759 kbp fragment (Xrs2-A/Xrs2-B primers) into pCRscript, and the ORF was disrupted by insertion of a *HIS3* marker into the *Xba*I site; the disruption fragment was liberated by *Nae*I/*Stu*I digestion. The *TEL1* disruption fragment was constructed by PCR cloning a 1.8 kbp fragment into pBS-SK+, using Tel1-1 and Tel1-2 primers, and a *HIS3* selectable marker was used to disrupt the ORF by inserting into *Eco*RI/*Pvu*II restriction sites unique within the *TEL1* fragment. The disruption fragment was excised using *Cla*I and was used to transform the appropriate strains. The *RAD10* disruption construct was generated by cloning a 2.029 kbp fragment (Rad10a/Rad10b) into pGEM-T, and the ORF was disrupted by inserting a *HIS3* fragment into the *Xba*I/*Eco*RV sites. The disruption fragment was excised using *Sac*II/*Not*I. All strains were checked by PCR screening as described previously (Boulton and Jackson 1996a).

Plasmids and DNA manipulation

All yeast–*E.coli* shuttle vectors used have CEN/ARS sequences for stable maintenance in yeast, an auxotrophic yeast selectable marker. pBTM:TRP1 selection (obtained from Stan Fields). pRS413/4/5/6 (Stratagene): *HIS3*, *TRP1*, *LEU2* and *URA3* selectable markers, respectively. Bacterial plasmid DNA extraction was performed using the QIAgen mini plasmid isolation kit (QIAgen). This DNA was used for automated sequencing (Applied Biosystems). Genomic and plasmid DNA from yeast was isolated as outlined in Ausubel *et al.* (1991). For telomere analysis, 1 μg of genomic DNA was digested overnight with 10 units of *Xho*I, then analysed on a 0.8% agarose gel, transferred to nitrocellulose, and used for hybridization to the telomere sequences with a $poly(GT)_{20}$ oligo. Telomere blots were hybridized in Church buffer (7% SDS, 1% BSA, 0.25M Na_2HPO_4, 1 mM EDTA) overnight at 60°C and washed in 0.2× SSC, 0.1% SDS at room temperature for 20 min before exposing to film.

Primers

Tel1-1	CTATGGTAGACAAAACATTGACC
Tel1-2	GCACTAAATGGTCTACATTTATGG
Rad50-1	CGCTTCTAAAGCGGCTTTCAAGCTTTGATC
Rad50-2	CAGAGTCATGGATTAATTTAGATCTGTCC
Mre11-A	CAAACATATGTTGGAAATGC
Mre11-B	AGCCCTTGGTTATAAATAGGA
Xrs2-A	GCAATGCGTAAACCAAATAAGC
Xrs2-B	GATCCGATACAGCTGAAACAA
Rad10a	GTCTACAATCTTACCTGGGATG
Rad10b	CTGAATTTCACATCATTGTCAGAGG

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